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Effect Different Concentrations ofmethanol (MeOH) and Dilution Rateson Motility Parameters of Beluga (*Huso huso*) Spermatozoa after Short-Term Storage

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Abstract: Semen obtained from four Beluga males (*Huso huso*) was cryopreserved using extender; Tris-sucrose-KCl (30mM Tris, 23.4mM sucrose, 0.25mM KCl, PH 8.0) supplemented with MeOHatconcentration of 5%, 10% and 20%. Semen was diluted, respectively, with ratios of 1:0.5, 1:1 and 1:2 with extender and frozen in liquid nitrogen vapor. Frozen sperms after 3 and 15 days were excluded from freezing. Experiment showed the highestmotility duration and the most motility percentage of post-thawed sperms after 3 days was related to the treatments with the concentration of MeOH10% and the dilution ratio of 1: 1 (297.15±23.52s and 26.62±5.40%; $P<0.05$), as well as the upmost mobility and the motility duration of post-thawed sperms after 15 days was related to the treatments with the concentration of MeOH10% and the dilution rates of 1: 1 (224.25±21.20 s and 18.21±5.10%; $P<0.05$).

Key words: *Huso huso* · Cryopreservation · Sperm · MeOH · Motility duration · Motility percentage

INTRODUCTION

Sturgeons (Order Acipenseriformes) are chondrostean fishes of classical origin that inhabit only the Northern hemisphere [1]. Several species are restricted to very little populations which in some cases are close to extinction due to exploitation of natural stocks for flesh and caviar as well as destruction of habitat [2]. It has been estimated that semen from 200 fish species have been Successfully cryopreserved [3], however, species-specific optimizations of technology are needed. Successful cryopreservation of fish spermatozoa is well established for many species [4]. Earlier works indicated that the cryopreservation of sturgeon sperm using DMSO-sucrose extender resulted in recovery of motile spermatozoa with basic mobility characteristics similar to those of fresh milt [5]. Main parameters for cryopreservation include types of extenders and cryoprotectants, the dilution rates, the freezing and thawing rates and kind of extender used for fertilization. The mostefficient permeating cryoprotectant for fish semen appears to be dimethyl sulfide (DMSO) and methanol (MeOH) [6]. Other cryoprotectants such as glycerol, ethylene glycol, propane-diol, dimethylacetamide and methanol are short popular or have been used with limited success. 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 mobility [5-7] or as fertilization success during early embryo growth [8]. Sturgeon (*Acipensersp., Chondrostei*) spermatozoa are significantly different from teleost fish sperm. These differences concern morphology (more complex structure, presence of acrosome), physiology (longer durationof mobility, acrosome reaction) and biochemistry (presence of acrosin, arylsulfatase) [9, 10]. Otherstriking difference between semen properties of sturgeons and teleost fish is the low osmolality of Sturgeon Seminal plasmacomposition [11]. The objectives of our work were to test the effect of: (1) MeOHin different concentrations on the motility percentage and motility duration of Belugasturgeon sperm; (2) several dilution rates in combination with different MeOH concentrations on the motility percentage and motility duration of Beluga sturgeon sperm.

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MATERIALS AND METHODS

Sperm Collection for Cryopreservation: Semen samples were collected from eight males of Beluga (*Huso huso*) in Shahid Marjani Sturgeon Hatchery located in Gorgan, Iran in March 2012. Spermiation was induced by injecting of sturgeon with pituitary extract in dose of 2-3mg kg^{-1} bodyweight [13]. Semen was collected within 16-24h (depending on the water temperature) post hormonal injection. Semen was transferred to Aquaculture Research Center of Gorgan University of Agricultural Sciences and Natural Resources. Milt was stored on ice and used within 2 h of storage forcryopreservation.

Assessment of Sperm Quality: Mobility of sperm samples was estimated under a light microscope at 400× magnification immediately after mixing of 5µL of sperm with 50µL of activation solution)NaCl3.5 mM, Tris-HCl12 mM, pH=8.5) [14] on a microscope slide. Sperm mobility and duration of sperm motility was recorded using a software gadmeitv home media v. 330 from note book connected to Nikon microscope (Optiphot-2, Japan) at 400× magnification that combined with CCD color video camera (model SPC-2000P, Japan). Sperm motility and duration of sperm motility were evaluated from sperm with forward movement. Immotile milt was 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 was determined during 0-10 s post-activation. Motility duration was evaluated by counting the time from sperm activation with activation solution until sperm stopped moving [15].

Extender and Sperm Cryopreservation: In this test using extenderTris- sucrose-KCl(30mM Tris, 23.4mM sucrose, 0.25mMKCl, PH 8.0) [12] supplement with 5%, 10% and 20% MeOH [16]. Semen and extender had a temperature of 4°C. Milt was diluted at ratios of 1:0.5, 1:1 and 1:2 with extender. Suspensions of extended milt were drawn into 0.25-ml straws.Semen-freezing was conducted in a styrofoam box filled with liquid nitrogen. Straws were placed on a 4-cm-high floating frame made of styrofoam. Straws were sealed and after 3 min of freezing in liquid nitrogen vapor, were plunged within liquid nitrogen [6].

Measurement Sperm Mobility, Duration of Sperm Motility and Concentration: Straws were thawed in a water bath with a temperature of 40°C for 15 s [6]. Sperm mobility and duration of sperm motility of thawed semen was observed after 3 and 15 day of storage in liguid N2. Post-thaw mobility and motility duration was observed and evaluated by the same operators using a monitor connected to a microscope.Milt concentration was measured by the LamNyvbarmethod [9].

Statistical Software: Microsoft Excel and SPSS version 16.0 were used for statistical analysis.

RESULTS

The duration of sperm motility used for cryopreservation exceeded 400 s (Table 1). Likewise, only sperm samples showing 80% mobility or higher were used for the experiments (Table 1).

Effect of Dilution Ratios with Concentrations of MeOH on Quality Post-Thawed Sperms after 3 Days: Highestmotility duration and the most motility percentage of post-thawed sperms after 3 days was related to the treatments with the concentration of MeOH10% and the dilution of 1: 1(297.15±23.52 s and 26.62±5.40%; Table 2). The least Duration and the lowest mobility of post-thawed sperms was observed in the treatments with the concentration of MeOH20% and thedilution of 1: 2. (221.54±29.48 s and 15.24±4.57%; $P<0.05$) Table 2. The maximum duration and the most mobility results were observed in treatments where the dilution rate was 1:1, as well as the lowest motility percentage and motilityduration of post-thawed sperm was observed in dilution rate 1:2.

Effect of Dilution Rates with Concentrations of MeOH 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 MeOH10% and the dilution of 1: 1 (224.25±21.20 s and 18.21±5.10%; Table 3).

Results shows the minimumduration and the lowest motility percentage of post-thawed sperms in the treatments with the concentration of MeOH20% and the dilution of 1: 2 (163.32±18.53 s and 11.74±4.21%; $P<0.05$). Table 3 showed the highest motility duration and the most motility percentage results in treatments where the dilution rate was 1:1, as well as minimum motility duration and the least mobility of post-thawed sperm observed indilution rate 1:2.

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	170	291	2.30	430.21±12.14	82.32±2.41
2	160	240	2.56	410.64±8.75	83.43±2.70
3	150	260	2.02	418.52±10.42	82.46±1.87
4	135	260	2.87	407.34±9.61	84.32±2.04
Total	153.75	262.75	2.41	416.68±12.72	83.13±2.11

Table 2: Effect of different concentrations of MeOH and dilution rates on post-thaw sperm motility and duration of sperm motility after 3 days of freezing

Cryoprotectant	Cryoprotectant concentration (%)	Diluted rates (sperm: extender)	Motility duration (s)	Motility percentage (%)
		1: 0.5	22.30^{a}±279.86	4.41^{abc}±20.24
MeOH	5	1: 1	22.50^{a}±293.00	4.08^{ab}±25.30
		1: 2	20.58^{b}±228.23	3.87^{c}±17.12
		1: 0.5	17.81^{a}±284.10	4.37^{abc}±21.00
MeOH	10	1: 1	23.52^{a}±297.15	5.40^{a}±26.62
		1: 2	31.30^{b}±233.33	3.84^{abc}±19.08
		1: 0.5	21.37^{ab}±263.37	3.88^{bc}±17.72
MeOH	20	1: 1	22.53^{a}±281.00	4.20^{abc}±20.23
		1: 2	29.48^{b}±221.54	4.57^{c}±15.24
Control	-	-	416.68±12.72	83.13±2.11

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

Table 3: Effect of different concentrations of MeOH and dilution rates on post-thaw sperm motility 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	23.52^{abc}±198.61	2.03^{ab}±.15.61
MeOH	5	1: 1	21.05^{ab}±220.30	1.11^{a}±17.18
		1: 2	25.65^{bc}±179.14	2.01^{c}±.12.13
		1: 0.5	19.37^{abc}±197.00	1.45^{ab}±15.51
MeOH	10	1: 1	21.20^{a}±224.25	5.10^{a}±18.21
		1: 2	18.44^{abc}±184.67	1.55^{bc}±13.50
		1: 0.5	28.35^{abc}±192.51	1.51^{bc}±12.61
MeOH	20	1: 1	17.37^{abc}±200.26	1.90^{bc}±14.00
		1: 2	18.53^{c}±163.32	4.21^{c}±11.74
Control	-	-	416.68±12.72	83.13±2.11

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

DISCUSSION

Decrease in stocks and limited number of potential breeders has led to the establishment of fish semencryobanks which play a crucial role in the genetic management and conservation of aquatic resources [17, 18]. The establishment ofsemen banks from valuable fish species including sturgeon is widely practiced in multiple countries [19, 20]. The cryopreservation remains one of the most attractive and quickly developing trends forthe sturgeon protection. Methods of cryopreservation of the sturgeon semen have been well established [6, 21]. However, the differences steps required for cryopreservation (cryoprotective agent loading, freezingthawing, cooling to a low sub zero temperature) may contribute individually or cumulatively in semen damage that in turn decreases fertilization and growth stages [22]. Recently, it has been shown, that profound freezingmechanically destroys cell membranes [23]. According to the above results, by comparing Table 2 and 3 the dilution rate has significant differences on the duration of sperm motility (P<0.05), as thehighestmotility duration related to dilution rate of 1:1of the treatments and the duration of sperm motilitywith increasing dilution significantly reduced as seminal plasma loses its protective effect, sperm viability reduced, the concentration of cryoprotectantin increased causing toxicity and reduced sperm viability [24]. The results showed that the quality ofsperm significantly reduced after thawing similar to the resultsof [25]. These researchers have reported that the quality of Ponto-Caspian sturgeonsemensharply decreased after thawing. [26] reported that methanol is an excellent cryoprotectant for cryopreservation of starlet milt.In this experiment, post-thawed sperms with MeOH concentration of 10% and the dilution of 1:1 has the highest mobility and

motility duration similar to the results [6]. These researchers have reported that the most suitable cryoprotectant for sperm cryopreservation Siberian sturgeon (*Acipenser baeri*) is MeOHconcentration of 10%. [26] reported that themost suitable cryoprotectant for sperm Cryopreservation paddle fish (*Polyodonspathula*) was with MeOH8% [27]. The reason ofdifference in suitable density of cryoprotectant in written result with experiment result may be for selected species, difference in extender solution or semen is specific characteristics of this species.

CONCLUSION

From the above study it can be concluded that storage of frozen semen has a negative impact on motility duration and motility percentage of post-thawed sperms. Results also showed that highest motility duration and motility percentage of post-thawed sperms was for treatments with the concentration of MeOH 10% and the dilution of 1:1.

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