ELSEVIER

CRYOPRESERVATION OF CHANNEL CATFISH SPERMATOZOA: EFFECT OF CRYOPROTECTANT, STRAW SIZE, AND FORMULATION OF EXTENDER

J. M. Christensen and T. R. Tiersch

School of Forestry, Wildlife, and Fisheries
Louisiana Agricultural Experiment Station
Louisiana State University Agricultural Center
Baton Rouge, LA 70803 USA

Received for publication: April 4, 1995
Accepted: May 17, 1996

ABSTRACT

Various aspects of the cryopreservation of spermatozoa of channel catfish (Ictalurus punctatus) were studied in relation to spermatozoa motility. The objectives were to evaluate 1) the efficacy of 5, 10 and 15% of methanol or n,n-dimethyl acetamide (DMA) as cryoprotectants; 2) the acute toxicity of 5, 10 and 15% methanol or DMA; 3) the use of 0.5-ml vs. 0.25-ml straws; 4) the efficacy of 5, 10 and 15% of methanol in Hanks' balanced salt solution (HBSS) or HBSS without glucose, and 5) the use of HBSS with or without 5% methanol. We found that use of 5% methanol as a cryoprotectant resulted in significantly higher post-thaw motility ($P = 0.0001$) than did 5, 10 or 15% DMA. The use of 5% of either cryoprotectant resulted in significantly higher post-thaw motility ($P = 0.0001$) than did 10 or 15% of the cryoprotectants. Samples containing 10 or 15% DMA had significantly lower motility ($P = 0.0001$) after 30 min exposure than did samples containing 5, 10 or 15% methanol. The use of 0.25-ml straws resulted in significantly higher post-thaw motility ($P = 0.0001$) than that of 0.5-ml straws. No difference was found in post-thaw motility between HBSS with and without glucose as the extenders. Cryopreservation in HBSS without addition of cryoprotectant resulted in post-thaw motility values of about 1%.

Key words: teleost, Ictalurus punctatus, methanol, n,n-dimethyl acetamide, glucose

INTRODUCTION

The cryopreservation of spermatozoa is an important tool for genetic improvement of livestock animals, including beef cattle, dairy cattle, swine and poultry. Successful cryopreservation of spermatozoa from improved livestock has, in turn, allowed these industries to greatly increase production capabilities. In the 50 yr that cryopreservation and artificial insemination have been employed, various livestock industries such as that of dairy cattle have come to rely heavily on artificial insemination using cryopreserved semen (7).

However, the use of cryopreservation as a means for genetic improvement in aquaculture is still in its infancy. The cryopreservation of salmonid spermatozoa has been studied for about 25 yr (13), but the use of cryopreservation for genetic improvement of commercial stocks has not been implemented. Numerous studies have shown that cryopreservation of salmonid spermatozoa (12)

Acknowledgments: This manuscript was approved for publication by the Director of the Louisiana Agricultural Experiment Station as manuscript number 94-22-8402. Supported in part by a grant from the Louisiana Catfish Promotion and Research Board, and by USDA special grant 93-34310-9057. We thank Tony's Seafood of Baton Rouge for providing catfish, and M. Bates and W. Wayman for assistance in collection of samples. We thank D. Culley, R. Malone, and C. Wilson for critical review.

0093-691X/97/$17.00
PII S0093-691X(97)00022-8

and clariid spermatozoa (11) has been optimized to provide fertilization success comparable to that of fresh spermatozoa; thus, commercial application may be feasible in the near future. Increased coordination of industry needs and research efforts will be required before commercial application of cryopreservation techniques can occur (16).

The farming of channel catfish (Ictalurus punctatus) is the largest food fish aquaculture industry in the United States, and yet there has been little published on the refrigerated storage and cryopreservation of spermatozoa of this species. Guest et al. (6) reported on the cryopreservation of channel catfish spermatozoa with glycerol, dimethyl sulfoxide (DMSO), and propylene glycol as the cryoprotectants. They found that DMSO resulted in the highest final motility after thawing. Tiersch et al. (14) studied several cryoprotectants, including methanol and DMSO, and reported on the production of channel catfish by artificial fertilization using cryopreserved spermatozoa. They found that methanol resulted in higher final motility after thawing than did DMSO.

The purpose of this study was to advance earlier research by evaluating various cryoprotectants, straw sizes, and formulations of extenders for use in the cryopreservation of channel catfish spermatozoa. Gallant and McNiven (4) reported n,n-dimethyl acetamide (DMA) to be significantly better than DMSO as a cryoprotectant for rainbow trout (Oncorhynchus mykiss) spermatozoa, and to our knowledge DMA has not been tested as a cryoprotectant in other fish species. Straw sizes of 0.5 and 0.25 ml are used widely for the cryopreservation of mammalian spermatozoa (9). The effect of straw size on cryopreservation is variable, and must be studied on a species-by-species basis. Hanks' balanced salt solution (HBSS) was used an extender by Tiersch et al. (14), and Christensen and Tiersch (2) observed that HBSS without glucose provided longer refrigerated storage times than did complete HBSS. Thus, by analysis of motility of channel catfish spermatozoa, the objectives of this study were to evaluate: 1) the efficacy of 5, 10 and 15% methanol or DMA as cryoprotectants; 2) the acute toxicity of 5, 10 and 15% of methanol or DMA; 3) the use of 0.5 or 0.25-ml straws; 4) the efficacy of HBSS with or without glucose as the extender; and 5) the use of HBSS with or without 5% methanol for cryoprotection.

MATERIALS AND METHODS

Spermatozoa Collection and Motility Estimation

Healthy, mature channel catfish (1.5 to 3 kg and 40 to 60 cm) were killed by overdose of tricaine methanesulfonate anesthetic (Argent Laboratories, Inc., Redmond, WA) or by electrical shock. The testes were removed, placed in HBSS (14), and kept chilled (4°C) until processing. The testes were crushed within 6 h to release spermatozoa into extender solutions. Spermatozoa were suspended at 1 g of testis/20 ml HBSS (or HBSS prepared without glucose), placed in plastic beakers covered with paraffin film, and refrigerated at 4°C. To insure sufficient osmotic pressure to prevent activation of spermatozoa during storage, the final osmolality of all solutions was adjusted to 295 mOsm, as measured with a vapor pressure osmometer (Model 5500, Wescor, Inc., Logan UT; 1).

Motility and fertility are currently the only evaluation techniques available for routine use with fish spermatozoa (5). Because channel catfish spawn only once a year but produce spermatozoa year round, sperm motility was chosen as the evaluation parameter so that evaluations could proceed throughout the year. Motility was estimated by placing a 5-µl sperm sample onto a glass microscope slide, activating with 50 µl of distilled water and viewing immediately at x100 magnification with dark-field microscopy. Only samples with motility of ≥50% were used for experimentation. The motility of each sample was estimated immediately before freezing (initial motility) and immediately after thawing (final motility).

Freezing and Thawing Procedures

Solutions of 50% HBSS and 50% cryoprotectant were used for addition of cryoprotectant to samples. Straws (IMV International Corp., Minneapolis, MN) were plugged with standard PVC powder (solidified by immersion in HBSS) or steel balls (Minitube of America, Madison, WI). Preliminary studies showed no difference between sealing with powder or balls (data not shown). The straws were placed in a computer-controlled freezer (Planar Kryo 10, Middlesex, England), where the temperature was held at 4°C for 10 min, lowered to -80°C at -40°C/min, and held at -80°C for 20 min. The samples were removed from the freezer and plunged into liquid nitrogen (-196°C) for storage. The straws remained frozen for at least 24 h prior to thawing.

For thawing, the straws were removed from liquid nitrogen and plunged immediately into a water bath (40°C) for 10 sec. The straws were dried, and clipped with scissors to release spermatozoa into microcentrifuge tubes.

Comparison of 3 Concentrations of Methanol or DMA, and 2 Straw Sizes

Methanol (Fisher Chemical Co., Fair Lawn, NJ) and DMA (Sigma Chemical Co., St. Louis, MO) were compared at concentrations of 5, 10 and 15%. Standard 0.5- and 0.25-ml straws were compared. Sperm suspensions from each of 4 males were removed from 4°C storage, and 2-ml samples were distributed into plastic petri dishes. Diluted (50%) methanol or DMA was added to each sample to produce the desired final concentration. The samples were drawn into straws and allowed to equilibrate for <15 min prior to freezing.

Analysis of Acute Toxicity of Methanol and DMA

Based on the results of the experiment above, we evaluated acute toxicity of the cryoprotectants (prior to freezing) as a source of reduced sperm motility. Sperm suspensions from each of 3 fish were distributed into plastic petri dishes, and methanol or DMA was added to produce final concentrations of 5, 10 or 15%. Motility was estimated for each sample before addition of methanol or DMA, and every 10 min for 30 min after addition of the cryoprotectant.

Comparison of 3 Concentrations of Methanol, and HBSS With or Without Glucose

Spermatozoa were cryopreserved in 5, 10 or 15% methanol in HBSS with or without glucose. Spermatozoa from each of 8 males were suspended in HBSS with or without glucose and stored at 4°C. Sperm suspensions were removed from 4°C, and 2-ml samples were distributed into plastic petri dishes. Methanol was added to each sample to produce final concentrations of 5, 10 or 15%. The samples were drawn into 0.5-ml straws and allowed to equilibrate for <15 min prior to freezing.

Comparison of No Added Cryoprotectant and Cryoprotection With 5% Methanol

As a control treatment, we compared freezing of semen in HBSS without added cryoprotectant with freezing in HBSS plus 5% methanol. Spermatozoa suspensions from each of 8 males were removed from 4°C storage, and 2-ml samples were distributed into plastic petri dishes. Methanol was added to each sample to produce a final concentration of 5%. The samples were drawn into 0.5-ml straws and allowed to equilibrate for <15 min prior to freezing.

Statistical Analyses

Statistical analyses were performed using SAS Statistical Analysis Software for IBM (SAS Institute, Cary, NC). Values for the final percentage of motility were arcsin square-root transformed. Treatment groups were analyzed using analysis of variance for comparison of the

final (post-thaw) percentage of motility. Specific differences between treatment groups were analyzed by Fisher least significant difference test. Cryoprotectant, concentration, and straw size were analyzed in a 2x3x2 factorial arrangement including all interactions for comparison of methanol or DMA at 5, 10 or 15% and of 0.5 and 0.25-ml straws. Concentrations and extenders were analyzed in a 3x2 factorial arrangement for comparison of 5, 10 and 15% methanol and HBSS with or without glucose. A P value of < 0.05 was considered to be significant.

RESULTS

The use of methanol as a cryoprotectant resulted in significantly higher final motility ($P = 0.0001$) than did DMA (Table 1). A concentration of 5% of either of the cryoprotectants resulted in significantly higher final motility ($P = 0.0001$) than did a concentration of 10 or 15% (Table 1), and the use of 0.25-ml straws resulted in significantly higher final motility ($P = 0.0007$) than that of 0.5-ml straws (Table 1). The interaction of cryoprotectant and concentration ($P = 0.0001$); cryoprotectant and straw size ($P = 0.0129$); concentration and straw size ($P = 0.0006$); and cryoprotectant, concentration and straw size ($P = 0.0070$) were all significant.

Table 1. Comparison of channel catfish spermatozoa frozen in 2 cryoprotectants at 3 different concentrations and in 2 different straw sizes. The use of methanol resulted in significantly higher final motility ($P = 0.0001$) than did the use of DMA. The use of 5% of either cryoprotectant resulted in significantly higher final motility ($P = 0.0001$) than did the use of 10% or 15%. The use of 0.25-ml straws resulted in significantly higher final motility ($P = 0.0007$) than did the use of 0.5-ml straws. Each value represents the mean ± SD of 4 fish. Initial motility was 55 ± 5% in all samples.

Cryoprotectant	Straw size	Concentration	Percent motility
Methanol	0.25 ml	5%	26.3 ± 4.8%
		10%	7.8 ± 4.5%
		15%	0.5 ± 0.6%
	0.5 ml	5%	8.0 ± 9.0%
		10%	5.5 ± 5.1%
		15%	0.8 ± 0.5%
DMA	0.25 ml	5%	3.0 ± 2.3%
		10%	1.7 ± 2.2%
		15%	0.3 ± 0.5%
	0.5 ml	5%	0.5 ± 0.6%
		10%	0.8 ± 0.5%
		15%	0.3 ± 0.5%

Sperm samples containing 10 or 15% DMA had significantly lower motility ($P = 0.0001$) after 30 min than did samples containing 5, 10 or 15% methanol (Table 2). The motility of spermatozoa cells after 30 min in 15% DMA was as low as 1% (reduced from 70%).

Table 2. Percent motility of spermatozoa suspended for as long as 30 min in Hanks' balanced salt solution containing 5%, 10%, or 15% of methanol (MeOH) or n,n-dimethyl acetamide (DMA). Spermatozoa samples containing 10% or 15% DMA had significantly lower motility (P = 0.0001) estimated after 30 min than did samples containing 5%, 10%, or 15% MeOH. Each value represents the mean ± SD of 3 fish. Initial motility (0 min) was 53 ± 6% for all treatments.

Cryoprotectant	Concentration	10 min	20 min	30 min
MeOH	5%	53 ± 6%	57 ± 12%	57 ± 12%
	10%	50 ± 0%	46 ± 6%	53 ± 6%
	15%	50 ± 10%	46 ± 6%	36 ± 21%
DMA	5%	53 ± 6%	52 ± 8%	53 ± 6%
	10%	48 ± 3%	47 ± 7%	37 ± 6%
	15%	31 ± 7%	20 ± 10%	1 ± 0%

No difference was found between extenders with or without glucose (P = 0.1805). Concentrations of 5 and 10% methanol resulted in significantly higher final motility (P = 0.0001) than did a concentration of 15% methanol. A significant interaction (P = 0.0138) was found between extender and the concentration of methanol (Table 3).

Table 3. Percent motility of thawed spermatozoa of channel catfish. Spermatozoa were suspended in Hanks' balanced salt solution (HBSS) or HBSS without glucose, and cryopreserved using 5%, 10%, or 15% methanol. No difference was found between the 2 HBSS formulations. The use of 5% or 10% methanol resulted in significantly higher final motility (P = 0.0001) than did the use of 15%. Each value represents the mean ± SD of 8 males. Initial motility was 70 ± 13% in all samples.

Concentration	Extender		Pooled (by concentration)
	HBSS	HBSS no glucose	
5%	35 ± 17%	30 ± 15%	33 ± 16%
10%	38 ± 18%	18 ± 10%	27 ± 18%
15%	5 ± 5%	10 ± 7%	7 ± 7%
Pooled (by extender)	25 ± 20%	19 ± 13%	---

The use of 5% methanol resulted in significantly higher final motility (P = 0.0001) than did the absence of cryoprotectant (Figure 1); however, even without cryoprotectant, final motility values of ~1% were observed.

DISCUSSION

Gallant and McNiven (4) found that 10 or 12% DMA was significantly better than 10 or 12% DMSO for cryoprotection of rainbow trout spermatozoa. In our study, we found DMA to be more toxic and less effective than methanol as a cryoprotectant for channel catfish spermatozoa. This lower effectiveness could be due to the toxic effects of DMA or to the lower cryoprotective abilities of DMA in channel catfish spermatozoa.

Straw size is an important factor in the cryopreservation of bull spermatozoa. Senger et al. (9) found that the use of 0.5 or 0.25-ml straws affected the success of cryopreservation but that the effect was dependent on the cooling rate and on the extender. The cattle industry has adopted the 0.5-ml straw as the standard size because of its ease of use. However, we found that the use of 0.25-ml straws resulted in higher final motility than 0.5-ml straws in the cryopreservation of channel catfish spermatozoa. This effect is likely due to differences in the freezing and thawing rates of spermatozoa in these straws (8). For example, faster freezing of spermatozoa in 0.25-ml straws could reduce the dehydration of cells. The 0.25-ml straw also consumes less space in storage, by reducing the void volume between straws. However, at present, given the availability of equipment through the cattle industry for automated straw packaging, the 0.5-ml straw may be more advantageous for use with channel catfish spermatozoa.

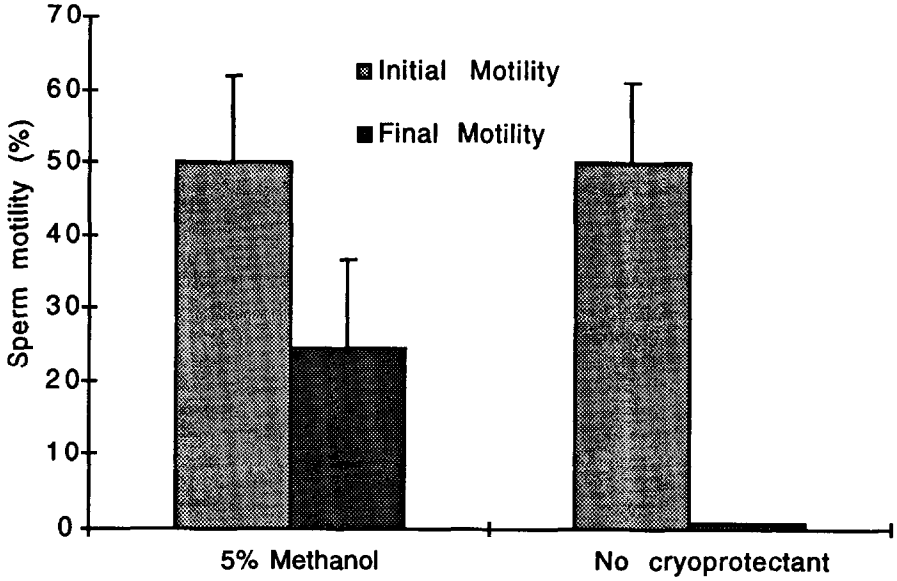

Figure 1. Initial (pre-freeze) motility and final (post-thaw) motility of channel catfish spermatozoa cryopreserved in Hanks' balanced salt solution with 5% methanol as the cryoprotectant or without additional cryoprotectant. The use of 5% methanol resulted in significantly higher final motility ($P = 0.0001$) than did the absence of methanol. Each column represents the mean ± SD of 8 males.

Christensen and Tiersch (2) observed that spermatozoa suspended in HBSS without glucose could be stored at 4°C for a longer period of time than spermatozoa suspended in complete HBSS. In our present study, we found no difference in the cryoprotective abilities of HBSS with and without glucose. Thus, the use of HBSS without glucose as a standard extender formulation for channel catfish spermatozoa could increase refrigeration storage time without affecting subsequent cryopreservation. Some motility (~1%) was observed after cryopreservation of channel catfish spermatozoa in HBSS without addition of cryoprotectant, suggesting that the extender formulation possesses some cryoprotective properties. Different extender formulations may increase the final motility of cryopreserved channel catfish spermatozoa, and further study is needed to improve extenders and to develop procedures for commercial-scale application of the cryopreservation of channel catfish spermatozoa.

REFERENCES

1. Bates MC, Wayman WR, Tiersch TR. The effect of osmotic pressure on activation and storage of channel catfish sperm. Trans Amer Fish Soc 1996; 125:798-802.
2. Christensen JM, Tiersch TR. Refrigerated storage of channel catfish sperm. J World Aqua Soc 1996; 27:340-346.
3. Cloud JG, Miller WH, Levanduski MJ. Cryopreservation of sperm as a means to store salmonid germ plasm and to transfer genes from wild fish to hatchery populations. Prog Fish Cult 1990; 52:51-53.
4. Gallant RK, McNiven MA. Cryopreservation of rainbow trout spermatozoa. Bull Aqua Assoc Can 1991; 3:25-27.
5. Graham EF. Cryopreservation and fertility. Proceedings of the 10th technical conference on artificial insemination and reproduction. Nat Assoc Anim Breeders 1984; 4-29.
6. Guest WC, Avault JW, Roussel JD. Preservation of channel catfish sperm. Trans Amer Fish Soc 1976; 105:469-474.
7. Herman HA. Improving Cattle by the Millions: NAAB and the Development and World Wide Application of Artificial Insemination. Colombia, MO: University of Missouri Press, 1981.
8. Jamieson GM. Fish Evolution and Systematics: Evidence From Spermatozoa. New York: Cambridge University Press, 1991.
9. Senger PL, Mitchell JR, Almquist JO. Influence of cooling rates and extenders upon post-thaw viability of bovine spermatozoa packaged in 0.25- and 0.5-ml French straws. J Anim Sci 1983; 56:1261-1268.
10. Smith LW. Projections of future productive efficiency. In: Putnam, PA, (ed) Handbook of Animal Science. New York: Academic Press, 1991; 385-396.
11. Steyn GJ, Van Vuren JHJ. The fertilizing capacity of cryopreserved sharptooth catfish (Clarius gariepinus) sperm. Aquaculture 1987; 63:187-193.
12. Stoss J. Fish gamete preservation and spermatozoan physiology. In: Hoar, WS, Randall, DJ, Donaldson, EM (eds) Fish Physiology, Behavior and Fertility Control, Volume IX, Part B. New York: Academic Press, 1983; 305-351.
13. Stoss J, Buyukhatipoglu S, Holtz W. Short-term and cryopreservation of rainbow trout (Salmo gairdneri Richardson) sperm. Ann Biol Anim Bioch Bioph 1978; 18:1077-1082.
14. Tiersch TR, Goudie CA, Carmichael GJ. Cryopreservation of channel catfish sperm: storage in cryoprotectants, fertilization trials, and growth of channel catfish produced with cryopreserved sperm. Trans Amer Fish Soc 1994; 123:580-586.
15. Tucker CS, Robinson EH. Channel catfish farming handbook. New York: Van Nostrand Reinhold, 1990.
16. Wolters W. Channel catfish breeding and selection programs: constraints and future prospects. In: Main, KL and Reynolds, E (eds) Selective Breeding of Fishes in Asia and the United States. Honolulu: The Oceanic Institute, 1993; 82-95.