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Cryopreservation of paddlefish sperm in 5-mL straws

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Summary

Experiments were conducted to test the feasibility of using 5-mL straws for the cryopreservation of paddlefish (*Polyodon spathula*) sperm. In experiment 1, the effects of 5% or 10% methanol as a cryoprotectant in combination with cooling times of 5 or 7 min on paddlefish sperm stored in 5-mL straws were evaluated for fertilization and hatching rates. Highest fertilization rate of 48 ± 5% (mean ± SE) and hatching rate of 47 ± 10% were observed using sperm cryopreserved with 5% methanol and a 5-min cooling time in liquid nitrogen vapors. However, fertilization and hatching rates were significantly lower with cryopreserved sperm than with fresh sperm (fertilization 77 ± 6%; hatching 66 ± 13%). In experiment 2, the effects of sperm : egg ratios on fertilization rates were investigated. When fresh sperm was used, fertilization rate was quadratically related to sperm : egg ratio ($y = -13.19x^2 + 55.90x + 38.44$, $r^2 = 0.823$) and the optimum range of sperm : egg ratios was between 1.379×10^6 and 2.758×10^6. When sperm were cooled for 5 min with 5% methanol, fertilization rate was linearly related to sperm : egg ratio ($y = 22.51x + 23.26$, $r^2 = 0.75$) but optimum sperm : egg ratio was not reached. In experiment 3, the hatching rates were not significantly different between the three-straw treatment and the control. With cryopreserved sperm, the relationship between the sperm and egg ratios and the hatching rates were best described by a quadratic equation ($y = -29.65x^2 + 119.2x - 51.04$, $r^2 = 0.837$). Therefore, when cryopreserved sperm is used, sperm : egg ratio should be increased significantly to optimize fertilization and hatching rates. This can be achieved either by increasing the total volume of cryopreserved sperm by at least 30% or by further research to improve the procedure to increase the viability of post-thawed sperm per straw.

Introduction

Cryopreservation methods for fish sperm have been developing over the last 50 years, but are not widely used in commercial aquaculture. Lack of application in the industry is due to the fact that most reported methods involve the use of small-volume storage containers such as French straws (i.e. 0.25–0.50 mL). Such sperm cryopreservation methods were developed for the terrestrial livestock industry, whereby one French straw (0.25 or 0.5 mL) would fertilize a single oocyte. However, with most fish species, large volumes of sperm and eggs are acquired and are used during propagation. Therefore, it is essential to adapt these methods for commercial aquaculture application.

Scientists developing protocols for fish sperm face a double challenge: their methods have to be suitable for the fertilization of large volumes of eggs, yet they still have to use the storage facilities available for livestock improvement centers. An alternative to the smaller French straws would be 5-mL cryostraws, however, fertilization and hatching rates between these two storage containers have been variable. Reduced fertilizing rates of sperm cryopreserved in 5-mL straws or macrotubes were observed in the rainbow trout (*Oncorhynchus mykiss*) (Wheeler and Thorgaard, 1991; Lahnsteiner et al., 1997). Cabrita et al. (2001) demonstrated a reduced fertilization rate of 73% using post-thawed rainbow trout sperm cryopreserved in 5-mL straws compared to 90% fertilization rate from post-thawed sperm stored in 0.5-mL French straws. They justified that the loss in fertility was compensated by the benefits of larger volumes of sperm.

Brown and Mims (1999) were the first to report the cryopreservation of paddlefish sperm using DMSO and stored in 5-mL straws that resulted in adequate post-thaw motility (25–50%) but low hatching rates (16 ± 2%). Additional studies on paddlefish (Horváth et al., 2006; Linhart et al., 2006) and closely related sturgeons (Glogowski et al., 2002; Horváth et al., 2005) have demonstrated significant improvements in fertilization and hatching rates when sperm were cryopreserved with methanol combined with an extender but stored in French straws. Although Linhart et al. (2006) reported the use of 2-mL cryovials as an alternative to French straws, cryopreservation of paddlefish sperm with methanol has not been attempted using containers of larger volumes.

The objectives of the present study were to determine the effects of methanol concentration and cooling time on the fertilization and hatching rates using cryopreserved paddlefish sperm stored in 5-mL straws; and to determine the relationship between post-thawed sperm to egg ratio and fertilization and hatching rates.

Materials and methods

Paddlefish broodstock were captured in the Ohio River below McAlpine Dam, Louisville, KY USA (longitude: 85°46′51.9″W, latitude: 38°17′07″N) and transported to the Aquaculture Research Center, Kentucky State University, Frankfort, USA. Each fish was held separately in a 3000-L circular tank with a water flow of 12 L min^{-1} and a controlled water temperature of 19°C. Males were given a single intraperitoneal injection of luteinizing hormone-releasing hormone analogue (LHRH*a*) [des-Gly 10, (D-Ala6) ethylamide] at a rate of 50 μg kg^{-1} of body weight (BW). Within 24 h, sperm were 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 were temporarily stored in 25-cm^2 cell

culture flasks on wet ice. Females were injected with LHRH*a* at a total dose of 100 μg kg^{-1} BW administered in a priming injection (10 μg kg^{-1}) and a resolving injection (90 μg kg^{-1}) 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).

In experiment 1, sperm samples from each of three males were collected and used. Sperm samples were diluted in modified Tsvetkova's extender (Horváth et al., 2005) composed of 23.4 mM sucrose, 0.25 mM KCl, 30 mM Tris (pH 8.0 set with concentrated HCl). Methanol was added to the extender in order to have a final (post-dilution) concentration of the cryoprotectant of either 5 or 10%. Dilution ratio of sperm : extender was 1 : 1. Diluted sperm samples were loaded into 5-mL straws (Minitube, Germany). Prior to loading one end of each straw was heat-sealed using a hot hemostat. Straws were laid onto a polystyrene frame which was placed onto the surface of liquid nitrogen in a polystyrene box. The straws were held at 3 cm above the surface of the liquid nitrogen for a cooling period of either 5 or 7 min, then plunged into the liquid. Straws were stored in a Dewar container for 24 h before being used for fertilization. Straws were thawed in a 40°C water bath for 40 s prior to fertilization.

The cooling rate in the 5-mL straws was measured using a thermocouple inserted into the open end of the straw as the straw was cooling. The thermocouple was connected to a computer and temperature readings were recorded per second.

Forty grams of eggs from a single female (105 ± 3 eggs per gram) were placed into each of 12 dry plastic bowls. Eggs in three of the bowls received post-thawed sperm from two straws (i.e. 2.5 mL of sperm / straw) of each of the four treatments. The treatments were 5 or 10% methanol with 5 or 7 min cooling time. For the control, eggs received 5 mL of fresh sperm. Gametes were activated with the addition of 1.0 L of hatchery water. After approximately 2 min a suspension of Fuller's earth was added to the eggs and were stirred gently for 30 min. Eggs were incubated in 7-L McDonald-type hatching jars. Fertilization percentages at 4–8 cell stage as well as hatching rates were recorded. Fertilization percentage was calculated for three randomly collected samples of 100–150 eggs per sample. Hatching rate was determined by dividing the number of hatched larvae (determined following hatching) with the number of fertilized eggs multiplied by 100.

In experiment 2, the concentration of sperm from a single male was measured by counting sperm cells using an improved Neubauer hemocytometer (200× magnification and 100× dilution with modified Tsvetkova's extender). A portion of the sperm were cryopreserved following the procedures of experiment 1, using 5% methanol as a cryoprotectant and 5-min cooling time. Four grams of eggs (92 ± 4 eggs per g) from a single female were placed into one of 30 dry glass bowls. Eggs in three of the bowls were fertilized with 0.063, 0.125, 0.250, 0.500 or 1.00 mL of fresh sperm or cryopreserved sperm from the same male. The sperm concentration was 1.015×10^9 spermatozoa per mL. The number of fertilized eggs was used to calculate fertilization rates at 4–8 cell stage and neurulation (developmental stage 22 according to Dettlaff et al., 1993).

In experiment 3, forty grams of eggs (103 ± 3 g^{-1}) were fertilized with 2.5, 5.0 and 7.5 mL of sperm from one, two or three straws of cryopreserved sperm, with corresponding sperm : egg ratios at 6.895×10^5, 1.379×10^6 and 2.758×10^6, respectively. The control group was fertilized with 5 mL of fresh sperm at the sperm : egg ratio of 1.379×10^6. Fertilization trials were conducted in four replicates. Procedures for fertilization and incubation were the same as described in experiment 1. Numbers of hatched larvae were used to calculate hatching rates.

Data were analyzed with Statistical Analysis System (SAS, Inc. Gary, NC). In the first experiment, fertilization and hatching rates were subjected to a one-way analysis of variance (ANOVA). Orthogonal contrasts were used to examine the difference between methanol concentration, between cooling times and the interaction between methanol concentration and cooling time.

In experiment 2, regression analysis was used to examine the relationship between the sperm and egg ratios and the fertilization rates. In experiment 3, one way ANOVA was also used to analyze the data with LSD for the comparison of the means. Orthogonal contrasts were used to compare the control with the other three treatment groups, the linear and quadratic relation between sperm and egg ratios and fertilization and hatching rates.

Results

In experiment 1, there was no significant interaction between methanol concentrations and cooling times, so only the main effects were reported. Fresh sperm gave the best fertilization (77.3 ± 6.0%) and hatching rates (66.2 ± 12.8%; Table 1). The lower methanol concentration (5%) resulted in higher fertilization (47.5 ± 5.2%) and hatching rates (47.1 ± 10.5%) than the higher methanol concentration (10%) ($P < 0.004$). The shorter cooling time (5 min) resulted in higher fertilization and hatching rates than the longer cooling time (7 min) ($P < 0.03$). Highest fertilization and hatching rates were observed with 5% methanol and 5 min cooling time (Table 1.), but were significantly lower ($P < 0.02$) than rates using fresh sperm. The cooling rate in this experiment (Fig. 1.) was 20.37°C min^{-1} with three distinct sections: a linear cooling at a rate of 32.93°C min^{-1}, a slow cooling section at a rate of 5.59°C min^{-1} and a final section starting at around 150 s at a rate of 24.02°C min^{-1}.

In experiment 2, with fresh sperm, fertilization rates at 4-8 cell stage and the neurulation stage increased significantly with increasing sperm to egg ratio, and the fertilization rates tended to plateau when the ratio was 1.379×10^6 or higher. The relationship between the fertilization rate and the sperm : egg ratio at the 4–8 cell stage was best described by a quadratic equation ($y = -13.19x^2 + 55.90x + 38.44$, $r^2 = 0.82$) (Fig. 2). With cryopreserved sperm, the fertilization rate was linearly related to the sperm egg ratio ($y = 22.51x + 23.26$, $r^2 = 0.75$). The relationship between the neurulation rate and the sperm : egg ratio was best described by a quadratic

Table 1
Fertilization and hatching rates of paddlefish eggs with sperm cryopreserved with methanol concentrations of either 5 or 10% and cooling times of either 5 or 7 min stored in 5-mL straws. Data are presented as mean ± SE (n = 3 males)

Methanol concentration (%)	Fertilization (%)		Hatching (%)	
	5 min	7 min	5 min	7 min
5	48 ± 5	34 ± 6	47 ± 11	29 ± 11
10	30 ± 4.9	20 ± 5	28 ± 11	25 ± 11

Control (fresh sperm) fertilization and hatching rates were 77 ± 6 and 66 ± 13%.

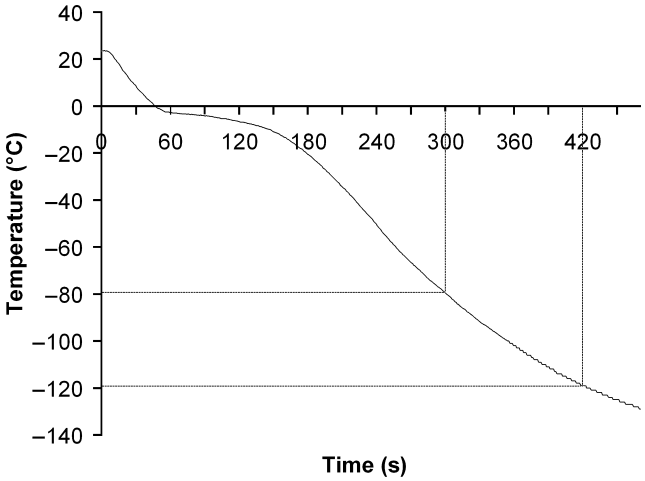

Fig. 1. The temperature profile employed during the freezing process when gradually transferring sperm samples to liquid nitrogen. Hatched lines represent the time and temperature of plunging the samples into liquid nitrogen

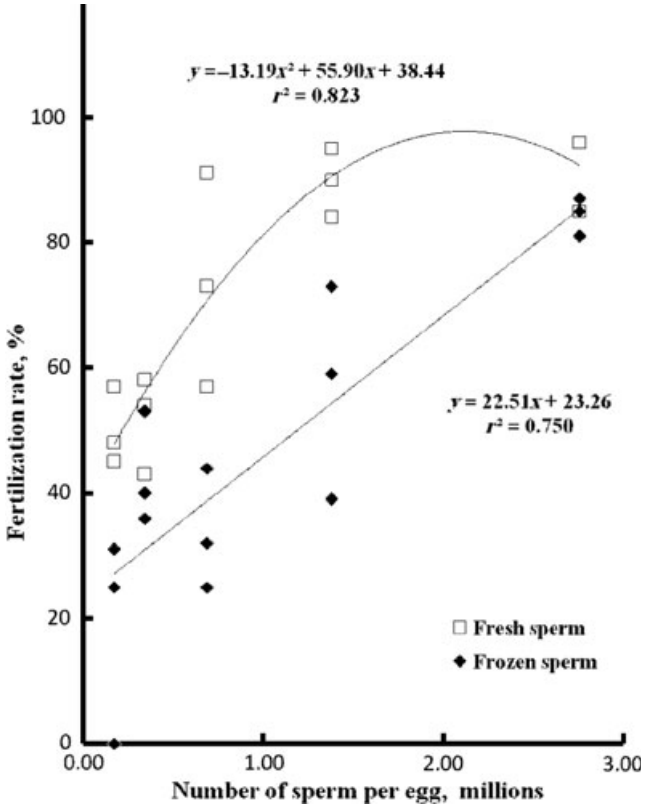

Fig. 2. Fertilization rates recorded using fresh and cryopreserved paddlefish sperm stored in 5-mL straws. Four grams of eggs (92 ± 4 eggs per g) used for fertilization (n = 3 replicates)

equation ($y = -13.63x^2 + 60.94x + 22.41$, $r^2 = 0.63$) (Fig. 3). With cryopreserved sperm, the neurulation rate was linearly related to the sperm egg ratio ($y = 21.06x + 18.15$, $r^2 = 0.513$). At the sperm : egg ratio of 2.758×10^6, fertilization rate achieved with cryopreserved sperm was comparable to that achieved with fresh sperm at sperm : egg ratio of 1.379×10^6.

In experiment 3, the hatching rates were not significantly different between the three-straw treatment and the control.

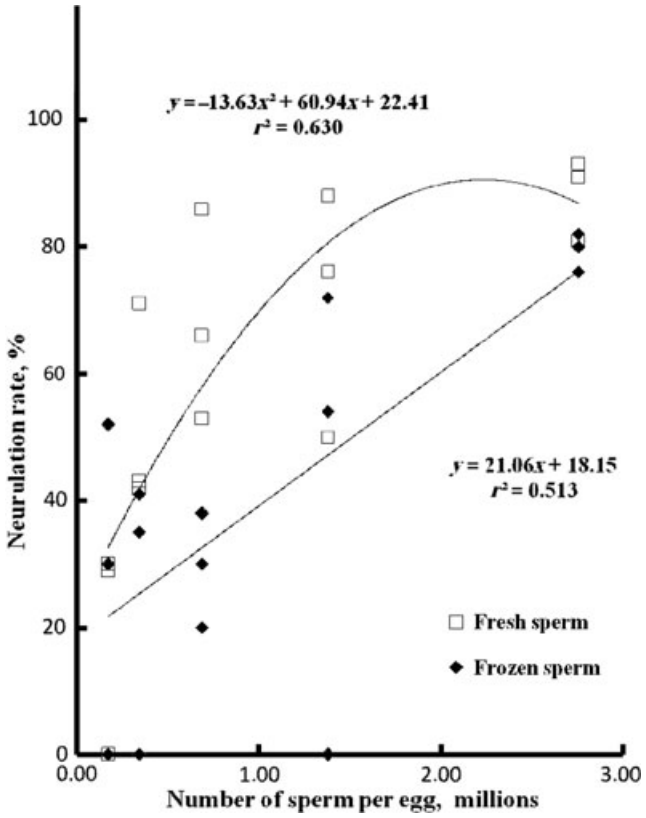

Fig. 3. Neurulation rates recorded using fresh and cryopreserved paddlefish sperm stored in 5-mL straws. Four grams of eggs (92 ± 4 eggs per g) used for fertilization (n = 3 replicates)

With cryopreserved sperm, the relationship between the sperm and egg ratios and the hatching rates were best described by a quadratic equation ($y = -29.65x^2 + 119.2x - 51.04$, $r^2 = 0.837$) (Fig. 4). The highest hatching rate of (69 ± 6%) was observed when three straws were used for fertilization, while the hatching rate for the fresh sperm was 77 ± 6%.

Discussion

Results from experiment 1 corresponded to those of other studies on the use of 5-mL straws. Fertilization and hatching rates with fresh sperm were higher than those observed with the use of cryopreserved sperm. Similar results were observed in the rainbow trout by Lahnsteiner et al. (1997) who found that the use of 5-mL straws yielded only 40% fertilization with 94% fertilization in the control and 90% fertilization with 0.5-mL straws. Cabrita et al. (2001) found that cell viability and fertility were slightly lower with sperm frozen in 5-mL straws. Reduced hatch percentages using 5-mL straws were also observed in the common carp (*Cyprinus carpio*) (Horváth et al., 2007). The overall fertilization rates observed in this study were similar to what we found with sperm stored in French straws (Horváth et al., 2006). Both methanol concentration and the cooling time significantly affected the fertilization and hatching rates. Even though further research is needed to optimize methanol concentration and the cooling time, 5% methanol and 5 min cooling time resulted in the best fertilization rate (48 ± 5%) and hatching rate (47 ± 11%). It is apparent that the temperature of sperm before being plunged into liquid nitrogen could be a critical factor. With 5-min cooling time, temperature of the sperm was about

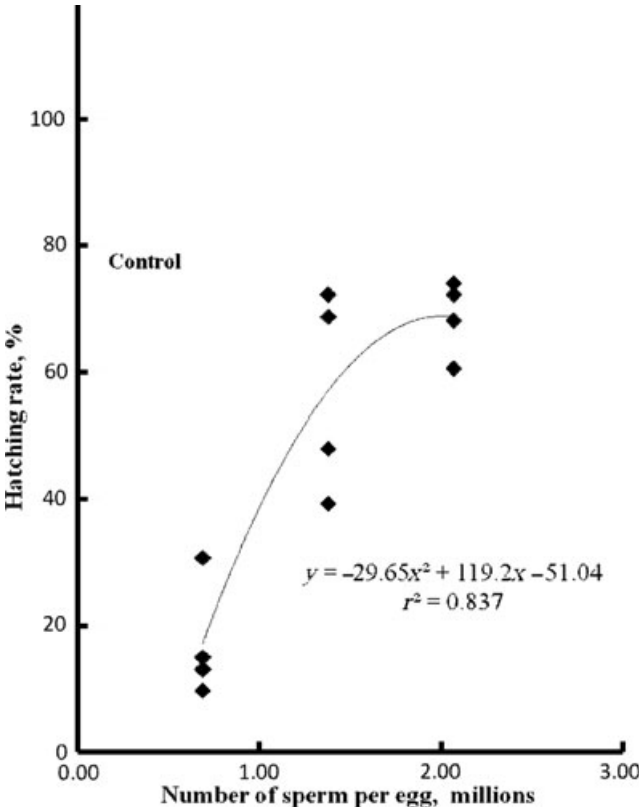

Fig. 4. Hatching rates of paddlefish eggs fertilized with cryopreserved sperm. Control sperm : egg ratio was 1.39×10^5 spermatozoa to one egg and hatching rate was 77 ± 6%. Forty grams of eggs (103 ± 3 eggs per g) used for fertilization (n = 4 replicates)

−80°C, whereas, with 7-min cooling time, this temperature was about −120°C.

Results from experiment 2 indicated that sperm : egg ratio was a critical factor determining fertilization rates. Optimal sperm : egg ratio appeared to be between 1.379×10^6 and 2.758×10^6. Cryopreserved sperm retained close to 70% of their fertilization capacity, even though some sperm cells were likely destroyed or damaged by the freezing process. It is evident that increasing the volume of cryopreserved sperm could compensate for the lower fertilization capacity. This was supported by further evidence from experiment 3 where hatching rate achieved with cryopreserved sperm at sperm : egg ratio of 2.07×10^6 was comparable to that achieved with fresh sperm at sperm : egg ratio of 1.379×10^6. This suggest that increasing the volume of cryoperserved sperm by 30% was adequate to achieve fertilization rate similar to what could be accomplished with fresh sperm.

Improvement in the cryoperservation procedure (such as changes in the cooling time) for paddlefish sperm could provide more viable spermatozoa capable of higher fertilization and hatching rates. The cooling rate measured in the present study (20.37°C min^{-1}) was considerably slower than that (70°C min^{-1}) reported for the same species using 0.5-mL straws (Horváth et al., 2006). Similar differences (20.8°C min^{-1}using 5-mL straws vs 63°C min^{-1} with 0.5-mL straws) were observed by Cabrita et al. (2001) who attributed the reduced cryopreservation success with larger straws to the prolonged exposure of cells to solvent effects before freezing. Problems associated with cryopreservation typically emphasize the hazards of fast cooling and subsequent formation of large ice crystals that damage the internal structure of cells. However, in case of fish spermatozoa slow cooling can be equally problematic as it results in a lethal dehydration of cells (Denniston et al., 2000).

From a practical standpoint, the addition of 30% more post-thawed sperm than fresh sperm to fertilize the eggs could provide similar hatching rates. This is possible because male paddlefish are able to produce large volumes of milt (up to 500 mL) over a 4-day period with sperm concentrations ranging from 0.5 to1.5×10^9 spermatozoa per mL (Linhart et al., 2000). This quantity of milt would permit about 250 5-mL straws to be stored. An average female (i.e.15–20 kg) ovulates about 1.5–2.0 kg of eggs requiring about 225–300 mL of fresh sperm (Mims and Shelton, 2005). If an additional 30% more cryopreserved sperm would be needed to have similar hatching rate as fresh sperm than 300–400 mL would be needed or 120–160 5-mL straws, respectively. Therefore it would be commercially practical to add additional cryopreserved sperm (post-thawed) to increase fertilization capacities and improve hatching rates.

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