J. Appl. Ichthyol. 22 (Suppl. 1) (2006), 384-388
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ISSN 0175-8659

Received: September 14 2005
Accepted: March 18, 2006

Cryopreservation of Chinese sturgeon *(Acipenser sinensis)* sperm

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Summary

This investigation studied the effectiveness of techniques to collect sperm from Chinese sturgeon males and developed examination methods fon the viability of sperm. Further, a series of experiments tested a variety of sperm diluents while also selecting suitable cryoprotectants and its concentration, determined optimal equilibrium time, cooling rate and freezing point. Additionally, the effects of the cryo-preservation methods on the viability of sperm were tested while also determining the fertilization success with sperm cryo-preserved with three different diluents. The results indicated that the diluent D-5 (8.85g/L NaCl; 0.20g/L and 0.40g/L $NaHCO_3$) was the best sperm diluent, and 12% DMSO was a suitable cryo-protectant keeping 0.5-2h as the equilibrium time at 4. Combining D-5 with DMSO under the described conditions seems to be the most suitable method for sturgeon sperm cyro-proservation while employing the cooling rate at 2/min and freezing point at -6 keeping the sperm for 10 min before placing into liquid nitrogen. Fertilization tests, D-3, D-5 and D-6, (whose effects were similar in the diluents tests), were used to compare the effects of cryo-preserved sperm on fertilization success. The results indicated that the effect of D-5 in thawed sperm fertilization was the best among all three diluents having the least negative effect. The resulting in a fertilization rate was 83.8% and survival rate of larvae reached up to 68.1%. However, comparing with the control, the survival rate of D-5 was still noticeably smaller. The reason needs to further study.

Introduction

The study aimed at determining optimum conditions for cryo-preservation of sperm of the Chinese sturgeon in order to obtain high survival and good fertilization success after long-term storage. There is a need to develop methodologies that assist in sturgeon conservation in particular cases where brood stocks are small and dwindling and also genetic diversity is endangered.

There have been many successful reports on the sperm cryo-preservation of fish since Mounib began to study the sperm cryo-preservation of coalfish in the 1950s (Mounib, 1978; Cheng et al., 1992; Fabbrocini et al., 2000; Babiak et al, 2000;). However, few studies are known to demonstrate the cryo-preservation of sturgeon sperm in general (Brown et al., 1998; Ciereszko et al., 1996; Cosson, 1997; Gallis et al, 1989; Ingermann et al, 2002; Linhart et al , 2002; Urbanyi et al., 2001), and large-scale, rare fish preservation techniques, especially for Chinese sturgeon are lacking. The declining population of the Chinese Sturgeon requires urgent measures for conservation, especially after completion of the Gezhouba Dam (Chang, 1999; Wei, 2003). One option is the artificial reproduction, however, the problem is invariably met by the lack of mature gonads of males and females which could not always be guaranteed to be available at the same time while artificial fertilization was intended. At least half of the problem can be solved if sperm could be preserved for immediate demand. The technology of sperm cryo-preservation had been successful employed in many fish species (Chao, 1987; Zhang, 1994; Ji, 2005; Jaehnichen et al., 1999; Tsvetkova et al 1996). Therefore, the objectives of this study were (a) to explore a useful recipe of a diluent suitable for Chinese Sturgeon sperm; (b) to develop a suitable diluent / cryoprotectant combination which also optimize the equilibrium time to deep freezing; (c) to determine an instant freezing standard before transferring the sperm to liquid nitrogen for long-time storage; and (d) to test the effectiveness of cryo-preserved sperm by fertilizing fresh eggs and monitoring the survival rate of fry.

Materials and Methods

Collection of spermatozoa and its assessed standard

The males for the experiment were caught from the naturally migrating spawning stock in the Yangtze River in the section below the Gezhouba Dam (between 1998 and 2000). At the riverside, immediately after catch the males were artificially induced for spermiation with LHRH-A (Lutinizing hormone releasing hormone A) and HCG (human chorionic gonadotropin) (Ningbo, Hormone Product Co., Ltd., China). When these males began to spermiate, the abdomen was extruded in order to collect their fresh milt. The fresh milt was put in a small hermetic plastic bag with oxygen and placed on ice and transferred to the laboratory. In the laboratory, the motility of the fresh sperm was assessed immediately under a microscopy (magnification: 4010). Only the milt from samples which showed motility over ++++ 90% was chosen for the experiments in this study. The sperm of two males was obtained in 1998, four males in 1999, and three males in 2000. The fresh milt was stored at 4 in a refrigerator prior to the beginning of the tests.

During the entire experiment, the sperm motility was evaluated by using 5 categories, as follows:

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(1) Drastic and extreme rapid movement: the path of sperm motion was so fast that it was impossible to clearly follow individual sperm under the 40x10 times microscope magnification. This motility level was denoted with ++++ while the number of active spermatozoa visible in the microscope field were estimated (%). These estimates were all made by the same observer so that the subjective error was always the same and the countings were comparable.
(2) Fast movement: the speed of moving sperm is very fast under microscope field at 40x10 magnification, but their path can be clearly seen. These movements were recorded as +++ and the active numbers were given.
(3)Slow movement: the speed of moving sperm is very slow but clearly noticable. These observations are recorded as ++ with the percentage of visible sperm in the observation field being determined.
(4) Vibration: sperm does not move forward but its tail shows right-and-left vibrations. This activity is expressed as + x% (with the percentage of vibrating sperms determined.
(5) Motionless: most sperm do not show any movement. This situation is expressed in the records by a negative sign (-).

Selection of the sperm diluents

In our preliminary tests, a total of 38 diluents were selected for evaluation as potential extenders for Chinese sturgeon sperm. However, Table 1 includes only the components and concentrations of 6 diluents, because they can represent the components of others while the results of most others were not satisfactory.

For selecting the most suitable diluent, semen and diluent were mixed at a ratio of 1:3 (vols semen: vols diluent) and placed into 2.5 ml small plastic vials. After a rapid shaking, the vials were put into a 4 refrigerator. Some mixtures in each vial were taken out from the refrigerator to assess the sperm motility under the microscope at this was done in 12 hrs time intervals for a total period of 86 hrs.

Cryoprotectants and suitable equilibrium time

DMSO (dimethyl sulfoxide) and glycerol were initially tested as potential cryoprotectants. The semen and the best diluent selected from Table 1 were mixed at a ratio of 1:3 (vol semen: vol diluent) and placed into 2.5 ml plastic vials and the total volume of the suspension was 2ml. The final concentration of the cryoprotectants added in each suspension was 8%, 10%, 12%, and 14% DMSO or 3%, 4%, 5% and 6% glycerol, respectively. All suspensions with added cryoprotectant were placed into a refrigerator at 4 to test the suitable equilibrium time by comparing sperm motility in fixed time intervals. At 0.5, 1, 2, 4, 6 and 8 hours after the beginning of the test, some samples were taken from the vials for assessing the sperm motility under the microscope. First the samples were prepared for observation by adding 0.4%NaCl to dilute the protectant and then freshwater was added to stimulate sperm motility while the mixture was microscopically assessed.

Cyropreservation and thawing of sperm

For testing the speed of the cooling rate, the microcomputer controlled low-temperature apparatus (Low-temperature Bioengineering laboratory of Shanghai Traffic University) was used to control the different cooling rates. According to the results of all above tests, the most suitable diluent / cyroprotectant mixture was selected in all tests. First, the sperm was mixed with the mixture at a ratio of 1:3 sperm/medium in 2.5 ml plastic vials containing in total 2 ml of suspension. Then, the suspensions were placed at 4 in a refrigerator for the most suitable equilibrium time, which also was previously determined from the results of above described initial tests. Last, sperm vials were cooled in the computer-controlled freezer from 4 to -6, -10, and -14 at a rate of 1.5/min, 2/min, 2.5/min and 3/min, respectively. After keeping the samples for 10 min at these temperatures, all treatment vials were placed directly into liquid nitrogen.

After 24 hours the deep frozen sperm samples were thawed by quickly immersing them in a water bath at 32 under constant shaking for about 2 min. Thereafter, 0.4% NaCl was added for diluting the thawed samples, and freshwater was added in excess to stimulate the sperm motility immediately before microscopic observation and assessment.

Fertilization tests

Based on the above results, the procedures and conditions have been adopted for continuation of the study. However, the best effective diluent at 4 may not necessarily be the best one to produce good results after preserving sperm in liquid nitrogen, and this suspicion is according to our experience with other fish. Therefore, in 2001, three additional suitable diluents (including the best one of the previous series) were selected to cryo-preserve the sperm for the subsequent fertilization tests and to compare their fertilization effects. The conditions (including the concentration of cryoprotectants, equilibrium time, and cooling rate) and the methods of cyro-preservation were the most suitable in the above tests. The time to keep cryopreserved sperm ranged from 24 hours to 15 days after which the fertilization tests were performed.

Table 1
Determining the effectiveness of various diluents for Chinese sturgeon sperm. Composition is given in terms of concentration of chemical components (g/L). The various diluents are denoted as D-1 to D-6 as they were used in the study.

Diluent identification	The components of the diluents (g/L)						
	NaCl	KCl	$CaCl_2$	$MgCl_2$	$NaHCO_3$	Glucose	EDTA-Na
D-1	8.75	0.20	0.20	-	0.30	-	-
D-2	8.75	0.20	-	0.20	0.40	-	-
D-3	7.25	0.40	-	-	0.80	2.0	-
D-4	8.75	0.20	0.10	0.10	0.40	-	-
D-5	8.85	0.20	-	-	0.40	-	-
D-6	8.75	0.20	-	-	0.40	-	0.10

The females for the tests also were caught from the naturally migrating spawning stock in the Yangtze River along the section below the Gezhouba Dam. All three females were induced for spawning with LHRH-A and HCG. When these females began to ovulate, the abdomen was slightly extended to collect their ova. The ova were placed into a small hermetic plastic bag with oxygen placed on ice (about 4) and transferred to lab in the same manner as described for previous trials above.

Fertilization tests were performed using two vials of cyropreserved sperm to fertilize about 200 eggs in each batch. The thawed sperm was immediately added to the eggs together and simultaneously mixed with 0.4%NaCl mixed. After thorough mixing with a goose feather for about 10 sec, water was added and after 5 minutes the eggs were rinsed and laid out in divided incubation trays. The water was changed twice every day, and at each changing time the dead eggs were taken out and their numbers was recorded. After hatching of the embryos, the number of fry were counted in each group. The viability of fry was determined as average from replicates.

Results

The diluents influence on sperm motility

Table 2 shows the sperm motility for 6 diluents at different times during the experimental observation period. The effects of D-5 preserved sperm seems to perform best, and results are most closely to those obtained for the control group. The results obtained with the D-3 and D-6 groups are also close to those of the controls, however, the longevity of their sperm active life (the terms of the time sperm movement occurred after stimulation) was shorter than that obtained with the D-5 series. Therefore, the D-5 diluent was settled to be the best for use in the following trials described below which were designed to evalutate cryoprotectant performance, equilibrium time and cooling rates. Besides D-3, D-5 and D-6, the effects of D-1, D-2, D-4 and other groups in the experiments were not considered satisfactory for sperm cyroproservation and were not used in the subsequent trials. The sperm motility in these diluents descended very fast comparing with the controls.

In the experiments it was also found that the sperm became fragile and could break when the diluents contained calcium ions, and the sperm could vibrate ceaselessly when the diluents contained magnesium ions. The higher the concentrations of calcium ions in the diluents were, the higher was the number of observed broken sperms. In the case of magnesium the results were similar. The reason for these phenomena are not well understood and need further study. Furthermore, if the diluents did not contain any potassium ions, it was found that the sperm could be instantly activated at the very moment when sperms were placed into the diluents. The instantaneous activation time was about 1-2 seconds only. It is concluded that the diluents must contain a certain amount of potassium ions.

Sperm durability for cyroprotectants and equilibrium time

DMSO and glycerol were selected as cyroprotectants. Figure 1 shows the results of sperm durability for the two cyroprotectants when being exposed over time. Comparing the two results, it was discovered that the sperm durability for DMSO was better than that for glycerol. In the glycerol test groups, though the sperm was treated even at 3% concentration of glycerol, which was the lowest concentration in all test groups, sperm motility began to descend only after 0.5 h. With the concentration of glycerol rising, the sperm motility obviously declined fasterr. In DMSO test groups, 8% and 10% concentrations seemed to gain lower values than the other concentrations, because the sperm motility holds above 70% after 8h treatment at these concentrations. It was assumed that the sperm could not have been sufficiently dehydrated in these concentrations of the cyroprotectant. However, the 14% concentration seemed to have a higher negative effect than all the other concentrations because the sperm motility began to decline clearly after 2 hours of treatment.

Overall the results indicate a 12% DMSO concentration to be suitable for the sperm cyroprotectants and 0.5 to 2 hours being acceptable for sperm equilibration time. Using this concentration and equilibration time, not only the sperm of sturgeon was sufficiently dehydrated, but also the toxicity of the cyroprotectant would be at negligible levels while the sperm motility can reach over ++++90%.

Cooling speed and freezing point influence on sperm motility

Four cooling speeds and three freezing points were tested in different combinations with the chosen cryoprotectant. Figure 2 shows the sperm motility obtained with sperm treated at different cooling speeds and freezing points and thawed thereafter.

Interpreting the data depicted in Figure 2, the best results for cyro-preservation of sturgeon sperm were obtained at a cooling rate of 2/min and a freezing point of -6 while keeping the sample at this temperature for 10 min. Using this method, the motility of thawing sperm could reach to motility to be categorized above ++++70% when sperm was preserved in liquid nitrogen for 24 hours. When the cooling speeds were higher or lower than 2/min, the motility of thawed sperm noticeably decline. When extending the equilibrium time (time before transfer to liquid nitrogen), and using lower freezing point temperatures would also result in declining sperm motility after thawing.

Table 2
Determining the effects of diluents on sperm motility after various storage times (hours. Explanations for semi-quantitative activity codes (+ to ++++) see text.

No. of diluents	The sperm motility after different exposure times						
	12 h	24 h	36 h	48 h	60 h	72 h	86 h
D-1	++++60%	+++80%	+++60%	+++40%	++70%	++50%	+70%
D-2	++++60%	+++80%	+++70%	+++60%	++90%	++70%	++50%
D-3	++++90%	++++80%	++++70%	+++90%	+++80%	+++70%	+++60%
D-4	++++50%	+++70%	+++40%	++80%	++50%	+70%	+50%
D-5	++++90%	++++80%	++++70%	+++90%	+++80%	+++70%	+++60%
D-6	++++90%	++++70%	++++60%	+++90%	+++70%	+++50%	+++50%
Control	++++90%	++++90%	++++70%	+++90%	+++80%	+++80%	+++60%

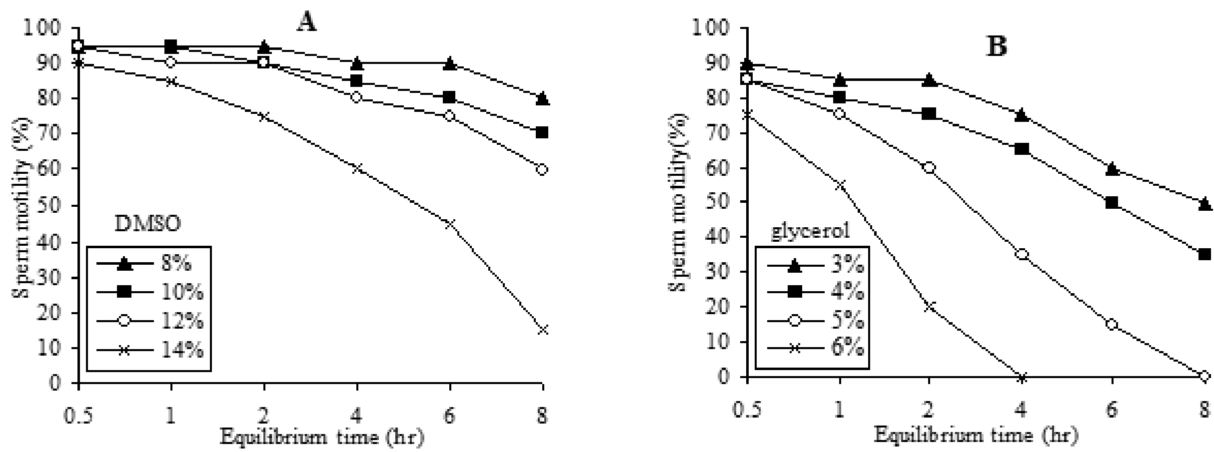

Fig. 1. Duration of sperm activity exposed to various cryoprotectants of different concentrations and tested for equilibrium time. (a) = DMSO; (b) = glycerol added at various amounts (%)

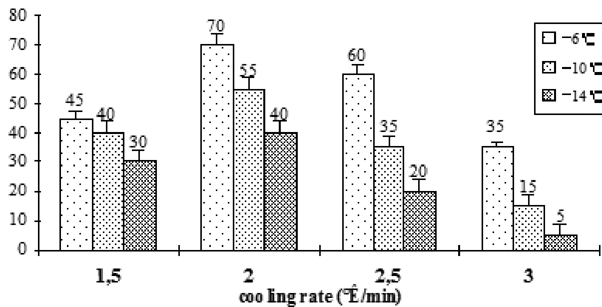

Fig. 2. Cryo-preservation trials with Chinese sturgeon sperm. Preservation tests at 4 different cooling rates reaching freezing points at 3 different temperatures. Data show the effect on sperm motility (%).

Fertilization test

The effects of the three diluents D-3, D-5and D-6 were also used in handling sperm with the selected cyroproservant to test the fertilization ability after thawing. After 24 hours storage in liquid nitrogen, the sperm were thawed and used in fertilization tests. Table 3 shows the results of the fertilization tests**.**

The effect of D-5 agaain proved to provide the best results among all three diluents also in the fertilization trials reaching 83.8% fertilized eggs and a survival rate of fry up to 68.1%. (Table 3). Although D-3 and D-6 were suitable for cryopreservation of Chinese sturgeon sperm, the fertilization and survival rates were all lower than those obtained with D-5. However, comparing with controls (fertilization rat 94.5%; survival 91.0%), the survival rate in the D-5 trial was still lower.

Discussion

The influence of diluents on sperm motility

There were a total of 38 diluents with different concentrations and component initially tested in this experiment. Only six diluents were chosen for the continuation of the experiments and are listed in Table 2. This is because they represent the best results in terms of motility and activity life span of sperm prior to freezing while others did not perform as expected. After further test trials with these six remaining diluents, it was discovered that D-3, D-5 and D-6 were performing better than the other three. Some suggested that the sperm of different fish needs different compositions in diluents, however, there is a tendency to minimize the number of components of diluents so that they become easy to prepare but still more effective (Scott 1980; Saad 1987; Glogowski, 2002). The results of the present study confirmed these observations. The components of the best tested diluent (D-5) contained only NaCl, KCl and $NaHCO_3$.

When sperm of Chinese Sturgeon was placed into diluents that did not contain any potassium ion, the sperm was instantly activated. This phenomenon is first described in this study for Chinese sturgeon as we did not find any publications to this effect. However, there may exist information elsewhere while the reasons for this observation are so far unknown and need further study.

Experience with studies on other fish species shows that the cooling rates and freezing points are key factors for successful sperm cryopreservation (Liu, 1997). This has also been comfirmed by many studies reported in the literature (e.g. Lahnsteiner, 2004; see references there). Different fish sperm have different cooling rate and freezing point requirements to obtain good results on viability and fertilization ability. At the present time, there exists no

Table 3
Fertilization and hatching rates achieved with cryopreserved sperm kept in three different diluents (D-3, D-5, D-6: composition see Material and methods) in relation to control samples. Numbers represent means of three replicates

Dilutents	No. of test eggs		Rate of fertilization (%)		No. surviving to hatching		Survival rate (%)	
	Test group	Control	Test group	Control	Test group	Control	Test group	Control
D-3	733	636	80.4	93.7	351	567	47.9	89.2
D-5	698	568	83.8	94.5	475	517	68.1	91.0
D-6	513	641	77.6	90.9	288	552	56.1	86.1

uniform standard for these factors in fish sperm cryopreservation. The methods using by scholars from different countries and their viewpoints are diverse. Many scientists (e.g. Babiak et al, 2000) considered these factors in relation to composition and concentration of cryoprotectants. Others emphasized the relations to species-specific characteristics of fish sperm (e.g. Viveiros et al., 2000) and our results confirm the need for considering both aspects.

The survival rates of embryos until hatching obtained with the use of thawed sperm reached levels sufficient to employ the cryopreservation technique for mass application in hatcheries while there is still room for improvement because the results of control experiments are better. Therefore, further studies to optimize the procedures are warranted.

Acknowledgements

The auhors extend special thanks to Prof. Dr H. Rosenthal, who made valuable comments and revised the inital manuscript. This study was supported by the fund Commonwealth Study of China (No. 2004DIB3J099), the key programm of Foundational Work of China (No. 2002DEH1004), the key project of Natural Science Foundation of China (No. 30490231) and the Fund for Minimisation of Impacts of Hydroelectric Projects on Natural Reserves of the Upper Yangtze River (No. 0714085). We are very grateful to Dr. Zhu Y J and Liu J Y, Yangtze River Fisheries Research Institute, for offering us all sperm and ova during the experiment, and Dr. Lu D C, Yangtze River Fisheries Research Institute, for his interest in this work and valuable comments.

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