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Effect Different Concentrations of DMSO and Dilution Rateson Motility Parameters of Stellate (*Acipenser stellatus*) Spermatozoa

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Abstract: Milt obtained from four stellate males (*Acipenser stellatus*) was cryopreservation using extender; Tris-sucrose-KCl (30mM Tris, 23.4mM sucrose, 0.25mM KCl, pH 8.0) supplemented with DMSO atconcentration of 5%, 10% and 20%. Semen was diluted, respectively, with ratios of 1:0.5, 1:1, 1:2 and 1:5 with extender and frozen in liquid nitrogen vapor.Frozen sperms after 2 and 15 dayswere excluded from freezing. Experiment showed thehighest motility duration and the mostmotility percentage of post-thawed sperms after 2 dayswas related to the treatments with theconcentration ofDMSO 10% and the dilution ratio of 1: 1 (260±25.62 s and 32.28±5.36%; $P<0.05$), as well as the upmost mobility and the motility duration of post-thawed sperms after 15 days wasrelated to the treatments with the concentration of DMSO 10% and the dilution ratio of 1:1(251.19±23.54s and 23.37±5.51%; $P<0.05$). Theresults showed that holdin time from 2 to 15 daysof frozen sperm has a negative impact on Motility duration and Motility percentage of post-thawed sperms. Results also showed that highestmotility duration and motility percentage of post-thawed sperms was for treatments that have been diluted with 1:1 ratio.

Key words: Stellate · Sperm · DMSO · Dilution Ratios · Motility Duration · Motility Percentage

INTRODUCTION

The creation of sperm banks of the selected stock to prevent outbreaks, catastrophes and geneticdrift is essential to develop genetic selection programs in commercial aquaculture, but it is alsonecessary for conservation of strains or species in danger of extinction, until the environmental conditions are recovered (1275 fish species threatened according to the International Union for Conservation of Nature, www.iucnredlist.org). Reproductive technologies are being developed to recover a population just from frozen sperm, applying further hybridization programs of using androgenic procedures [1, 2]. Biotechnology, ecotoxicology and basic sciences could also benefitfrom cryobiology through the preservation of gametes or embryos from model species or strains as well as from sperm or embryos genetically modified with research or commercial production purposes (polyploidy, transgenic).

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 [3].

Cryopreservation induces severe stress to fish spermatozoa that in turn affect sperm quality in termsof fertilization ability. Sperm quality is influenced by several factors such as temperature, diet [4], time of sampling [5] and delays caused after the injection of hormones [6].

Important parameters for cryopreservation include types of extenders and cryoprotectants, the dilution ratio, the freezing and thawing rates and type of extender used for fertilization.

The most efficient permeating cryoprotectant for fish milt appears to be dimethyl sulfoxide (DMSO). Other cryoprotectants such as glycerol, ethylene glycol, propane-diol, dimethyl acetamide and methanol are less popular or have been used with limited success.

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Unlike most teleost fish, information concerning reliable technology for cryopreservation of sturgeon semen is not available.

Cryopreservation success was usually measured as post-thaw sperm motility [7, 8] or as fertilizationsuccess during early embryo development [9]. DMSO or ethylene glycol was used as a cryoprotectant. Sturgeon (*Acipenser* sp., *Chondrostei*) spermatozoa are significantly different from freshwater teleost fish sperm. These differences concern morphology (more complex structure, presence of acrosome), physiology (longer duration of motility, acrosome reaction) and biochemistry (presence of acrosin, arylsulfatase) [10, 11].

Another striking difference between milt properties of sturgeons and teleost fish is the lowosmolality of sturgeon seminal plasma [12]. These peculiar characteristics of sturgeon milt mayexplain the lack of success in sturgeon of cryopreservation methods established for teleost fish. The objectives of our work wereto test the effect of(1) DMSO in different concentrations on the mobilityand motility duration of stellatesturgeon sperm; (2) several diluted rates in combination with different DMSO concentrations on the mobility and motility duration of stellatesturgeon sperm.

MATERIALS AND METHODS

Sperm Collection for Cryopreservation: Milt was collected from eight stellate (*Acipenser stellatus*) males using a syringe with an attached polyethylene rigid tubing inserted into the urogenital opening. Semen from these males was obtained in March. Before stimulation, fish were transferred from ponds to tanks with a watertemperature of 18°C for 3 days and the temperature was raised to 20°C for an additional 7days.

Spermiation was induced by injecting of sturgeon pituitary extract in dose of 2-3mgkg^{-1} body weight [6]. Spermatozoa were collected within 15-24h (depending on the water temperature) post hormonal injection. Semen was stored on ice and used within 2 h of storage for cryopreservation.

Assessment of Sperm Quality: Motility of sperm samples was estimated under a light microscope at 100× magnification immediately after mixing of 5µL of sperm with 50µL of activation solution)NaCl 3.5 mM, Tris-HCl 12 mM, pH=8.5) [13] on a microscope slide. Sperm mobility and duration of sperm motility was recorded using a software gadmei tv home media v. 330 from note book connected to Nikonmicroscope (Optiphot-2, Japan) at 100× magnification that combined with CCD color video camera (model SPC-2000P, Japan). Sperm motility and duration of spermmotilitywere evaluated fromsperm with forward movement. Immotile sperm were defined as sperm that did not show forwardmovement after activation. Video records were set at 30 frames/s using video camera mounted on a microscope. Percentage of sperm motility wasdetermined during 0-15 s post-activation. Motility duration was evaluated by counting the timefrom sperm activation with activation solution until sperm stopped moving [14]. Analyses were repeated three times for each treatment.

Extender and Sperm Cryopreservation: in these experiment using extender Tris- sucrose-KCl(30mM Tris, 23.4mM sucrose, 0.25mM KCl, PH 8.0) [15] were supplement with 5%, 10% and 20% DMSO [16]. Semen and extender had atemperature of 4°C. Milt was diluted at ratios of 0.5:1, 1:1, 2:1 and 5:1 with extender.

Suspensions of extended milt were drawn into 0.25-mlstraws. Sperm freezing was conducted in a Styrofoam box with liquid nitrogen. Straws (of 0.25 ml volume) with diluted sperm, prior kept toequilibrate for 5 minutes at room temperature, were placed forslow freezing during 3 minutes on a 3 cm high floating frame made from the same material as the box. Straws were subsequentlyplunged into liquid nitrogen [17].

Measurement Sperm Mobility, Duration of Spermmotility and Concentration: Thawing of sperm was conducted in a water bath at 40°C for 5 sec [17]. Sperm mobility and duration of sperm motility of thawed semen was observed after 2 and 15 day of storage in liquidN_2.

Post-thaw mobility and motility duration was observed and evaluated by three operators using a monitor connected to a microscope.Sperm concentration was measured by the optical density method [18].

Statistical Software: All values are shown as mean±SD. Data for percentage and duration of sperm motility were transformed by angular transformation prior to statistical analysis by SPSS 10.0 software. The effects of concentrations DMSO and diluted rates on post-thaw sperm motility and duration of sperm motility were analyzed using two-way analysis of variance (ANOVA). Means were separatedby Duncan's New Multiple Range test and were considered significantly different at $P<0.05$.

RESULTS

The duration of sperm motility used for cryopreservation exceeded 320 s (Table 1). Likewise, only sperm samples showing 80% motility or higher were used for the experiments (Table 1).

Effect of Dilution Rates with Concentrations of DMSO on Quality Post-Thawed Sperms After 2 Days: Highestmotility duration and the most motility percentage of post-thawed sperms after 2 days was related to the treatments with the concentration of DMSO 10% and the dilution of 1: 1 (260±25.62 s and 32.28±5.36%; Table 2). The least Duration and the lowest mobility of post-thawed sperms was observed in the treatments with the concentration of DMSO 20% and the dilutionof 1: 5 (183.24±18.86 s and 18.31±4.95%; P<0.05) Table 2.

Table 2 showed the maximum duration and the most mobility results were observed intreatments where the diluted rate was 1:1, as well as the lowest motility percentage and motilityduration of post-thawed sperm observed in diluted rate was 1:5.

Effect of Dilution Rates with Concentrations of DMSO on Quality Post-Thawed Sperms After 15 Days: Maximum motility duration and the upmost mobility of post-thawed sperms after 15 days wasrelated to the treatments with the concentration of DMSO 10% and the dilution of 1: 1 (251.19±23.54 s and 23.37±5.51%; Table 3).

Table 3 shows the minimum Duration and the lowest motility percentage of post-thawed spermswas observed in the treatments with the concentration of DMSO 20% and the dilution of 1: 5(137.54±19.84 s and 13.38±4.94%; P<0.05).

Table 1: Males used for sperm cryopreservation process

Male	Body weight (g)	Total length (cm)	Sperm concentration ($\times 10^9$ ml^{-1})	Motility duration (s)	Motility percentage (%)
1	10	144	3.12	342.24±8.24	80.64±1.84
2	12	143	2.59	330.11±11.70	82.41±2.70
3	14	155	2.25	340.67±6.94	81.20±2.34
4	13	166	2.46	325.62±8.42	80.42±2.06

Table 2: Effect of different concentrations of DMSOand diluted rates on post-thaw sperm motility and duration of sperm motility after 2 days of freezing

Cryoprotectant	Cryoprotectant concentration (%)	Diluted rates (sperm: extender)	Motility duration (s)	Motility percentage (%)
		1: 0.5	$31.10^{a} \pm 249.24$	$5.10^{bc} \pm 25.36$
DMSO	5	1: 1	$27.62^{a} \pm 258.54$	$5.04^{ab} \pm 29.54$
		1: 2	$23.42^{ab} \pm 234.72$	$5.10^{cd} \pm 21.00$
		1: 5	$22.34^{bc} \pm 198.20$	$4.87^{cd} \pm 19.23$
		1: 0.5	$23.54^{a} \pm 251.43$	$5.35^{ab} \pm 28.41$
DMSO	10	1: 1	$25.62^{a} \pm 260.00$	$5.36^{a} \pm 32.28$
		1: 2	$24.61^{ab} \pm 240.28$	$4.97^{cd} \pm 21.28$
		1: 5	$19.34^{bc} \pm 200.47$	$5.10^{cd} \pm 19.00$
		1: 0.5	$25.59^{ab} \pm 237.54$	$5.24^{cd} \pm 21.28$
DMSO	20	1: 1	$23.21^{ab} \pm 241.47$	$5.28^{cd} \pm 22.02$
		1: 2	$22.47^{ab} \pm 232.26$	$5.37^{cd} \pm 21.24$
		1: 5	$18.86^{c} \pm 183.24$	$4.95^{d} \pm 18.31$

Values within column followed by different superscript letters were significantly different (P<0.05)

Table 3: Effect of different concentrations of DMSO and diluted rates on post-thaw spermmotility and duration of sperm motility after 15 days of freezing

Cryoprotectant	Cryoprotectant concentration (%)	Diluted rates (sperm: extender)	Motility duration (s)	Motility percentage (%)
		1: 0.5	$24.12^{bc} \pm 199.28$	$5.27^{abc} \pm .18.54$
DMSO	5	1: 1	$21.24^{ab} \pm 219.14$	$4.92^{ab} \pm 20.35$
		1: 2	$21.07^{abc} \pm 207.67$	$5.10^{abc} \pm .17.00$
		1: 5	$24.98^{d} \pm 154.18$	$4.24^{bc} \pm 15.27$
		1: 0.5	$20.73^{bc} \pm 204.29$	$5.12^{ab} \pm 20.51$
DMSO	10	1: 1	$23.54^{a} \pm 251.19$	$5.51^{a} \pm 23.37$
		1: 2	$22.30^{bc} \pm 206.38$	$4.94^{abc} \pm 17.00$
		1: 5	$24.27^{cd} \pm 163.42$	$4.27^{bc} \pm 16.28$
		1: 0.5	$23.27^{bc} \pm 200.74$	$5.10^{abc} \pm 17.57$
DMSO	20	1: 1	$30.18^{ab} \pm 215.64$	$5.37^{ab} \pm 20.85$
		1: 2	$21.34^{bc} \pm 205.67$	$4.90^{abc} \pm 18.04$
		1: 5	$19.84^{d} \pm 137.54$	$4.94^{c} \pm 13.38$

Values within column followed by different superscript letters were significantly different (P<0.05)

Table 3 showed the highest motility duration and the most motility percentage results wereobserved in treatments where the diluted rate was 1:1, as well as minimum motility duration and the least mobility of post-thawed sperm observed in diluted rate was 1:5.

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 [19, 20]. The establishment of sperm banks from valuable fish species including sturgeon is widely practiced in manycountries [21, 22].

According to the above results, by comparing Table 2 and 3 were determined the dilutionratios has a significant differences on the duration of sperm motility ($P<0.05$), as most of the motility duration related to dilution ratio of 1: 1of the treatments and the duration of sperm motility with increasing dilution significantly reduced. Because of the high dilution of the sperm plasma loses its protective effect, sperm viability reduced and by increasingresulting in reduced sperm viability [23]. The results showed that thequality spermsignificantly reduction after thawing was similar to the results [24].

These researchers have reported that the quality of Ponto-Caspian sturgeon spermsharply decreased after thawing. In this experiment, post-thawed sperms with DMSOconcentration of 10%and the dilution rate 1:1 has the highest mobility and motility duration was similar to theresults [25]. These researchers have reported that the most suitablecryoprotectant for sperm cryopreservation Italian Cobice sturgeon (*Acipensernaccarii*), isDMSO concentration of 10%. Also, Lahnsteiner *et al.* [23], announced the most motility of post-thawed sperm Starlet (*Acipenser ruthenus*) was with DMSO 10% (80±7.4%). Horvath *et al.* [16], reported themaximum motility and motility duration ofpost-thawed sperm of pallid sturgeon (*Scaphyrinchusalbus*) was with DMSO concentration of 5% (26±13%). Liu *et al.* [25], reported that themost suitablecryoprotectant for sperm Cryopreservation Chinesesturgeon (*Acipenser persicus*), was with DMSO 12%. Thereason ofdifference in suitable density of cryoprotectant in written result with experiment result may be for selected species, difference in extender solution or semen isspecific characteristics of this species.

CONCLUSION

From the above study it can be concluded thatstorage of frozen sperm has a negative impact on motilityduration and motility percentage of post-thawed sperms. Results also showed that the highest motility duration andmotility percentage of post-thawed sperms was fortreatments with the concentration of DMSO 10% and thedilution of 1:1.

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