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Effect of cryoprotectants and male on motility parameters and fertilization rate in paddlefish (*Polyodon spathula*) frozen-thawed spermatozoa

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Summary

A sperm cryopreservation method using different cryoprotectants and sperm from different males was developed. Different percentages of pure cryoprotectants (methanol and DMSO) were added to extender 1 or 2 (20 mM tris pH 8 + 30 mM sucrose + 0.5 mM KCl or 20 mM tris pH 8 + 50 mM sucrose + 0.5 mM KCl, dilution 1:1) or non-extended sperm and every 1 ml of mixture was transferred to a 2-ml cryotube. The cryotubes were directly transferred to a pre-programmed PLANER Kryo 10 series III freezer at 0 ^{o}C and cooled from 0 ^{o}C to –5 ^{o}C at a rate of 3 ^{o}C.min^{-1}, from –5 ^{o}C to –15 ^{o}C at a rate of 5 ^{o}C.min^{-1}, from –15 ^{o}C to –25 ^{o}C at a rate of 10 ^{o}C .min^{-1}, from –25 ^{o}C to –80 ^{o}C at a rate of 20 ^{o}C .min^{-1}. Thereafter the samples were held for 5 min at -80 ^{o}C and finally transferred into LN_2 until next morning. The sperm was then thawed in a water bath at 40 ^{o}C for 105 s. Fertilization rate of control sperm was 81.5% (kept unfrozen; samples tested after 24-h storage at 3 ^{o}C), indicating that the gametes were of good quality. Percentage and the velocity of motile sperm from were evaluated in fresh and post-thawed sperm using video frames and subsequent image analysis. The results on hatching rates were significantly correlated with post-thawed sperm motility (r=0.49, P=0.035) and velocity (r=0.55, P=0.014) and not correlated with velocity of post-thawed spermatozoa (r=0.32, P=0.177). The best fertilization rates were obtained with 64-75 % in post-thawed sperm ($3.6.10^5$ spermatozoa per egg) when sperm were treated either without any extender or with both extenders with methanol concentrations of 8 or 10 %. These results were not significantly different compared with those obtained using fresh sperm control samples. Hatching rate was very low, only 8-15 %, when sperm was frozen with 8 or 10 % DMSO. ANOVA showed a significant effect of males on sperm motility, velocity and fertilization rate in post-thawed sperm.

Introduction

The paddlefish *Polyodon spathula* Walbaum, 1792 is an important species in the United States (Carlson and Bonislawsky, 1981; Mims et al., 1993). The basic reproductive methods to reproduce paddlefish have been developed by Needham (1965) and Graham et al. (1986). Paddlefish females are highly valued for blackish roe, which is processed into caviar. The cryopreservation study is part of a series of detailed studies to develop a complete breeding programme for production of all-female paddlefish populations in aquaculture. Two biotechnological methods to induce gynogenesis and hormonal sex inversion, have already been developed (Mims et al., 1997). Genome banking of frozen sperm from sex-inverted gynogenetic males (neo-males) is one of the final steps in this breeding programme..

Storage of fish sperm for aquaculture purposes and preservation of biodiversity in fish steadily increases in importance. In recent years extensive new data and knowledge on this topic has accumulated. Deep freezing of fish spermatozoa is a complex but frequently used method, the success of which depends on a number of variables affecting the capacity of spermatozoa to fertilize an egg. Cryopreservation techniques for spermatozoa in liquid nitrogen (-196^{o}C) are among the modern methods applied in aquaculture (Stoss, 1983; Ranna, 1995; Gwo, 2000). It has been estimated that sperm from 200 fish species are already successfully cryo-preserved (Billard et al., 1995), but for sturgeons and paddlefish species the optimization of this technology is still needed. The present state of knowledge on cryopreservation of sturgeon sperm was recently summarized by Billard et al. (2004). Unfortunately, only limited hatching rate have been reported for sturgeons or paddlefish using cryopreserved sperm (Brown and Mims, 1999; Jahnichen et al., 1999; Mims et al., 2000; Glogowski et al., 2002). DMSO, ethylene glycol or methanol were commonly used as cryoprotectants in these trials.

At the present time, several sperm banks were established in Russia, Ukraine, Hungary and the Czech Republic to serve the genetic conservation of sturgeon species (Billard et al., 2004; Flajshans et al., 1999).

Spermatozoa of chondrostean fishes (*Chondrostei*) are quite different from teleostean fish (*Teleostei*). These differences concern (a) their morphology (in particular the presence of an acrosome, Ginsburg et al., 1968; Dettlaff et al., 1993), (b) their physiology exhibiting a relatively long duration of the sperm motility (Cosson et al., 2000, Linhart et al., 2002; Allavi et al., 2004) and finally (c) their biochemistry with the presence of acro-

sin being involved in the acrosome reaction (Piros et al., 2002). Spermatozoa of sturgeons and paddlefish are essentially immotile in the seminal plasma (Linhart et al., 1995; Cosson and Linhart 1996; Cosson et al., 2000; Linhart et al., 2003; Alavi et al., 2004). Ionic concentrations in seminal fluid of paddlefish and sturgeons are lower than for teleosts (Linhart et al., 1991; Piros et al., 2002). The level and ratio of ions in seminal fluid is very important for the maintenance of energetic content. When the quantity or ratio between Na^+ and K^+ change, the sperm motility can be initiated (Cosson et al., 1999, Linhart et al., 2002, Alavi et al., 2004). Osmolality of seminal fluid in paddlefish (33.0 - 62.7 mOsmol.kg^{-1}, Linhart et al., 2003) was higher than that for Siberian sturgeon (33.0-46.3 mOsmol.kg^{-1}, Gallis et al., 1991).

In this study, a sperm cryopreservation method using different cryoprotectants and sperm from different normal males (not sex-inverted males) was developed. Velocity, percentage of post-thawed sperm motility and fertilization yield were used to evaluate the success of each tested protocol.

Material and Methods

The experiments were conducted at the Aquaculture Research Center, Kentucky State University (KSU), Frankfort, Kentucky, USA. Paddlefish males from 4.0 to 8.0 kg were captured in Ohio River, Kentucky. Broodfish were transported to the hatchery of the Aquaculture Research Center at KSU. Males were selected for good condition and held in circular metal tanks (1 000 l) with a water flow rate of 12 l.min^{-1}, dissolved oxygen of 9.0 mg 0_2.l^{-1} and at water temperature between 15 and 19^{0}C. Maturity of males was judged presence of milt after abdominal compression. The spermiation and ovulation were stimulated with LHRH analogue, des-Gly^{10} (D-Ala^6) LHRH ethylamide (Sigma Chemical Company) injected intramuscularly at the dose of 50 µg.kg^{-1} b.w. Sperm was collected 24 h after stimulation. Ovulated oocytes were stripped from individual females 30 hours post stimulation. Eggs from 3 females were then pooled, short-term stored at a temperature of 17^{o}C and directly used for experiments.

Sperm Collection and Dilution

For stripping, males were fished out from the tank, fixed in dorsal position and urogenital pore was wiped. A 10-mL plastic syringe with 5 cm of Tygon tubing was used to collect sperm. The tube was inserted into the urogenital pore to fill up with sperm, and then sperm was transferred to 100 ml containers which were stored on ice for 1.5 hour until examination. Quality of the extended sperm was checked for the percentage of sperm motility from video records. Only sperm with motility higher than 80% was used for cryopreservation. Sperm concentration was counted in Thoma cell haemocytometer under Nicon microscope (400x) and the value was expressed as mean number of spermatozoa counted in 20 squares of the Thoma cell.

Cryopreservation and Thawing

Different percents of pure cryoprotectants (methanol and DMSO) were added in extender 1 (20 mM tris pH 8 + 30 mM sucrose + 0.5 mM KCl, dilution 1:1), extender 2 (20 mM tris pH 8 + 50 mM sucrose + 0.5 mM KCl, dilution 1:1) or non-extended sperm and every 1 ml of mixture without equilibration time was transferred to a 2-ml cryotube. The cryotubes were directly transferred to a pre-programmed PLANER Kryo 10 series III at 0 ^{o}C and cooled from 0 ^{o}C to –5 ^{o}C at a rate of 3 ^{o}C.min^{-1}, from –5 ^{o}C to –15 ^{o}C at a rate of 5 ^{o}C.min^{-1}, from –15 ^{o}C to –25 ^{o}C at a rate of 10 ^{o}C .min^{-1}, from –25 ^{o}C to –80 ^{o}C at a rate of 20 ^{o}C .min^{-1}, then held for 5 min at -80 ^{o}C and finally transferred into LN_2 until next morning. The sperm was thawed in a water bath at 40 ^{o}C for 105 s and used for fertilization and video recording of sperm motility.

Experimental Design

Pooled eggs (the same number of eggs per one gram of eggs) was used for all experiments. For each treatment or different experimental conditions (different extenders, croprotectants and males), six cryotubes with sperm were frozen and after thawing of the sperm (next morning) the following parameters were evaluated for each: velocity of sperm, % of sperm motility, % of fertility and % of hatching. The same parameters were measured in the control sample of unfrozen fresh sperm.

In the first series of experiment, effect of two different cryoprotectants of extended or non-extended sperm were studied. The cryoprotectants studied were DMSO at final concentrations of 8 and 10 and methanol at final concentration of 8 and 10 %. One ml of extended or non-extended sperm with cryoprotectants were always gently homogenized and transferred into cryotubes of 2 ml and frozen.

In the second series of experiment, methanol as cryoprotectant was tested. Eight and 10 % of DMSO was added to the non-extended sperm and 8 % of extended sperm with extender 1 (20 mM tris pH 8 + 30 mM sucrose + 0.5 mM KCl, dilution 1:1), the solution was gently homogenized. Latter, one ml of the solution was transferred into cryotubes of 2 ml and frozen.

In the third series of experiment, three different males were tested. Eight % of Me_2SO was added to extended sperm with extender 1 and 2, respectively. The mixture was gently homogenized and volumes of 1 ml were transferred into cryotubes and frozen. Unfortunately for last fertilization at male no. 3, extender 2 was not sufficient quantity of eggs. It was the reason why there is no fertilization rate at male 3 in Fig. 3.

Fertilization and Hatching Trial

The suitability of different methodologies of cryopreservation was assessed by fertilization and hatching rates (additionally to other parameters of sperm quality as mentioned above). This was done as followed: twenty grams of eggs (80 eggs per 1 g) were placed into a 50 ml dish; an accurate volume of sperm (thawed or non-frozen as control) with 360,000 spermatozoa per one egg was dropped on them from a micropipette. Twenty ml of hatchery water at 17 ^{o}C was added. One minute later additional 20 ml water was added and 2 minutes later around 200 eggs were placed into a glass dish of 200 ml supplied with UV-treated recirculated tap water at 17 ^{o}C, 9 mg.l^{-1} O_2. For each experiment, the procedure was replicated three times. The fertilization was counted in each dish at embryogenesis stage of 32 blastomers.

The percentage of fertilization rate (F_r) was calculated for each dish from the total No. of eggs placed in the dish (E_t) minus eggs without cleavage (E_d) as followed:

$$F_r = [(E_t - E_d)/E_t]x100$$

Observation of sperm motility and velocity

Spermatozoa were evaluated by percentage of motility and by velocity. Measurements used dark field microscopy and a Nikon camera setup as described by Cosson et al. (2000) and Linhart et al. (2002). Percentage motility and velocity was examined under 200x magnification immediately after mixing 0.5 µl of sperm with 49.5 µl of swimming medium (SM = 20 mmol.l^{-1} NaCl + 20 mmol.l^{-1} TRIS-HCl, pH 8.2). The sample was chequed on a glass slide previously prepositioned on the microscope stage. The final dilution was 1:100. Within 10 s after mixing, a video recording was started for 1-min to be used in the evaluation of spermatozoa swimming activity. The focal plane was always positioned near the glass slide

surface. The movements of spermatozoa were recorded at 60 frames.s^{-1} using a 3CCD video camera (SONY DXC-970MD) mounted on a dark-field microscope (NIKON Optiphot 2, Japan) and visualized on a video monitor illuminated with stroboscopic lamp of Strobex (Chadvick-Helmut, 9630, USA). The adjustable frequency stroboscopic flash illumination was set in automatic register with video frames (60 Hz) for sperm velocity measurement.

Evaluation of the velocity and percentage of motility

Velocity and motility were assessed at 20 s after activation: the successive positions of the video - recorded sperm heads (thawed or non frozen as control) were analyzed from video frames by means of Olympus MicroImage software (Version 4.0.1. for Windows with a special macro by Olympus C&S). The velocity and percentage of motility were measured by evaluating spermatozoa head positions on five successive frames with three different colours (frame 1 - blue, frames 2 - 4 - green and frame 5 - red). The analyses were repeated 3 times from 3 records at 20-second intervals, i.e. frames 1-5. Thirty to forty spermatozoa were evaluated from each frame. Motile spermatozoa were visible in three colours, while non-moving spermatozoa were white. Percentage of motile spermatozoa was easily calculated from white versus red cells. Velocity of spermatozoa was calculated as μm.s^{-1} based on length traces of spermatozoa from blue to green and red heads, calibrated for magnification. Excel 97 automatically calculated both values.

Data analysis

Average values of the acquired parameters and standard deviations (SD) were determined from 3 replicates for each fertilization trial, including the velocity and percentage of sperm motility. Statistical significance was assessed using multiple analysis of variance (ANOVA, Statgraphics version 5), followed by Tukey HSD multiple range test comparisons. Correlations were evaluated from the nine average values of the following parameters: velocity of post-thawed sperm, percentage of post-thawed sperm motility and percentage of fertility assessed with Microsoft Excel 97. Probability values at < 0.05 were considered as significant.

Results

Fertilization rates of control sperm (unfrozen) samples after 24-h storage at 3 ^{o}C ranged from 65 to 90%, indicating that the gametes were of good quality. The best cryoprotectant for having live post-thawed sperm ($3.6.10^5$ spermatozoa per egg) was methanol (8%) resulting in fertilization rates ranging from 61 to 76%. ANOVA showed a significant effect of males on post-thawed sperm velocity and fertilization rate.

Effect of extender and cryoprotectant

The results of individual parameters (percentage of motile sperm and average velocity of spermatozoa at 20 s after activation as well fertilization rates) in extended or non extended frozen/thawed sperm with using two different cryoprotectants (either the product or concentration) are shown in Figures 1-2 with comparison to the control sperm (non frozen). Fertilization rate of control sperm (unfrozen) samples after 24-h storage at 3 ^{o}C was 81.5 % (Figs 1-3).

The excellent fertilization rates 64-75 % were obtained on post-thawed sperm when sperm was either without dilution or diluted with extender 1 and treated by methanol in concentrations 8 or 10 % (Fig. 1). These results were not significantly different compared with the fresh sperm control sample. Hatching rate was very low, only 8-15 %, when sperm was frozen with 8 or 10 % DMSO (Fig. 1). At second test, 8 % of methanol with Extender 1 was the best combination for storage fertilization rate in thawed sperm. That combination was slightly but not significantly different with non-extended fresh sperm (Figure 2). ANOVA showed a significant influence of males on fertilization rate oscillating from 38 to 64 %, when fertilization rate of thawed sperm in all males were not statistically different from the fresh sperm.

No significant difference were found in terms of percentages of motility (70-86 %) at 20 sec after activation of frozen/thawed sperm, when sperm was treated with DMSO or methanol (Figure 1) and also when different sperm samples from three males were tested (Figure 3). The average value in fresh sperm (control) was 98%. A solution of 8% methanol used as the cryoprotectant combined with the extender 1 yielded the highest percentage of motility in post-thawed sperm (82%, Figure 2). ANOVA showed no influence of post-thawed sperm derived from different males on sperm motility. The results of post-thawed sperm motility (without fresh sperm) were significantly correlated with the hatching rate of eggs obtained from post-thawed sperm ($r=0.49$, $P=0.035$) and did not correlate with the velocity determined in post-thawed spermatozoa ($r = 0.32$, $P = 0.177$).

Velocity of post-thawed spermatozoa ranged from 118 to 157 μm.s^{-1} and these were considered to be high for the trials using sperm frozen in methanol of 8 or 10% (Figures 1 and 2). Methanol of 8 % with extender 1 was the best cryoprotectant for saving the high velocity (157 μm.s^{-1}) in post-thawed sperm. Also when sperm was stored only without extender with 8-10 % of methanol, the velocity of post-thawed spermatozoa was on the level 118-135 μm.s^{-1} (Figure 2). Similar insignificant velocity of spermatozoa was found between the fresh none extended sperm and thawed sperm extended with methanol (Figures 1 and 2). ANOVA showed a significant influence of males on sperm velocity of post-thawed sperm. Post-thawed sperm velocity correlated with hatching rate ($r=0.55$, $P=0.014$).

Regarding all parameters it can be concluded that methanol of 8 or 10 % with extenders 1 or 2 are useful for keeping of good quality of frozen/thawed sperm.

Discussion

A complete freezing procedure to preserve the sperm of paddlefish consisted of four different cooling rates, four steps during cooling and the temperature at which the super-cooled sperm were plunged into LN_2 (see figure 4). This methodology was highly modified from cryopreservation studies on common carp (Linhart et al., 2000) and European catfish (Linhart et al., 2006). The first step of the freezing program was a slow cooling from 0 ^{o}C to –5 ^{o}C at a rate of 3 ^{o}C.min^{-1}. When ice started to form in the external medium, the program was accelerated at the rate of 5^{o}C .min^{-1} from –5 ^{o}C to –15 ^{o}C, then at rate of 10 ^{o}C from –15 ^{o}C to –25 ^{o}C and finally at the rate of 20 ^{o}C .min^{-1} from –25 ^{o}C to –80 ^{o}C. The sperm was at the temperature of -80°C held for 6 min and then transferred into LN_2. In programmable freezer Jahnichen et al. (1999) used a two step freezing regime at the rate of 3.5- 5^{o}C .min^{-1} from +2 ^{o}C to –14 ^{o}C, then at rate of 10-20 ^{o}C from –14 ^{o}C to –70 ^{o}C and direct transfer to liquid nitrogen. The freezing rate was also controlled according to different size of straws by the height above the surface of liquid nitrogen at the level 3-5 cm (Horvath and Urbanyi, 2000; Glogowski et al., 2002).

When experiments with hatching rate in post-thawed sperm are conducted to detect the optimal freezing protocol, it is necessary to use low or medium number of spermatozoa per egg. Number of fresh spermatozoa ranging from 10,000 to 100,000 per egg is required for artificial insemination in sturgeon's species (Ginsburg, 1968; Dettlaff et al., 1993). If high number of spermatozoa per egg (millions) is used, low viability and bad quality of post-thawed

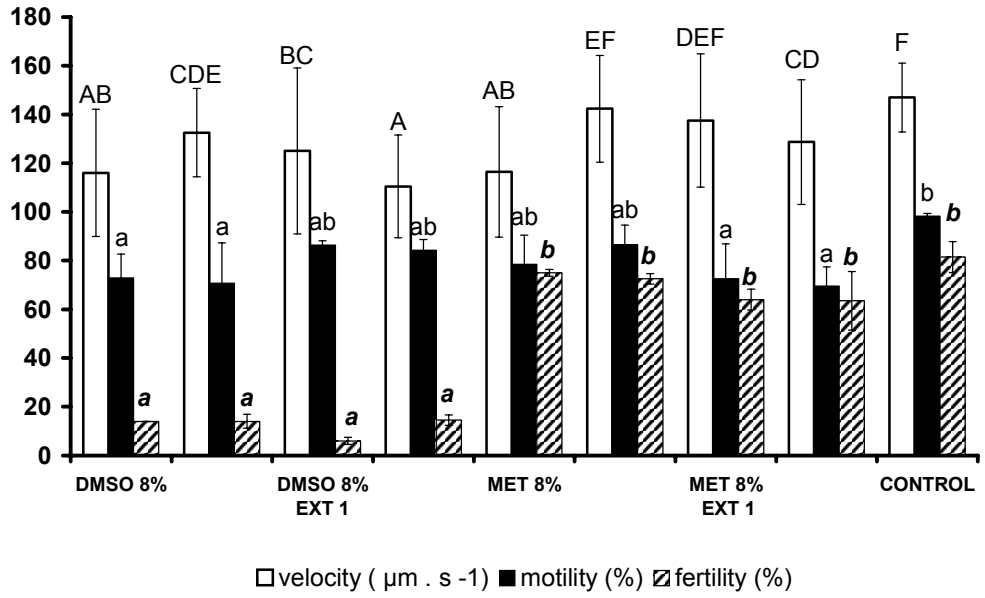


Fig. 1. Effect of 2 different types of cryoprotectants was evaluated. The columns express average values of data found in post-thawed sperm and all different concentration for DMSO and methanol (8 and 10 %) with or without extender 1 in fertilization rates, percentage of sperm motility at 20 s after activation and velocity of spermatozoa at 20 s after activation. Fresh non-extended sperm was used as control. Groups with a common superscript for each parameter do not differ significantly (P<0.05).

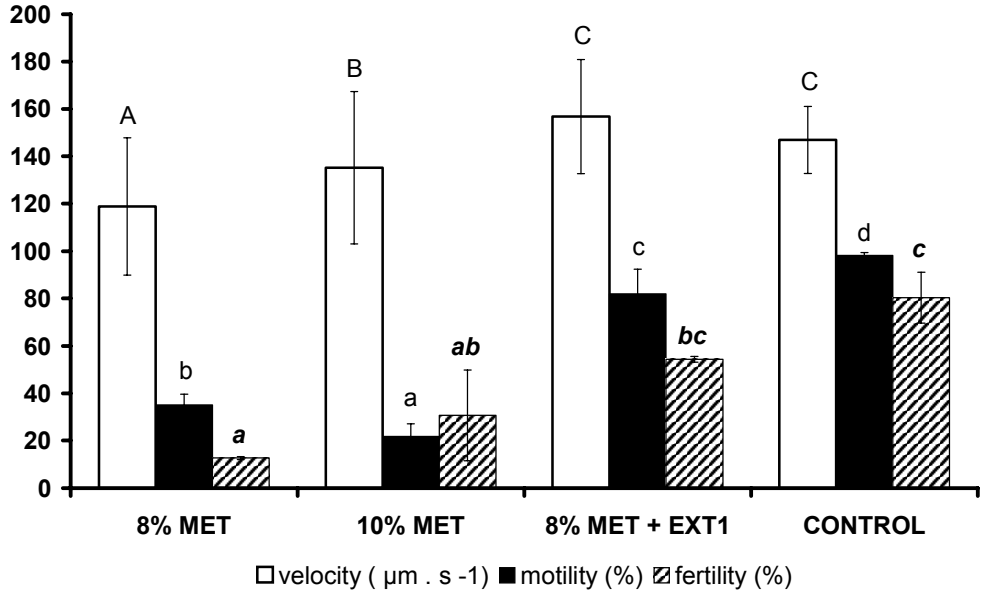


Fig 2. Effect of methanol as cryoprotectant was evaluated. The columns express average values of data found in post-thawed sperm and all different concentration for methanol (8 and 10 %) with or without extender 1 in fertilization rates, percentage of sperm motility at 20 s after activation and velocity of spermatozoa at 20 s after activation. Fresh non-extended sperm was used as control. Groups with a common superscript for each parameter do not differ significantly (P<0.05).

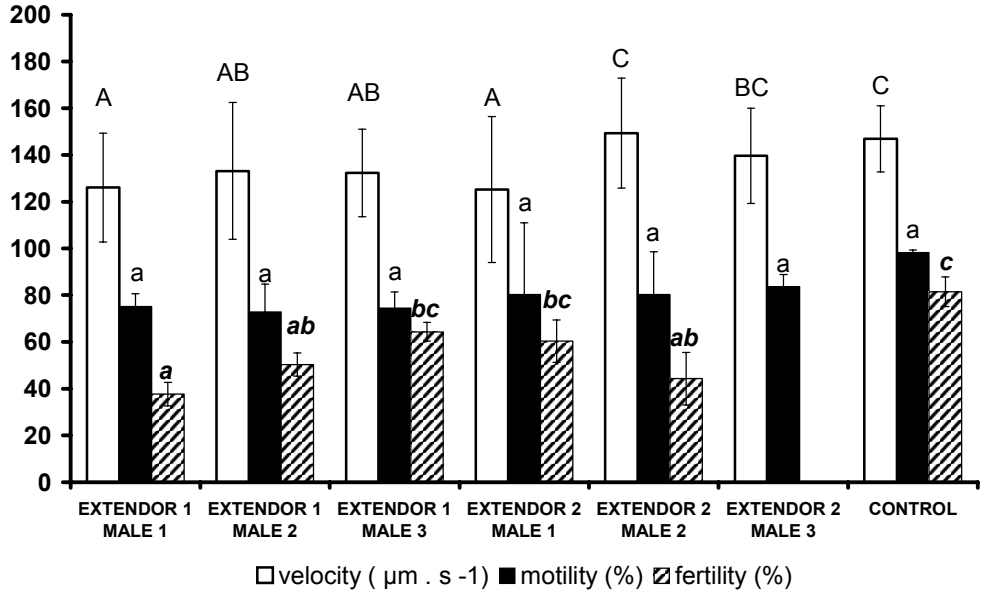
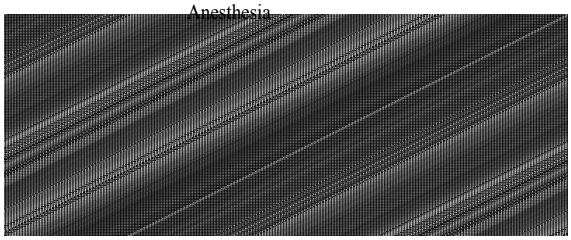
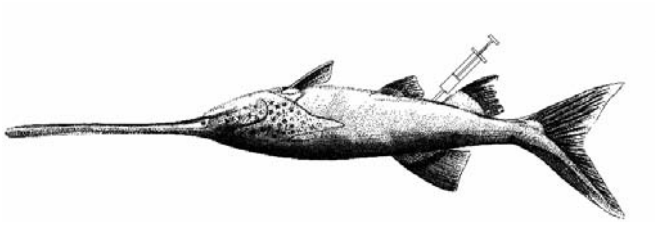


Fig. 3. Effect of 3 different males on cryopreservation success of sperm was evaluated. The columns express average values of data found in post-thawed sperm of three different males with extenders 1 and 2, and methanol (8 %) in fertilization rates, percentage of sperm motility at 20 s after activation and velocity of spermatozoa at 20 s after activation. As control was used fresh not extended sperm. Groups with a common superscript for each parameter do not differ significantly (P<0.05).

Anesthesia

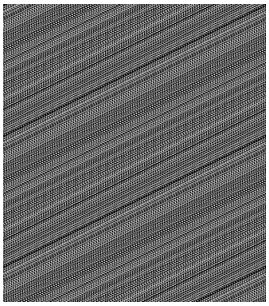




Collection of sperm

1.5 hours storage sperm in plastic containers on ice at temperatures 0-4°C.

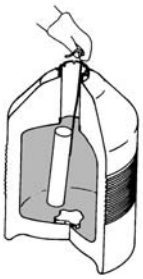
Minimum 80 % of spermatozoa motility



Methanol (8%) was added in extender 1 (20 mM tris pH 8 + 30 mM sucrose + 0.5 mM KCl, dilution 1:1) or extender 2 (20 mM tris pH 8 + 50 mM sucrose + 0.5 mM KCl, dilution 1:1) and every 1 ml of mixture without equilibration time was transferred to a 2-ml cryotube

Freezing in the PLANER KRYO 10 III at 0 °C to –5 °C at a rate of 3 °C .min-1, from –5 °C to –15 °C at a rate of 5 °C .min-1, from –15 °C to –25 °C at a rate of 10 °C .min-1, from –25 °C to –80 °C at a rate of 20 °C .min-1, then held for 5 min at -80 °C

Finally, transfer to liquid nitrogen (LN2)



Frozen sperm were thawed in a water bath 40 ^{O}C for 105 s.

Fig. 4. Procedure for cryopreservation of paddlefish sperm after strapping from hormonally treated broodstock, identifying indiviual steps in terms of media application and temperature exposures during the freezing and thawing process.

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