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Effects of Use of Combinations of Permeating Cryoprotectant (MeOH, DMSO) and Non Permeating Cryoprotectant (BSA) on Viability of Beluga (*Huso huso*) Post-Thawed Sperm

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Abstract: This experiment wascarried out to explore the effect of use of combinations of permeating cryoprotectant and non permeating cryoprotectant on thequality of frozen-thawed Beluga (*Huso huso*) spermas compared to the use of permeating cryoprotectant as a control. For this work methanol (MeOH) and dimethyl sulfoxide (DMSO)are used aspermeatingcryoprotectantsand bovine serum albuminas a non permeating cryoprotectant. Semen samples were pooled and divided into aliquots. MeOH and DMSO each at concentration of 10% was added to the extender; Tris-sucrose- KCl (30mM Tris, 23.4mM sucrose, 0.25mM KCl, PH 8.0) and then semen was diluted with ratio 1:1 with extender and frozen in liquid nitrogen vapor. Onthe sametime BSA at concentration of 2.5,5 and 10 mg/ml and MeOH and DMSO at concentrations 10% was used that combined with the extender and then semen was diluted with ratio 1:1 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 treatment combinatorial BSA at concentration of 10 mg/ml with DMSO10% (158.24±30.41 s and 20.41±2.18%; $P<0.05$), as well as the upmost mobility and the motility duration of post thawed sperms after 60 days was related to the treatments combinatorial BSA at concentration of 10 mg/ml with DMSO10% (135.58±20.47 s and 17.41±1.90%; $P<0.05$).

Key words: Beluga · BSA · MeOH · DMSO · 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 mostefficient permeating cryoprotectant for fish semen appears to be dimethyl sulfide (DMSO) and methanol (MeOH) [2]. The highest efficiency ofDMSO and MeOH is at approximately 10% concentration. Other cryoprotectants such asglycerol, ethylene glycol, propane-diol, dimethyl acetamide and are short popular or have been used with limited success. Also most efficient non permeating cryoprotectantfor fish semen appears to be bovine serum albumin (BSA).

One of the most important features of bovine serum albumin (BSA) is known as the elimination of free radicals generated by oxidative stress and the protection of membraneintegrity of sperm cells [3]. Oxidative stress originates from heat shock during freezing-thawing of Cryopreserved sperm.In addition, fish species can be replaced from the cryopreserved sperm using artificially-induced androgenesis, providing only the male inheritance. 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 [4-5] or as fertilization success duringprimary embryo growth [6]. 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

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mobility, acrosome reaction) and biochemistry (presence of acrosin, arylsulfatase) [7, 8]. Other striking difference between semen properties of sturgeons and teleost fish is the low osmolality of sturgeon seminal plasma composition [9]. Sperm cryopreservation is one way to overcomethe problems associated with brood stock supply. Sperm collection for cryopreservation should be carriedout during the spawning seasons because the quality andquantity of spermatozoa is highest at this time. In Seabass, for example, the concentration of spermatozoadecreased as the spawning season progressed [10]. Similarly, [11] reported lower motility rates, fertilization rates and reduced short-term storagecapacity at the end of the reproductive period of the fish. The objective of our work were totest the effects ofapplication combination permeating cryoprotectant (DMSO, MeOH) andnonpermeating cryoprotectant (BSA) on sperm mobility and motility duration of Beluga (*Huso huso*) post-thawed sperm.

MATERIALS AND METHODS

Semen Collection for Cryopreservation: Semen samples were collected from eight males of Beluga (*Huso huso*) that were transferredto the Shahid Marjani Sturgeon Hatchery located in Gorgan, Iran. Milt from these males wasobtained in March. Spermiation was induced by injecting of sturgeon pituitary extract in dose of 2-3mg kg^{-1} body weight [12]. Spermatozoa were collected within 16-24h (depending on the water temperature) post hormonal injection. Milt was stored on ice and used within 2 hof 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 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 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 sperm weredefined as sperm that did not show forwardmovement after activation. Video records wereset at 30 frames/s using video camera mounted one 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 untilsperm 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, 0.25mMKCl, PH 8.0) [15] to do that once the MeOH and DMSOeach atconcentration of 10% was addedto the extender and onthe same time the BSA at concentration of 2.5, 5 and 10mg/mland MeOH and DMSO at concentrations 10% was used thatcombinedwith another portion of theextender. To prevent bacterial growth intheantibiotic G500 unitspermL ofthe extender was added. Sperm and extender had a temperature of 4°C. Milt was diluted atratio 1:1 withextender. Suspensions of extended milt were drawn within 0.25-ml straws.

Semen-freezing wasconducted in a Styrofoam box filled with liquid nitrogen. Straws wereplaced on a 4-cm-high floating frame made ofstyrofoam. Straws were not sealed and after 3 min of freezing in liquid nitrogen vapor, wereplunged in 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 liguid N2. Post-thaw mobility and motility duration was observed and evaluated by same operators using a monitor connected to a microscope. Semen concentration was measured by the Lam Nyvbar method [16].

Statistical Analysis of Data: All results are expressed as means ± SD. Statistical analyses were carried out using the computerized package SPSS 16.0 for Windows. Prior to statistical analysis, percentage data were arcsine transformed. Data were analyzed by one-way and the means were comparedusing the Tukey test. Differences were considered to be significant when $p < 0.05$.

RESULTS

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

Effect of Different Concentrations of BSA Onquality Post-Thawed Sperms after 30 Days: Inthisexperiment, 8treatments were [1. Control (1): extender + DMSO10%; 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±41.12	82.32±2.41
2	160	240	2.56	410.64±75.80	83.43±2.70
3	150	260	2.02	418.52±42.10	82.46±1.87
4	135	260	2.87	407.34±61.90	84.32±2.04

Table 2: Motility duration and Motility percentages of post-thawed Sperms after 30 days of freezing (means% ± S.E.M)

	Treatment	Motility duration (s)	Motility percentages (%)
1	Control: DMSO 10%	130.00±25.46^{a}	12.63±3.80^{b}
2	Control: MeOH 10%	112.00±24.52^{a}	12.58±4.52^{b}
3	DMSO 10% + BSA 2.5 mg/ml	138.41±21.19^{a}	16.00±2.11^{ab}
4	DMSO 10% + BSA 5 mg/ml	147.20±24.13^{a}	17.70±3.10^{ab}
5	DMSO 10% + BSA 10 mg/ml	158.24±30.41^{a}	20.41±2.18^{a}
6	MeOH 10% + BSA 2.5 mg/ml	121.36±27.41^{a}	13.80±2.14^{b}
7	MeOH 10% + BSA 5 mg/ml	127.11±24.52^{a}	14.30±1.80^{b}
8	MeOH 10% + BSA 10 mg/ml	135.47±20.14^{a}	16.40±2.50^{ab}

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

Table 3: Motility duration and Motility percentages of post-thawed Sperms after 60 days of freezing (means% ± S.E.M)

	Treatment	Motility duration (s)	Motility percentages (%)
1	Control: DMSO 10%	98.18±22.52^{ab}	10.31±2.10^{c}
2	Control: MeOH 10%	80.33±22.52^{b}	10.80±3.32^{c}
3	DMSO 10% + BSA 2.5 mg/ml	124.19±24.12^{a}	13.50±1.74^{bc}
4	DMSO 10% + BSA 5 mg/ml	130.45±27.18^{a}	15.24±2.10^{ab}
5	DMSO 10% + BSA 10 mg/ml	135.58±20.47^{a}	17.41±1.90^{a}
6	MeOH 10% + BSA 2.5 mg/ml	102.41±21.50^{ab}	12.10±1.34^{bc}
7	MeOH 10% + BSA 5 mg/ml	106.11±17.19^{ab}	13.00±1.91^{bc}
8	MeOH 10% + BSA 10 mg/ml	114.19±24.37^{ab}	13.40±1.20^{bc}

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

Control (2): extender + MeOH 10%; 3. extender + DMSO 10% + BSA 2.5 mg/ml; 4. extender + DMSO 10% +BSA 5 mg/ml; 5. extender + DMSO 10% + BSA 10 mg/ml; 6. extender + MeOH 10% +BSA 2.5 mg/ml; 7. extender +MeOH 10% + BSA 5 mg/ml and 8. extender + MeOH 10% +BSA 10 mg/ml]. Highestmotility duration and the most motility percentage of post-Thawedsperms after 30 days was related to the treatment 5) extender + DMSO10% + BSA 10 mg/ml((158.24±30.41 s and 20.41±2.18%; Table 2). The least duration and the lowest mobility of post-tha wed sperms was observed in the treatment 2) extender + MeOH) (112.00±24.52 s and 12.58±4.52%; P<0.05) Table 2.

Effect of Different Concentrations of BSA Onquality Post-Thawed Sperms after 60 Days: Inthisexperiment, 8treatments were [1. Control (1): extender + DMSO10%; 2. Control (2):extender + MeOH10%; 3. extender + DMSO10% + BSA 2.5 mg/ml; 4. extender + DMSO10%+ BSA 5 mg/ml; 5. extender + DMSO10% + BSA 10 mg/ml; 6. extender + MeOH10% +BSA 2.5 mg/ml; 7. extender +MeOH10% + BSA 5 mg/ml and 8. extender + MeOH10% +BSA 10 mg/ml]. Highest motility duration and the most motilitypercentage of post.

Thawedsperms after 60 days was related to the treatment 5) extender + DMSO10% + BSA 10 mg/ml (135.58±20.47 s and 17.41±1.90%; Table 3). The least duration and the lowest mobility of post-thawedsperms wasobserved in the treatment 2) extender + MeOH) (80.33±22.52 s and 10.80±3.32%; P<0.05).

DISCUSSION

BSA is known to eliminate free radicals generated by oxidative reactions and thereforeto protect the membrane integrity of sperm cells from lipid peroxidation during the semenfreezing process [17]. The cryopreservation remains one of the most attractive and quickly developing trends for the sturgeon protection. Methods of cryopreservation of the sturgeon semen have been well established [18-2]. However, the different steps required for cryopreservation (cryoprotective agent loading, freezing/thawing, cooling to a low sub zero temperature) may contribute individually or cumulatively to semen damage that in turn decreases fertilization and growth stages [19]. Recently, it has been shown, that pro found freezing mechanically destroys cell membranes [20]. BSA protects especially the functional

integrity but only for the concentration of 5mg/ml, higher concentrations leading to a decrease in all parameters. Albumin is an important extra cellular antioxidant because of its property to link transitional metal ions (Fe^{2+} and Cu^{+}), thus minimizing the formation of OH radical, the promoter of sperm lipid peroxidation [21].

In our investigation, various concentrations of BSA were observed to improve post-thawingsperm motility and viability, but the best results were obtained from 10 mg/ml of BSA. It can be concluded that concentrations lower than 10 mg/ml of BSA are not sufficient to protect sperm cells from lipid peroxidation. The results showed that the spermquality significantly reduced after thawing was similar to the results [22].

These researchers have reported that the quality of Ponto-Caspian sturgeon semensharply decreased after thawing. In this experiment, post-thawed sperms with BSA concentration of 10% highest mobility and motility duration. Also least duration and the lowestmobility of post-thawed sperms was observed in the treatment with the concentration of DMSO 10% and this was in contrast to the results obtained by [23]. These researchers have reported that the most suitable cryoprotectant for sperm cryopreservation Italian Cobicesturgeon (*Acipenser naccarii*), is DMSO concentration of 10%. Also, [24], announced themost motility of post-thawed sperm Starlet (*Acipenser ruthenus*) was with DMSO 10% (80±7.4%). Also [23] reported thatmethanol is an excellent cryoprotectant forcryopreservation of starlet milt. In this experiment, post-thawed sperms with MeOHconcentration of 10% and the dilution of 1:1 has the highest mobility and motility durationwas similar to the results [2]. These researchers have reported that themost suitablecry oprotectant for sperm cryopreservation Siberian sturgeon (*Acipenser baeri*), is MeOHconcentration of 10%. [25] reportedthat 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 selectedspecies, 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 onmotility duration and motility percentage of post-thawed sperms. Results also showed that highest motility duration and motility percentage of post-thawed sperms was for treatment combinatorial BSA 5 mg/ml with MeOH 10%.

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