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Cryopreservation of Stellate (*Acipenser stellatus*) Sperm: Effect of Different Concentrations of DMSO and Dilution Rates on Sperm Mobility and Motility Duration After Long-Term Storage

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Abstract: Semen obtained from four stellate males (*Acipenser stellatus*) was cryopreserved using extender; Tris-sucrose-KCl (30mM Tris, 23.4mM sucrose, 0.25mM KCl, PH 8.0) supplemented with DMSO at concentration 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 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 related to the treatments with the concentration of DMSO 10% and the dilution ratio of 1: 1 (189±21.86 sand 17.86±4.13%; $P<0.05$), 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 DMSO 10% and the dilution rates of 1: 1 (145.47±20.76 s and 15.02±3.27%; $P<0.05$).

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

INTRODUCTION

Stellate sturgeon (*Acipenser stellatus*) are among the commercially precious sturgeon species in the Caspian Sea that their stocks have declined drastically in the recent decades [1]. Long-term storage of deep-frozen sturgeon spermatozoa has received worldwide attention in the recent years because of the loss of adequate and appropriate brood stock for restocking programme and also for sturgeon aquaculture [2, 3]. Semen quality is influenced by several factors such as temperature, food [4], time of sampling [5] and delays caused after the injection of hormones [6]. Main parameters for cryopreservation include types of extenders and cryoprotectants, the dilution rate, the freezing and thawing rates and kind of extender used for fertilization. The most efficient permeating cryoprotectant for fish semen appears to be dimethyl sulfide (DMSO) and methanol (MeOH) [7]. Other cryoprotectants such as glycerol, ethylene glycol, propane-diol, dimethyl acetamide and methanol are less popular or have been used with limited success. Unlike mostteleost fish, information concerning reliable technology for cryopreservation of sturgeon milt is not available. Cryopreservation success was usually measured as post-thaw sperm mobility [8-9] or as fertilization success during early embryo development [10]. Sturgeon (*Acipenser sp., Chondrostei*)spermatozoa are significantly different from teleost fish sperm. These differences concern morphology (more complex structure, presence of acrosome), physiology (longer duration of mobility, acrosome reaction) and biochemistry (presence of acrosin, arylsulfatase) [11, 12]. Other striking difference between semen properties of sturgeons and teleost fish is the lowosmolality of sturgeon Seminal plasmacomposition [13]. The objectives of our work were to test the effect of:

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(1) DMSO in different concentrations on the motility percentage and motility duration of stellate sturgeon sperm; (2) several dilution rates in combination with different DMSO concentrations on the motility percentage and motility duration of stellate sturgeon sperm.

MATERIALS AND METHODS

Semen Collection for Cryopreservation: Semen samples were collected from four males of stellate (*Acipenser stellatus*) in Shahid Marjani Sturgeon Hatchery located in Gorgan, Iran in March 2012. Spermiation was induced by injecting of sturgeon pituitary extract in dose of 2-3mg kg^{-1} body weight [4]. Spermatozoa were 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 for cryopreservation.

Assessment of Sperm Quality: Mobility 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) [14] 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 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 fromsperm with forward movement. Immotile sperm was defined as sperm that did not show forward movementafter activation. Video records were set at 30 frames/s using video camera mounted on a microscope. 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 [15].

Extender and Sperm Cryopreservation: In this experiment using extender Tris- sucrose-KCl(30mM Tris, 23.4mM sucrose, 0.25mMKCl, PH 8.0) [16] supplement with 5%, 10% and 20% DMSO [17]. Semen and extender were kept for 5 minutes at room temperature Milt was diluted at ratios of 1:0.5, 1:1, 1:2 and 1:5with extender. Suspensions of extended milt at temperature of 4°C.were drawn into 0.25-ml strawsSemen freezing was conducted in aStyrofoam box with liquid nitrogen. Straws (of 0.25 ml volume) of diluted semen were placed for slow freezing during 3 minutes on a 3 cm high floating frame made from the same material as the box. Straws were subsequently plunged within liquid nitrogen [18].

Measurement Sperm Mobility, Duration of Sperm Motility and Concentration: Thawing of semen was conducted in a water bath at 40°C for 5 sec [18]. 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 same operators using a monitor connected to a microscope. Milt concentration was measured by the LamNyvbar method [19].

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

RESULTS

Semen with duration of sperm motility exceeded 320 s and, only sperm samples showing 80% mobility or higher were used for cryopreservation (Table 1).

Effect of Dilution Rates with Concentrations of Dmso on Quality Post-Thawed Sperms after 30 Days: Highestmotility duration and the most motility percentage of post-thawed sperms after 30 days was related to the treatments with the concentration of DMSO 10% and the dilution of 1: 1 (189±21.86 sand 17.86±4.13%; Table 2). The least Duration and the lowest mobility of post-thawed sperms was observed in the treatments with the concentration of DMSO 5% and the dilution of 1:5 (73.29±17.84 s and 8.52±3.76%; $P<0.05$) Table 2.

Results showed the maximum duration and the most mobility results were observed in treatmentswhere 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 1:5.

Effect of Dilution Rates with Concentrations of Dmso on Quality Post-Thawed Sperms after 60 Days: Maximum motility duration and the upmost mobility of post-thawed sperms after 60 days wasrelated to the treatments with the concentration of DMSO 10% and the dilution of 1:1 (145.47±20.76 s and 15.02±3.27%; Table 3).

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
Total	12.25	152	2.60	334.66±10.59	81.16±2.08

Table 2: Effect of different concentrations of DMSOand 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	86.38±20.37 ^{b}	11.52±3.90 ^{abc}
DMSO	5	1: 1	185.67±21.87 ^{a}	17.00±4.86 ^{a}
		1: 2	172.28±21.39 ^{a}	14.26±4.90 ^{abc}
		1 : 5	73.29±17.84 ^{b}	8.52±3.76 ^{c}
		1: 0.5	88.74±18.78 ^{b}	11.00±3.27 ^{abc}
DMSO	10	1: 1	189.00±21.86 ^{a}	17.86±4.13 ^{a}
		1 : 2	179.64±20.51 ^{a}	15.47±3.37 ^{abc}
		1: 5	80.64±16.86 ^{b}	9.67±4.52 ^{bc}
		1: 0.5	85.37±20.18 ^{b}	10.61±3.10 ^{abc}
DMSO	20	1: 1	152.34±22.84 ^{a}	13.45±2.81 ^{abc}
		1: 2	104.67±19.26 ^{b}	11.48±3.94 ^{abc}
		1 : 5	74.24±19.32 ^{b}	9.42±3.91 ^{bc}
Control	-	-	334.66±10.59	81.16±2.08

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

Table 3: Effect of different concentrations of DMSOand diluted 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	66.10±17.37 ^{cde}	6.34±2.83 ^{de}
DMSO	5	1: 1	120.64±23.41 ^{ab}	14.64±2.28 ^{a}
		1: 2	103.32±18.94 ^{bc}	12.00±3.21 ^{abc}
		1 : 5	58.67±18.41 ^{e}	4.78±1.86 ^{e}
		1: 0.5	71.12±19.38 ^{cde}	8.68±2.36 ^{cde}
DMSO	10	1: 1	145.47±20.76 ^{a}	15.02±3.27 ^{a}
		1 : 2	113.64±21.82 ^{ab}	14.00±2.86 ^{ab}
		1: 5	62.39±16.68 ^{de}	5.26±2.10 ^{e}
		1: 0.5	68.67±19.78 ^{cde}	6.57±2.41 ^{de}
DMSO	20	1: 1	100.28±23.34 ^{bcd}	10.29±2.70 ^{abcd}
		1: 2	89.54±20.41 ^{cde}	9.64±2.82 ^{bcde}
		1 : 5	60.68±19.51 ^{e}	5.36±1.61 ^{e}
Control	-	-	10.59±334.66	2.08±81.16

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

Results showthe minimum duration and the lowest motility percentage of post-thawed sperms in the treatments with the concentration of DMSO 5% and the dilution of1:5 (58.67±18.41 s and 4.78±1.86%; 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 in dilution rate 1:5.

DISCUSSION

The cryopreservation remains one of the most attractive and quickly developing trends forthe sturgeon protection. Methods of cryopreservation of the sturgeon sperm have been well established [7, 20]. However, the different steps required for cryopreservation (cryoprotective agent loading, freezing/thawing, cooling to a low subzero temperature) may contribute individually

or cumulatively to semen damage that in turn decreases fertilization and growth stages [21]. Recently, it has been shown, that profound freezing mechanically destroys cell membranes [22]. According to the above results, by comparing Table 2 and 3, the dilution rateshave significant differences on the duration of sperm motility ($P<0.05$), as the highest motility duration related to dilution rate of 1:1 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 cryoprotectant increased causing toxicity and reduced sperm viability [23]. The results showed that the sperm quality significantly reduced after thawing was similar to the results of Lahnsteiner *et al*. [24]. These researchers have reported that the quality of Ponto-Caspian sturgeon semen sharply decreased after thawing. In this experiment,post-thawed sperms with the dilution rate 1:1 andconcentration of 10% has the *et al*. motility durationsimilar to the results ofDzubahighest mobility and [25]. These researchers have reported that the most suitable cryoprotectant for sperm cryopreservation Italian Cobice sturgeon (*Acipensernaccarii*), is DMSO concentration of 10%. Also, Lahnsteiner *et al*, 2004 announced the most motility of post-thawed sperm Starlet (*Acipenser ruthenus*) was with DMSO 10% (80±7.4%) [23].

In this experiment, post-thawed sperms with DMSO concentration of 5% has the lowestmobility and motility duration and this was in contrast to the results obtained by [17]. These reserchers have Announced that the maximum motility and motility duration of post-thawed sperm of pallid sturgeon (*Scaphyrinchus albus*) was with DMSO concentration of 5% (26±13%) [12] reported that themost suitable cryoprotectant for sperm Cryopreservation Chinesesturgeon (*Acipenser persicus*), waswith DMSO 12%.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 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 DMSO10% and the dilution of 1:1.

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