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Improved Cryopreservation of Sperm of Paddlefish (*Polyodon spathula*)

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Abstract

Experiments were performed to improve protocols for sperm cryopreservation of paddlefish (*Polyodon spathula*), a species for which there has been limited study. The first experiment was conducted to investigate the effects of two extenders (modified Tsvetkova's extender: mT and modified Hanks' balanced salt solution: mHBSS) in combination with methanol (MeOH) and dimethyl sulfoxide in two concentrations (5 and 10%) on the postthaw motility and fertilization rates of cryopreserved sperm. The highest postthaw motility (85 ± 5%) was observed when sperm were frozen using mT extender with 10% MeOH as cryoprotectant. Extenders (*P* = 0.0018) and cryoprotectants (*P* = 0.0040) each had a significant effect on the postthaw motility of paddlefish sperm. The highest fertilization (80 ± 3%) was found when eggs were fertilized with sperm frozen with mT extender in combination with 10% MeOH. However, there was no significant difference among fertilization rates when MeOH was used as a cryoprotectant in either concentration or in combination with either mT or mHBSS extenders. In the second experiment, 4000 eggs were fertilized with the pooled contents of five straws of thawed sperm (total volume of 1.25 mL) using mT extender in combination with 5% MeOH, and hatch rates as high as 79 ± 5% were observed. A third experiment was also conducted to clarify the role of MeOH concentration; however, no significant difference was found among fertilization and hatch rates when either 5 or 10% MeOH was used as a cryoprotectant. These results suggest that MeOH is a safe and reliable cryoprotectant for freezing of paddlefish sperm and obtaining viable postthaw sperm for consistent fertilization and hatch rates. Further, this experimental protocol is relatively simple and applicable for commercial hatchery production of paddlefish.

Paddlefish are members of the family Polyodontidae and are closely related to sturgeon of the order Acipenseriformes. There are only two species in this family: the American paddlefish (*Polyodon spathula*) and the Chinese paddlefish (*Psephurus gladius*). The American paddlefish is indigenous to the Mississippi River basin and adjacent gulf drainages and has a variable history as a food fish from its capture fishery. Most recently, popularity has increased through value-added products (such as smoked meat) and the global demand for caviar, which has placed considerable pressure on natural populations. Culture techniques such as artificial propagation, intensive nursery production, pond and tank culture, and advances in monosex production have been developed and provide the methodology for development of a commercial paddlefish industry (Mims 2001; Mims and Shelton 2005).

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A necessary biotechnology for paddlefish aquaculture is the ability to cryopreserve milt from broodstock of diverse genetic populations and with unique gametes (such as sex-reversed females; Mims and Shelton 1999). The cryopreservation of milt from acipenseriform fish dates back to the late 1960s when scientists of the Soviet Union conducted experiments on sturgeons (Dettlaff et al. 1993; Mims et al. 2000). Several methods have been described since then for sturgeon and paddlefish milt (Drokin et al. 1991; Ciereszko et al. 1996; Mims et al. 2000) but they share a common problem. Thawed sperm is observed to have high motility (>50%) but the fertilization rates remain low (<20%), often with inconsistent results.

Although there has been some success for cryopreservation of sperm from two Eurasian sturgeons, sterlet (*Acipenser ruthenus*) and Siberian sturgeon (*Acipenser baeri*) (Tsvetkova et al. 1996; Jähnichen et al. 1999; Glogowski et al. 2002), there has been limited success achieved with cryopreservation of paddlefish sperm (Mims et al. 2000). Dimethyl sulfoxide (DMSO) was used as a cryoprotectant with paddlefish sperm in a 1:3 mixture of a freezing medium containing DMSO and sperm (medium : sperm; Brown and Mims 1999). The final concentration of the cryoprotectant was 0.6 M or about 4% of solution. Motility of thawed spermatozoa was about 50%. The hatching rate was 16 ± 2%, which was significantly lower than the control rate (fresh sperm) of 90 ± 3%. The goal of this study was to test the effects of methanol (MeOH) and DMSO as cryoprotectants, in combination with two extenders, on the motility of spermatozoa and fertilization and hatching rates using thawed paddlefish sperm.

Materials and Methods

Paddlefish broodstocks were captured in the Ohio River below McAlpine Dam, Louisville, Kentucky, USA (38°17′07″N, 85°46′51.9″W), in 2 consecutive yr, 2003 and 2004. Each year, three males and two females were selected and transported to the Aquaculture Research Center, Kentucky State University, Frankfort, Kentucky, USA. Each fish was held separately in a 3000-L circular tank with a water flow of 12 L/min and a controlled water temperature of 19 C. Males were given a single intraperitoneal injection of luteinizing-hormone-releasing hormone analogue (LHRHa) (des-Gly 10/(D-Ala6) ethylamide) at a rate of 50 μg/kg of body weight (BW) to increase the volume of sperm (Linhart et al. 2000). Within 24 h, sperm was collected using Tygon tubing (5 mm in diameter, 5 cm in length) attached to a 10-mL plastic syringe. The tubing was inserted into the urogenital pore and the syringe filled with sperm. The sperm was temporarily stored in 25-cm^2 cell culture flasks on wet ice until motility analysis was performed. Females were injected with LHRHa at a total dose of 100 μg/kg BW administered in a priming injection (10 μg/kg) and a resolving injection (90 μg/kg) 12 h apart. Ovulation typically occurred 12–16 h after the resolving injection. Eggs were removed using the MIST (minimally invasive surgical removal of ovulated eggs) method (Stech et al. 1999).

Two sets of experiments were conducted on paddlefish sperm in the year 2003. In the first set, motility after thawing and fertilization rate were tested. In this set, sperm of three males were used separately (not pooled). Sperm were diluted 1:1 in two extenders: modified Tsvetkova's extender (mT; 30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl, pH 8.0; osmolality: 82 mOsmol/kg; Glogowski et al. 2002) and modified Hanks' balanced salt solution (mHBSS; 8.0 g NaCl, 0.4 g KCl, 0.16 g $CaCl_2$ $2H_2O$, 0.20 g $MgSO_4$ $7H_2O$, 0.12 g Na_2HPO_4 $7H_2O$, 0.06 g KH_2PO_4, 0.35 g $NaHCO_3$, and 1.0 g glucose per 1 L of solution) with distilled water to an osmolality of 100 mOsmol/kg. Osmolality of all solutions used in the experiments was measured with a vapor pressure osmometer (Wescor 5500, Logan, UT, USA). MeOH or DMSO (5 or 10%) was used as a cryoprotectant (v/v, final concentration). The sperm samples were loaded into 0.5-mL French straws. They were distributed on a 3-cm Styrofoam frame and frozen in the vapor of liquid nitrogen in a Styrofoam box. After 3 min, the straws were plunged into liquid nitrogen. Thus, based on thermocouple readings, the average cooling rate for the cryoprotectant MeOH was 70 C/min, while for DMSO it was 66 C/min (Horváth et al.

2005). The straws were stored in plastic goblets attached to canes in shipping dewars charged with liquid nitrogen (−196 C) for 24 h before thawing and use for fertilization. There were three replicates per treatment (using sperm samples from individual males as replicates). Concentration of spermatozoa in each sperm sample was calculated using a Buerker-type counting chamber at 200× magnification and 100× dilution of sperm.

In the first set of experiments, eggs from a single female were used. Four grams of eggs (approximately 400 eggs) were distributed into dry glass bowls. The sperm samples were thawed in a 40-C water bath for 13 sec. Two microliters of sperm were mixed on a glass slide with an activating solution composed of 20 mM Tris–HCl (pH 8.2) and 20 mM NaCl (Cosson et al. 2000). Motility was estimated using dark-field microscope at 200× magnification. Egg batches were fertilized with one straw (500 μL) of thawed sperm. Fresh sperm from the same males were used as a control to monitor egg quality in a volume equal to the volume of frozen milt prior to dilution (250 μL). Gametes were activated by the addition of 5 mL of hatchery water. Approximately 2 min after activation, eggs were spread in a monolayer in the glass bowls and were incubated in a fiberglass tank with flow-through water (12 L/min). Fertilization percentage was recorded at the four- to eight-cell stages under a dissecting microscope at 5× magnification.

In the second set of experiments, fertilization and hatching tests were conducted with thawed sperm from three males with four replicates per male. The sperm were diluted 1:1 in mT extender (described above). Sperm samples were frozen in the presence of 5% MeOH as cryoprotectant (v/v, final concentration). Conditions for freezing and thawing were the same as described above. Frozen sperm were stored in a storage dewar in liquid nitrogen (−196 C) for 3 wk. Batches of 40 g of eggs (approximately 4000 eggs) were used for fertilization in this experiment. One batch was fertilized with the pooled contents of five 0.5-mL straws of thawed sperm. Adhesiveness of eggs was eliminated by gently stirring in a suspension of Fuller's earth for 30 min. Eggs were incubated in 7-L, McDonald-type hatching jars. Fertilization rates at the four- to eight-cell stages as well as hatching rate were recorded.

A third set of experiments was conducted in the year 2004 to clarify the influence of cryoprotectant concentration on fertilization and hatch rates. In this set, fertilization and hatching tests were carried out with frozen sperm from three males with three replicates per male. The sperm was diluted 1:1 in mT extender. The samples were frozen in the presence of 5 and 10% MeOH as cryoprotectant (v/v, final concentration). Conditions of freezing and thawing were the same as described in the first and second sets. Samples were stored in a canister storage dewar for 24 h. Four grams of eggs were fertilized with one straw of thawed sperm as described in the first set of experiments, and fertilization rate was recorded at four- to eight-cell stages under a dissecting microscope. Simultaneously, five straws of thawed sperm were used to fertilize 40 g of eggs. The conditions of fertilization, adhesiveness removal, and incubation were the same as in the second set. The hatching rate of larvae was recorded.

Postthaw motility and fertilization results from the first set of experiments were subjected to two-way ANOVA with Bonferroni's posttest to investigate the main effects of extenders, cryoprotectants, and concentrations. Fertilization and hatching data of sperm samples of each male from the second set of experiments were subjected to a two-sample *t* test. In the third set, fertilization and hatch rates were subjected to one-way ANOVA with Bonferroni's posttest to investigate the effect of cryoprotectant concentration. Pearson's correlation was used to investigate correlations between motility and fertilization rates in the first set of experiments and between fertilization and hatch rates in the second set. All statistical analyses were conducted using GraphPad Prism 4.0 statistical software (GraphPad Software, Inc., San Diego, CA, USA).

Results

The highest postthaw motility (85 ± 5%) in the first set of experiments was observed when milt was frozen with mT extender in the

presence of 10% MeOH as cryoprotectant (Table 1). Overall, extenders ($P = 0.0018$) and cryoprotectants ($P = 0.004$) each had a significant effect on the postthaw motility of paddlefish sperm. The highest fertilization rate at the four- to eight-cell stages (80 ± 3%) was found for eggs fertilized with sperm frozen with mT extender in combination with 10% MeOH as cryoprotectant (Table 1). However, there was no significant difference among fertilization rates when MeOH was used as cryoprotectant in any concentration or in combination with either mT or mHBSS extenders. Overall, cryoprotectant had a significant effect ($P < 0.0001$) on fertilization rates, while the type of extender or cryoprotectant concentration did not affect fertilization success. A significant ($P = 0.002$) correlation ($r^2 = 0.6334$) was found between motility of thawed sperm and fertilization rates when MeOH was used as a cryoprotectant, however, no significant correlation was observed ($P = 0.7833$; $r^2 = 0.0079$) between motility and fertilization data with DMSO as cryoprotectant. The concentrations of spermatozoa in sperm samples were $8.5 \pm 0.7 \times 10^8$ cells/mL (male 1), $6.9 \pm 0.9 \times 10^8$ cells/mL (male 2), and $7.5 \pm 0.4 \times 10^9$ cells/mL (male 3). Thus, the sperm to egg (spermatozoa : egg) ratios in this experiment were 1.06×10^6:1 (male 1), 8.6×10^5:1 (male 2), and 9.4×10^5:1 (male 3).

In the second set of experiments, eggs fertilized with the thawed sperm of male 3 clumped, likely because of human error (incubation in hatching jars before complete elimination of adhesiveness of eggs), and thus one replicate of this group was discarded. The highest fertilization (84 ± 9%) and hatching (79 ± 5%) rates were observed with the sperm of male 1 (Table 2). There was no significant difference between fertilization and hatching rates of eggs fertilized with thawed sperm of males 1 and 3, but there was a significant difference ($P = 0.0049$) between these percentages for male 2. A significant ($P = 0.0018$) correlation ($r^2 = 0.7252$) was found between fertilization and hatch rates. Relative hatch rates were 94% (male 1), 76% (male 2), and 72% (male 3) of the corresponding fertilization rates.

In the third set of experiments, the highest fertilization (69 ± 14%) and hatch (33 ± 13%) rates were observed when 5% MeOH was used as a cryoprotectant (Table 3). There were no significant differences among fertilization and hatch rates when either 5 or 10% MeOH was used. However, there was a significant difference ($P < 0.01$) that was observed between the hatch rates of eggs fertilized with sperm frozen in the presence of 10% MeOH and the control.

Discussion

Previous reports on the cryopreservation of paddlefish milt suggest that an increase in postthaw motility of cryopreserved sperm would result in better fertilization and hatching rates (Brown and Mims 1999; Mims et al. 2000). However, in our experiments, we observed that postthaw motility was not a direct predictor of fertilization success.

TABLE 1. *Postthaw motility and fertilization rates (mean ± SD) of cryopreserved sperm of paddlefish (n = 4 males).*

Parameter	Extender	DMSO 5%	DMSO 10%	MeOH 5%	MeOH 10%
Motility (%)	mHBSS	23 ± 12[a1]	17 ± 6[a1]	17 ± 10[a1]	30 ± 30[a1]
	mT	30 ± 10[a1]	20 ± 10[a1]	50 ± 26[a1]	85 ± 5[b2]
	mHBSS	32 ± 18[a1]	18 ± 6[a1]	57 ± 2[a2]	71 ± 2[a2]
Fertilization (%)	mT	37 ± 18[a1]	17 ± 10[a1]	79 ± 9[a2]	80 ± 3[a2]

DMSO = dimethyl sulfoxide; MeOH = methanol; mHBSS = modified Hanks' balanced salt solution; mT = modified Tsvetkova's extender; 5 and 10% = cryoprotectant concentrations.

Values sharing a superscript letter within a column (for motility and fertilization individually) were not significantly different ($P > 0.05$). Values sharing a superscript number within a row were not significantly different ($P > 0.05$).

Motility of fresh sperm ($n = 3$ males) was 90 ± 5%. Control fertilization was 85 ± 4% ($n = 3$ males).

TABLE 2. *Fertilization and hatching rates (mean ± SD) of paddlefish eggs fertilized with thawed sperm of three males (*n = 4 *replicates per male).*

Parameter	Male 1	Male 2	Male 3
Fertilization (%)	84 ± 9[a]	84 ± 6[a]	52 ± 30[a]
Hatching (%)	79 ± 5[a]	64 ± 7[b]	49 ± 27[a]

Values sharing a superscript letter within a column were not significantly different ($P > 0.05$). Control ($n = 3$ males) fertilization rate was 94 ± 5%, and hatch rate was 88 ± 6%.

In the first set of experiments, the use of MeOH as cryoprotectant yielded higher motility and fertilization rates than did DMSO regardless of concentration or the type of extender. A similar effect of MeOH was observed with Eurasian sturgeon species such as sterlet (Horváth and Urbányis 2000) and Siberian sturgeon (Glogowski et al. 2002). MeOH has been used for the cryopreservation of sperm from cyprinid species such as zebrafish (*Danio rerio*; Harvey et al. 1982) and common carp (*Cyprinus carpio*; Horváth et al. 2003) and other fishes such as salmonids (Lahnsteiner et al. 1997), catfish (Steyn and Van Vuren 1987; Steyn 1993; Tiersch et al. 1994), and tilapia (Harvey 1983). MeOH is often nontoxic when used as a cryoprotectant in a number of cell types and was found to be superior to DMSO or glycerol for mammalian tissue culture (Harvey et al. 1982). MeOH is known for its rapid penetration of cells (Ashwood-Smith 1980), but the details of its action as a cryoprotectant are not clear. The finding that sperm samples cryopreserved with DMSO had postthaw motility similar to those frozen with MeOH indicates that the cells survived the freezing and thawing processes and retained the ability to move, but they lost the ability to fertilize eggs. As it was confirmed by the results of the third set of experiments, MeOH concentration does not have a significant effect on fertilization or hatch rates.

It has been observed that the addition of DMSO to an extender increases its osmolality. When 5, 10, and 15% DMSO were added to Mounib's solution, the osmolality increased from the original 289 mOsmol/kg to 1022, 1768, and 2456 mOsmol/kg, respectively (Ogier de Baulny et al. 1997). This was also observed when DMSO was added to HBSS, while the addition of MeOH resulted in a slight decrease of osmolality (Tiersch et al. 1994). According to our earlier observations, addition of DMSO to mT extender (73 ± 2 mOsmol/kg) significantly increased osmolality to 719 ± 9 mOsmol/kg (5% DMSO), 1421 ± 26 mOsmol/kg (10% DMSO), and 1985 ± 12 mOsmol/kg (15% DMSO), whereas addition of MeOH caused almost no alteration in osmolality: 75 ± 1 mOsmol/kg (5% MeOH), 78 ± 15 mOsmol/kg (10% MeOH), and 84 ± 14 mOsmol/kg (15% MeOH) (Horváth et al. 2005). The physiological osmolality of paddlefish seminal plasma ranged from 33 to 63 mOsmol/kg (Linhart et al. 2003). Our observations on the sperm of shortnose sturgeon (*Acipenser brevirostrum*), a close relative of paddlefish, suggest that hyperosmotic conditions of the combined extender and cryoprotectant can have a negative effect on fertilization success of sperm without decreasing motility of sperm (Horváth et al. 2005) although the physiological details of this effect are not known. This observation is also supported by the strong correlation between postthaw motility and fertilization rates when MeOH was used as cryoprotectant.

In the second set of experiments, we found that five straws of sperm (total of 1.25 mL of sperm) were suitable for fertilization of 40 g of paddlefish eggs. We also found that the fertilization rate at the four- to eight-cell stages was suitable for the prediction of hatching rate in most cases as there was a strong correlation

TABLE 3. *Fertilization and hatching rates (mean ± SD) of paddlefish eggs fertilized with sperm frozen in presence of 5 or 10% MeOH as cryoprotectant (*n = *3 males with three replicates per male, except for the hatch rate with 5% MeOH where* n = *1 with three replicates per male).*

Parameter	5% MeOH	10% MeOH	Control
Fertilization (%)	69 ± 14[a]	52 ± 21[a]	68 ± 10[a]
Hatching (%)	33 ± 13[ab]	20 ± 16[a]	57 ± 7[b]

MeOH = methanol.

In fertilization tests, one straw (500 μL) of thawed sperm was used to fertilize 4 g of eggs, while in hatching tests five straws were used to fertilize 40 g of eggs. Values sharing a superscript letter within a row were not significantly different ($P > 0.05$).

between these data. MeOH, a close isosmotic cryoprotectant relative to paddlefish milt, is safe for freezing its sperm and for obtaining viable postthaw sperm for consistent fertilization and hatch rates. Further, these results suggest the potential application of cryopreserved paddlefish sperm for commercial hatchery practices.

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