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Cryopreservation of Atlantic sturgeon *Acipenser sturio* L., 1758 sperm: First results and associated problems

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ABSTRACT

We present our attempts to adapt the established cryopreservation techniques for sturgeon sperm to *Acipenser sturio* L., 1758, using the sperm of a wild male which matured *in vivo*. The sperm, from a ripe male caught in the Gironde estuary in 1996 and obtained 4 h after its catch and transport, was diluted 1:1 with media containing 56.0-76.0 % tris-HCl-buffer (49.5 mM tris(oxymethyl)aminomethane) (pH 8.0), 14.4-24.0 % dimethylsulfoxide and 9.6-20.0 % egg yolk. The suspension was poured into 1.5 ml tubes, sealed and frozen in -196 °C liquid nitrogen vapour in a three-stage programme. Thawing took place in a 40 °C water bath. The motility of thawed sperm was 10-15 %, whereas the motility in native sperm before cryopreservation was 50 %. After one month storage time in liquid nitrogen, sperm was thawed and used for fertilization of sterlet *Acipenser ruthenus* L., 1758 eggs with the help of two activator media. After fertilization in a medium containing 3.1 mM tris(oxymethyl)aminomethane, 5.3 mM NaCl and 58.3 mM sucrose, 38.3 % of the embryos were developing on the second day, compared with 23.2 % when activator without sucrose was used. In the control group, fertilization of sterlet eggs with fresh sterlet sperm resulted in 43 % developing embryos.

Key words: Fertilization, reproduction, conservation, recovery.

RESUMEN

Criopreservación del esperma de esturión atlántico* Acipenser sturio *L., 1758: primeros resultados y problemas asociados

Presentamos nuestros intentos de adaptar las técnicas establecidas de criopreservación al esperma de Acipenser sturio *L., 1758, usando el esperma de un macho silvestre madurado* in vivo. *El esperma, procedente de un macho maduro capturado en el estuario del Gironda en 1996 y obtenido cuatro horas después de su captura y transporte, fue diluido 1:1 en un medio que contenía 56.0-76.0 % tris-HCl-buffer (49.5 mM tris(oximetil)aminometano) (pH 8.0), 14.4-24.0 % dimetilsulfóxido y 9.6-20.0 % yema de huevo. La suspensión fue vertida en tubos de 1.5 ml, sellada y congelada en nitrógeno líquido a -196 °C en un programa de tres fases. La descongelación tuvo lugar en un baño de agua a 40 °C. La movilidad del esperma descongelado fue del 10-15 %, mientras que la movilidad del esperma nativo antes de la criopreservación fue del 50 %. Después de un mes de almacenamiento en nitrógeno líquido, el esperma fue descongelado y empleado para la fertilización de huevos de esterlete* Acipenser ruthenus *L., 1758 con la ayuda de dos medios activadores. Después de la fertilización en un medio conteniendo 3.1 mM tris(oximetil)aminometano, 5.3 mM NaCl y 58.3 mM sacarosa, el 38.3 % de los embriones se desarrollaron al segundo día, frente al 23.2 % obtenido cuando se usó un activador sin sacarosa. En el grupo control, la fertilización de huevos de esterlete con esperma fresco de esterlete produjo un 43 % de embriones desarrollados.*

Palabras clave: *Fertilización, reproducción, conservación, recuperación.*

INTRODUCTION

The Atlantic sturgeon *Acipenser sturio* L., 1758 was caught in many European rivers in large quantities until the onset of the 20th century (Kinzelbach, 1977). In recent years, only a few specimens have been reported (Elvira and Almodovar, 1993; Debus, 1995). For future work on the recovery of this species, it is important to create a storage stock of cryopreserved gametes, embryos and embryo cells, because only limited numbers of mature specimens are available from controlled conditions (Williot *et al.*, 1997). Availability of such a storage stock would enable us to restore the genome of Atlantic sturgeon at any time, with the help of androgenesis or substitution crossing, even in the absence of any males and females of this species. Only limited experience with sturgeon sperm cryopreservation is available to date. The first research was done by Burtsev and Serebryakova (1969), who used glycerol in concentrations of 5-14 % in combination with egg yolk and sucrose (or lactose) or salts as cryoprotectant media. The sperm of beluga *Huso huso* (L., 1758), kaluga *Huso dauricus* (Georgi, 1775), sterlet *Acipenser ruthenus* (L., 1758), and bastard *H. huso* × *A. ruthenus* had a motility from 10-100 % after freezing and thawing, but fertilised not more than 1 % of the eggs.

Kasimov *et al.* (1974) reported that stellate *Acipenser stellatus* Pallas, 1771 and Russian *Acipenser gueldenstaedtii* Brandt & Ratzeberg, 1833 sturgeon sperm had 40 % of motile cells and fertilised 35 % of the eggs after freezing in 5 ml tubes in media with urea, chloralhydrat, sucrose, glycerol and egg yolk extract at -55 °C.

Kopeika (1982) demonstrated the possibility of cryopreserving sturgeon (beluga, sterlet, sturgeon, stellate) sperm with dimethylsulfoxide (DMSO), DMSO + polyethylenoxide-3000 (PEO-3000), DMSO + sucrose, and ethylenglycole. DMSO and DMSO + PEO-3000 were determined to be the best of these cryoprotectants (Kopeika, Belous and Pushkar, 1981). Cryopreserved *A. stellatus* sperm was stored for 4-22 days and had 40-60 % of motile cells, which fertilised 64.1 ± 8.8 % of the eggs and controls, reaching 77.5 ± 5.8 % using fresh sperm of identical species (Kopeika and Novikov, 1983). Frozen-thawed sperm of *A. ruthenus* fertilised 87.1 ± 2.9 % (control 97 %) of the eggs. The high sensitivity of sturgeon sperm to osmotic pressure changes was decreased by adding 20 % egg yolk and the gradual increase of osmotic pressure in suspension. After 6-year storage in liquid nitrogen, the sturgeon sperm, frozen according to this procedure in a medium with tris-HCl-buffer, egg yolk, and DMSO, revealed good motility (*A. gueldenstaedtii*, 20-40 %; *H. huso*, 25-40 %; *A. stellatus*, 55 % and 60 %; *A. ruthenus*, 35 % and 50 %; *Acipenser nudiventris* Lovetzky, 1828, 60 %) (Dzuba *et al.*, 1999).

Because of the importance of cell cryopreservation for conservation, we performed our experiments upon the incidental catch of a mature male Atlantic sturgeon in Gironde River in April 1996. This experiment was designed to transfer the method established for other sturgeon species, and to adapt the cryoprotectant medium to the sperm of this male.

MATERIALS AND METHODS

Immediately after catching the male *A. sturio* (23 April 1996) and its subsequent delivery to the farm, its weight and size were determined. The sperm was obtained at 23:00 h (4 h after its catch and arrival at the farm). In order to prevent interaction of water and faecal mass with sperm, a soft transparent polyethylene tube (5 mm diameter) was inserted into the genital hole. The effluent of the tube was placed in glass beakers for sperm storage. Sperm was flowing freely without previous massage of the abdomen. The procedure took place without anaesthesia. During the time of manipulation water was passed through fish gills. The sperm obtained was put into a refrigerator at 5 °C, where it was stored prior to cryopreservation for 1-4 h. Estimation of sperm quality was carried out on the basis of spermatozoa motility with the help of a ×800 microscope. For sperm motility activation, 0.2 ml of river water was put onto a glass slide, and then 0.005 ml of sperm was added. This suspension was thoroughly mixed with the tip of a pipette. We visually estimated the quantity of cells that were moving straight as well as all cells on the 3-5 segments of the slide. The ratio of these counts was expressed as a percentage. The duration of sperm moving straight was estimated in the first experiment. The concentration of spermatozoa was counted in a Nikon Chamber**.** Media for sperm cryopreservation were created *ex tempero* (table I). Cooled buffer medium (5 °C) was slowly added to the sperm via side of the rotating glass. Medium/

Table I. Cryoprotective media and freezing regimens used in different experiments

No. of experiment and cryo - preservation regimen	Medium	Compound of cryoprotective medium			Duration of first stage of cryopreservation (min)
		49.5 mM tris-(oxymethyl)-aminomethane-HCl (%)	DMSO (%)	Hen egg yolk (%)	
1	A	68	22	10	15
2	B	70	18	12	8
	C	56	24	20	
3	B	70	18	12	17
	C	56	24	20	
	D	76	14.4	9.6	
4	D	76	14.4	9.6	15

sperm ratio was in a 1:1 proportion. Sperm equilibration in media with cryoprotectants was for 30 min; then 1 ml portions of the cell suspension were poured into 1.5 ml cylindrical ampoules. These units were closed with plugs.

We used a scheme of multifactor experiments in our experiments 2 and 3 (media B and C) where we varied two factors (osmolarity of medium and duration of first stage of cryopreservation) on two levels. In the third experiment, the medium for sperm dilution had the lowest DMSO and yolk concentration tested (D).

Sperm cryopreservation

Ampoules with diluted sperm were placed above the surface of liquid nitrogen in the Dewar's liquid. One of the tubes containing sperm suspension with media A was equipped with the thermo-couple in all experiments (P510, Cole-Parmer Instrument Co., Dostman Electronic GmbH, Wertheim, Germany) and connected to the computer. Cryopreservation used a three-stage freezing regimen: (1) speed of 1-5 grad/min from 5 °C to -10 and -15 °C; (2) speed of 20-25 grad/min till -70 °C; (3) the tubes with suspension were plunged into liquid nitrogen. Temperature for transferring to the second stage of cryopreservation depended on the duration of stage 1 (table I). The speed of freezing could be changed by altering the distance of the tubes from the nitrogen surface. The temperature changes in the tubes were recorded on the display every 30 s.

Thawing

Sperm was thawed in a static water bath at 40 °C until liquid medium became visible. We checked the quality of thawed sperm after freezing in activator 1 for each cryoprotective medium utilised in order to estimate the suitability of the regimen for the sperm used (table II). In these cases, the sperm was in liquid nitrogen for 10 min.

Egg fertilization

The sperm destined for fertilization was stored in liquid nitrogen for one month. For fertilization, we used only the sperm (experiments 3, 4) which was cryopreserved in the cryoprotectant medium D containing 76 % 49.5 mM tris-oxymethyl-aminomethan-HCl-buffer (pH 8.0) + 14.4 % DMSO + 9.6 %

Table II. Sperm activators used for estimation of *A. sturio* sperm motility and fertilising ability. (*): this activator was used only once in experiment No. 1

Sperm activators used for	
Motility testing	Fertilization
1. 9.3 mM tris-HCl + 12 mM $NaHCO_3$	3. 3.1 mM tris-HCl+ 5.3 mM NaCl + + 58.3 mM sucrose
2. 9.3 mM tris-HCl + 12 mM $NaHCO_3$ + + 146 mM sucrose *	4. 3.1 mM tris-HCl + 5.3 mM NaCl

egg yolk. Fertilising ability of thawed *A. sturio* sperm was tested using eggs of 3 females of sterlet *A. ruthenus* due to the absence of *A. sturio* eggs. We then added 2 ml of thawed cell suspension and 2 ml of activator 3 or 4 to the batches (200-250 eggs/batch) of *A. ruthenus* eggs and mixed for 3-4 min, after which sperm was rinsed off, and the batch subsequently incubated in river water. In the control, 0.4 ml of fresh *A. ruthenus* sperm activated with 4 ml of water was added to the *A. ruthenus* eggs. The insemination results were estimated on the basis of developing embryos on the second day after fertilization.

RESULTS

A. sturio weight was 25.3 kg, and length was 170 cm. We obtained 35 ml of sperm. The cell concentration determined in sperm was 1.1×10^8 spermatozoids/ml. Initial motility was 50 % after activation with river water. Straight moving of spermatozoa was for 3 min. The results of cryopreservation experiments are presented in table III. After thawing the sperm in the first experiment, only 1 % of cells were activated in activator no. 1 and 10-15 % –in activator no. 2 with sucrose. But motility of sperm after cryoconservation was short, not exceeding 90 s.

Table III. Motility of spermatozoa of *A. sturio* after equilibration in cryoprotectant media and freezing-thawing

No. of experiment and cryopreservation regimen	Medium	Sperm motility % in activator 1 after	
		Equilibration in cryoprotectant medium	Thawing
1	A		1
2	B	40	10
	C	1	1
3	B	20	10
	C	10	1
	D	20-30	10-15
4	D		10-15

With the help of regression analysis of spermatozoa motility after cryopreservation in media B and C (experiments 2 and 3), we have calculated the regression equation:

$$Y = 22 - 18X_1$$

where Y = spermatozoa motility, and X_1 = first studied factor (osmolarity of cryoprotectant medium). It could be concluded from this equation that differences in the regimens of cryopreservation do not influence sperm motility. The negative coefficient of X_1 is evidence of the need to lower medium osmolarity in order to increase sperm survival. This was confirmed in experiments 3 and 4, by using medium D. Here, we observed the maximum survival of cells, although the increased storage time prior to cryopreservation led to a decline in sperm quality.

These results were confirmed in the fourth experiment, using medium D (table I) for cryopreservation of the sperm. One month after cryopreservation, the sperm was thawed in order to test its fertilising ability. Eggs of three *A. ruthenus* females were inseminated with this sperm (table IV). Using the eggs of two females (no. 1 and no. 3), neither the control nor the experiment set were successful.

Table IV. Fertilizing ability of frozen-thawed sperm of *A. sturio*

Eggs *A. ruthenus* 2nd female	Sperm	Activator	Quantity of developing embryos on the second day after fertilization (%)
Experiment	*A. sturio*	3	38.3
Experiment	*A. sturio*	4	23.2
Control	*A. ruthenus*	H_2O	43.0

DISCUSSION

Over the course of their reproductive life, males do not produce spermatozoa with identical genomes (Ayala, 1984). Therefore, the limited availability of reproductive material makes it important to store the maximum quantity of cells in order to decrease the possibility of inbred depression. The quality of cryopreserved sperm is dependant upon various factors. Their relevance could vary according to intrinsic (e.g. degree of gonadal maturation, initial quality of sperm) as well as extrinsic factors (critical factors in cryopreservation and cryoprotection). Initially, obtaining sperm with high quality and cryoresistance seems critical, due to the unpredictable availability of ripe males, hindering proper preparation for obtaining sperm. It could be supposed that in the present study the stress conditions imposed on the fish by catch and transportation, as well as the time when sperm was obtained, had an adverse impact on sperm quality. This is supported

by the findings on Siberian sturgeon *Acipenser baerii* Brandt, 1869 (Williot, Kopeika and Goncharov, 2000), in which the degree of maturation, as well as the time of obtaining sperm, had significant influence on sperm quality**.** The sperm motility and cryoresistance changes show a bell-curve after hormonal stimulation (Kopeika, Williot and Goncharov, 1999a). The peak of motility has been identified at 5 a.m. 2-3 days after hormonal stimulation of males (Kopeika, Williot and Goncharov, 1999b). Therefore, half of the potentially available cells were lost during our first stage of obtaining sperm. Additionally, degradation of immobile cells could have imposed a negative influence on the remaining live cells, thereby affecting their cryoresistance. This interaction has been reported for mammal sperm (Bugrov and Sidashov, 1991). We obtained only 35 ml of sperm with cell concentration 1.1×10^8 spermatozoids/ml, i.e. close to the minimum. Ginzburg (1968) reported that sturgeons give from 25-500 ml of sperm with concentrations ranging from 0.14 to 7.55×10^9 spermatozoids/ml. Concentration of *A. baerii* sperm varied from 0.5×10^8 to 5×10^8 (Gallis *et al.*, 1991). With the increasing of delay after hormonal stimulation, there was quantitative growth of mature motile spermatozoa, as well as in general concentration (Williot, Kopeika and Goncharov, 2000). Therefore, we could suppose that we obtained sperm from males with low reproductive ability, or sperm that was immature. The latter supposition was confirmed, begining from our first experiment on cryopreservation, in which we obtained only 1 % of motile cells. Although we used cryoprotective media and universal three-stage freezing regimens that have provided good results for cryopreservation of sperm in other sturgeon species (Kopeika, Belous and Pushkar, 1981; Kopeika, 1982). This regimens was also used with good results for cryopreservation of sperm from carp *Cyprinus carpio* L., 1758 (Kopeika, 1986), salmon, and other fish (Kopeika, unpublished). In order to obtain reliable results, we investigated thawed sperm under critical conditions –in river water or activator no. 1, thus providing lower osmolarity than the usual activator for thawed sturgeon sperm (Kopeika, 1982; Kopeika and Novicov, 1983; Tsvetkova *et al.*, 1996; Jähnichen *et al.*, 1999). The increased sperm motility, 10-15 % upon activation with sucrose activator, compared to water activation, might be attributable to the higher osmolarity of the activator. The increased osmolarity led to a lower possibility of post-hypertonical shock of the thawed sperm membrane. Spermatozoa, like all other cells, become more sensitive to osmolarity deviations in the medium. This sperm immaturity could also be confirmed by the short time of sperm motility (90 s) after cryopreservation. Similar effects have been observed for carp sperm (Kopeika, unpublished). The duration of motility in immature carp sperm was 10-15 s after dilution with the cryoprotectant medium, compared to 60-90 s in mature sperm. Therefore, in the case of immature sperm, in which cryoresistance is lower, the need exists, as we assumed, to decrease the osmolarity of the cryoprotectant medium by lowering the concentration of cryoprotector (B). To exclude possible errors in our supposition, we also used media with higher osmolarity (C), and other methods, in each experiment. Crystallization in medium B, with a lower content of cryoprotectant, was expected to be at a higher temperature than that for medium C, with higher cryoprotectant concentration. Therefore, in medium B, we began increasing our freezing speed during the second stage earlier and at a higher temperature than in medium C. It is important to take into account here that increased freezing speed before the end of crystallization can cause increased cell destruction (Kopeika, unpublished). Therefore, we used two regimens of cryopreservation. However, the results of our multifactor experiment showed that these changes of regimens did not influence sperm motility. This could be evidence for the fact that crystallization was over before the suspension was transferred into the second stage of cooling. A negative coefficient of X_1 shows that for better results, it is necessary to decrease the cryoprotective medium's osmolarity. In order to confirm the significance of our obtained regression equation, it appeared necessary to repeat the experiment. But we did not do this, in the end, because we obtained additional confirmation regarding the relevance of our conclusion. The survival of sperm after cryopreservation was the highest in medium D, with lower osmolarity, than in the multifactor experiment media. The delay of sperm storage in the refrigerator could lead to the growth of sperm sensitivity to extreme factors.

The fertilization ability of thawed sperm was dependent on the activator used. Higher fertilization rates (expressed in the quantity of developed embryos) were observed in activator no. 3 with sucrose,

compared to the activator without sucrose. Our results correlate with data that freshwater fish sperm (sturgeon, carp) becomes more sensitive to variation in osmotic pressure after cryopreservation (Kopeika, 1982; Kopeika and Novicov, 1983). Additionally, the fertilising ability of frozen-thawed sperm depends on the quality and quantity of the activating medium and insemination conditions. Comparing the average fertilization ability of cryopreserved sperm of *A. sturio* with that of control sperm from *A. ruthenus*, our results are still better than other published experiments (Tsvetkova *et al.*, 1996; Jähnichen *et al.*, 1999).

Our short and uncompleted (due to the restricted availability of sperm) study indicates that this material could successfully be stored, even with sperm of initially low quality, with the given method (Kopeika, 1982) of cryopreservation in tris-HCl-buffer with DMSO and egg yolk. The total survival of fish spermatozoa during cryopreservation could be increased if the causes of variability in sperm quality could be influenced. In the future, it will be necessary to create optimal conditions for obtaining high-quality sperm, even from reared species. In order to reach this objective, the males should be reared under optimal temperatures before and after injection. Only mature sperm should be obtained. The best time is early morning, but not earlier than 24-36 hours after hormonal stimulation, in order to increase the survival of cells during cryopreservation. The use of antifreeze fish proteins also provides possibilities of improving the results by increasing the sperm's initial cryoresistance. In any case, additional research efforts are urgently required in this field to eliminate the existing uncertainties.

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