Cryopreservation of Sturgeon and Paddlefish Sperm

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Introduction

Sturgeons and paddlefish belong to the infraclass Chondrostei, one of the most primitive groups of Osteichthyes. There are only about 25 sturgeon species and two paddlefish species in the order of Acipenseriformes that exist in the world (Rochard et al. 1991, Birstein 1993, Birstein and Bemis 1997). For centuries, these fishes have been highly valued for their role as commercial caviar and for their mild tasting, boneless meat. Today, some of these fishes remain a valuable natural resource; unfortunately, wild populations have declined due to pollution, habitat destruction and in some cases overexploitation for the international caviar trade. Development of induced spawning and husbandry techniques have permitted restoration programs for sturgeons and paddlefish as well as the establishment of farming some of these fishes for food (Mims et al. 2005). Therefore, sperm cryopreservation protocols need to be further developed and optimized so they can provide viable sperm for restoration, conservation of threatened and endangered chondrostei, hybridization, sex manipulation (i.e. gynogenesis and androgenesis) and development of genetically superior broodstock. This chapter will provide information on the chondrostean spermatozoa and a review of past and current cryopreservation protocols.

Morphology of Chondrostean Sperm and Milt Characteristics

Chondrostei spermatozoa are similar to those of other primitive fishes (Afzelius 1978). In general, the spermatozoon has a long cylindrical head capped with a functioning acrosome, a short-mid-piece with several mitochondria and a flagellum with fin-like extensions on both sides of flagellum (Figure 1) (Ginzburg 1972, Brown and Mims 1995, Horvath et al. 2009). The shape of the head is conducive to penetration through one of several narrow micropyle canals in the dense, thick egg membrane (Ginzburg 1972, Linhart and Kudo 1997). The head contains the nucleus and an apical acrosmome that is shaped like a cap with a rounded top. The mid-piece contains the mitochondria and is connected to a sheath surrounding the proximal portion of the flagellum with opposite lateral fins. The presence of a functional acrosome is an important consideration in the freezing and thawing of chondrostean sperm, because a damaged acrosome (i.e. premature acrosomal reaction) causes the sperm not to be viable (Dan 1956, Cieresko et al. 1996a, 1996b, 2000, Brown and Mims 1999).

Chondrostrean males can release large volumes of milt with average concentration of sperm up to 1-1.5 billion spermatozoa per ml. Milt is considered dilute compared to milt of teleost (Linhart et al. 2000, Mims et al. 2000, Piros et al 2002).

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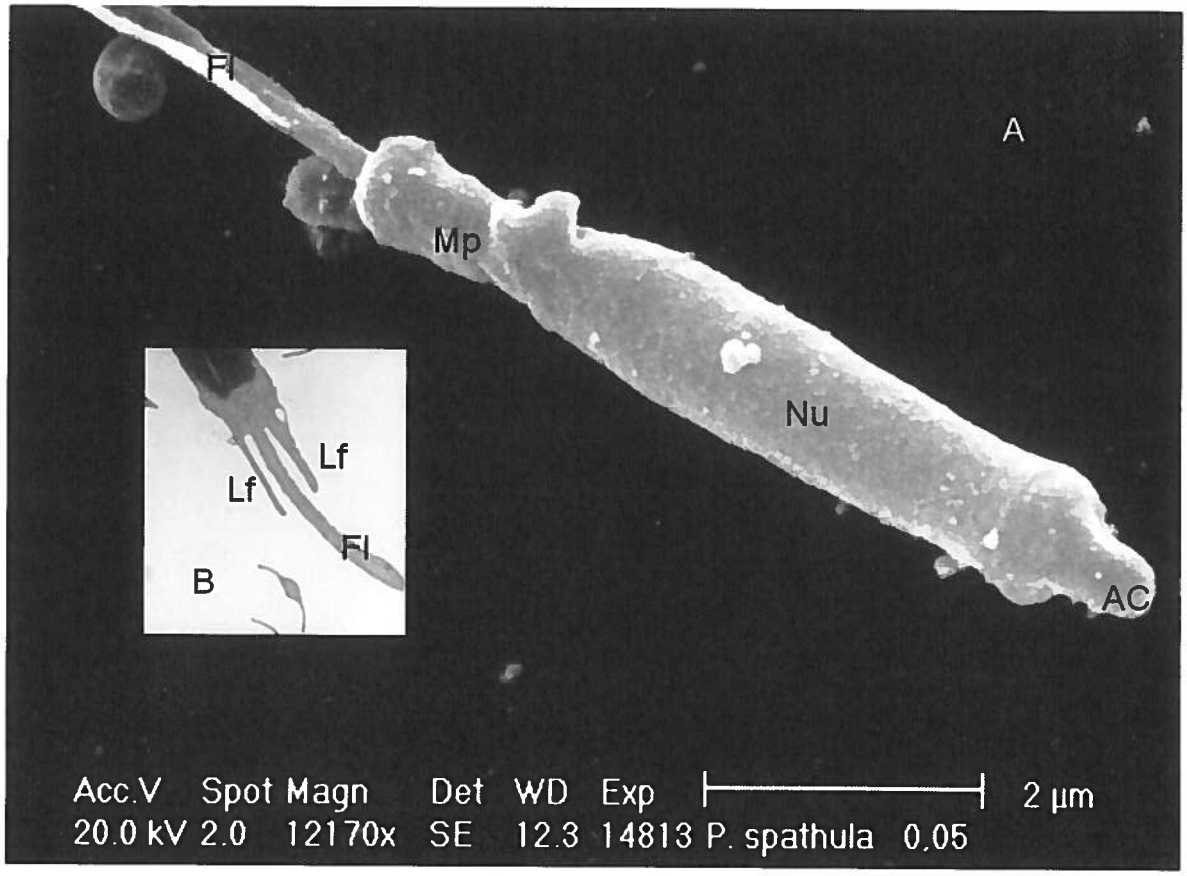

Figure 1. (A) Scan electron micrograph of a paddlefish *Polyodon spathula* spermatozoon (bar=2µm) acrosome (Ac), nucleus (Nu), midpiece (Mp) and flagellum (Fl), (B) Micrograph illustrates the two lateral fins on opposite sides of the flagellum.

Cryopreservation Protocols for Sturgeon Sperm

This section is an updated historical review of the literature as it pertained to research on cryopreservation of sturgeon sperm in the 20th and 21st centuries. During the 20th century, most of the initial research on sperm cryopreservation was done entirely on European sturgeon species, and primarily, if not exclusively, in the former Soviet Union. Published information on sturgeon sperm cryopreservation techniques during this time period was limited but has been reported in reviews by Kopeika and Novikov (1983), Dettlaff et al. (1993) Mims et al. (2000) and Billard et al. (2004).

Burtsev and Serebryakova (1969) first attempted sperm cryoperservation of three sturgeon species: beluga *Huso huso,* kaluga *Huso dauricus* and sterlet *Acipenser ruthenus.* Milt was collected and cryopreserved in 5% glycerol in a medium containing 7% saccharose or lactose solution with chicken egg yolk. Also, milt of bester (beluga x sterlet) was cryopreserved using 14% glycerol in a 0.4% NaCl solution. The cryoprotectant medium and milt mixture was frozen in granulated dry ice and stored in liquid nitrogen (LN_2; quick-freeze method). After 15 to 34 d of storage, the samples were thawed in a saline solution and were activated by Ringer's

solution, 0.4% NaCl solution or seawater. The motilities of post-thawed beluga sperm were 50 to 80%, kaluga and sterlet sperm were 10%, and bester (beluga x sterlet) sperm were 40%. However, fertilization rates of samples used to fertilize sturgeon eggs of the same species did not exceed 1%.

Kasimov et al. (1974) froze and thawed sperm of stellate (sevruga) *Acipenser stellatus.* They observed 40% motility of post-thawed sperm and obtained 35% fertilization. Their cryoprotectant medium contained 10% glycerol in a medium of 0.003% chloral hydrate, 0.03% urea and 10% egg yolk. The milt was diluted 1:1 with the cryoprotectant medium, poured into 5 mL centrifuge tubes with caps, frozen in LN_2 vapor to -55 °C for 2 hr and thawed in a water bath at 40 °C.

Pushkar et al. (1979) investigated the influence of dimethyl sulfoxide (DMSO), ethylene glycol and glycerol as cryoprotectants and the speed of freezing on sperm cryopreservation for stellate and Russian sturgeon *Acipenser guldenstadti.* DMSO was found to be the best cryoprotectant for sturgeon sperm and when used as a diluent with tris-buffer and egg yolk, 50 to 60% of the post-thawed sperm maintained forward motility. Post-thawed sperm of stellate sturgeon were frozen at -196 °C and stored for 7 to 23 d, fertilized 64% of eggs, as compared with 77% of eggs fertilized with fresh sperm (control).

In later studies, Pushkar et al. (1980a) reported testing of cryoprotectant medium containing 25% DMSO in 0.2 M (32.5 %) tris-HCl-buffer, 0.1 M (32.5%) HCl, and 10% of egg yolk. The milt was diluted 1:1 in the cryoprotectant medium. The mixture was poured into 0.8 mL ampules and cooled with LN_2 to -12 to -14 °C at a rate of -0.5 to -5 °C/min and frozen to -196 °C. The sperm were thawed in a water bath at 40 °C; sperm motility was 60%. Pushkar et al. (1980b) used cryoprotectant medium containing 18 to 25% DMSO in 0.1M (65 to 75%) tris-HCI-buffer, 7 to 10% egg yolk and 0.004 to 0.008% of silver iodide. They concluded: 1) tris-HCI-buffer solution was better than Ringer's or Ott-Horton's solution for deactivation and survival of sperm; 2) egg yolk was important to increase the activity and fertilization rate of sperm; and 3) silver iodide decreased the ice crystallization during freezing thereby decreasing the number of damaged sperm. Thus, the following procedure was recommended. Chilled milt (5 °C) should be mixed continually into the chilled cryoprotectant medium in a 1:1 dilution ratio. The mixture should be allowed to equilibrate for 40 min at 5 °C. Ampules should be cooled at a rate of 3 to 12 °C/min by lowering into LN_2 vapor until the samples reach -35 to -45 °C; thereafter, the ampules should be cooled to the final temperature of -196 °C at a rate of -130 to -150 °C/min.

Drokin et al. (1991, 1993) reported motility of 20% for post-thawed sperm of sakhalin sturgeon *Acipenser mikadoi* after cryopreservation with 12% DMSO and 12% yolk in 20 mM tris-HCI buffer (pH 7.5 or 8.0). The milt was chilled to 5 °C and diluted 1:1 in a chilled cryoprotectant medium. The mixture was allowed to equilibrate for 90 min in 1-mL plastic ampules. The samples were frozen in LN_2 vapor at a rate of 5 °C/min to -15°C and at -25 °C/min to -70 °C; thereafter, the samples were submerged into LN_2. The samples were thawed in a water bath at 40 °C for 30 sec.

Cherepanov et al. (1993) reported long-term storage of cryopreserved sperm of sturgeons including beluga, sterlet, Russian sturgeon, bester and ship *Acipenser nudiventris* (Aral Sea origin) from Don River basin. The composition of the cryoprotectant medium and the optimum freeze-thaw regime were similar to the protocols of Pushkar et al (1980b) and Drokin et al. (1991, 1993). Sperm motility was activated by KCl in a tris-HCl buffer. Motility ranged from 10 to 90% after thawing. Samples of frozen sperm from these different sturgeons were stored at the

Low-Temperature Bank of the Institute for Problems of Cryobiology and Cryomedicine of the Ukrainian Academy of Sciences. The motility of the post-thawed sperm after two yr in storage was similar to fresh samples prior to freezing and storage.

Tsvetkova et al. (1996) reported successful fertilization using cryopreserved milt from Siberian sturgeon (*Acipenser baeri*) and sterlet. The milt was diluted in a 1:1 ratio with a cryoprotective medium containing 15% DMSO, 23.4 mM saccharose, 118 mM tris-HCl) pH 8.0 and 20% yolk. The mixture in the tubes was frozen immediately in LN_2 vapor. After thawing for 25 sec at 40 °C and activating with 50 mM solution of tris-HCI (pH8.0), the sperm motility for Siberian sturgeon was 23 ± 9% compared to 88 ± 4% for fresh sperm (control). For sterlet sperm, post-thawed motility was 15 ± 11% and fresh sperm motility was 68 ± 19%. The fertilization rates using post-thawed cryopreserved sperm were 53 ± 8% (control 89 ± 8%) for Siberian sturgeon and 23 ±11% (control 53 ± 9%) for sterlet.

Andreev et al (1996) determined that 12% DMSO as a cryoprotectant and a slow, three-stage freezing regime provided best results for cryopresrvation of beluga sperm. They found the addition of antifreeze proteins to the medium and quick freezing were not effective. Post-thawed sperm fertilized 43% of beluga eggs compared to 68% fertilization by fresh sperm.

Cierezko et al. (1996) reported the use of 10% DMSO and 0.6M sucrose at a dilution ratio of 1:3 (v:v) for the cryopreservation of lake sturgeon sperm (*Acipenser fulvescens*). Sperm were equilibrated for 1 min and frozen as 0.1-ml pellets on dry ice. After 3 d of storage in liquid nitrogen, the samples were thawed in an activating solution of 20 mM Tris, 30 mM glycine buffer, and 10 mM NaCl and analyzed using computer-assisted sperm analysis. Cryopreservation reduced motility by 70% (fresh - 46%, post-thawed - 14%). Motility characteristics of post-thawed sperm were similar to fresh sperm immediately after activation, but showed decreased straight line velocity and linearity after 5 min compared to fresh sperm.

In 1999, the Third International Symposium on Sturgeon published abstracts that provided information on efforts to develop cryopreservation techniques for sturgeon sperm at the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. Cherepanov and Kopeika (1999) summarized cryopreservation of sperm from 7 species of sturgeon. The best protocol developed was 10 - 15% DMSO, 100-150 mM Tris-HCl at pH 8.1, and 10 - 15% chicken egg yolk. Sperm were frozen in LN_2 using a three-step process in 0.5- to1.5-mL polypropylene ampoules. No information was given regarding post-thaw motility or fertilization rates. Drokin and Kopeika (1999) reported on the cryopreservation of Siberian sturgeon, Sakhalin sturgeon, and stellate. The sperm of each species were cryopreserved by diluting 1:1 in a 20 mM Tris-HCl, pH 8.0, 15% DMSO, 18% yolk solution, pouring into plastic ampoules and freezing in a three-step program. Ampoules were thawed at 40 °C for 30 sec. Siberian sturgeon sperm had post-thaw motility of 30 - 40%. Sakhalin sperm sturgeon had 15-20%, and stellate sperm had 30 - 40%. The authors speculated that higher phospholipase activity in the Sakhalin sturgeon sperm was causing damage to the acrosomes, and thereby causing reduced post-thaw motility. Dzuba et al. (1999) reported motility of post-thaw sperm from five species of sturgeon that have been stored for six yr. Post-thaw motility ranged from 20-40% in Russian sturgeon to 60% in ship sturgeon.

Jähnichen et al. (1999) reported a protocol on the cryopreservation of sterlet sperm. Ethylene glycol (12.5% or 17.5% final concentration) was added to an extender composed of 25 mM NaCl, 10 mM Tris, pH 8.5. Sperm were diluted 1:1 with the mixture and loaded into 0.25-mL straws. Samples were frozen in a programmable freezer using two cooling profiles: Profile I. 2 °C to -7 °C: -5 °C/min; -7 °C to -30 °C: -3 °C/min; -30 to -80 °C: -2 °C/min; and, Profile II. 2

°C to -7 °C: -0.5 °C/min; -7 °C to -30 °C: -1 °C/min; -30 to -80 °C: -2 °C/min. Sperm were stored in LN_2 for 1 d or 355 d. Samples were thawed in a 40 °C water bath for either 3 or 5 sec. Highest post-thaw motility was 28 ± 7% using computer-assisted sperm analysis (CASA) and hatch rates were as high as 91%.

Progress in sturgeon sperm cryopreservation has been significant in the 21st century. Sturgeon sperm cryopreservation research has expanded to the rest of Europe, the United States, the Middle East, and East Asia.

Horváth and Urbányi (2000) were the first to report the use of methanol as a suitable cryoprotectant for sturgeon sperm. Sperm of sterlet were diluted at a 1:1 ratio in three basic extenders [sucrose, NaCl and a combination (details were not reported)]. Three cryoprotectants (DMSO, dimethyl-acetamide (DMA) and methanol) were tested at various concentrations. Highest post-thaw motility (46 ± 23%) was reported using the sucrose extender and 10% methanol. The use of methanol also yielded the best fertilization results (22 ± 16% vs. 28 ± 16% in the control); whereas, only 2 ± 4% fertilization was reported for DMSO and none for DMA.

Glogowski et al. 2002 provided a more detailed report on the use of methanol as a cryoprotectant for the cryopreservation of Siberian sturgeon sperm. Three extenders were tested in combination with 10% methanol: 30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl (pH 8.0) modified Tsvetkova's (mT) extender, Jähnichen's extender (Jähnichen et al. 1999) and 20 mM Tris, 400 mM sucrose (pH 8.0) extender. Sperm were diluted at a 1:1 ratio with the freezing diluents containing one of the three extenders and 10% methanol and loaded into 0.25-mL straws. Freezing was conducted in a programmable freezer using a cooling rate of -3.5 °C/min from 4 °C to -15 °C with seeding induced at -7 °C. Straws then were removed from the freezer and placed between two blocks of dry ice for 5 min, then stored in liquid nitrogen. A hatch rate of 30 ± 5% was observed using the Tris-sucrose-KCl extender and was not significantly different from the hatch rate observed with Jähnichen's extender (18 ± 2%) or the control (22 ± 6%).

Urbányi et al. (2004) demonstrated that post-thawed sperm of Siberian, Russian and European sturgeon (*Acipenser sturio*) could successfully be used to fertilize sterlet eggs (3000 eggs per batch) and produced hybridization of three Acipenser species. Post-thawed sterlet sperm were used also to fertilize sterlet eggs (3000 eggs per batch) as a control. The sperm were diluted 1:1 in a diluent containing both mT extender and 10% methanol (cryoprotectant) as reported by Glogowski et al. (2002). Sperm were frozen in 0.5-mL straws placed on 3-cm high Styrofoam frame which was in turn placed onto the surface of liquid nitrogen. Freezing time was 3 min. Hatch rates were 31% for sterlet sperm (control), 50% for Siberian sturgeon sperm, 17% for Russian sturgeon sperm, and 34% for European sturgeon sperm.

Lahnsteiner et al. (2004) evaluated multiple factors for their effects on equilibration motility, post-thaw motility, acrosome reactions, and fertilizing ability of sterlet sperm. The addition of 10% DMSO, 10% methanol, 0.5% sucrose or 0.5% lactose to an extender consisting of 50 mM/L NaCl, 5 mM/L KCl, and 10 mM/L Tris had no effect on equilibration motility after 5 min of exposure. The addition of 0.5% glycine decreased sperm motility. Freezing was found to be optimal at 3-5 cm above the surface of liquid nitrogen. Thawing was optimal at 25 °C for 30 sec. Ten percent DMSO was found to give the highest post-thaw motility rates, followed by 7.5 - 10% methanol, and 7.5% DMSO. Post-thaw motility was decreased by sucrose, lactose, and propandiole. Propandiole also increased acrosome reactions, whereas all other combinations had no effect. Factors for evaluation in the fertilization trials were selected based on the results from the previous experiments. DMSO (10%) and methanol (7.5%) were selected as cryoprotectants

and the addition of 2 mM/L or 5 mM/L KCl to the extender was also examined. Best fertilizations rates were obtained using 7.5% methanol and 5 mM/L KCl.

Tsvetkova et al. (2004, 2006) reported a basic cryoprotective medium consisting of 0.1% of sucrose, 0.08% KCl and 8% methanol for five different sturgeon species (beluga, Russian sturgeon, Siberian sturgeon, sevruga and sterlet) that was developed at the Laboratory of Cryobiology of Institute of Freshwater Fisheries, Russia. The composition of the medium had additional components such as amino acids, sugars, antioxidants or antifreeze glucoproteins for improving the freezing success. Fertilization rates of fresh sperm compared to post-thawed sperm combined in the basic cryoprotectant medium with these additives are indicated in Table 1.

Table 1. Fertilization rates of cryopreserved sperm of five sturgeon species.

Fish Species	Fertilization rate, %	
	Fresh sperm	Post-thawed sperm
Siberian sturgeon	62-88	52-81
Russian sturgeon	35-85	62-78
Beluga (giant sturgeon)	70-98	49-67
Sterlet	79-95	40-62
Sevruga (stellate)	81-93	73-83

Melehova et al. (2004) have developed a patented method for evaluating the rate of sperm damage based on intensity of free-radical reactions. This method permits a more efficient storage of higher quality frozen sperm at the Laboratory of Cryobiology of Institute of Freshwater Fisheries, Russia.

Horváth et al. (2005) reported comparisons of several cryoprotectants and extenders on the sperm of shortnose sturgeon (*A. brevirostrum*) and pallid sturgeon (*Scaphirhynchus albus*). Two experiments were conducted on the shortnose sturgeon and one on pallid sturgeon. The first experiment with shortnose sturgeon sperm tested the modified Tsvetkova's (mT) extender in combination with DMSO or methanol at concentrations of 5, 10 or 15%. The sec experiment tested three extenders mT extender, original Tsvetkova's extender [(oT), Tsvetkova et al. 1996] and modified Hanks' balanced salt solution [(mHBSS), diluted from a commercially available concentrate to an osmolality of 100 mosM/ Kg] and three concentrations of methanol (5, 10 and 15%). The experiment with pallid sturgeon sperm tested mT and mHBSS extenders in combination with 5 or 10% methanol. Sperm samples were frozen according to the protocol described above (Urbányi et al. 2004). In the first experiment with sperm from shortnose sturgeon, the use of 5% DMSO resulted in the highest post-thaw motility (26 ± 13%) however, the highest fertilization (40 ± 15%) and hatch (32 ± 12%) percentages were observed using 5% methanol. In the sec experiment, the highest post-thaw motility (18 ± 10%), fertilization (18 ± 11%) and hatch (17 ± 12%) percentages were found with mT extender in combination with 5% methanol.

In general, the use of DMSO in the first experiment or oT extender in the sec experiment resulted in higher or similar post-thaw motility to methanol or mT extender, however, fertilization and subsequently hatch percentages were significantly lower when DMSO or oT extender were used. Both components are known to have a higher osmolality than that of sturgeon milt plasma, thus, authors speculated that maintaining iso-osmotic conditions is

important for successful fertilization with sturgeon sperm. Extender mHBSS with its osmolality close to that of the seminal plasma yielded intermediate results. This was later confirmed in the experiment on pallid sturgeon sperm when the mT extender resulted in higher post-thaw motility (70 ± 10% with 10% methanol) and fertilization (88 ± 6% with 5% methanol) percentages than that with mHBSS, however, there were no significant differences.

Ciereszko et al. (2006) used a similar protocol on lake sturgeon sperm however, the KCl concentration was increased to 5 mM in the mT extender combined with 10% methanol and stored in 0.25-mL straws. Freezing was conducted on a 3-cm platform floating on the surface of LN_2 for 5 min. Samples were thawed in a 40 °C water bath for 6 sec. Post-thaw motility of cryopreserved sperm was 19 ± 18% with high variability among individual samples. The percentage of pre-hatch embryos was 1.8 ± 0.7% (with 14.3 ± 2.7% in the control). This low hatch rate with cryopreserved sperm as well as in the control indicates that further studies should be conducted and individual species might require slight adjustments of the protocol. In this study, the comet assay was used to determine the level of damage to sperm DNA. Although DNA damage was higher in cryopreserved than in fresh sperm, there was no significant difference.

Liu et al. (2006) used DMSO (12%) as the cryoprotectant for cryopreservation of Chinese sturgeon (*A. sinensis*) sperm. Sperm were diluted 1:3 (v:v) in a diluent containing 8.85 g/L NaCl, 0.20 g/L KCl, 0.40 g/L $NaHCO_3$, and 12% DMSO, and 2-ml of diluted sperm were frozen in 2.5-mL plastic vials. Equilibration times of 0.5-2 hr were determined to be best. A freezing rate of -2 °C/min to a temperature of -6 °C and holding for 10 min produced the highest post-thaw motility (70%). Samples were thawed at 32 °C for about 2 min and two vials were used to fertilize ~200 eggs. Fertilization rate was 84% and hatch rate was 68%.

Mirzoyan et al. (2006) reported positive effects of ascorbic acid and lysine added to the cryo-diluent used in freezing Russian sturgeon sperm. These substances are known to suppress the activity of oxygen-free radicals thus; their addition to the cryoprotective media was considered to improve the survival of cells. Sperm were cryopreserved using an extender that contained a Tris-HCl buffer, egg yolk, sucrose (concentrations of chemicals were not reported) and 15% DMSO with or without 0.01 M ascorbic acid or 0.05 M lysine. Samples were frozen in 2-ml test tubes using a three-step cooling protocol: 5 °C to -5 °C, -5 °C to -70 °C in the vapor of LN_2 (cooling rate was not reported), and -70 °C to -196 °C by plunging into LN_2. Addition of lysine or ascorbic acid improved post-thaw motility and fertilization results, although actual data were not reported.

Grunina et al. (2006) reported the induction of dispermic androgenesis using cryopreserved sperm in stellate sturgeon. For genetic inactivation of maternal chromosomes eggs were irradiated with X-rays; fusion of male pronuclei were stimulated by heat shock. Portions of irradiated eggs were inseminated with cryopreserved and fresh sperm. Sperm samples were frozen in media containing 10% methanol, 7% sucrose and Tris-HCl (pH 7) in 1.5-ml tubes and thawed at 40 °C for 1 min. Hatching rate of 7 and 17% and one-mo survival of 58 and 68% of diploid androgenic larvae were observed after using cryopreserved and fresh sperm, respectively. Combined approach consisting of the dispermic androgenesis and sperm cryopreservation can be used for restoration of endangered sturgeon species.

In 1988, the Low-Temperature Gene Bank of Fish Sperm (i.e. Bank of Cryopreserved Sperm) was founded at the All-Russian Research Institute of Freshwater Fisheries (Rybnoe, Moscow Province, Russia). One of the main objectives of the Gene Bank was to preserve the genetic diversity (gene pool) of sturgeons. In August 2008, the total volume of approximately

400 sperm samples from different sturgeon species was about 8000 mL (Tsvetkova, unpublished data). Regular monitoring of frozen sperm has been performed; periodic tests have shown that the quality of sperm does not decrease during storage in the Bank (Tsvetkova et al. 2006; Dokina et al. 2007).

Tsvetkova et al. (2007) reported the protective ability of antifreeze proteins (AFP) or antifreeze glycoproteins (AFGP) extracted from the blood of Barents Sea cod (*Gadus morhua*). Addition of AFGP into cryoprotective media at concentrations from 5 to 25 mg per mL provided a threefold or more increase in fertilizing ability of Siberian sturgeon sperm (Table 2).

Table 2. Dependence of fertilization rate on concentration of antifreeze glycoproteins (AFGP) in cryoprotective media for Siberian sturgeon sperm.

Concentration of AFGP in cryoprotective media, mg/mL	Fertilization rates with post-thawed sperm	
	% relative to fresh sperm	% relative to basic cryoprotective media
5	93±8	355±32
10	78±18	298±69
25	75±21	288±79

Wayman et al. (2008) reported that pallid sturgeon sperm were cryopreserved using varied concentrations of methanol (5, 10, or 15%) and mHBSS at 100 mosM/Kg. Samples were diluted 1:1 (v:v), and were frozen in 0.5-mL straws with an equilibration time of 2 min. The straws were loaded into goblets and placed at the bottom position on aluminum canes and then lowered into a nitrogen-vapor shipping dewar. The average rate of freezing was -22 °C/min. Straws were thawed at 40 °C for 9 sec, and used to fertilize ~150 eggs. Highest post-thaw motility (25 - 28%), fertilization rate (91 - 92%), and hatch rate (77%) occurred using 5% or 10% methanol as the cryoprotectant.

Psenicka et al. (2008) analyzed the motility and acrosomal staining characteristics of cryopreserved sterlet sperm in an effort to determine why DMSO when used as a cryoprotectant of sturgeon sperm produced high post-thaw motility, but low fertilization rates as compared to methanol (as shown in previous studies). The sperm were cryopreserved as in Glogowski et al. (2002). After thawing, motility was analyzed using a computer-assisted sperm analysis program, and sperm were stained with SBTI-Alexa Fluor 488. There were no significant differences in the motility characteristics of sperm cryopreserved with methanol or DMSO. However, sperm cryopreserved with DMSO had a significantly higher (12.7%) specific acrosomal staining than did sperm cryopreserved with methanol (6%). The authors postulated that the increased staining indicated damage to the acrosome caused by DMSO during the cryopreservation process. However, overall staining was low, and they could not definitively conclude acrosomal damage was the cause for the low fertilization rates seen with DMSO. The authors stated that the DMSO may be causing preliminary effects that lead to the acrosomal reaction which are not detectable with the current staining technique, but that may be the cause for the reduced fertility. Further studies were warranted.

Horváth et al. (2008) evaluated the relationship between membrane integrity and fertilizing ability for cryopreserved sperm samples that had been previously frozen as reported in Horvath et al. (2005). Sperm from two sturgeon species shortnose sturgeon (*Acipenser brevirostrum*) and pallid sturgeon (*Scaphirhynchus albus*) were tested. Flow cytometry and two

fluorescent dyes were used to assess the membrane integrity of post-thaw sperm. A membrane permeable green fluorescent dye was used to stain all the cells, and then a non-permeable red fluorescent dye was used to counter stain cells with damaged membranes. In most cases membrane integrity correlated with post-thaw motility, but sometimes was independent of fertilization rates.

In conclusion, research of sturgeon sperm cryopreservation has increased dramatically since the publication of the last chapter. Research has progressed from developing basic methods with low fertilization results for only a few species to the refinement of these basic methods for numerous species and resulting in higher levels of fertilization. The newer investigations into alternative approaches to determine sperm quality should also lead to the development of protocols yielding even higher cryopreservation efficiencies.

Cryopreservation Protocols for Paddlefish Sperm

Brown and Mims (1999) were the first to report cryopreservation of paddlefish (*Polyodon spathula*) sperm. Milt was mixed with a cryoprotectant medium containing DMSO (2.4 M) in a ratio of 3:1 (milt:medium; final concentration of DMSO 0.6 M). The medium was composed of 1.6 mL of DMSO, 4.0 mL of trehalose and 4.4 mL of extender. The composition of the extender was 0.205 g $CaCl_2$ H2O, 0.440g $MgCl_2$ $6H_2O$, 0.470 g $NaHCO_3$, 5.115 g KCl, 11.560g NaCl, 20g glucose, 0.200g citric acid, 4.760g HEPES, 2,000 mL double-distilled H_2O, 20 mL KOH, 20mL penicillin-streptomycin, pH 7.6 and 300 mOsmol/Kg. The mixture was stored in 5mL straws, frozen on dry ice for 15 min and then stored in liquid nitrogen. For thawing, straws were immersed in a water bath at 20 °C for 15 sec. Motility of post-thawed sperm was 25 to 50%, compared to 100% motility for fresh sperm. Post-thawed samples of two straws (about 5.25 mL of pooled milt and 1.75 mL of medium) were mixed with about 3,500 eggs per trail following standard fertilization procedure (i.e. clay-coating of adhesive eggs) and incubation technique (i.e. McDonald jars). The ratio of motile, post-thawed sperm to egg was about 6.17×10^5 sperm egg^{-1}. Hatching rate was 16 ± 2% from eggs fertilized with post-thawed sperm and was significantly lower than the hatching rate of 90 ± 3% using fresh sperm. The authors postulated that acrosomal damage could have been a major culprit in the low hatching rate and that increasing the post-thaw sperm to egg ratio could mask the low viability of post-thawed sperm. Observation by electron microscopy (Mims and Brown unpublished data) indicated that acrosomal damage probably occurred to paddlefish sperm either during freezing or thawing and probably resulted in premature acrosomal reaction, thus preventing higher rates of fertilization and ultimately higher hatching rates (Figure 2).

Horváth et al. (2006) reported a series of experiments with the goal to improve protocol for sperm cryopreservation of paddlefish. Similar methods that were successfully developed for cryopreservation of sturgeon sperm (Horváth and Urbányi, 2000; Glogowski et al. 2002; Urbányi et al. 2004) were tested on paddlefish sperm. The first experiment was to evaluate the effects of two extenders [mT extender and modified Hank's balance salt solution (mHBSS)] in combination with methanol and DMSO in two concentrations (5 and 10%) on the post-thaw motility and fertilization rates of cryopreserved sperm. The highest post-thaw motility (85 ± 5%) and highest fertilization (80 ± 3%) were observed when sperm was frozen with mT extender and 10% methanol as the cryoprotectant. In sec experiment, 4,000 eggs were fertilized with the pooled samples of five straws per male (total = three males; four replicates per male) of post-thawed sperm representing a volume of 1.25 mL using mT extender and 5% methanol. Hatch

rates were as high as 79 ± 5%. The third experiment was conducted to clarify the role of methanol concentrations. There was no significant difference found among fertilization or hatch rates when either 5 or 10% methanol was used as a cryoprotectant. The authors stated that methanol was found to be a safe and reliable cryoprotectant for freezing of paddlefish sperm and obtaining viable postthaw sperm for consistent fertilization and hatch rates. Further, the experimental protocol is relatively simple and applicable for commercial hatchery production of paddlefish.

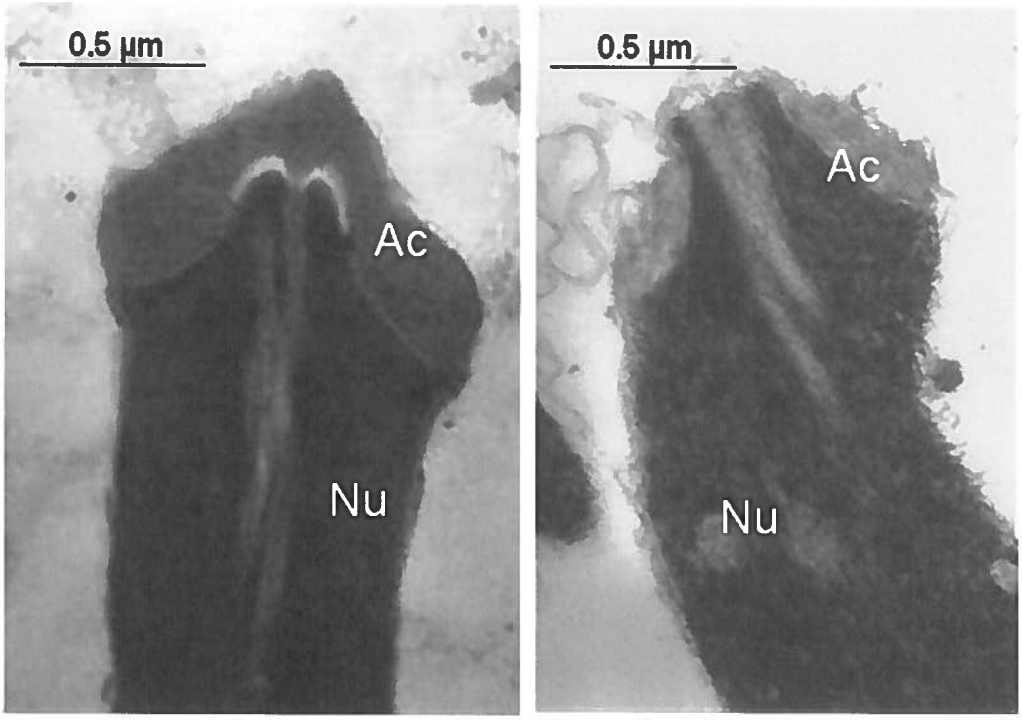

Figure 2. (A) Longitudinal section demonstrating the intact acrosome (Ac) and nucleus (Nu) at the apical tip of fresh paddlefish *Polyodon spathula* spermatozoon, (B) Longitudinal section demonstrating the damaged acrosome (Ac) at the apical end of post-thawed spermatazoon (bar=0.5 µm).

Linhart et al. 2006 compared different percentages (8 and 10%) of cryoprotectants (DMSO and methanol) added to extender 1 (20mM tris pH 8, 30mM sucrose and 0.5mM KCl) or extender 2 (20mM tris pH 8, 50mM sucrose and 0.5mM KCl, dilution 1:1) or non-extended sperm. One mL of the mixture was stored in a 2-mL cryotube. The cryotubes were directly loaded into a pre-programmable PLANER Kryo10 series III freezer at 0 °C and cooled from 0 °C to -5 °C at a rate of -3 °C/min, from -5 °C to -15 °C at a rate of -5 °C/min, from -15 °C to -25 °C at a rate of -10 °C/min, from -25 °C to -80 °C at a rate of -20 °C/min. The samples were held for 5 min at -80 °C and then plunged into LN2 for 24 hr. The sperm was thawed in a water bath of 40 °C for 15 sec. Fertilization rates of 64 to 75% were obtained on post-thawed sperm (3.6×10^5 sperm/egg) when sperm was either without dilution or diluted with extender 1 and treated with methanol in concentrations of 8 or 10%. These results were not significantly different compared

with fresh sperm (stored at 3 °C for 24 hrs). Fertilization rates were only 8-15%, when sperm was frozen with 8 and 10% DMSO.

Bean (2007) reported the hatching rates of paddlefish eggs fertilized with post-thawed sperm previously frozen in different size storage containers. The three cryocontainer sizes were 0.5-mL straws, 2.0-mL cryovials and 4.5-mL cryotubes. Milt was pooled from two males and diluted 1:1 with mT extender and 10% methanol. Containers were placed on a floating tray and were frozen at 3-cm from the surface of LN_2 as described by Horvath et al. (2006) for 3, 4 and 5 min, respectively. Containers were then stored in LN_2 for 24 hrs. Samples in the different containers were thawed by submerging in a 40 °C water bath for 13, 90 and 120 sec, respectively. Eggs were pooled from two females and weighed into twelve 40 g batches (about 4,000 eggs/batch; 4 replicates/treatment). Five mL of post-thawed sperm from each storage container treatment was added and activated with dechlorinated water. Hatching rates with 0.5-mL straw treatment (control) was 25 ± 6%, with 2.0-mL cryovial treatment was16 ± 12% and with 4.5-mL cryotube was 16% ± 12%. Usage of storage containers of different types and capacities did not have a significant impact on hatching rate of paddlefish.

Horvath et al. (2010) tested the feasibility of using 5-mL straws for the cryopreservation of paddlefish (*Polyodon spathula*) sperm for mass production. In the first experiment 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 when fresh sperm (fertilization 77±6 %; hatching 66±13%). In the sec experiment 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 ($y = -13.19x^2 + 55.90x + 38.44$; $r^2=0.82$) 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 linear 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 when using 7.5 mL of post-thawed sperm from three 5 mL straws (69 ± 6%) and 5.0 mL of fresh sperm (77 ± 6%). With cryopreserved sperm, the relationship between the sperm/egg ratios and the hatching rates were best described by a quadratic equation ($y=-29.65x^2 + 119.2x -51.04$, $r^2=0.84$). The authors suggested the volume of cryopreserved sperm should be increased by at least 30% to optimize fertilization and hatching rates.

Horvath et al. 2008 evaluated motility and viability (cell membrane integrity) of cryopreserved sperm from paddlefish. Paddlefish sperm was frozen in mT or mHBSS extenders with methanol or DMSO as cryoprotectants (5 or 10%; v:v). Paddlefish sperm had the highest post-thaw motility (85% ± 2%) and fertilization (80 ± 3%) percentages using 10% methanol; however, the highest viability (59 ± 2%) was observed with mHBSS combined with 5% DMSO. Statistically, post-thaw motility and viability were correlated, but independent of fertilization. The interaction of cryoprotectant and its concentration was significant for fertility, with all fertilization rates higher with 10% methanol than the other combinations and with 10% DMSO yielding significantly the lowest fertilization rate. The decreasing fertility rate with DMSO could be explained by the effect of acrosomal reaction because there is a dramatic increase in osmolaity (i.e. osmotic shock process) and a rapid penetration of the acromosal membrane causing release of acrosin enzyme (Ciereszko et al.1996a,b, 2000; Otomar et al. 2006).

Conclusion

Sturgeon and paddlefish spermatozoa are unique among the male gametes of other fishes because of the presence of a functional acrosome. The majority of the research on cryopreservation of chondrostean sperm had developed protocols that resulted in high motility but low fertilization rates (Horvath et al. 2009). As summarized in this chapter, most of the protocols used dimethyl sulfoxide (DMSO) as the cryoprotectant. However, in the last decade methanol has resulted in significantly higher fertilization and hatching rates than DMSO. Horvath et al. (2009) clearly reported that DMSO greatly increased the osmolality of the extender while methanol caused only a slight increase in osmolality. In addition, when sperm was frozen in hyperosmotic extender (i.e. original Tsvetkova extender as described in Tsvetkova et al. 1996) containing either DMSO or methanol as cryoprotectant, poor fertilization also occurred. Therefore, iso-osmotic freezing diluents (i.e. combined extender and cryoprotectant) are necessary to use for cryopreserving chondrostean sperm in order to obtain consistent fertilization success.

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