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Viability and fertilizing capacity of cryopreserved sperm from three North American acipenseriform species: a retrospective study

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Summary

Populations of sturgeon across the globe are threatened due to unregulated harvest and habitat loss, and the status varies among species across North America. Ready access to viable and functional sperm would contribute to recovery programmes for these species. In this study, we examined the motility, viability (cell membrane integrity) of cryopreserved sperm from three North American acipenseriform species and fertilizing capacity. Milt samples were collected from captive shortnose sturgeon (*Acipenser brevirostrum*), wild paddlefish (*Polyodon spathula*) and pallid sturgeon (*Scaphirhynchus albus*) and cryopreserved using combinations of Modified Tsvetkova's (MT) extender, Original Tsvetkova's extender, and modified Hanks' balanced salt solution, along with the cryoprotectants methanol (MeOH) or dimethyl sulfoxide (DMSO). A dual-staining technique using the fluorescent stains SYBR-14 and propidium iodide was employed with flow cytometry to determine the percentages of spermatozoa that were viable by virtue of having intact membranes. The percentage of viable spermatozoa ranged from 5% to 12% in shortnose sturgeon, 30–59% in paddlefish, and 44–58% in pallid sturgeon. In the first experiment with shortnose sturgeon sperm, methanol allowed for higher values for dependent variables than did DMSO, and sperm viability generally correlated with post-thaw motility. However, fertilization rate, neurulation, or hatching rates were independent from these factors. In the second experiment with shortnose sturgeon, 5% MeOH combined with MT yielded higher values for all parameters tested than the other combinations: viability was correlated with motility, fertilization rate, and hatching rate. Overall, viability and post-thaw motility was not affected by the use of hyperosmotic extenders (OT) or cryoprotectants (DMSO), but their use decreased fertilization percentages. For paddlefish sperm (experiment 3), MT combined with 10% MeOH was clearly a good choice for cryopreservation; viability and motility results were correlated, but independent of fertilization. For pallid sturgeon sperm (experiment 4), MT with 5–10% MeOH showed significantly higher sperm quality and fertilization parameters. Membrane integrity can be used as a predictor of fertilization by cryopreserved sperm, however additional sperm quality parameters, supplementary to motility and membrane integrity, would be useful in the refining and optimizing cryopreservation protocols with acipenseriform sperm.

Introduction

Sturgeons and paddlefishes (order Acipenseriformes) are chondrostean fish of ancient origin (Birstein, 1993). Most of these species are highly endangered or near extinction (Billard and Lecointre, 2001). Recovery plans throughout Europe and North America include supplementation of wild stocks with hatchery reared progeny (Schrey and Heist, 2007). In North America, recovery plans by the U.S. Fish and Wildlife Service call for the development of sperm banks to aid artificial propagation of these species (DiLauro et al., 2001). Cryopreservation of sturgeon sperm from the Eastern hemisphere has been studied since the 1960s (Dettlaff et al., 1993), with several ensuing protocols (Drokin et al., 1991; Ciereszko et al., 1996; Tsvetkova et al., 1996), whereby the relatively good post-thaw motilities consistently resulted in low fertilization rates, often with inconclusive results. Our recent studies have shown that one of the key factors of successful sperm cryopreservation in Acipenseriformes is combining an appropriate extender with a cryoprotectant (Glogowski et al., 2002; Horváth et al., 2005, 2006). Horváth et al. (2005) observed that higher fertilization rates were observed when the osmolality of this combination was similar to that of the seminal plasma. When extenders with higher osmolalities were used for sperm cryopreservation, fertilization rates were low, although post-thaw motility remained satisfactory.

The quality of cryopreserved sperm is often defined by post-thaw motility, fertilization rates, or hatching rates. However, protocols for assessing sperm quality prior to performing fertilization tests will be vital in the advancement of cryopreservation science in aquatic species. As shown with studies of many aquaculture species, sperm motility is not always suitable for predicting fertilization success. One assessment of sperm quality, especially useful with cryopreserved cells, is the determination of membrane integrity or viability (Segovia et al., 2000; Jenkins and Draugelis-Dale, 2006). In fact, the efficiency of the protocol has been proven with several fish species including rainbow trout *Oncorhynchus mykiss* (Ogier de Baulny et al., 1997; Cabrita et al., 2001), Nile tilapia *Oreochromis niloticus* (Segovia et al., 2000), European catfish *Silurus glanis* (Ogier de Baulny et al., 1999), common carp (*Cyprinus carpio*) (Jenkins and Draugelis-Dale, 2006), mosquitofish (*Gambusia affinis*) (Jenkins and Goodbred, 2005) and other aquatic invertebrate species such as the eastern oyster *Crassostrea virginica* (Paniagua-Chávez et al., 2006) and red

abalone (Salinas-Flores et al., 2005). In these studies, membrane integrity was assessed by cell viability staining when nucleic acids inside intact membranes were stained fluorescent green by SYBR-14, whereas none-intact or permeable membranes allowed a red counterstain (propidium iodide) to enter the cells so that 'dead' or moribund cells stained fluorescent red (Jenkins and Draugelis-Dale, 2006). This dual staining technique has been tested on sturgeon spermatozoa in combination with microscopic image cytometry (Flajshans et al., 2004). Flow cytometry allows rapid assessments of high numbers of cells per replicate sample.

In order to extend the work presented in Horváth et al. (2005, 2006), this study was designed to measure the viability of spermatozoa, previously cryopreserved with combinations of extenders and cryoprotectants, from three North-American acipenseriform species: the shortnose sturgeon (*Acipenser brevirostrum*), the pallid sturgeon (*Scaphyrhinchus platorhynchus*) and the paddlefish (*Polyodon spathula*). In order to fully appraise results, we investigated possible relationships between membrane integrity and other sperm quality assessments performed with the same samples, including post-thaw motility, fertilization and hatching rates previously reported by Horváth et al. (2005, 2006).

Materials and methods

Sample collection and cryopreservation

Broodstock management, induced spawning, gamete collection, and fertilization tests are described in Horváth et al. (2005, 2006). Briefly, using sperm from captive shortnose sturgeon broodstock, cells were cryopreserved in two consecutive experiments (Table 1). In the first experiment, sperm were frozen in Modified Tsvetkova's extender (MT) (Glogowski et al., 2002) (30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl, pH 8.0, osmolality, 73 mOsmol kg^{-1}) with added cryoprotectants of methanol (MeOH) or dimethyl sulfoxide (DMSO) at 5, 10 or 15% (v/v). In the second experiment, sperm from the same group of fish were frozen in the presence of three different extenders: MT, Original Tsvetkova's (OT) extender (23.4 mM sucrose, 118 mM Tris, pH 8.0, 204 mOsmol kg^{-1}) (Tsvetkova et al., 1996), or modified Hanks' balanced salt solution (mHBSS) (H4385; Sigma-Aldrich Chemical Company, St Louis, MO) diluted 24 times from the concentrate with distilled water to an osmolality of 100 mOsmol kg^{-1}. The cryoprotectant in this experiment was MeOH at 5, 10 or 15%. In the third and fourth experiments, sperm from paddlefish and pallid sturgeon were obtained from wild fish and cryopreserved near sites of capture. Paddlefish sperm were frozen in MT or mHBSS extenders with MeOH or DMSO as cryoprotectants (5 or 10% v/v). Pallid sturgeon sperm were cryopreserved with MT or mHBSS in combination with 5 or 10% MeOH (Table 1).

Conditions of freezing and thawing were uniform for each experiment, whereby sperm were diluted 1 : 1 in the extender with cryoprotectant and loaded into 0.5 ml French straws. Straws were placed on a 3 cm high styrofoam frame which was in turn placed onto the surface of liquid nitrogen in a styrofoam box. Straws were allowed to cool in the vapor of liquid nitrogen for 3 min and then plunged directly into liquid nitrogen. After a period of storage (24–36 h for shortnose sturgeon and paddlefish, and 4 weeks for pallid sturgeon cells) at −196°C in storage dewars filled with liquid nitrogen, samples were thawed in a 40°C water bath for 13 s and used for the estimation of post-thaw motility and fertilization trials (Horváth et al., 2005, 2006).

Cryopreserved sperm samples were transported in shipping dewars to the Aquaculture Research Station (ARS) of the Louisiana State University Agricultural Center and thawed prior to membrane integrity analyses. Because this instrumentation was not in proximity of the sampled animals collected for experiments 3 and 4, overnight shipped samples of cold-stored captive shortnose sperm were used as controls in experiments 1 and 2, and in the membrane integrity assay validation using duplicates (Fig. 1).

Sperm quality assessments

For each sample, motility estimations were conducted by darkfield miscrosopy (Horváth et al., 2005, 2006). Cell concentrations were measured by hemocytometer in order to calculate sperm to egg ratios in the fertilization trials (Horváth et al., 2005, 2006). In the first experiment, either a full straw or a half-full straw of thawed sperm was used for fertilization scored by per cent fertilization at the 4-cell stage, neurulation, and hatch (eggs were incubated in experimental 300 ml McDonald-type hatching jars at 16°C). Because straw size was not a significant variable ($P > 0.05$), full straws were used thereafter. In the second experiment, fertilization was measured at the 4-cell stage and survival identified until hatching. In the third experiment with paddlefish, fertilization percentage

Table 1
Experimental design for cryopreservation of acipenseriform spermatozoa

Origin of mature fish	Experiment	Species	Extender	Cryoprotectant (%)
Captive[1]	1	Shortnose sturgeon	MT	Methanol (5, 10, 15) DMSO (5, 10, 15)
Captive	2	Shortnose sturgeon	MT OT mHBSS	Methanol (5, 10, 15)
Field[2]	3	Paddlefish	MT mHBSS	Methanol (5, 10) DMSO (5, 10)
Field[3]	4	Pallid sturgeon	MT mHBSS	Methanol (5, 10)

OT, original Tsvetkova's extender; mHBSS, modified Hanks' balance salt solution extender; MT, modified Tsvetkova's extender; DMSO, cryoprotectant dimethyl-sulfoxide.
[1]Captive shortnose sturgeon broodstock were sampled at the Bears Bluff National Fish Hatchery (Wadmalaw Island, South Carolina) of the US Fish and Wildlife Service. Experiments and two used samples from this population.
[2]Paddlefish were captured in the Ohio River below McAlpine Dam, Louisville, Kentucky (longitude: 85°46′51.9″W, latitude: 38°17′07″N).
[3]Pallid sturgeon were collected from the Mississippi River Outflow Channel at the Old River Control Complex, Concordia Parish, Louisiana.

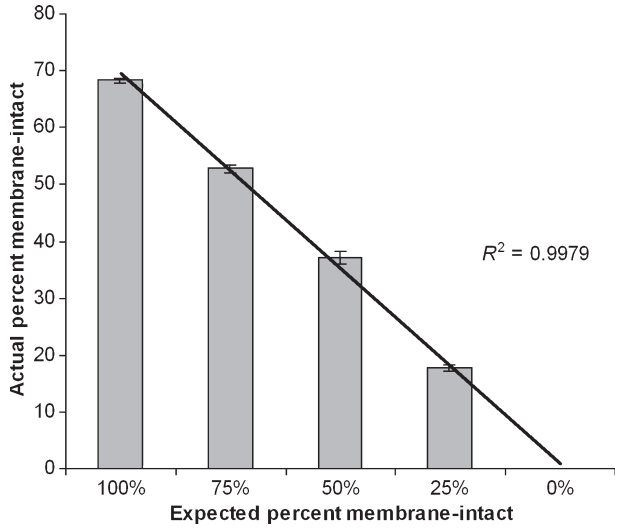

Fig. 1. Standard curve (line) demonstrating verification of a flow cytometric membrane integrity (viability) assay for use with shortnose sturgeon. Data were generated by using known proportions of live and heat-killed sperm samples in duplicate from one fish. Columns represent means and bars standard error

at the 4-cell stage was recorded, and in the fourth experiment with pallid sturgeon survival rates were measured at the 4-cell stage and at stage 34 (tail reaching the tip of the head) according to Dettlaff et al. (1993) which in the present case is labelled as heartbeat stage (eggs were incubated at 18°C in rectangular plastic boxes immersed in a 1.5 m^3 fiberglass tank with recirculated water) (Horváth et al., 2005, 2006).

Thawed cells were suspended in 10 ml of the extender originally used for cryopreservation. Sperm concentrations approximated 1×10^6 for a rate of analysis of 300 cells per second by flow cytometry (FACSCalibur®; Becton Dickinson Immunocytometry Systems, San Jose, CA) [BDIS]. Five microlitres of SYBR-14 (20 μM) were added to 250 μl of the cell suspensions, and incubated at 24°C in the dark. After 10 min, 2.5 μl of propidium iodide (PI; 1 mM) was added and incubated similarly. Instrument calibration had been done using FACSComp® software (BDIS) and labeled fluorescent beads (Calibrite Beads, BDIS). Ungated data were generated using dot or density plots, and a total of 10 000 events were collected per sample in duplicate. Data were analyzed using Cell Quest® software (BDIS).

Assay validation was done using spermatozoa from shortnose sturgeon that had been collected from one male at the Warm Springs Fish Technology Center, U.S. Fish and Wildlife Service and shipped overnight to the ARS. Cells at 1×10^6 ml^{-1} were killed by heating in a waterbath at 80°C for 10 min. Cell preparation and analysis was done as described above on duplicates of known viable and non-viable mixtures (Fig. 1) (Segovia et al., 2000).

Statistical analyses

Percentages of post-thaw motility, fertilization parameters, and viabilities in each experiment were checked for normality and homogeneity and transformations were applied to normalize data where necessary. The level of significance was 0.05 for all analyses. Correlations were investigated between independent variables including straw size, extender, cryoprotectant type, and cryoprotectant concentrations.

A three-way multivariate analysis of variance (MANOVA) using Wilk's lambda was used to determine the main effects of the independent variables. The highest order significant interactions were then investigated using Least Mean Squares analyses. In experiment 1 with captive shortnose sturgeon sperm, a two-way MANOVA was used to analyze motilities and viabilities to investigate the main effects of cryoprotectants and their concentrations, whereas a three-way MANOVA was used to clarify the main effects of cryoprotectants, their concentrations and straw size on fertilization, neurulation and hatch percentages. In experiment 2, a four-way MANOVA analyzed motilities, fertilization, hatch and viabilities to investigate the main effects of extenders, cryoprotectant, and cryoprotectant concentrations.

In experiment 3 with paddlefish data, a three-way MANOVA was used to clarify the main effects of extenders, cryoprotectants and their concentrations on post-thaw motility and viability percentages, and a three-way ANOVA was run on fertilization data to check the main effects of the same variables.

In experiment 4 on pallid sturgeon sperm, two MANOVA were run, one to investigate the main effects of extender type and methanol concentrations on post-thaw motilities and viabilities, and another to clarify the same main effects on fertilization at cleavage and at heartbeat stage.

Results

The concentration of spermatozoa of shortnose sturgeon in the present study was $1.3 \pm 1.1 \times 10^9$ cells per ml which resulted in a sperm to egg ratio of $1.5 \pm 1.3 \times 10^6$ spermatozoa to one egg when a half - straw was used for fertilization and $3.3 \pm 2.8 \times 10^6$ sperm cells per egg with a full straw. Sperm concentration of paddlefish sperm was $7.6 \pm 0.8 \times 10^8$ cells per ml, thus, the sperm to egg ratio was $9.5 \pm 1.0 \times 10^5$ cells per egg. The sperm density of the pallid sturgeon sample was $1.3 \pm 0.9 \times 10^9$ spermatozoa per ml, resulting in a sperm to egg ratio of $3.9 \pm 2.2 \times 10^6$ cells to one egg.

Using duplicates of shortnose sturgeon sperm in assay validation with heat-killed cells and cold-stored fresh sperm, mixtures ranging from 100% to 0% dead sperm were analyzed by flow cytometry. A linear relationship was found at $R^2 = 0.9979$ (Fig. 1).

In the first experiment using sperm from shortnose sturgeon only, the highest post-thaw motility (26 ± 13%) was observed with 5% DMSO as cryoprotectant (Table 2), whereas the highest survival rates were observed using 5% MeOH with 40 ± 15% at the 4-cell stage, 38 ± 13% at neurulation and 32 ± 12% at hatching (Table 2). The highest viability (12 ± 8%) of cryopreserved sperm was achieved in 10% DMSO. Fertilization, neurulation and hatching percentages were independent from post-thaw motilities and viabilities. No significance effect was found with straw size on all of these parameters. Cryoprotectants and their concentrations were significantly related to post-thaw motility and viability ($P = 0.0001$; Table 2), with 5% methanol yielding the highest post-thaw motility, while 10% DMSO yielded higher viability. Generally, no significant differences were observed among post-thaw motility and viability percentages with different treatments with the exception of 15% DMSO giving the lowest results.

For shortnose sturgeon fertilization at the 4-cell stage, neurulation and hatching percentages, cryoprotectants, their concentrations, and their interactions were significant at

Table 2
Parameters of cryopreserved and fresh (control) shortnose sturgeon sperm from experiment 1

Cryoprotectant	Cryoprotectant concentration (%)	Straw used for fertilization	Motility (%)	Fertilization (%)	Neurulation (%)	Hatch (%)	Viability (%)
MeOH	5	Half	16 ± 7^{a}	40 ± 15^{a}	36 ± 17^{a}	31 ± 15^{a}	5 ± 2^{ab}
		Full		39 ± 11^{a}	38 ± 13^{a}	32 ± 12^{a}	
	10	Half	13 ± 8^{a}	19 ± 13^{b}	22 ± 17^{b}	18 ± 16^{b}	6 ± 3^{ab}
		Full		21 ± 12^{b}	21 ± 11^{b}	16 ± 8^{b}	
	15	Half	10 ± 5^{a}	3 ± 5^{c}	3 ± 4^{c}	2 ± 2^{c}	7 ± 6^{ab}
		Full		8 ± 9^{c}	7 ± 6^{c}	5 ± 4^{c}	
DMSO	5	Half	26 ± 13^{a}	0 ± 0^{c}	1 ± 1^{c}	1 ± 1^{c}	7 ± 4^{ab}
		Full		1 ± 2^{c}	0 ± 1^{c}	0 ± 1^{c}	
	10	Half	17 ± 6^{a}	2 ± 2^{c}	0 ± 1^{c}	0 ± 0^{c}	12 ± 8^{a}
		Full		1 ± 1^{c}	1 ± 1^{c}	0 ± 1^{c}	
	15	Half	2 ± 3^{b}	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{c}	4 ± 1^{b}
		Full		0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{c}	
Control[1]	–	Half	77 ± 8	78 ± 26	69 ± 24	51 ± 22	68.3 ± 0.5[2]
		Full		79 ± 29	71 ± 20	53 ± 21	

Percentages of post-thaw motility, fertilization, neurulation, hatch and viability (mean ± SD; n = 6 males). Modified Tsvetkova's extender was used at the extender, half: sperm from a half a straw was used for fertilization of a given amount of eggs, full: sperm from a full straw was used for fertilization of a given amount of eggs (in case of control, half: 0.125 ml fresh sperm, full: 0.25 ml fresh sperm). Values sharing a superscript letter within a column are not significantly different ($\alpha = 0.05$).
MeOH, cryoprotectant methanol; DMSO, cryoprotectant dimethyl-sulfoxide.
[1]Fresh sperm was used without cryoprotectant.
[2]Fresh sperm was used in viability assay validation (see Fig. 2).

$P < 0.0001$. The 5% MeOH cryoprotectant yielded significantly higher percentages for each of the three fertility parameters, and 10% MeOH yielded higher results than 15% MeOH. The 5 and 10% MeOH concentration resulted in higher fertility percentages than DMSO.

In experiment 2, the post-thaw motility (18 ± 10%), fertilization rate (18 ± 11%) and hatching rate (17 ± 12%) percentages using shortnose sturgeon sperm were found with the MT extender combined with 5% MeOH. However, the highest viabilities (10 ± 8%) were found with mHBSS combined with 5% MeOH (Table 3). Post-thaw motility, fertilization, hatch, and viabilities were correlated. The main effects of extender and cyroprotectant were significant ($P < 0.001$ and $P = 0.0064$, respectively). The 5% MeOH yielded significantly higher post-thaw motility, fertilization, and hatch percentages than the 10% and 15% concentrations, however, viability was not affected. The OT extender resulted in the highest post-thaw motility and viability, but the use of MT extender yielded significantly higher fertilization and hatch results (Table 3).

Table 3
Parameters of cryopreserved shortnose sturgeon sperm from experiment 2 using the cryoprotectant methanol

Cryoprotectant (%)	mHBSS	Extenders MT	OT
Percent motility			
5	11 ± 7^{a2}	18 ± 10^{a12}	13 ± 6^{a1}
10	8 ± 7^{b2}	8 ± 5^{b12}	11 ± 2^{b1}
15	3 ± 2^{b2}	7 ± 4^{b12}	8 ± 3^{b2}
Control*		73 ± 16	
Percent fertilization			
5	12 ± 10^{a2}	18 ± 11^{a1}	3 ± 7^{a12}
10	4 ± 5^{b2}	7 ± 11^{b1}	3 ± 3^{b12}
15	0 ± 1^{c2}	1 ± 2^{c1}	0 ± 1^{c12}
Control*		40 ± 22	
Percent hatch			
5	11 ± 12^{a2}	17 ± 12^{a1}	2 ± 2^{a12}
10	5 ± 6^{b2}	3 ± 5^{b1}	3 ± 4^{b12}
15	0 ± 0^{b2}	1 ± 2^{b1}	1 ± 1^{b12}
Control*		27 ± 13	
Percent viability			
5	10 ± 8^{a1}	5 ± 3^{a2}	9 ± 4^{a1}
10	7 ± 4^{a1}	5 ± 3^{a2}	9 ± 5^{a1}
15	8 ± 4^{a1}	5 ± 3^{a2}	7 ± 5^{a1}

Mean percentages of post-thaw motility, fertilization rate, hatching rate, and viability (±SD; n = 6 males). Values sharing a superscript letter within a column are not significantly different; values sharing a superscript number within a row are not significantly different ($\alpha = 0.05$).
OT, original Tsvetkova's extender; mHBSS, modified Hanks' balance salt solution extender; MT, modified Tsvetkova's extender.
*Control: fresh sperm used without extender or cryoprotectant.

In experiment 3 with paddlefish sperm, the highest post-thaw motility (85 ± 2%) and fertilization (80 ± 3%) percentages occurred using MT with 10% MeOH; however, the highest viability (59 ± 2%) was observed with mHBSS combined with 5% DMSO (Fig. 2). Statistically, post-thaw motility and viability were correlated, but independent of fertilization. The interaction of cryoprotectant and extender was significant at $P = 0.0011$, and the interaction of concentration and cryoprotectant was significant at $P = 0.0021$. For motility as well as viability using 10% methanol as the cryoprotectant, MT gave higher values than mHBSS extender at $P = 0.0125$. Using MT as the extender, 10% MeOH gave higher motility rates than did DMSO, at $P = 0.0125$. Viabilities using MeOH at 10% were significantly higher than at 5% MeOH ($P = 0.0125$). Overall for fertility, the control values were higher than those of MT which was higher than for mHBSS ($P = 0.0248$). The interaction of cryoprotectant and its concentration was significant for fertility ($P = 0.0025$), with all fertilization results generated with 10% MeOH higher than the other combinations, with 10% DMSO yielding significantly lower fertilization values with paddlefish sperm.

In experiment 4 using sperm from pallid sturgeon, the highest post-thaw motilities (70 ± 10%) and viabilities (58 ± 13%) were observed with MT combined with 10% MeOH, while the highest fertilization percentage at 4-cell stage (88 ± 6%) and at heartbeat stage (73 ± 14%) was found with MT combined with 5% MeOH (Fig. 3). Post-thaw motility was correlated with viability, and fertilization at cleavage was correlated with fertilization at heartbeat stage. For fertilization at the heartbeat stage, MT (72%) gave

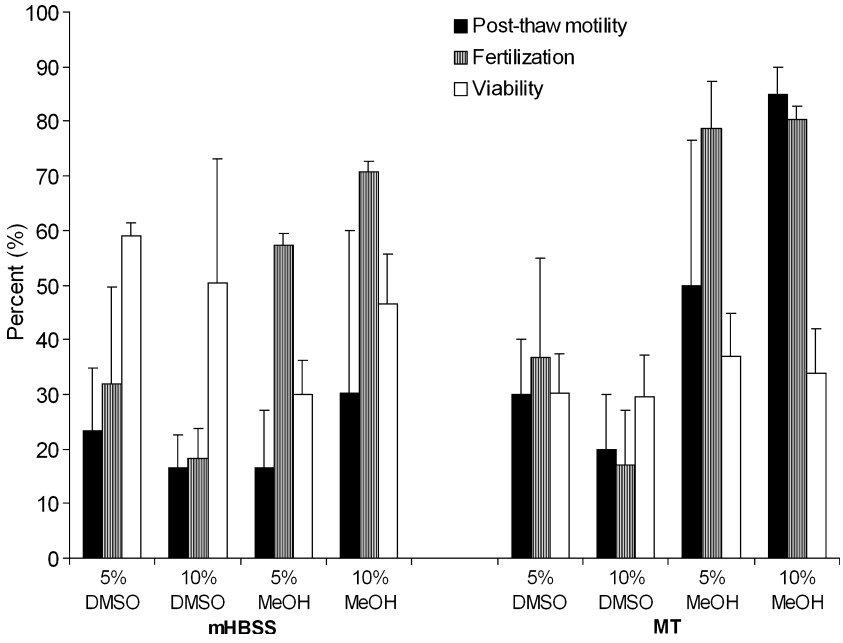

Fig. 2. Percentages of post-thaw motility, fertilization rates determined at 4-cell stage and viability (mean = columns; bars = ±SD) of cryopreserved paddlefish sperm (n = 3 males, 3 replicates per male) in experiment 3. MeOH, cryoprotectant methanol; DMSO, cryoprotectant dimethyl-sulfoxide; mHBSS, modified Hanks' balance salt solution extender; MT, modified Tsvetkova's extender; 5 and 10% represent cryoprotectant concentration. Control fertilization rate at 4-cell stage: 85 ± 4%

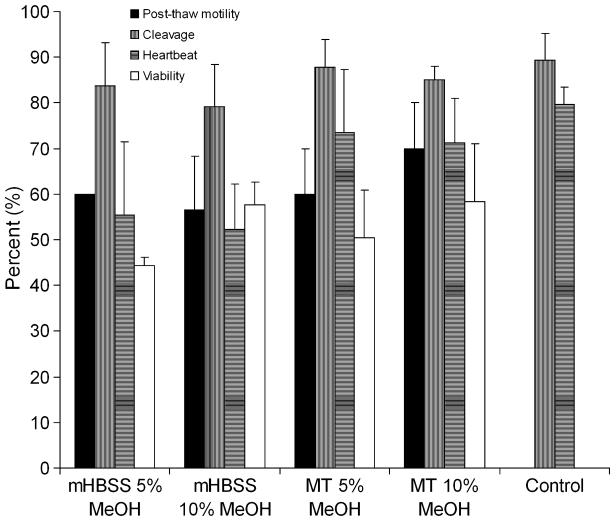

Fig. 3. Percentages of post-thaw motility, fertilization rate determined at 4 cell stage (cleavage), at heartbeat stage [at stage 34 (tail reaching the tip of the head) according to Dettlaff et al. (1993)] and viability (membrane integrity; columns = means, bars = ±SD) of cryopreserved and fresh pallid sturgeon sperm (n = 3 males, 2 replicates per male). MT, modified Tsvetkova's extender; mHBSS, modified Hanks' balance salt solution extender; MeOH, cryoprotectant methanol; 5 and 10% represent cryoprotectant concentration. All abbreviations as in Fig. 2

significantly higher overall percentage than mHBSS (54%; P = 0.0072). Motilities were significantly higher with MT (46%) than with mHBSS (22%; P = 0.0056), but no significant differences were seen with viability percentages and extender type.

Discussion

The integrity of the plasma membrane of spermatozoa is required to maintain fertilizing capacity (Graham et al., 1990). In one of the few studies on semen characteristics in a natural population, sperm viability was not associated directly with male fertility because cell viability is so essential for fertilization that males with lower values have been intensively selected against in natural populations (Malo et al., 2005). However, the processes occurring during sperm cryopreservation can cause traumatic injury to cell membranes, where negative impacts have been demonstrated in several studies either on the percentage of cells with intact membranes (Cabrita et al., 1998) or on the structure of the cell membranes (Drokin et al., 1998). Therefore, studies of membranes are useful in cryopresevation science.

In the present study, cryopreserved sperm of shortnose sturgeon showed low percentages of intact membranes (5–12%), independent of the choice of extender, cryoprotectant or its combination. These values reflect our earlier observations where the post-thaw motility and fertilization rates were also low (Horváth et al., 2005). Sperm viability data obtained from the cryopreserved pallid sturgeon (40–70%) and paddlefish (21–77%) showed higher percentages of membrane-intact post-thaw spermatozoa than the shortnose sturgeon, where the fertilization percentages were generally much higher in the other two species, as well. A similar trend was also observed among these species with post-thaw motilities and fertilization rates (Horváth et al., 2005, 2006), whereby values for the shortnose sturgeon were lower. Thus, these observations confirm the hypothesis that membrane integrity is a key factor in preserving the fertilizing capacity of cryopreserved spermatozoa.

However, results also show that membrane integrity was in most cases correlated with post-thaw motility yet was sometimes independent of fertilitization. In a study with Siberian sturgeon (*Acipenser baerii*), post-thaw motility did not predict fertilization success (Glogowski et al., 2002). Fertilization with sturgeon and paddlefish sperm largely depends on the extender and cryoprotectant used. It has been demonstrated in our previous studies on these species that extenders and cryoprotectants that are iso-osmotic to the milt plasma of sperm resulted in significantly higher fertilization and hatching rates without having an effect on post-thaw motility (Horváth et al., 2005, 2006). The cryoprotectant DMSO is known to increase the osmolality of the basic extender (Ogier de Baulny et al., 1997) whereas MeOH

results in a slight decrease of osmolality (Tiersch et al., 1994). Similarly, the osmolality of the extender OT (204 mOsmol kg^{-1}) was almost three times of that of MT extender (73 mOsmol kg^{-1}). Extender mHBSS was deliberately diluted to an osmolality of 100 mOsmol kg^{-1}. Various combinations of extenders with concentrations of cryoprotectant result in different osmolarities. Following the general pattern of our results on shortnose sturgeon hyperosmotic conditions (the use of DMSO or OT extender) resulted in higher post-thaw motility and viability but in lower fertilization, neurulation or hatch percentages. Results on paddlefish contradict this pattern, although the use of extenders and cryoprotectants with lower osmolality resulted in significantly higher fertilization in this species, too. In the pallid sturgeon, even the slight difference in osmolalities between MT and mHBSS extenders resulted in a higher percentage of live embryos at heartbeat stage without having an effect on post-thaw motility or viability. These results suggest that spermatozoa survive cryopreservation retaining most of their cellular functions such as motility and membrane integrity, however, favorable osmotic conditions of the extender and croprotectant are required for fertilization.

Spermatozoa of sturgeons and paddlefishes are unique among the male gametes of fish in several respects. They possess a functioning acrosome which is absent from those of other fish species. Although several details of the acrosome reaction – such as the effect of a 66 kDa glycoprotein present in the egg envelope upon contact with water in triggering acrosome reaction – have been clarified (Cherr and Clark, 1985) the exact role of this acrosome in the fertilization process of sturgeon eggs remains unclear. Similarly, to the best of our knowledge, there have been no studies on the impact of cryopreservation and osmolality on the acrosome of sturgeon and paddlefish sperm. Selective staining of the acrosome by use of lectins (D'Cruz and Haas, 1996), and investigation into calcium exchange within sperm cell compartments would provide further information for the better understanding of the effect of cryopreservation methods on the fertilizing capacity of sperm in the investigated species.

In this study, the viabilities of the paddlefish sperm were generally higher than the motilities, except when using methanol at 5 and 10%. This is different from the pallid sturgeon results, where motilities were about equal between the two parameters. Therefore, the combinations of extenders and cryoprotectants used with paddlefish appear to reduce motility which is fueled by mitochondria. The use of a mitochondrial assay to see the potential increases in function with treatments may be particularly useful for paddlefish sperm cryopreservation. Generally, the results obtained with the pallid sturgeon are good.

The present study suggests that membrane integrity can be a useful tool for indication of the survival of cryopreservation in sturgeons and paddlefish but should be used with caution for the prediction of fertilization success in the development of cryopreservation methods.

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