

Preliminary Researches of Sperm Cryopreservation on Poliodon (*Polyodon spathula*) for Optimization of Spawning Biotechnology

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Abstract

The paper presents the first experiments of sperm cryopreservation on poliodon (*Polyodon spathula*), recently acclimated sturgeon in Romania. The accomplished research has been to determine the effect of using methanol (MeOH) and dimethyl sulfoxide (DMSO) as cryoprotectants in combination with two extenders (modified Tsvetkova's extender: mT and modified Hanks' balanced salt solution: mHBSS) on the spermatozoa motility and fertilization and hatching percentage of eggs by use of thawed sperm.

The best post thawed motility (78±5%) was recorded using modified Tsvetkova's extender: mT with 1% MeOH, and the best fertilization and hatching percentage of eggs were achieved by using mT extender in combination with 5% MeOH. The results confirm data from literature (Detlaf et al. 1993, Mims et al. 2000, Akos Horvath et al. 2006) reporting that MeOH is a proper cryoprotectant for freezing poliodon sperm.

Keywords: cryopreservation, fertilization, motility, poliodon, spawn

1. Introduction

Polyodon spathula (Acipenseriformes-Polyodontidae) is a sturgeon native of North-America, basin of Mississippi and Missouri rivers. It is a large fish reaching at maturity 1.5–2 m length and 50–70 kg. Present a rapid growth and economic feeding behavior (is a filtered feeder, predilection consumes zooplankton). The two biological characteristics plus the quality of meat and eggs has made the species come into the heed of aquaculture specialists around the world. Hereby, since 1980's species has been the subject of acclimation in many countries from Europe and China. In Romania, the species was introduced in 1992 and in 2002, with reaching maturity of first breeders batch was achieved successfully the

artificial spawning. The results achieved at artificial spawning and rearing of fry in different systems during 2002–2011 led to dissemination of species in many fish farms from the country.

For preservation and development of population genetic biodiversity from Nucet and optimizing spawning biotechnology, in 2010 and 2011 have initiated a series of sperm cryopreservation experiments.

On accomplish of the experiments have considered the data from literature considering sperm cryopreservation on sturgeons generally [1] and poliodon mainly: cryoprotectants and extenders used, their influence on the motility and fertilization rates [2-4].

The accomplished research has been to determine the effect of using methanol (MeOH) and dimethyl sulfoxide (DMSO) as cryoprotectants in combination with two extenders (modified Tsvetkova's extender: mT and modified Hanks'

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balanced salt solution: mHBSS) on the spermatozoa motility and eggs fertilization and hatching rates by using frozen sperm.

2. Materials and methods

The experimental batch consists of four males and four females reared at the experimental Base of SCDP Nucet. During the development of experiments males and females were stoked separately by gender in 4000 l tanks with 10 l/min water flow and 15 °C water temperature situated inside of the artificial spawning station.

Hormonal induction was accomplished using Nerestin 5A as follow: males were injected intramuscular with Nerestin 5A at a dose of 0.1 ml/kg body weight; sperm was collected after about 24 hours using a Tygon duct (5 mm diameter, 5 cm length) attached to a 10 ml plastic syringe. After harvesting, sperm was temporarily stoked 25 cm³ straws placed on wet ice until achieving the motility analysis.

Females were injected with Nerestin 5A in a total dose of 1.2 ml/kg administered in a preliminary injection (0.2 ml/kg) and a releaser injection (1.0 ml/kg) within 12 hours. The eggs were harvest by MIST method [5]. The rate of motile spermatozoa was determined using a microscope with 200× magnification.

Sperm samples having > 85 % motility were preserved in crushed ice and send to the laboratory for future use.

The eggs used in fertilization experiments were harvest only from a single female.

The following experiments were accomplished:

First was testing the post thawing motility and fertilization rate. Thus, each male's sperm was diluted 1:1 in two extenders: modified Tsvetkova extender (mT; 30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl, pH 8.0, osmolarity: 82 mOsmol/kg) and modified Hank's balanced salt solution (mHBSS; 8.0 g NaCl, 0.4 g KCl, 0.16 g CaCl₂ 2H₂O, 0.20 g MgSO₄ 7 H₂O, 0.12 g Na₂HPO₄ 7H₂O, 0.06 g KH₂PO₄, 0.35 g NaHCO₃ and 1.0 % glucose per liter of solution) with distilled water at a osmolarity of 100 mOsmol/kg. As cryoprotectant was used MeOH or DMSO (5 or 10 %). Sperm samples were loaded into straws and frozen into liquid nitrogen vapors. After 3 minutes straws were immersed in liquid nitrogen.

The average cooling rate for MeOH was 70°C/min, while for DMSO was 66°C/min. Before thawing and use for fertilization straws were stored for 24 h in plastic cups attached on sticks disposed in Dewar vessels filled with liquid nitrogen (- 196°C). Each treatment was made in three replications (using as replication sperm samples from each male). In each sperm sample spermatozoa concentration was determined using a Bruker counting camera at 200× and 100× magnification for diluted sperm.

In the first set of experiments were used the eggs from a single female. Ten grams of eggs (about 1000 eggs) were distributed in dry glass vessels. Sperm samples were thawed in a water bath at 40°C for 15 seconds and then were assess in terms of motility by fertilization experiments.

Motility was estimated using dark field microscopy at 200× magnification. The eggs were fertilized with three straws (1500 µL) of thawed sperm. Fresh sperm from the same males (the same amount as frozen sperm before dilution) was used as control to monitor the quality of eggs. Gametes were activated by adding 10–12 ml of technological water. Eggs were incubated in a flow-through fiber glass hatchery (12 l/min).

Fertilization rate was determined in development stages 4–8 by microscopic analysis with 5× magnification.

In the second set of experiments fertilization and hatching tests were accomplish with frozen sperm from four males with four replications per male. Sperm samples were diluted 1:1 in mT extender (described above). Sperm samples were frozen in the presence of 5% MeOH as cryoprotectant. Freezing and thawing conditions were those described previously. Sperm samples were stored in a Dewar storage, in liquid nitrogen (- 196°C) for 20 days. In this experiment were used samples of 50 g of eggs each (about 5000 eggs). A sample of eggs was fertilized with mixed content of five 0.5 ml straws of thawed sperm. Stickiness of eggs was drop out by desizing in a mud suspension for 45 minutes. Eggs were incubated in special hatchery. Fertilization rates were recorded between four and eight stages and also hatching rates.

Results of post thaw motility and fertilization from the first set of experiments were subjected to two-way ANOVA test with Bonferroni's post test to study the main effects of extenders, cryoprotectants and concentrations.

Data considering fertilization and hatch of sperm samples from each male in the second set of experiments were subjected at two t-test assays.

3. Results and discussion

In the first set of experiments the highest post thawed motility (78±5%) was observed when sperm was frozen with mT extender in the presence of 10 % MeOH as cryoprotectant (Table 1). Both extenders (P=0.0018) and cryoprotectants (P=0.004) had a significant effect on the poliodon

sperm after thawed. The highest fertilization rate between four and eight stages (70±3%) was observed on eggs fertilized with sperm frozen in the presence of mT extender combined with 0.5% MeOH as cryoprotectant (Table 1). Regarding the fertilization rates determined in the experiment in which was used MeOH have found that they registered close values for any of extenders. Have found that the cryoprotectant had a significant effect (P<0.0001) on the fertilization rates, but the concentration of extender or cryoprotectant type does not affect the fertilization result.

Table 1. Motility and fertilization rates (average ± SD) after thaw the cryopreserved sperm of poliodon (n = 4 males)

Parameter	Extender	DMSO		MeOH	
		5 %	10 %	5 %	10 %
Motility (%)	mHBSS	21 ± 11 ^{a1}	15 ± 5 ^{a1}	15 ± 9 ^{a1}	28 ± 29 ^{a1}
	mT	28 ± 9 ^{a1}	18 ± 9 ^{a1}	48 ± 24 ^{a1}	78 ± 5 ^{b2}
Fertilization (%)	mHBSS	30 ± 17 ^{a1}	16 ± 5 ^{a1}	55 ± 2 ^{a2}	69 ± 2 ^{a2}
	mT	35 ± 17 ^{a1}	15 ± 9 ^{a1}	70 ± 9 ^{a2}	68 ± 3 ^{a2}

DMSO=dimethyl sulfoxide; MeOH=methanol; mHBSS=modified Hank's balanced salt solution; mT=modified Tsvetkova's extender; 5% and 10% = concentrations of cryoprotectants.

Means folowed by superscript letter (only for motility and fertilization) were not significantly differentiated (P>0.005). Means on the raw folowed by a superscript number were not significantly differentiated (P>0.005).

Motility of fresch sperm (n = 4 males) was 90 ± 5 %. Fertilization of control was 85±4 % (n=3 males).

In the second experiment the highest fertilization (82 ± 8 %) and hatching (77 ± 4 %) rates were observed at sperm of male 1 (Table 2). There was no significant difference between fertilization and hatching rates of eggs fertilized with thawed sperm from male 1 and 4, but there was a significant difference (P = 0.0049) between these

rates for the male 3.

Regarding the issues of sperm cryopreservation on poliodon has been an increase of post thaw motility on cryopreserved sperm. This will led to achieving better fertilization and hatching rates. Also has noted that post thawed motility had not a direct influence on the fertilization rate.

Table 2. Fertilization and hatching rates (average ± SD) of fertilized eggs with thawed sperm from 4 males (n = 4 replications per male)

Parameter	Male 1	Male 2	Male 3	Male 4
Fertilization (%)	82 ± 8 ^a	82 ± 5 ^a	72 ± 6 ^a	80 ± 8 ^a
Hatch (%)	74 ± 4 ^a	62 ± 6 ^b	69 ± 6 ^a	73 ± 5 ^a

Means folowed by superscript were not significantly differentiated (P > 0.005). Fertilization rate of control (n=4 males) was 90±5% and hatching rate was 85±6 %.

4. Conclusions

Using MeOH as cryoprotectant assists in achieving higher percentages of motility and fertilization comparing to DMSO regardless of extender concentration or type.

The MeOH concentration showed no significant effect on the fertilization and hatching rates.

Sperm samples cryopreserved with DMSO showed post thawed motility similarly to those frozen with MeOH indicating that the cells

survived to the frozen and thawed processes but have lost the ability to fertilize eggs.

It was found that 1.50 ml of sperm is sufficient to fertilize 50 g of polyodon eggs.

The results of experiments have shown that currently MeOH is an adequate cryoprotectant for sperm of polyodon resulting in obtaining of some high percentages of fertilization and hatching.

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