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Cryopreservation of Beluga (*Huso huso*) Sperm: Effect of Different Concentrations of Methanol (MeOH) and Dilution Rates on Sperm Mobility and Motility Duration after Long-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 MeOH at concentration 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 30 and 60 days were excluded from freezing. Experiment showed the highest motility duration and the most motility percentage of post-thawed sperms after 30 days was relatedto the treatments with the concentration of MeOH10% and the dilution ratio of 1:1 (157±25.46 s and 12.58±4.94%; $P<0.05$), as well as the upmost mobility and the motility duration of post-thawed sperms after 60 days was related to the treatments with the concentration of MeOH10% and the dilution ratio of 1:1 (119.18±23.52 s and 10.31±4.12%; $P<0.05$).

Key words: *Huso huso* % Cryopreservation % Sperm % MeOH. Motility Duration % Motility Percentage

INTRODUCTION

It has been estimated that semen from 200 fish species have been successfully cryopreserved [1], however, species-specific optimizations of technology are needed. Main parameters for cryopreservation include types of extenders and cryoprotectants, the dilution ratio, the freezing and thawing rates. The most efficient permeating cryoprotectant for fish semen appears to be dimethyl sulfide (DMSO) and methanol (MeOH) [2]. Other cryoprotectants such as glycerol, ethylene glycol, propane-diol, dimethyl acetamide and methanol are short popular or have been used with limited success. Unlike most teleost fish, information concerning reliable technology for cryopreservation of sturgeon milt is not available. Cryopreservation success was usually measured as post-thaw sperm mobility [3-4] or as fertilization success during primary embryo growth [5]. Sturgeon (*Acipenser sp., Chondrostei*) spermatozoa are significantly different from teleost fish semen. These differences concern morphology (more complex structure, presence of acrosome), physiology (longer duration of mobility, acrosome reaction) and biochemistry (presence of acrosin, arylsulfatase) [6, 7]. Other striking difference between semen properties of sturgeons and teleost fish is the low osmolality of sturgeon seminal plasma composition [8]. The objectives of our work were to test the effect of: (1) MeOH in different concentrations on the motility percentage and motility duration of Beluga sturgeon sperm; (2) several dilution rates in combination with different MeOH concentrations on the motility percentage and motility duration of Beluga sturgeon sperm.

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 [9]. Semen was collected within 16-24h (depending on the water temperature) post hormonal injection. Semen was transferred to Aquaculture Research

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Center of Gorgan University of Agricultural Sciences and Natural Resources. Milt was stored on ice and used within 2 h of storage for cryopreservation.

Assessment of Sperm Quality: Mobility of sperm samples was estimated under a light microscope at 400× magnification immediately after mixing of 5µL of semen with 50µL of activation solution)NaC l3.5 mM, Tris-HCl 12 mM, pH=8.5) [10] 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 sperms were defined as sperm that did not show forward movement after activation. Video records were set at 30 frames/s using video camera mounted on amicroscope. Percentage of sperm motility was determined during 0–15 s post-activation. Motility duration was evaluated by counting the time from sperm activation with activation solution until sperm stopped moving [11].

Extender and Sperm Cryopreservation: In this experiment extender Tris- sucrose-KCl (30mM Tris, 23.4mM sucrose, 0.25mMKCl, PH 8.0) [12] was supplement with 5%, 10% and 20% MeOH [13]. Semen and extender had atemperature of 4°C. Milt was diluted at ratios of 1:0.5, 1:1 and 1:2with 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 [2].

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 [2]. Sperm mobility and duration of sperm motility of thawed semen was observed after 30 and 60 day of storage in liguidN2. Post-thaw mobility and motility duration was observed and evaluated by the same operators using a monitor connected to a microscope.Semen concentration was measured by the Lam Nyvbar method [14].

Statical 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 semen samples showing 80% mobility or higher were used for the experiments (Table 1).

Effect of Dilution Rates with Concentrations of MeOH on Quality Post-Thawed Sperms after 30 Days: Highest motility duration and the most motility percentage of post-thawed sperms after 30 days was related to the

Table 1: Males used for sperm cryopreservation process

Male	Body weight (g)	Total length (cm)	Sperm concentration × 10^9 mlG^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 30 days of freezing

Cryoprotectant	Cryoprotectant concentration (%)	Diluted rates (sperm: extender)	Motility duration (s)	Motility percentage (%)
		1: 0.5	11.78^{c}±111.79	1.80^{ab}±10.00
MeOH	5	1: 1	17.00^{ab}±146.12	1.60^{a}±12.38
		1: 2	24.72^{c}±104.52	4.28^{b}±7.03
		1: 0.5	16.97^{abc}±134.71	1.21^{a}±11.42
MeOH	10	1: 1	25.46^{a}±157.00	4.94^{a}± 12.58
		1: 2	17.60^{c}±112.20	1.40^{ab}±9.69
		1: 0.5	15.57^{bc}±123.47	1.45^{ab}±9.58
MeOH	20	1: 1	17.85^{abc}±131.39	2.13^{a}±10.78
		1: 2	18.30^{c}±109.77	1.31^{b}±7.37
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 60 days of freezing

Cryoprotectant	Cryoprotectant concentration (%)	Diluted rates (sperm: extender)	Motility duration (s)	Motility percentage (%)
		1: 0.5	15.66^{b}±88.42	2.96^{ab}±7.67
MeOH	5	1: 1	14.33^{ab}±105.56	3.50^{ab}±9.00
		1: 2	23.57^{b}±75.27	3.68^{b}±.4.61
		1: 0.5	21.65^{ab}±95.00	3.21^{ab}±8.10
MeOH	10	1: 1	23.52^{a}±119.18	4.12^{a}±10.31
		1: 2	18.39^{b}±80.76	2.40^{ab}±7.60
		1: 0.5	17.25^{b}±77.19	1.85^{ab}±6.87
MeOH	20	1: 1	17.80^{ab}±104.67	2.08^{ab}±8.14
		1: 2	15.81^{b}±80.48	1.23^{ab}±6.58
Control	-	-	416.68±12.72	83.13±2.11

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

treatments with the concentration of MeOH10% and the dilution of 1: 1(157±25.46 s and 12.58±4.94%; Table 2). The least duration and the lowest mobility of post-thawed sperms was observed in the treatments with the concentration of MeOH5% and the dilution of 1:2 (104.52±24.72 s and 7.03±4.28%; P<0.05) Table 2.

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 motility duration of post-thawed sperm was observed in dilution rate 1:2.

Effect of Dilution Rates with Concentrations of MeOH on Quality of Post-Thawed Sperms after 60 Days: Maximum motility duration and the upmost mobility of post-thawed sperms after 60 days was related to the treatments with the concentration of MeOH 10% and the dilution of 1: 1(119.18±23.52 s and 10.31±4.12%; Table 3).

Table 3 shows the minimum Duration and the lowest motility percentage of post-thawed sperms in the treatments with the concentration of MeOH 5% and the dilution of 1:2 (75.27±23.57 s and 4.61±3.68%; P<0.05).

Results showed the highest motility duration and the most motility percentage in treatments where the dilution rate was 1:1, as well as minimum motility duration and the least mobility of post-thawed sperm was observed in dilution rate1:2.

DISCUSSION

Sturgeons (Order Acipenseriformes) are chondrostean fishes of classical origin that inhabit only the Northern hemisphere [15]. 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 [16]. Successful cryopreservation of fish spermatozoa is well established for many species [17]. Earlier works indicated that the cryopreservation of sturgeon spermatozoa using DMSO-sucrose extender resulted in recovery of motile spermatozoa with basic mobility characteristics similar to those of fresh milt [3]. 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 the highest motility duration related to dilution rate of 1:1 of the treatments and the duration of sperm motility with 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 [18]. The results showed that the sperm quality significantly reduced after thawing was similar to the results of Dzuba *et al*. [19]. These researchers have reported that the quality of Ponto-Caspian sturgeon semen sharply decreased after thawing. [13] 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 of [2]. These researchers have reported that the most suitable cryoprotectant for sperm cryopreservation Siberian sturgeon (*Acipenser baeri*), is MeOH concentration of 10%. [20] reported that the most suitable cryoprotectant for sperm Cryopreservation paddle fish (*Polyodon spathula*), was with MeOH8%. The reason of difference 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 sperm 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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