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Optimization of activation, collection, dilution, and storage methods for zebrafish sperm

Rongyan Jing [a,c], Changjiang Huang [a,*], Chenglian Bai [a], Robert Tanguay [a,b], Qiaoxiang Dong [a,*]

[a] Institute of Watershed Science and Environmental Ecology, Wenzhou Medical College, Wenzhou 325035, PR China
[b] Environmental Health Sciences Center, Oregon State University, Corvallis, Oregon 97331, USA
[c] Institute of Marine Biology, Shantou University, Shantou 515063, PR China

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ABSTRACT

In the present study, we optimized activation, collection, dilution, and storage methods for zebrafish (*Danio rerio*) sperm. Our findings revealed that zebrafish sperm was motile in Hank's balanced salt solution (HBSS), glucose, and sucrose at an osmolality range of 25–270 mOsm/kg with the highest motility observed at 150–210 mOsm/kg. We compared three activation solutions: 0.3% NaCl, de-ionized water, and HBSS at 170 mOsm/kg. Our results indicate that the longevity of sperm motility depends on the final osmolality of the sperm suspension medium (extender) and activation solution combined. Evaluation of sperm collection methods suggested that higher percent motility is obtained when collected through dissecting without crushing (89 ± 3%, mean ± SD) or abdominal massage (90 ± 4%) than dissecting with crushing (65 ± 13%). The total number of motile sperm was higher for dissecting without crushing ($147.0 \pm 102.3 \times 10^5$/male) than abdominal massage ($7.1 \pm 11.9 \times 10^5$/male). Sperm suspended in HBSS had higher motility than those suspended in buffered sperm motility-inhibiting solution (BSMIS). Sperm retained motility longer when samples were stored at 4 °C than those stored at room temperature (25 °C). Motility which could be activated after storage decreased with increased dilution ratio and it is the resultant sperm density rather than the dilution factor that determines sperm motility. Our findings provide useful information on handling procedures of zebrafish sperm.

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1. Introduction

The zebrafish (*Danio rerio*) is a popular freshwater aquarium fish, and was initially used as model for experimental embryology studies (Mertens, 1973). Over the past few decades, their small size, fecundity, embryonic transparency, and rapid development have led to their selection as one of the most prominent model for a wide variety of research fields, including functional genomics (van Eeden et al., 1999), physiology (Briggs, 2002), environmental and high-throughput toxicology screening (Langheinrich et al., 2002; Hill et al., 2005) as well as specific human diseases (Berghmans et al., 2005). Despite its popularity as a vertebrate model organism, possible areas of interaction between zebrafish and the aquaculture research community have not been recognized until more recently (see Dahm and Geisler, 2006 for review). In this review, the zebrafish was foreseen to become a model organism for the improvement of aquacultural animals and husbandry based on the genetics-driven approaches. Results such as identification of genes involved in the development of certain organs, the metabolism of nutrients, disease and stress pathways, as well as research approaches such as morpholino-induced knock-down and transgenesis established in zebrafish can be transferred to aquacultural species for commercial interest (Dahm and Geisler, 2006). This study predicted that in the near future more interactions will occur between the zebrafish community and researchers studying fish species of aquacultural interest.

In contrast to their importance as an experimental model and its associated significant economic costs, the scientific rigour of zebrafish husbandry techniques is poorly developed (Lawrence, 2007). Gamete handling is one of those husbandry techniques that have received little attention while it often constitutes as an important prerequisite for many research activities such as in vitro fertilization, sperm cryopreservation, polyploidy manipulation, and androgenesis. In our laboratory, we are particularly interested in sperm cryopreservation as this is the only reliable tool currently available to store important genetic materials of mutant strains and transgenic lines that are derived from the wide use of zebrafish (Brand et al., 2002). Proper sperm handling will minimize the motility loss during the pre-freezing steps (e.g., sperm collection, dilution, and storage) and the reduction of motility loss in each step involved in the series of sequential steps for sperm cryopreservation would help reduce the cumulative loss upon thawing. In addition, studies of proper gamete handling in zebrafish will also provide useful information for other teleost fishes of aquaculture interest. Therefore, the goal of this project is to optimize the handling procedures of zebrafish sperm, and specifically we evaluated various factors affecting the sperm motility in the handling process: sperm activation (osmolality), sperm collection methods, choice of extenders, pH, storage temperature, and dilution ratio.

* Corresponding authors. Tel./fax: +86 577 86699135.
E-mail addresses: cjhuang5711@163.com (C. Huang), dqxdong@163.com (Q. Dong).

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2. Materials and methods

2.1. Sperm collection

Wild-type zebrafish of 3–4 month age were purchased from pet market in Wenzhou, China and kept at standard laboratory conditions of 28 °C on a 14:10 dark/light photoperiod (Westerfield, 1995) in a recirculating system. The fish were fed twice daily with either the zebrafish diet (Zeigler, Aquatic Habitats, Apopka Florida) or live artemia (Jiahong Feed Co., Tianjin, China). Upon sperm collection, fish were anesthetized in 0.01% tricaine-methane sulfonate for ~2 min, and their standard lengths (tip of snout to the crease of the caudal peduncle) and wet weight were measured after blotting excess water with a paper towel. Except for the experiment used to evaluate collection method, sperm were collected by surgical removal of the testis. Adherent tissue was dissected away and testes were placed in 0.5-ml tube and weighed. Unless otherwise specified, testes were pooled and Hanks' balanced salt solution at 300 mOsm/kg (NaCl 8.0 g, KCl 0.4 g, $CaCl_2 \cdot 2H_2O$ 0.16 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, Na_2HPO_4 0.06 g, KH_2PO_4 0.06 g, $NaHCO_3$ 0.35 g, $C_6H_{12}O_6$ 1.0 g in 1000 mL distilled water, pH 7.5) was added at the ratio of testis to HBSS (mass:volume) of 1:50 before stirring testes to release sperm. Within this report, HBSS at specific osmolalities such as 300 mOsm/kg are abbreviated as HBSS300. Sperm numbers per testis were obtained from the average of duplicate counts using a hemocytometer. Blood samples were collected in micro-hematocrit tubes with an internal diameter of 0.5–0.6 mm (VWR Scientific, Niles, IL) by severing the tail, and blood plasma were collected after centrifugation at 15,000 rpm for 10 min. Osmolality was measured by a freezing point depression osmometer (Osmomat 030; Genotec GmbH, Berlin, Germany).

2.2. Motility estimation

Like most teleost fish, zebrafish sperm were activated under hypotonic conditions. Thus, except for the evaluation of activation solution experiment, de-ionized water was used for sperm activation. In detail, sperm were initially diluted with HBSS 300 to achieve a sperm density of 5×10^7/ml (corresponding to ~1:50 dilution, mass:volume). A 10-µl aliquot was removed from each sperm sample and placed on a microscope slide, and 10-µl de-ionized water was added to the drop to activate the sperm. Sperm motility was estimated visually at 200× magnification using dark-phase microscopy (Nikon Corporation, Japan), and was expressed as the percentage of sperm that moved actively in a forward direction within 30 s after activation at room temperature. Motility was recorded in increments of 5%, and samples with motility below 5% but greater than 0% were recorded 1%, and samples with motility below 100% but greater than 95% were recorded as 99%. Sperm vibrating in place were not considered to be motile. Unless specified otherwise, all experiments were conducted at room temperature (25°).

In the present study, experiments were routinely conducted with milt pooled from several males due to limited sperm volume from individual fish. It is noteworthy that significant variations were observed among individual males. To avoid possible confounding effect of poor sperm quality used in one treatment group, but not in other groups, samples were evaluated for initial sperm motility after collection, and fish from the same batch with similar initial motility were pooled and used for the same experiment.

2.3. Determination of optimal osmolality for motility activation

There were four trials in this experiment. In the first trial, a small amount of undiluted non-motile sperm (the tip of 10 uL pipette tip) from three males was diluted in 10 µl of de-ionized water (25 mOsm/kg), and HBSS at 100, 200, 300, 400, 500, 600 mOsm/kg before assessment of motility. The purpose of using a tiny volume of concentrated undiluted sperm was to avoid the interaction effects of predilution media, thus the effect of osmolality could be estimated accurately. This also resembles the natural conditions where fish shed undiluted sperm into the aquatic environment. In the second trial, HBSS at a narrower range of 100, 150, 170, 200, 240, 270, 300, 400 mOsm/kg were evaluated with undiluted sperm from seven males. For these two trials, HBSS at different osmolalities was prepared by mixing HBSS900 with different ratios of de-ionized water. The remaining two trials evaluated the effect of osmolality of non-ionic solutions (glucose and sucrose) on zebrafish sperm motility, and osmolality values of 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360 mOsm/kg was tested with three fish.

2.4. Selection of activation solutions

Based on the above experiments, HBSS300 (the lowest osmolality that caused 100% sperm immobilization) was selected for sperm suspension. Testes from three males were suspended in HBSS300, and sperm were activated by mixing 10 µl sperm suspension with 10 µl different activation solutions: de-ionized water (25 mOsm/kg), 0.3% NaCl (95 mOsm/kg), and HBSS170. Motility was estimated at room temperature (25 °C) at 0.5, 1, 2, 5, 10, 15, 20 min after activation.

2.5. Sperm collection methods

There were two trials in this experiment. In the first trial, three different sperm collection methods were compared for motility yield. Specifically, sperm were collected through 1) dissecting without crushing: testes were removed and suspended in HBSS300 as described above (see Section 2.1), and sperm were released by gently but repeatedly disrupting the testis with a pipette tip; 2) dissecting with crushing: testes were placed in a tared resealable plastic bag (3×5 in., NASCO Whirl-Pak, USA) and HBSS300 at the ratio of testis to HBSS (mass:volume) of 1:50 was added before squeezing the testis to release sperm; 3) abdominal massage: gentle finger pressure was applied to the fish abdomen, and sperm were collected with capillary tubes directed to the anal pore, and resulted semen (1–2 µL) were diluted with HBSS300 for a final yield of 10 µl sperm suspension. For sperm collected through dissecting, the pairs of testes from the same fish were used with each of the pair allocated to each of the two dissecting methods (with or without crushing). At least four fish were used for each method. Sperm suspensions were stored at 4 °C in 1.5 ml centrifuge tubes with a sample volume ranging from 100 to 400 µl at a sperm density of 5×10^7 cells/ml, and motility was evaluated at 5 min, 2 h, 4 h, 10 h, 24 h, and 48 h after storage.

In the second trial, motility and the yield of total motile sperm were compared between the dissecting without crushing and the abdominal massage methods. Ten fish from the same batch were randomly selected for each method. Sperm motility, suspension volume, and density were evaluated. The total number of motile sperm was calculated by multiplying sperm motility with sperm density and suspension volume.

2.6. Selection of extender

In this experiment, we compared the extenders of HBSS 300 and buffered sperm motility-inhibiting solution (BSMIS) (NaCl 4.38 g, KCl 5.22 g, $CaCl_2 \cdot$ 0.22 g, $MgSO_4 \cdot$ 0.12 g, Tris 2.42 g in 1000 mL de-ionized water, pH 8.0, 300 mOsm/kg). Sperm were collected with the dissecting without crushing method, and testes from six fish (one replicate with pooled sperm from two fish, and a total of three replicates) were used for each extender. Sperm suspensions were stored at 4 °C, and motility was estimated at 5 min, 2 h, 4 h, 10 h, and 24 h. The term "extender" used in this report refers to solutions used for sperm suspension without cryoprotectants.

2.7. Effect of pH on sperm motility

There were three trials in this experiment. In the first trial, the pH of activator was evaluated with testis collected from three fish by

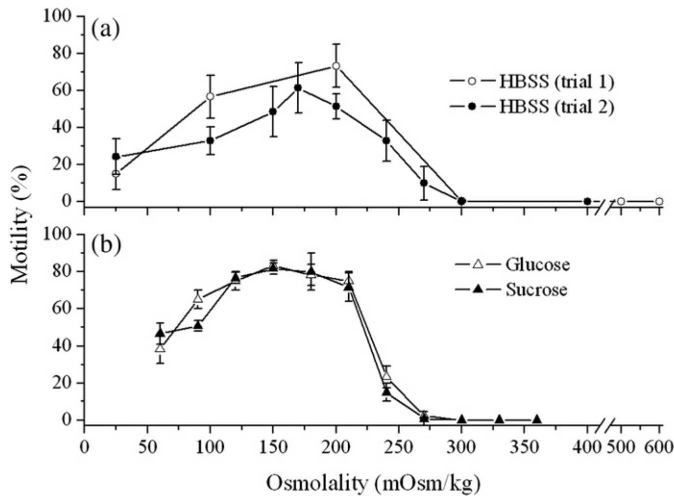

Fig. 1. Motility (mean ± SD) of undiluted zebrafish sperm after being activated with (a) de-ionized water (25 mOsm/kg) and HBSS at 100, 200, 300, 400, 500, and 600 mOsm/kg (trial 1, $n = 3$) and 100, 150, 170, 200, 240, 270, 300, 400 mOsm/kg (trial 2, $n = 7$), and (b) non-ionic solutions of glucose ($n = 3$) and sucrose ($n = 3$) at different osmolalities ranging from 60 to 360 mOsm/kg.

equilibrating a small amount of undiluted non-motile sperm (the tip of 10 uL pipette tip) in 10 uL de-ionized water at pH of 5.0, 6.0, 6.5, 7.0 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0. Motility was estimated immediately after activation. In the second trial, the pH of extender was tested on three fish. Similarly, a small amount of undiluted sperm were equilibrated in 5 uL HBSS300 at pH of 5.0, 6.0, 6.5, 7.0 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0 for 10 min at room temperature, and were then mixed with 5 uL de-ionized water (pH 7.5) for activation. In the third trial, testes were suspended in HBSS300 at pH of 6.5, 7.0, 7.5, and 8.0, and resulting sperm suspensions were stored at 4 °C. Motility was estimated at 5 min, 4 h, 10 h, and 24 h after storage. This experiment was replicated three times using a total of fifteen fish where each replicate constitutes sperm pooled from five fish.

2.8. Effect of storage temperature on sperm motility

Testes from four fish were collected by the dissecting without crushing method, and were suspended in HBSS300 as described above. Resulting sperm suspensions were divided into two sets of samples with one set stored at room temperature (25 °C) and the other stored at 4 °C. Motility was estimated at 5 min, 2 h, 4 h, 10 h, and 24 h after storage.

2.9. Effect of dilution ratio on sperm motility

To maximize the ability to evaluate effects of multiple factor treatments with limited number of sperm, one possible solution is to increase the dilution ratio to obtain larger sample volumes. Thus, the ratio of sperm to HBSS 300 was evaluated for refrigerated storage (4 °C) at dilution ratios of 1:50, 1:100, 1:200, 1:300, 1:400, and 1:500 with samples from seven males (trial 1), and at dilution ratios of 1:10, 1:20, 1:50 and 1:100 with samples from 11 males (trial 2). Motility was estimated at 5 min, 2 h, 4 h, 10 h, and 24 h. Sperm density was estimated using hemocytometer for each dilution.

2.10. Data analysis

Data were analyzed using simple linear regression, one-way ANOVA, and repeated measure ANOVA (SAS 9.1). When a significant difference ($P = 0.05$) was observed among treatments, Tukey's studentized range test was used for post-test comparisons. Percent motility was arcsine-square root transformed prior to analysis. Values presented are means ± SD.

3. Results

3.1. Basic parameters

The average measurements of the male zebrafish were 3.68 ± 0.35 cm ($n = 75$) for standard length and 321 ± 69 mg ($n = 75$) for body wet weight. The standard length was positively ($P<0.001$) related to the body wet weight. The average testis wet weight was 3.5 ± 1.4 mg ($n = 75$), and was positively ($P<0.001$) related to the body wet weight. There were $1.23 \pm 1.01 \times 10^7$ sperm cells per testis ($n = 20$), and sperm density was $2.59 \pm 1.42 \times 10^9$ cell/g of testis. Osmolality of the blood plasma was 315 ± 1 mOsm/kg ($n = 3$).

3.2. Determination of optimal osmolality for motility activation

In the first trial, sperm motility increased with the osmolality of HBSS to the highest level at 200 mOsm/kg (73 ± 12%) and declined thereafter to 0 ± 0% at 300 mOsm/kg and above (Fig. 1a). There were no differences between HBSS100 and HBSS200 ($P>0.05$), but sperm had higher motility when samples were activated with HBSS200 than those activated with de-ionized water ($P = 0.01$). The second trial revealed similar trends of the activation curve with the highest motility (61 ± 13%) observed at HBSS170, and followed by the HBSS200 (51 ± 7%). No significant differences were observed among HBSS150, HBSS170, and HBSS200 ($P>0.05$) (Fig. 1a).

When sperm were activated with non-ionic solutions such as glucose and sucrose, the highest motility was observed at 150 mOsm/kg for both glucose (83 ± 3%) and sucrose (82 ± 3%) (Fig. 1b). However, there were no differences among samples activated by glucose or sucrose solutions at 120, 150, 180, and 210 mOsm/kg ($P>0.05$), which had higher ($P<0.05$) motility than samples activated at either lower (equal or below 100 mOsm/kg) or higher osmolalities (240 mOsm/kg and above) (Fig. 1b).

3.3. Selection of activation solutions

At 30 s after activation, the highest motility (98 ± 4%) was found with 0.3% NaCl, followed by de-ionized water (93 ± 4%), and HBSS170 (90 ± 14%), however, there were no significant differences among de-ionized water, 0.3% NaCl and HBSS170 ($P>0.05$) (Fig. 2). Sperm retained motility longer when activated with de-ionized water than others, for example, approximately 20% sperm were still motile at 15 min after activation with de-ionized water while sperm motility was below 5% for all other treatments ($P<0.05$) (Fig. 2).

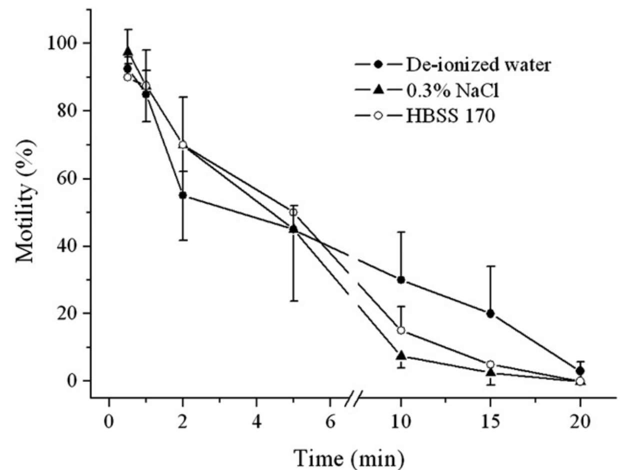

Fig. 2. Motility (mean ± SD, $n = 3$) of zebrafish sperm suspended in HBSS300 and activated by three activation solutions: de-ionized water, 0.3% NaCl, and HBSS170. Motility was estimated at room temperature (25 °C) at 0.5, 1, 2, 5, 10, 15, 20 min after activation.

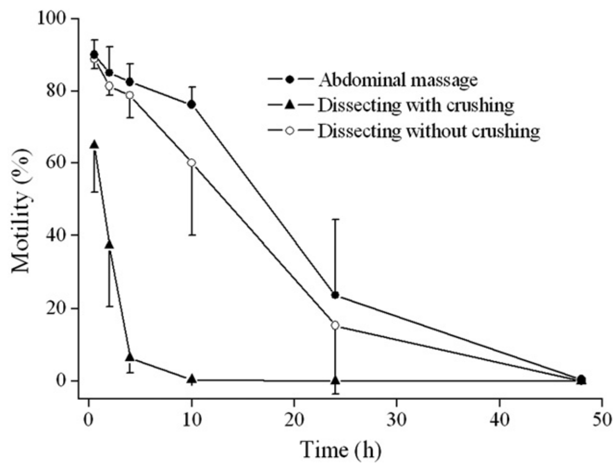

Fig. 3. Zebrafish sperm samples were collected through abdominal massage ($n=4$) and dissecting with or without crushing ($n=4$), suspended in HBSS 300, and stored at 4 °C. Motility was evaluated at 5 min, 2 h, 4 h, 10 h, 24 h, and 48 h after storage.

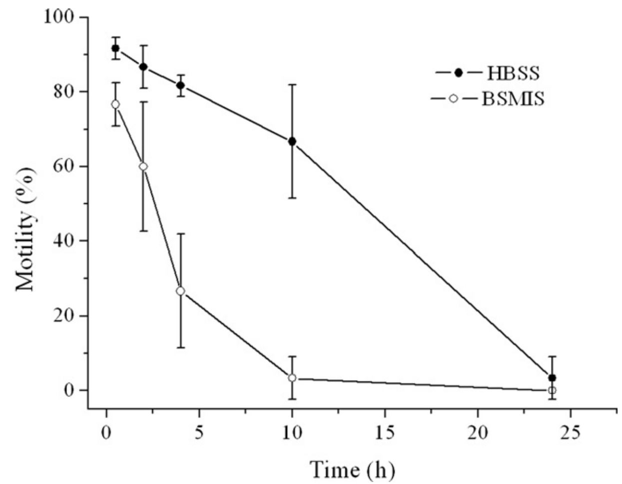

Fig. 4. Motility (mean ± SD, $n=3$) of zebrafish sperm suspended in HBSS and buffered sperm motility-inhibiting solution (BSMIS) at 4 °C for 5 min, 2 h, 4 h, 10 h, and 24 h.

3.4. Sperm collection methods

Sperm had higher ($P<0.05$) motility when collected through dissecting without crushing (89 ± 3%) or abdominal massage (90 ± 4%) than dissecting with crushing (65 ± 13%) at 5 min after refrigerated storage (4 °C) (Fig. 3). Sperm retained lower motility throughout a storage period of 24 h for samples collected through dissecting with crushing than dissecting without crushing or abdominal massage ($P<0.001$). Although there was no difference ($P>0.05$) in sperm motility between dissecting without crushing and abdominal massage, the yield of total number of motile sperm was approximately 20 times higher ($P<0.01$) for dissecting without crushing than abdominal massage (Table 1).

3.5. Selection of extender

At 5 min of refrigerated storage (Fig. 4), the motility of samples suspended in HBSS300 (92 ± 3%) was higher ($P<0.01$) than those suspended in BSMIS (77 ± 6%). Throughout a 24 h storage period, sperm retained higher motility in samples suspended with HBSS300 than with BSMIS ($P<0.01$). For example, samples suspended in HBSS300 had 67 ± 15% motile sperm after 10 h refrigerated storage, while there was 3 ± 6% motile sperm for samples suspended in BSMIS ($P<0.001$). Extended storage of 24 h or 48 h at 4 °C resulted in zero sperm motility (Fig. 4).

3.6. Effect of pH on sperm motility

In the first trial, no significant difference ($P=0.65$) was found for sperm motility in the pH range between 6.5 and 8.5 for the de-ionized water, while motility in this pH range was higher than those activated at pH 5.0 or 10.0 ($P=0.004$). The highest motility (68 ± 8%) was found with the de-ionized water at pH of 7.5 (Fig. 5a). In the second trial, similar trends were observed for the pH of extender with the highest motility (73 ± 6%) found at pH 7.5 for HBSS 300 (Fig. 5b). In the third trial, there were significant differences ($P<0.01$) among four pH values tested, and the highest motility at 5 min after refrigerated storage was found with pH 8.0 (90 ± 5%), followed by pH 7.5 (87 ± 6%), pH 7.0 (83 ± 3%) and pH 6.5 (73 ± 3%) (Fig. 6). Motility decreased with extended storage and the significant loss occurred at 24 h after refrigerated storage (Fig. 6).

3.7. Effect of storage temperature on sperm motility

At 5 min after initiation of the experiment, motility was the same (93 ± 4%) for samples stored at room temperature and 4 °C (Fig. 7). However, motility declined more rapidly for samples stored at room temperature than at 4 °C after 2 h storage. At 10 h, the

Table 1
Parameters of zebrafish sperm collected through two different methods.

Parameter	Collection method	
	Dissecting without crushing ($n=10$)	Abdominal massage ($n=6$)
Motility (%)	86 ± 7[a]	84 ± 13[a]
Volume (µl)	240 ± 90	10 ± 0
Sperm density (× 10^7 cell/ml)	6.5 ± 3.0	7.9 ± 12.4
Total number of sperm (× 10^5)	164.1 ± 106.6[a]	7.9 ± 12.4[b]
Total number of motile sperm (× 10^5)	147.0 ± 102.3[a]	7.1 ± 11.9[b]

Numbers in rows sharing the same superscript were not different at $P=0.05$.

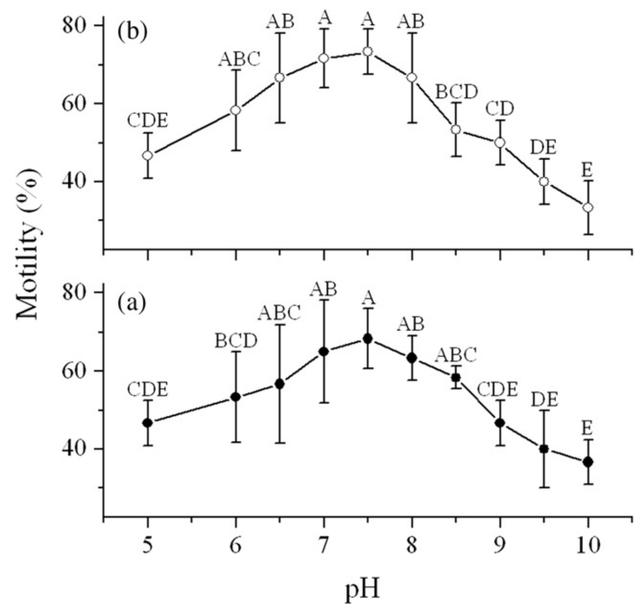

Fig. 5. Motility (mean ± SD) of undiluted zebrafish sperm (a) after activated with de-ionized water at various pH ($n=3$); or (b) activated with de-ionized water after sperm were suspended in HBSS 300 of various pH for 10 min at room temperature ($n=3$). Dots sharing the same letter indicate no significant difference at $P=0.05$.

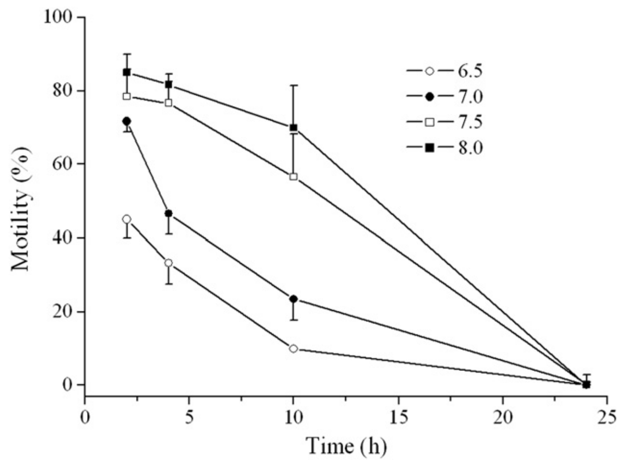

Fig. 6. Motility (mean ± SD, $n = 3$) of zebrafish sperm collected and suspended in HBSS300 at pH of 6.5, 7.0 7.5, and 8.0, and stored at 4 °C for 5 min, 4 h, 10 h, and 24 h.

motility of samples stored at 4 °C (65 ± 7%) were significantly higher ($P < 0.05$) than those stored at room temperature (20 ± 14%). Motility ceased at 24 h for all samples regardless of storage temperature (Fig. 7).

3.8. Effect of dilution ratio on sperm motility

For the first trial (Fig. 8a), the average sperm density was $1.09 \pm 0.46 \times 10^7$ cell/mL for the volume ratio (sperm: extender) of 1:50, and concentration estimation confirmed the accuracy of successive dilution from 1:100 to 1:500. For example, the sperm density for samples of the volume ratio of 1:500 was 1×10^6 cell/mL. With prolonged storage time, the motility of samples at higher dilution ratio declined faster than that of samples at lower dilution ratio. At 10 h storage, the highest motility (60 ± 10%) was found with the dilution ratio of 1:50 and the lowest motility (17 ± 6%) was at 1:500. Samples at the volume ratio of 1:50 had higher motility than samples within the grouping of 1:100, 1:200 and 1:300 ratios ($P < 0.05$) or the grouping of 1:400 and 1:500 ($P < 0.001$) although there were no significant differences within each grouping. For the second trial (Fig. 8b), the average sperm density was $8.90 \pm 5.12 \times 10^6$ cell/mL for the volume ratio of 1:10. Within a storage period of 10 h, samples at volume ratio of 1:10 and 1:20 retained higher motility than samples

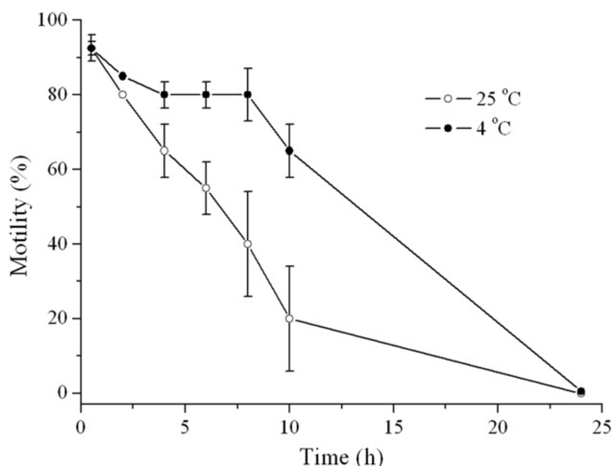

Fig. 7. Motility (mean ± SD, $n = 4$) of zebrafish sperm suspended in HBSS 300 and stored at room temperature (25 °C) or 4 °C for 5 min, 2 h, 4 h, 10 h, and 24 h.

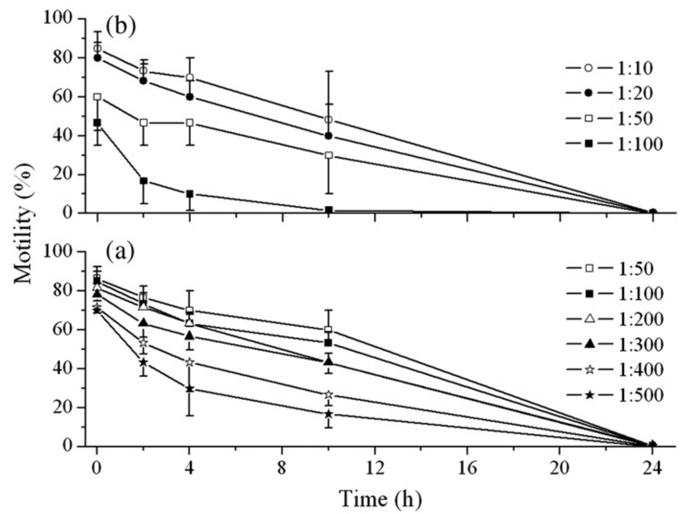

Fig. 8. Motility (mean ± SD) of zebrafish sperm suspended in HBSS 300 at various dilution ratio and stored at 4 °C for 5 min, 2 h, 4 h, 10 h, and 24 h: (a) trial 1 with a ratio of sperm to HBSS 300 of 1:50, 1:100, 1:200, 1:300, 1:400, and 1:500 ($n = 3$); (b) trial 2 with a ratio of sperm to HBSS 300 of 1:10, 1:20, 1:50, and 1:100 ($n = 3$).

at 1:50 or 1:100 ($P < 0.001$). All samples lost motility at 24 h refrigerated storage.

4. Discussion

Fish sperm is immotile in the testis and the initiation of motility can be controlled by changes in ion concentration or osmolality of the external medium (see Alavi and Cosson, 2006 for review). In cyprinids, motility is activated by suspending sperm in hypotonic solution (Morisawa and Suzuki, 1980). The present study showed that zebrafish sperm could be activated in hypotonic solutions at a wide range of osmolalities (25–270 mOsm/kg), but the highest motility was observed at 150–210 mOsm/kg. The osmolality for complete motility inhibition in zebrafish (≥300 mOsm/kg) was similar to its blood plasma (315 mOsmol/kg) or the seminal plasma osmolality observed in cyprinids (~300 mOsm/kg) (Alavi and Cosson, 2006). In addition, ion-free solutions solely containing glucose or sucrose were found to exhibit the same activation pattern as that of HBSS, which suggests that osmolality is the major factor for controlling zebrafish sperm motility. However, it is possible that K^+ efflux may also account for the initiation of sperm motility followed by the first trigger of osmolality change (Takai and Morisawa, 1995).

In laboratory settings, fish sperm have seldom been used in an undiluted form. Often, sperm are collected in an extender or motility inhibition medium for species with short sperm motility duration after activation. Therefore, motility activation often involves mixing the suspended sperm solution with an activation solution—a so called "2-step activation procedure" (Billard and Cosson, 1992). In zebrafish, egg water (Morris et al., 2003), distilled water (Yang et al., 2007), and 0.3% NaCl (Z. Zhong, personal communication) have all been used as activation solutions. Our experiment of activation with undiluted sperm suggested a suitable activator of HBSS 170 (Fig. 1a). We consequently compared the effect of these activation solutions on sperm motility and its longevity. There were no significant differences for the instant motility (at 30 s) upon activation, but the decline in motility over time was the slowest in de-ionized water. This may be due to the differences in the final osmolality of mixtures of various activation solutions because a volume ratio of 1:1 dilution of sperm-HBSS300 to activation solution resulted in a final osmolality of ~170 mOsm/kg for de-ionized water treatment, and 200–235 mOsm/kg for 0.3% NaCl and HBSS 170. This would agree with the activation curve results, in which de-ionized water was not as effective as HBSS 170 in motility

activation of undiluted sperm. These findings suggested that instant initiation of zebrafish sperm motility could be achieved with hypotonic solutions in a wide range of osmolalities, but sustaining motility over time requires an optimal osmolality in a narrower range. Our findings also emphasize the importance of each factor involved in the activation process such as the choice of extender, activation solution, dilution ratio, and the resulting final osmolality of sperm and activation solution mixtures. Because de-ionized water provided high motility over a long duration for zebrafish sperm suspended in HBSS300, we consequently chose de-ionized water as the activator in the subsequent experiments.

It is worth noting that the motility duration of 15 min for samples activated with de-ionized water observed in this study was unusually long for freshwater species. We ourselves subsequently had less success repeating the same experiments, especially with fish raised under laboratory conditions. For fish purchased from local pet market, however, we did observe a considerable batch to batch variation in sperm quality, and extended motility duration of 10–15 min were occasionally observed in some males. Future studies with rigorous experimental trials are necessary to further explore variations related to fish sources.

Sperm collection is the very first step of fish sperm cryopreservation process, and it plays an important role in sperm quality for subsequent steps. In general, fish sperm can be collected either by abdominal massage or from testes removed by dissection. Squeezing sperm from live zebrafish males preserves the donor and yields high sperm motility, but this method can be ineffective and time-consuming when large volumes are needed for experiments with multiple factors at many different levels. Because abdominal massage generally produces low yields (0.8–2 uL/fish), it requires a large sample size in zebrafish. For example, sperm were collected from only half of all fish tested by abdominal massage in a previous study (Morris et al., 2003). We also can only collect sperm from six out of ten fish in this study. On the contrary, for small fish with small testis, the dissection of testes is a simple and rapid method to obtain a large volume of sperm (e.g., Huang et al., 2004a,b). However, testes were often crushed or ground to release sperm after dissection (Wayman and Tiersch, 2000), resulting in the inclusion of immature sperm, and thus often leading to low initial motility. In this study, we devised a new method for sperm collection. Instead of crushing or grinding testes, gentle and repeated disruptions in the form of stirring were applied to testes in HBSS. This dissecting without crushing method not only produced motility similar to that of the abdominal massage, but importantly had a yield of total motile sperm 20 times higher. The high motility yield in this study may be attributed to the gentle disruption of testes with fewer immature sperm extracted. Our results also demonstrate that sperm collected by abdominal massage or dissecting without crushing is preferred because sperm motility is retained for a longer duration. Therefore, squeezing sperm through abdominal massage remains a good choice for large fish where sperm volume is not a concern (e.g., Akcay et al., 2004; Horváth et al., 2005).

The choice of extender is a primary focus in fish sperm cryopreservation (Stoss, 1983). In zebrafish, Morris et al. (2003) evaluated the toxicity of five diluents (Ginzburg Ringer solution, HBSS, BSMIS, Mounib's solution, and sperm-extender solution) and found BSMIS provided a high level of average sperm percent motility and a low and stable decrease in percent motility over time. The same study also reported that sperm incubated in HBSS possesses higher percent motility than sperm incubated in Ginzburg Ringer solution. Comparisons between BSMIS and HBSS in this study however, indicated lower motility and rapid decline of motility over time for samples suspended in BSMIS. It is possible that different activation solution/methods account for these conflicting findings. Currently, Ginzburg Ringer solution (Harvey et al., 1982), BSMIS, and HBSS 300 are the main extenders used in zebrafish sperm cryopreservation. Future studies should compare these three extenders directly for their effect on the motility, fertilization and hatch rate of thawed sperm.

In addition to osmolality, the pH and temperature are also major factors affecting sperm motility. Recently, Alavi and Cosson (2005) reviewed the effect of these two factors on fish sperm motility. Evaluation of pH for the activation solution as well as the extender in our study confirmed favorable alkaline conditions (pH 7.5–8) generally observed for fish sperm (Alavi and Cosson, 2005). Evaluation of storage temperature on zebrafish sperm motility revealed a rapid decline of motility when samples were stored at room temperature after 2 h, and suggested refrigeration (4 °C) for prolonged storage. This same temperature-dependence is consistent with results from other species such as *Cyprinus carpio* (Ravinder et al., 1997) and *Xiphophorus helleri* (Huang et al., 2004b). Thus, the practical implication for zebrafish sperm cryopreservation is to finish the pre-freezing process within 2 h after sperm collection at room temperature or collect and store sperm at 4 °C (e.g., walk-in cooler) when extended processing time is required for complicated experiments involving multiple factors.

Similar to all other small aquarium fish such as the platyfish *X. couchianus* (Huang et al., 2004a), green swordtail *X. helleri* (Huang et al., 2004b,c) and medaka *Oryzias latipes* (Aoki et al., 1997; Krone and Wittbrodt, 1998), zebrafish are characterized by small body and testis sizes, and limited sperm volumes. While sufficient volumes of sperm sample were required for systematic procedure optimization involving multiple factors at various treatment levels, reducing the loading volume in the French straws (Huang et al., 2004b) would help to maximize the ability to evaluate treatment effects. Unfortunately the small sperm volume (<5 µl/fish) in zebrafish necessitates pooling sperm from different males. Even with pooling, dilution was still necessary to maximize the volume for experiments involving multiple factors. However, dilution could reduce sperm motility by removing protective components of seminal plasma as suggested for rainbow trout *Oncorhynchus mykiss* (Lahnsteiner et al., 2004; Lahnsteiner, 2007). The present study also showed decreased motility (percent motile sperm and motility duration) with increased dilution ratio. In addition, we found that the rapid motility decline occurred at a sperm density below $\sim 2 \times 10^6$ cells/mL, which corresponded to a dilution ratio of 1:300 in trial 1 (Fig. 8a), and 1:50 in trial 2 (Fig. 8b). Thus, it is the sperm density rather than dilution factor that determines the final outcome of sperm motility in zebrafish. Since sperm density varies from male to male, it is important to use sperm concentration to adjust the dilution ratio for sperm freezing in future studies. Our findings also add further evidence of using sperm concentration instead of dilution ratio to minimize the inconsistency observed among various studies in aquatic organisms, which was recently proposed by Dong et al. (2007) derived from findings with oyster sperm cryopreservation.

In conclusion, the present study provides useful information on handling procedures for zebrafish sperm. Specifically, sperm collection is better done with dissecting without crushing; motility activation depends on the final osmolality of the mixtures of sperm suspension medium (extender) and activation solution; HBSS 300 is a better choice of extender; alkaline conditions (pH 7.5–8.0) of activation solution and extender are favorable for sperm motility; refrigerated storage helps retain sperm motility after prolonged storage; and it is the sperm density rather than dilution factor that contributes to sperm motility and longevity.

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References

Akcay, E., Bozkurt, Y., Secer, S., Tekin, N., 2004. Cryopreservation of mirror carp semen. Turk. J. Vet. Anim. Sci. 28, 837–843.
Alavi, S.M.H., Cosson, J., 2005. Sperm motility in fishes. I. Effects of temperature and pH: a review. Cell Biol. Int. 29, 101–110.
Alavi, S.M.H., Cosson, J., 2006. Sperm motility in fishes. (II). Effects of ions and osmolality: a review. Cell Biol. Int. 30, 1–14.
Aoki, K., Okamoto, K., Tatsumi, K., Ishikawa, Y., 1997. Cryopreservation of medaka spermatozoa. Zool. Sci. 14, 641–644.
Berghmans, S., Jette, C., Langenau, D., Hsu, K., Stewart, R., Look, T., Kanki, J.P., 2005. Making waves in cancer research: new models in the zebrafish. Biotechniques 39, 227–237.
Billard, R., Cosson, M.P., 1992. Some problems related to the assessment of sperm motility in freshwater fish. J. Exp. Zool. 261, 122–131.
Brand, M., Granato, M., Nüsslein-Volhard, C., 2002. Keeping and raising zebrafish. In: Nüsslein-Volhard, C., Dahm, R. (Eds.), Zebrafish: A Practical Approach. Oxford University Press, Oxford, pp. 7–37.
Briggs, J.P., 2002. The zebrafish: a new model organism for integrative physiology. Am. J. Physiol. Regul. Integr. Comp. Physiol. 282, 3–9.
Dahm, R., Geisler, R., 2006. Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. Mar. Biotechnol. 8, 329–345.
Dong, Q., Huang, C., Tiersch, T.R., 2007. Control of sperm concentration is necessary for standardization of sperm cryopreservation in aquatic species: evidence from sperm agglutination in oysters. Cryobiology 54, 87–98.
Harvey, B., Kelley, R.N., Ashwood-Smith, M.J., 1982. Cryopreservation of zebra fish spermatozoa using methanol. Can. J. Zool. 60, 1867–1870.
Hill, A.J., Teraoka, H., Heideman, W., Peterson, R.E., 2005. Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol. Sci. 86, 6–19.
Horváth, A., Wayman, W.R., Urbányi, B., Ware, K.M., Dean, J.C., Tiersch, T.R., 2005. The relationship of the cryoprotectants methanol and dimethylsulfoxide and hyper-osmotic extenders on sperm cryopreservation of two North-American sturgeon species. Aquaculture 247, 243–251.
Huang, C., Dong, Q., Tiersch, T.R., 2004a. Sperm cryopreservation of a live-bearing fish, the platyfish *Xiphophorus couchianus*. Theriogenology 62, 971–989.
Huang, C., Dong, Q., Walter, R.B., Tiersch, T.R., 2004b. Initial studies on sperm cryopreservation of a live-bearing fish, the green swordtail *Xiphophorus helleri*. Theriogenology 62, 179–194.
Huang, C., Dong, Q., Walter, R.B., Tiersch, T.R., 2004c. Sperm cryopreservation of green swordtail *Xiphophorus helleri*, a fish with internal fertilization. Cryobiology 48, 295–308.
Krone, A., Wittbrodt, J., 1998. A simple and reliable protocol for cryopreservation of Medaka (*Oryzias latipes*) spermatozoa. Fish Biol. J. Medaka 9, 47–48.
Lahnsteiner, F., 2007. Characterization of seminal plasma proteins stabilizing the sperm viability in rainbow trout (*Oncorhynchus mykiss*). Anim. Reprod. Sci. 97, 151–164.
Lahnsteiner, F., Mansour, N., Berger, B., 2004. Seminal plasma proteins prolong the viability of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Theriogenology 62, 801–808.
Langheinrich, U., Hennen, E., Stott, G., Vacun, G., 2002. Zebrafish as a model organism for the identification and characterization of drugs and genes affecting p53 signaling. Curr. Biol. 12, 2023–2028.
Lawrence, C., 2007. The husbandry of zebrafish (*Danio rerio*): a review. Aquaculture 269, 1–20.
Mertens, J., 1973. Year-round controlled mass reproduction of the zebrafish *Brachydanio rerio* (Hamilton-Buchanan). Aquaculture 2, 245–249.
Morisawa, M., Suzuki, K., 1980. Osmolality and potassium ions: their roles in initiation of sperm motility in teleosts. Science 210, 1145e7.
Morris, J.P.I.V., Berghmans, S., Zahrieh, D., Neuberg, D.S., Kanki, J.P., Look, A.T., 2003. Zebrafish sperm cryopreservation with *N*, *N*-dimethylacetamide. Biotechiniques 35, 956–968.
Ravinder, K., Nasaruddin, K., Majumdar, K.C., Shivaji, S., 1997. Computerized analysis of motility, motility patterns and motility parameters of spermatozoa of carp following short-term storage of semen. J. Fish Biol. 50, 1309–1328.
Stoss, J., 1983. Fish gamete preservation and spermatozoan physiology. In: Hoar, W.S., Randall, D.J., Donaldson, E.M. (Eds.), Behavior and Fertility Control. Fish Physiology, vol. 9. Academic Press, San Diego, pp. 305–350. part B.
Takai, H., Morisawa, M., 1995. Change in intracellular K^+ concentration caused by external osmolality change regulates sperm motility of marine and freshwater teleosts. J. Cell Sci. 108, 1175–1181.
van Eeden, F.J.M., Granato, M., Odenthal, J., Haffer, P., 1999. Developmental mutant screens in the zebrafish. Methods Cell Biol. 60, 21–41.
Wayman, W.R., Tiersch, T.R., 2000. Research methods for cryopreservation of sperm. In: Tiersch, T.R., Mazik, P.M. (Eds.), Cryopreservation in Aquatic species. World Aquaculture Society, Baton Rouge, pp. 264–275.
Westerfield, M., 1995. The Zebrafish Book. University of Oregon Press, Eugene.
Yang, H., Carmichael, C., Varga, Z.M., Tiersch, T.R., 2007. Development of a simplified and standardized protocol with potential for high-throughput for sperm cryopreserva-tion in zebrafish *Danio rerio*. Theriogenology 68, 128–136.