

Fertilizing ability of cryopreserved spermatozoa in the Persian sturgeon (*Acipenser persicus*) and stellate sturgeon (*A. stellatus*)

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Abstract

Motility of spermatozoa was studied on 12 and 7 specimens of *Acipenser persicus* and *A. stellatus*, respectively. The density measured to be $2.22 \pm 0.65 \times 10^9 \text{ ml}^{-1}$ in *A. persicus* and $2.21 \pm 0.55 \times 10^9 \text{ ml}^{-1}$ in *A. stellatus*. Semen samples were diluted with two extenders containing tris 118mM, sucrose 23.4mM, pH=8, egg yolk (20%), dimethyl sulfoxide (15%) and penicillin potassium (500IU/ml) and biociphus extender containing glycerol as a cryoprotectant at a ratio of 1:1 and then transferred to 0.5ml straws and frozen in a computer controlled low temperature apparatus and stored in liquid nitrogen for one week. To study fertilizing ability, the spermatozoa were then used to inseminate eggs after thawing. Mean sperm motility in fresh spermatozoa was 86.6% in *A. persicus* and 73.75% in *A. stellatus* which decreased to 32.2% ($P < 0.001$) and 37.5% ($P < 0.001$) in frozen spermatozoa, respectively. Also mean fertilization rate decreased from 90.4% to 30.7% in *A. persicus* and from 72% to 36.8% in *A. stellatus*.

Keywords: *A. persicus*, *A. stellatus*, Spermatozoa, Cryopreservation, DMSO, Glycerol

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Introduction

Persian sturgeon (*Acipenser persicus*) and stellate sturgeon (*Acipenser stellatus*) are among the commercially valuable sturgeon species in the Caspian Sea that their stocks have declined drastically in the recent decades (Pourkazemi, 2006). Long-term storage of deep-frozen sturgeon spermatozoa has received worldwide attention in the recent years because of the lack of adequate and appropriate brood stock for restocking programme and also for sturgeon aquaculture (Baradaran Noveiri *et al.*, 2003; Billard *et al.*, 2004).

Cryopreservation induces severe stress to fish spermatozoa that in turn affect sperm quality in terms of fertilization ability. Sperm quality is influenced by several factors such as temperature, diet (Billard *et al.*, 1999), time of sampling (Kopeika *et al.*, 1999) and delays caused after the injection of hormones (Williot *et al.*, 2000).

Motility is an appropriate indicator of fertilizing ability of spermatozoa in sturgeons (Billard *et al.*, 1995). Considering that sperm activity in fresh spermatozoa as well as frozen-thawed spermatozoa decreased after activation (Tsvetkova *et al.*, 1996), researchers are of the opinion that fertilizing ability using frozen-thawed spermatozoa could be a more reliable criterion to evaluate this technology.

Fertilizing ability using frozen-thawed spermatozoa have been investigated in a few sturgeon species, including the Sakhalin sturgeon, *A. medirostris micadoli* (Drokin *et al.*, 1993), ship sturgeon, *A. nudiiventris*

(Cherepanov *et al.*, 1993), paddlefish, *Polyodon spathula* (Linhart *et al.*, 2006).

The objective of the present study was to compare the effect of two cryoprotectant on cryopreservation of spermatozoa and activation of frozen-thawed spermatozoa and to investigate the fertilizing ability of frozen-thawed spermatozoa in *A. persicus* and *A. stellatus*.

Material and methods

Sperm samples were collected from seven males of *A. stellatus* and twelve males of *A. persicus* that were transferred to the Shahid Beheshti Sturgeon Hatchery located in Rasht, Iran. Handling, transport and maintenance of spawners from the catch stations to the hatchery was carried out under the same conditions. Spermiation was induced by injecting of sturgeon pituitary extract in dose of 2-3mg kg⁻¹ body weight (Williot *et al.*, 2000). Spermatozoa were collected within 15-24h (depending on the water temperature) post hormonal injection.

Sperm from small individuals was collected by abdominal massage, while that from larger ones was collected using a 50ml syringes connected to a plastic vial and immediately stored in a refrigerator at a temperature of 5°C. Spermatozoa polluted by urine or excrement were not used (Linhart *et al.*, 1995). Sperm was collected from males by use of dry syringe with a silicon tube attached to it. The tube was inserted into the urogenital opening of the fish.

Sperm motility was visually determined by a microscope at 400 magnification immediately after mixing 10 μ l of fresh sperm with hatchery water (1:50) (Linhart *et al.*, 1995). Motility (percentage of spermatozoa with forward movement) was estimated using 400 magnification microscopy by mixing 50 μ l of hatchery water with 1 μ l of sperm on a glass slide.

100 μ l of spermatozoa samples placed on a hemocytometer were examined after 10min under a microscope at 400 magnifications to determine cell density (Hashemi, 1998). Good quality spermatozoa with suitable density were diluted at a ratio of 1:1 with extender and stored in a refrigerator for 60 min and allowed to equilibrate.

The extender was prepared with Tris buffer (118mM, pH=8), sucrose (23.4mM), egg yolk (20%), dimethyl sulfoxide (DMSO)(15%) and penicillin potassium (500IU/ml) (Tsvetkova *et al.*, 1996; Cherepanov & Kopeika, 1999; Lahnsteiner *et al.*, 2004). Another diluting solution Biociphus prepared by IMV Company (formula not known) in combination with glycerol was also

used as an extender. Diluted sperm samples were stored in 0.5ml straws (Cherepanov & Kopeika, 1999). Straws were filled using a three pinned injector manufactured by the IMV Company, France. The samples were subjected to a freezing protocol in a computer controlled low temperature freezer (Table 1).

Straws were thawed in a water bath at the temperature of 40°C for 25sec. Fertilization rate of cryopreserved spermatozoa was examined one week after storage of samples in liquid nitrogen. For this purpose, about 3ml of frozen-thawed spermatozoa (12 straws) were used to fertilize 100g eggs.

Fertilizing ability of frozen-thawed spermatozoa was tested with three replicates using eggs harvested from three different female spawners, separately. To avoid adhesion, eggs were treated with suspension of water and clay for 35 minutes. Eggs were assumed to be fertilized when they exhibited the four-cell stage, which occurred 4-5h after fertilization (Detlaff *et al.*, 1993). Results were statistically processed using SPSS and analyzed using ANOVA and Tukey test.

Table 1: Freezing protocol used to store sperm samples in a computer controlled low temperature freezer

Stage	Initial temperature (°C)	Final temperature (°C)	Freezing intensity (°C/min)
I	+5	-10	3
II	-10	-70	20
III	-70	-130	25
IV	-130	-196	immediately

Results

On the basis of motility and density of spermatozoa studied in the 12 cryopreserved sperm samples harvested from *A. persicus*, good motility was recorded in five samples, moderate motility was observed in five samples and poor motility was recorded in two samples (Table 2). Mean sperm density in the *A. persicus* specimens studied was estimated at 2.22×10^9 cells ml^{-1} .

Mean percentage motility in fresh spermatozoa in *A. persicus* specimens ($n=5$) was $86.6 \pm 5\%$ and that in frozen-thawed

spermatozoa (DMSO) was $32.2 \pm 4.4\%$ (Fig. 1). Mean fertilization rate in fresh spermatozoa and frozen-thawed spermatozoa (DMSO) were 90.4 ± 3.8 and $31.0 \pm 7.6\%$, respectively (Fig. 2).

In the seven cryopreserved sperm samples in *A. stellatus*, sperm motility was good in two samples, moderate in three samples and poor in two samples. Mean sperm density in cryopreserved sperm samples was 2.21×10^9 cells ml^{-1} (Table 3).

Table 2: Density and quality of spermatozoa in *A. persicus*

No.	Spermatozoa number ($\text{N} \times 10^9 \text{ ml}^{-1}$)
1	1.33
2	2.08
3	3.42
4	2.70
5	2.87
6	2.81
7	2.29
8	1.42
9	2.69
10	2.27
11	3.43
12	1.95

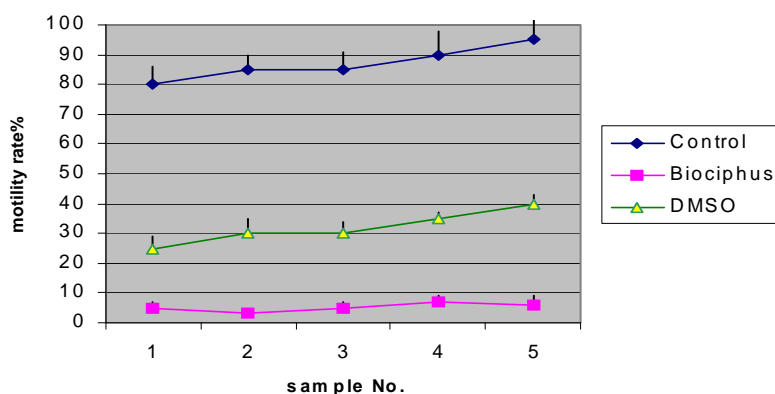


Figure 1: Comparison of percentage motility in fresh (control) and frozen-thawed (Biociphus and DMSO) spermatozoa in *A. persicus*

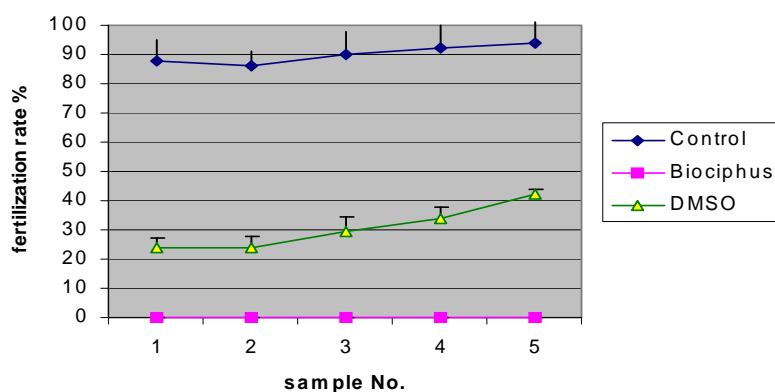


Figure 2: Comparison of fertilization rate in fresh (control) and frozen-thawed (Biociphus and DMSO) in spermatozoa *A. persicus*

Table 3: Density and quality of spermatozoa in *A. stellatus*

No.	Spermatozoa number ($N \times 10^9 \text{ ml}^{-1}$)
1	3.12
2	1.9
3	2.59
4	1.88
5	2.46
6	2.25
7	1.25

Mean sperm motility in fresh spermatozoa harvested from *A. stellatus* specimens ($n = 4$) was $73.75 \pm 6\%$ and that in frozen-thawed spermatozoa was $37.5 \pm 5.5\%$ (Fig. 3). Mean fertilization rate in fresh and frozen-thawed

spermatozoa was 72 ± 2.4 and $34.6 \pm 8\%$, respectively (Fig. 4).

The mean movement time in spermatozoa was compared with the time that spermatozoa moved forward (Table 4).

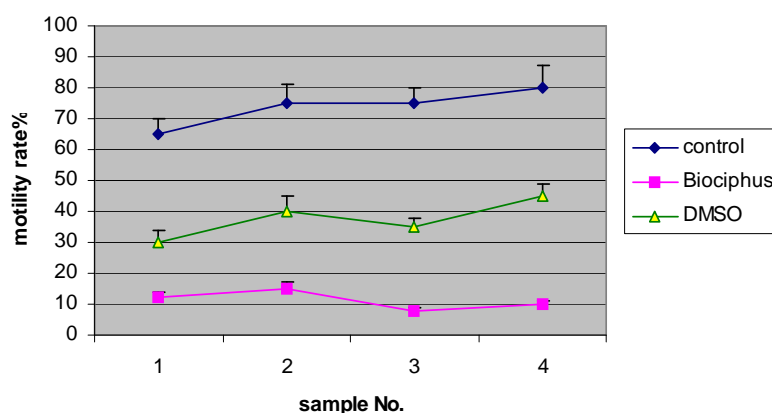


Figure 3: Comparison of percentage motility in fresh (control) and frozen-thawed (Biociphus and DMSO) spermatozoa in *A. stellatus*

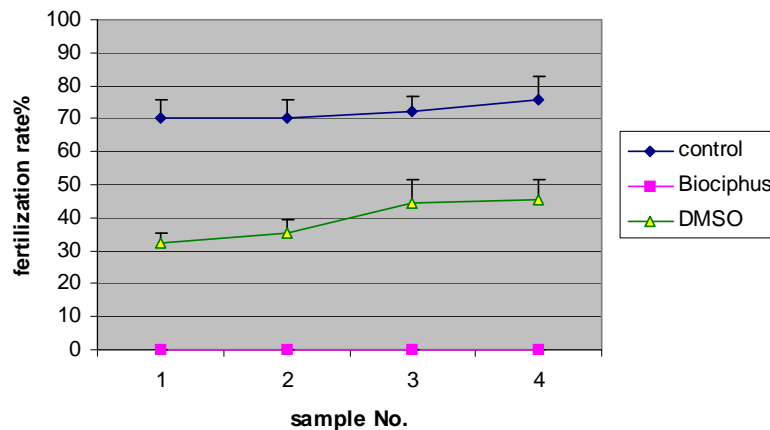


Figure 4: Comparison of fertilization rate in fresh (control) and frozen-thawed (Biociphus and DMSO) spermatozoa in *A. stellatus*

Table 4: Duration period of motility of fresh and frozen-thawed spermatozoa of *A. persicus* and *A. stellatus*

Movement	<i>A. persicus</i>		<i>A. stellatus</i>	
	Fresh sperm	Frozen-thawed sperm	Fresh sperm	Frozen-thawed sperm
Forward movement (sec)	230	175	165	145
Total movement (sec)	350	240	270	210

Discussion

Decline in stocks and limited number of potential breeders has led to the establishment of fish sperm cryobanks which play a crucial role in the genetic management and conservation of aquatic resources (Linhart *et al.*, 1993; Billard & Zhang, 2001). The establishment of sperm banks from valuable fish species including sturgeon is widely practiced in many countries (Kopeika *et al.*, 2000; Chao & Liao, 2001). Such practices were initiated with simple freezing facilities in Iran and have now been developed to

sophisticated cooling systems (Baradaran Noveiri *et al.*, 2003).

In the present study, the duration of forward movement in spermatozoa in *A. persicus* and *A. stellatus* were 340sec and 165sec, respectively which is considered to be good for Acipenseridae spermatozoa (Linhart *et al.*, 1995; Tsvetkova *et al.*, 1996; Alavi *et al.*, 2004). In another investigation, the duration of forward movement in spermatozoa in *A. persicus* was reported at 143 sec (Alavi *et al.*, 2004). So when required and based on the

existing conditions, lower quality spermatozoa can also be used for cryopreservation. For example, in the Atlantic sturgeon (*A. sturio*) spermatozoa with a density of 1.1×10^8 cells ml^{-1} and with a motility of 50% were cryopreserved (Kopeika *et al.*, 2000). Low sperm motility in fresh spermatozoa is because spermatozoa have not yet attained sexual maturity in the gonads of the male spawners (Kopeika *et al.*, 2000).

A significant decrease in sperm motility and duration of forward movement and total movement of frozen-thawed spermatozoa are observed in *A. persicus* and *A. stellatus* which have been seen to affect fertilization rate. Similar results were reported in green sturgeon (*A. medirostris*) (Drokin & Kopeika, 1999). In a previous investigation, where spermatozoa in *A. persicus* and *A. stellatus* were cryopreserved using a manual protocol, sperm motility was 50% in *A. persicus* and 60% in *A. stellatus* and fertilization rate was 44% in *A. persicus* and 48% in *A. stellatus* (Baradaran Noveiri *et al.*, 2003), which were higher than the values obtained in the present study. Better quality of breeders could be the possible explanation for this (Kopeika *et al.*, 2000). While the manual method for the cryopreservation of spermatozoa cannot be reproduced easily (Chao & Liao, 2001), cryopreservation of spermatozoa using the computer controlled low temperature apparatus can be repeated

until similar conditions (Lahnsteiner *et al.*, 2000).

The use of Biociphus extender prepared by IMV Company (formula not known) in combination with glycerol as an extender as compared to DMSO decreased permeability of spermatozoa membrane in tilapia (Leung, 1991). This extender is more widely used to preserve spermatozoa in mammals (Okada *et al.*, 2001). The unsuitability of this solution to cryopreserve sperms in salmon (Gwo, 1994) and in sturgeons (Cherepanov & Kopeika, 1999) has been previously reported. The application of DMSO in combination with egg yolk produced 20-40% motility in frozen-thawed spermatozoa in *A. gueldenstaedtii* and 55-60% motility in frozen-thawed spermatozoa in *A. stellatus* of the north Caspian Sea (Dzuba *et al.*, 1999). Although having motility in frozen - thawed spermatozoa of *A. persicus* (3-7%) and *A. stellatus* (8-15%) using the diluting solution 'biociphus', fertilization rate in these treatments was zero, which correspond with results reported on *Acanthopagrus latu* (Gwo, 1994).

Different protocols for the cryopreservation of spermatozoa in sturgeons have been suggested by different researchers who have worked with a particular species and have found the method successful for

that particular species (Billard *et al.*, 2004). Highest fertilization rate using frozen-thawed spermatozoa was reported at 74-94% (94% in fresh spermatozoa) in *A. ruthenus* (Jahnichen *et al.*, 1999). Highest fertilization rate using frozen-thawed spermatozoa of the Caspian Sea sturgeons was reported as 63% (77% in fresh spermatozoa) (Pushkar *et al.*, 1979). Maximum fertilization rate using frozen-thawed spermatozoa in *A. sturio* was 53% (89% in fresh spermatozoa) (Tsvetkova *et al.*, 1996).

With regard to the results of the present study, it is suggested that artificial fertilization of eggs using frozen-thawed spermatozoa should be carried out immediately after thawing. If fertilization is delayed for whatever reason, it is recommended to store the thawed spermatozoa in thin layers at 5°C (Kopeika *et al.*, 1997). Spermatozoa are not adapted to tolerate very low temperatures;

therefore during the process of freezing and thawing, spermatozoa are subject to a series of markedly changes.

The results of this study could be recommended as a useful method for sperm cryopreservation of the Persian and stellate sturgeons of the Caspian Sea and establishing of their sperm bank.

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