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Effect of Different Concentrations of Methanol (MeOH) and Dilution Rates on Viability of Stellate (*Acipenser stellatus*) Post-Thawed Sperm

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Abstract: Semen samples collected from four males of stellate (*Acipenser stellatus*) 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, 1:2 and 1: 5 with extender and frozen in liquid nitrogen vapor. Frozen sperms after 2, 15, 30 and 60 days were excluded from freezing. Experiment showed the highest motility duration and the most motility percentage of post-thawed sperms after 2, 15, 30 and 60 days was related to the treatments with the concentration of MeOH 10% and the dilution ratio of 1: 1 (273±24.81s and36.51±5.20%; 254.36±24.81 s and 27.54±5.12%; 197.30±22.81 s and20.58±5.30%; 153.41±22.81 s and 16.58±5.20%, respectively). The results showed that storage of frozen sperm has a negative impact on motility duration and motility percentage of post-thawed sperms.

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

INTRODUCTION

Extender composition and cryoprotectant concentration are known to affect cryopreservation success [1-2]. Cryopreservation of sperm is a simple technique that is useful for species conservation, preservation of biodiversity [3], protection of valuable breeding lines and as a helpful tool in facilitating animal reproduction. Cryopreservation induces severe stress to fish spermatozoa that in turn affect sperm quality in terms of fertilization ability. Semen quality is influenced by several factors such as temperature, food [4], time of sampling [5] and retardation caused after the injection of hormones [6]. 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 [7]. 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 [8-9]. It has been estimated that semen from 200 fish species have been successfully cryopreserved [10]; however, species-specific optimizations of technology are needed.Successful cryopreservation of fish spermatozoa is well established for many species [11]. 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 [11].

The main parameters for cryopreservation include types of extenders and cryoprotectants, the dilution ratio, 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) [12]. Other cryoprotectants such as glycerol, ethylene glycol, propane-diol, dimethyl acetamide and 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 [11-13] or as fertilization success during primary embryo growth [14]. Sturgeon (*Acipenser sp., Chondrostei*) spermatozoa are significantly different from teleost fish sperm. These differences concern morphology (more complex structure, presence of acrosome), physiology

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(longer duration of mobility, acrosome reaction) and biochemistry (presence of acrosin, arylsulfatase) [15, 16]. The objectives of our work were to test the effect of: (1) MeOHin different concentrations on the motility percentage and motility duration of stellate sturgeon sperm; (2) several dilution rates in combination with different MeOH concentrations on the motility percentage and motility duration of stellate sturgeon sperm.

MATERIALS AND METHODS

Sperm Collection for Cryopreservation: Semen samples were collected from eight 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 [6]. Spermatozoa were collected within16-24h (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) [17] 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 100× 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 sperm 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 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 [18].

Extender and Sperm Cryopreservation: In this experiment using extender Tris- sucrose-KCl (30mM Tris, 23.4mM sucrose, 0.25mM KCl, PH8.0) [19] supplement with 5%, 10% and 20% MeOH [20]. Semen and extender had a temperature of4°C. Milt was diluted at ratios of 1:0.5, 1:1, 1:2 and 1:5 with extender. Suspensions of extended milt were drawn within 0.25-ml straws. Sperm freezing was conducted in a Styrofoam box with liquid nitrogen. Straws (of 0.25 ml volume) with diluted sperm, prior kept to equilibrate for 5minutes at room temperature, 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 into liquid nitrogen [21].

Measurement Sperm Mobility, Duration of Sperm Motility and Concentration: Thawing of semen was conducted in a water bath at 40°C for 5 sec [21]. Sperm mobility and duration of sperm motility of thawed semen was observed after 2, 15, 30 and 60 days 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.Semen concentration was measured by the Lam Nyvbar method [22].

Statistical Analysis of Data: All values are shown as mean± S.D. 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 MeOH and dilution rates on post-thaw sperm motility and duration of sperm motility were analyzed using two-way analysis of variance (ANOVA). Means were separated by Duncan's New Multiple Range tests 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% mobility or higher were used for the experiments (Table 1).

Effect of Dilution Rates with Concentrations of MeOH on Quality Post-Thawed Sperms after 2 Days: Highest motility duration and the most motility percentage of post-thawed sperms after 2 days was related to the treatments with the concentration of MeOH10% and the dilution of 1: 1 (273±24.81 s and36.51±5.20%; Table 2). The least duration and the lowest mobility of post-thawed sperms was observed in the treatments with the concentration of MeOH20% and the dilution of 1: 5 (196.58±27.63 s and 20.48±6.21%; $P<0.05$) Table 2.

Results showed the maximum duration and the most mobility results 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 1:5.

Table 1: Males used for sperm cryopreservation process

Male	Body weight (g)	Total length (cm)	Sperm concentration ($x10^9 ml^{-1}$)	Motility duration (s)	Motility percentage (%)
1	10	144	3.12	342.24±24.80	80.64±1.84
2	12	143	2.59	330.11±70.11	82.41±2.70
3	14	155	2.25	340.67±49.60	81.20±2.34
4	13	166	2.46	325.62±42.80	80.42±2.06
Total	12.25	152	2.60	334.66±59.10	81.16±2.08

Table 2: Effect of different concentrations of MeOH and dilution 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	29.58 ^{abc}±261.27	5.60 ^{abc} ±.30.00
MeOH	5	1 : 1	30.50 ^{a}±271.34	4.51 ^{ab} ±33.40
		1 : 2	30.58 ^{abc} ±247.39	4.90 ^{bc} ±.26.36
		1 : 5	24.50 ^{cb} ±211.20	4.87 ^{bc} ±24.23
		1 : 0.5	23.80 ^{ab} ±264.67	5.42 ^{ab} ±33.21
MeOH	10	1 : 1	24.81 ^{a} ±273.00	5.20 ^{a} ±36.51
		1 : 2	22.30 ^{abc} ±253.54	4.86 ^{bc} ±25.34
		1 : 5	31.21 ^{bcd} ±213.33	5.24 ^{c} ±23.00
		1 : 0.5	29.48 ^{abc} ±250.84	5.08 ^{bc} ±25.72
MeOH	20	1 : 1	22.07 ^{a} ±266.10	5.10 ^{bc} ±26.68
		1 : 2	24.51 ^{abcd} ±222.37	4.50 ^{c} ±23.08
		1 : 5	27.63 ^{d} ±196.58	6.21 ^{c} ±20.48
Control	-	-	10.59 ±334.66	2.08 ±81.16

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	22.29 ^{bc}±202.53	4.15 ^{abc} ±.19.61
MeOH	5	1 : 1	21.30 ^{ab}±222.37	5.21 ^{abc} ±21.28
		1 : 2	24.58 ^{bc} ±210.30	4.90 ^{ab} ±.22.00
		1 : 5	27.07 ^{de} ±157.58	4.86 ^{bc} ±15.00
		1 : 0.5	22.80 ^{bc} ±207.67	5.12 ^{abc} ±21.00
MeOH	10	1 : 1	24.81 ^{a} ±254.36	5.12 ^{a} ±27.54
		1 : 2	21.30 ^{bc} ±209.47	5.40 ^{ab} ±23.42
		1 : 5	20.21 ^{cde} ±166.00	4.97 ^{bc} ±17.34
		1 : 0.5	22.48 ^{bcd} ±192.10	5.08 ^{bc} ±17.66
MeOH	20	1 : 1	23.07 ^{bc} ±206.59	4.82 ^{abc} ±18.43
		1 : 2	25.51 ^{cde} ±164.34	4.58 ^{bc} ±15.24
		1 : 5	30.41 ^{e} ±140.14	5.02 ^{c} ±11.85
Control	-	-	10.59 ±334.66	2.08 ±81.16

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

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 was related to the treatments with the concentration of MeOH 10% and the dilution of 1: 1 (254.36±24.81 s and 27.54±5.12%; Table 3). Results shows the minimum duration and the lowest motility percentage of post- thawed sperms in the treatments with the concentration of MeOH 20% and the dilution of 1: 5 (140.14±30.41s and 11.85±5.02%; 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.

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 treatments with the concentration of MeOH 10% and the dilution of 1: 1 (197.30±22.81 s and 20.58±5.30%; Table 4). The least duration and the lowest mobility of post-thawed sperms in the treatments with the

Table 4: 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	20.48 ^{b}±81.84	4.86 ^{b} ±.8.51
MeOH	5	1 : 1	21.50 ^{a}±193.24	4.10 ^{ab} ±17.00
		1 : 2	20.58 ^{a} ±180.05	4.80 ^{ab} ±.15.54
		1 : 5	21.50 ^{b} ±194.51	4.35 ^{ab} ±11.34
		1 : 0.5	20.42 ^{b} ±88.67	5.10 ^{b} ±9.63
MeOH	10	1 : 1	22.81 ^{a} ±197.30	5.30 ^{a} ±20.58
		1 : 2	19.30 ^{a} ±187.00	4.86 ^{ab} ±16.21
		1 : 5	18.21 ^{b} ±196.39	5.12 ^{b} ±11.00
		1 : 0.5	18.29 ^{b}±82.36	4.41 ^{b} ±.9.25
MeOH	20	1 : 1	22.07 ^{a} ±182.58	5.10 ^{ab} ±15.28
		1 : 2	18.51 ^{a} ±177.00	5.30 ^{ab} ±14.67
		1 : 5	19.07 ^{b} ±93.27	4.95 ^{b} ±10.24
Control	-	-	10.59 ±344.66	2.08 ±81.16

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

Table 5: 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	19.21 ^{c}±50.52	3.54 ^{c} ±.4.66
MeOH	5	1 : 1	25.21 ^{ab}±128.24	5.12 ^{ab} ±14.37
		1 : 2	18.24 ^{b} ±111.42	4.90 ^{abc} ±.12.00
		1 : 5	20.24 ^{c} ±58.00	4.39 ^{bc} ±6.28
		1 : 0.5	22.74 ^{c} ±54.21	4.35 ^{bc} ±5.29
MeOH	10	1 : 1	22.81 ^{a} ±153.41	5.20 ^{a} ±16.58
		1 : 2	23.28 ^{ab} ±121.57	4.97 ^{ab} ±14.00
		1 : 5	20.11 ^{c} ±63.24	5.18 ^{abc} ±8.68
		1 : 0.5	19.48 ^{c} ±54.00	3.94 ^{bc} ±5.23
MeOH	20	1 : 1	24.14 ^{ab} ±132.24	5.12 ^{abc} ±13.56
		1 : 2	21.06 ^{ab} ±115.36	4.86 ^{abc} ±11.71
		1 : 5	18.25 ^{c} ±60.27	4.98 ^{bc} ±6.53
Control	-	-	10.59 ±344.66	2.08 ±81.16

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

concentration of MeOH 5% and the dilution of 1: 0.5(81.84±20.48 s and 8.51±4.86%; P<0.05) Table 4. Results showed the maximum duration and the most mobility results in treatments where the dilution rate was 1:1, as well as the lowest motility percentage and motility duration of post-thawed sperm observed in dilution rate 1:0.5.

Effect of Dilution Rates with Concentrations of MeOH on Quality 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 (153.41±22.81 s and 16.58±5.20%; Table 5). Results 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: 0.5 (50.52±19.21 s and 4.66±3.54%; P<0.05). Table 5 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 1:0.5.

DISCUSSION

Sturgeons (Order Acipenseriformes) are chondrostean fishes of classical origin that inhabit only the Northern hemisphere [23]. 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 [24]. Decrease in stocks and limited number of potential breeders has led to the establishment of fish semen cryobanks which play a crucial role in the genetic management and conservation of aquatic resources [25, 26]. The establishment of sperm banks from valuable fish species including sturgeon is widely practiced in multitude countries [27, 28]. According to the above results, by comparing Table2 to 5 the dilution ratios has

a significant differences on the duration of sperm motility ($P<0.05$), as the highest motility duration related to dilution rate of 1:1of 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 [29]. A significant decrease in sperm motility and duration of forward movement frozen-thawed spermatozoa are observed in *Acipenser stellatus* and similar results were reported in green sturgeon (*Acipenser medirostris*) [30]. Horvath and Urbanyi [21] reported that methanol is an excellent cryoprotectant for cryopreservation of starlet milt. In this experiment, post-thawed sperms with MeOH concentration of 10% andthe dilution of 1:1has the highest mobility and motility duration similar to the results [12]. These researchers have reported that the most suitable cryoprotectant for sperm cryopreservation Siberian sturgeon (*Acipenser baeri*), is MeOH concentration of 10%. Linhart *et al*. [31] reported that the most suitable cryoprotectant for sperm Cryopreservation paddle fish (*Polyodon spathula*), was with MeOH 8%. 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 methanol (MeOH) 10% and the dilution of 1:1.

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