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Effect of Extenders and Different Concentrations of Methanol on Motility Parameters of Goldfish (*Carassius auratus gibelio*) Spermatozoa after Short-Term Storage

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Abstract: Semen samples were collected from eight males of Goldfish (*Carassius auratus gibelio*) and cryopreserved using four extenders (Goldfish sperm extenders; GFSE 1– GFSE 4) supplemented with Methanol (MeOH) at concentration of 5%, 10% and 20%. Semen was diluted with ratio of 1:1 with extenders and frozen in liquid nitrogen vapor. Frozen sperms after 2 and 5 days were excluded from freezing. Experiment showed the highest motility duration and the most motility percentage of post-thawed sperms after 2 days for sperm frozen at extender GFSE 2 with the concentration of MeOH 10%(31.66±8.02 s and 16.66±3.51%; $P<0.05$), as well as the upmost mobility and the motility duration of post-thawed sperms after 5 days was observed for sperm frozen at extender GFSE 2 with the concentration of MeOH10% (22.66±3.78 s and 13±2.64%; $P<0.05$).

Key words: Goldfish · Sperm · Methanol · Motility Duration · Motility Percentage

INTRODUCTION

The Goldfish (*Carassius auratus gibelio*) is a freshwater fish in the Cyprinidae family that is similar to Common carp (*Cyprinus carpio*) in biological and nutritional aspects [1]. The Goldfish associated with the culture and beliefs of people around the world and is also an important fish aspects of research and economically. It has been estimated that sperm from 200 fish species have been successfully Cryopreserved [2], however, species-specific optimizations of technology are needed. Important parameters for cryopreservation include types of extenders and cryoprotectants, the dilution ratio, the freezing and thawing rates and type of extender used for fertilization. The most efficient permeating cryoprotectant for fish milt appears to be Dimethyl Sulfoxide (DMSO) and Methanol (MeOH). The highest efficiency of DMSO and MeOH is at approximately 10% concentration. Other cryoprotectants such as Glycerol, Ethylene glycol, Propane-diol, Dimethyl Acetamide and are less popular or have been used with limited success.

Sperm cryopreservation is one way to overcome the problems associated with brood stock supply. Sperm collection for cryopreservation should be carried out during the spawning seasons because the quality and quantity of spermatozoa is highest at this time. In Sea bass, for example, the concentration of spermatozoa decreased as the spawning season progressed [3]. Similarly, Suquet *et al*. [4] reported lower motility rates, fertilization rates and reduced short-term storage capacity at the end of the reproductive period of the fish. Extender composition and cryoprotectant concentration are known to affect cryopreservation success [5, 6]. Cryopreservation of sperm is a simple technique that is useful for species conservation, preservation of biodiversity [7], protection of valuable breeding lines and as a helpful tool in facilitating animal reproduction. During cryopreservation, a number of factors may alter the physiological status of spermatozoa. The main factors are: cryoprotectant used, its concentration, composition of the diluent, milt dilution ratio, equilibration time and rates of freezing and thawing [8, 9]. Cryopreservation success was usually measured as post-thaw sperm mobility [10, 11] or as fertilization success during primary embryo growth [12]. The objective of the present study was to determine the most suitable extender and concentration of Methanol for Goldfish (*Carassius auratus gibelio*) spermatozoa dilution for cryopreservation.

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Table 1: The compositions of various extenders used in the experiments

	Type of extenders			
Chemicals	GFSE1	GFSE2	GFSE3	GFSE4
NaCl	-	0.3427 g	-	-
KCl	0.4620 g	-	0.4620 g	-
Fructose	1.9374 g	-		3.8749 g
Sucrose	-	3.4314 g	3.4314 g	-
Distilled water	100mL	100mL	100mL	100mL
NaoH solution[a]	21µL	16µL	20µL	17µL
Antibiotic[b]	0.5mL	0.5mL	0.5mL	0.5mL
pH	7.7	7.7	7.7	7.7

GFSE: Goldfish sperm extenders

[a] NaOH solution (NaOH 1 g + distilled water 100 mL) for adjusting pH.

[b]Antibiotic 10,000 Unit/mL penicillin and 10,000_g/mL streptomycin (Pen Strep from GIBCO BRL, cat. no 15140)

MATERIALS AND METHODS

Sperm Collection for Cryopreservation: Semen samples were collected from eight males of Goldfish (*Carassius auratus gibelio*) in the Research Center of Aquaculture of Fazli Barabadi martyr, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran.

Assessment of Sperm Quality: Motility of sperm samples was estimated under a light microscope at 400× magnification immediately after mixing of 5µL of sperm with 50µL of distilled water on a microscope slide. Sperm motility 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 were defined as sperm that did not show forward movement after activation. Video records were set at 30 frames/sec using a 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 distilled water until sperm stopped moving. Analyses were repeated three times for each treatment. Only sperm samples showing 80% motility or higher were used for the experiments [13].

Extenders and Sperm Cryopreservation: Extenders were developed from sugars such as Fructose and Sucrose and ion based chemicals such as NaCl and KCl. The extenders were simplified as Goldfish sperm extender (GFSE) consisting of various components (CCSE1–CCSE4) shown in Table 1. All extenders were adjusted to a pH of 7.7 with NaOH and kept at 2°C before use. All extenders supplemented with MeOH at concentration of 5%, 10% and 20%. Pooled semen was prepared by mixing equal portions of semen collected from eight males. Pooled semen was then diluted 1:1 with extenders. Suspensions of extended milt were drawn within 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 [14].

Measurement Sperm Mobility and Duration of Sperm Motility: Straws were thawed in a water bath with a temperature of 60°C for 10 s [13]. Sperm mobility and duration of sperm motility of thawed semen was observed after 2 and 5 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.

Data Analysis: All values are shown as mean±S.D. Data for percentage and duration of sperm motility statistical analysis by SPSS v16.0 software. The effects of extenders on post-thaw sperm motility and duration of sperm motility were analyzed using one-way analysis of variance (ANOVA).

RESULTS

Effects of Extenders and Concentrations of MeOH on Quality Post-Thawed Sperms after 2 Days: Sperm frozen at GFSE 2 with the concentration of MeOH 10% had the highest post-thaw motility (16.66±3.51%) while other treatments had average post-thaw sperm motility between 2.33 and 15.66% (Table 2). Longest duration of post-thaw sperm motility (31.66±8.02s) was observed from sperm

Table 2: Effects of extenders and different concentrations of MeOHon post-thaw sperm motility and duration of sperm motility after 2 days of freezing

Type of extenders	Cryoprotectant concentration (%)	Cryoprotectant	Motility duration (s)	Motility percentage (%)
	5		19.33±3.05^{bcde}	13.66±3.51^{a}
GFSE1	10	MeOH	23.00±5.56^{bcd}	14.33±2.51^{a}
	20		20.66±3.05^{bcde}	12.33±2.08^{ab}
	5		27.33±3.05^{ab}	15.33±3.05^{a}
GFSE2	10	MeOH	31.66±8.02^{a}	16.66±3.51^{a}
	20		25.00±4.58^{abc}	15.66±5.13^{a}
	5		17.00±3.00^{cde}	11.00±2.00^{abc}
GFSE3	10	MeOH	18.66±4.72^{cde}	8.00±4.58^{bcd}
	20		21.00±3.00^{bcde}	6.33±1.52^{cde}
	5		14.00±4.00^{e}	2.33±1.52^{e}
GFSE4	10	MeOH	15.33±4.16^{de}	4.33±1.52^{de}
	20		16.33±3.05^{de}	7.33±2.51^{bcde}
Control	-	-	116.89±14.90	87.50±6.18

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

Table 3: Effects of extenders and different concentrations of MeOHon post-thaw sperm motility and duration of sperm motility after 5 days of freezing

Type of extenders	Cryoprotectant concentration (%)	Cryoprotectant	Motility duration (s)	Motility percentage (%)
	5		20.00±2.00^{ab}	9.33±2.08^{b}
GFSE1	10	MeOH	19.66±1.52^{ab}	9.66±0.57^{b}
	20		17.33±3.51^{b}	10.00±3.00^{ab}
	5		20.66±1.52^{ab}	11.33±2.30^{ab}
GFSE2	10	MeOH	22.66±3.78^{a}	13.00±2.64^{a}
	20		20.33±3.05^{ab}	10.66±1.15^{ab}
	5		8.33±1.52^{c}	4.00±1.00^{c}
GFSE3	10	MeOH	9.66±2.08^{c}	2.66±0.57^{c}
	20		11.00±2.00^{c}	3.33±2.51^{c}
	5		8.66±1.52^{c}	1.66±1.15^{c}
GFSE4	10	MeOH	7.00±2.00^{c}	2.00±1.00^{c}
	20		7.66±2.51^{c}	2.33±0.57^{c}
Control	-	-	116.89±14.90	87.50±6.18

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

frozen at GFSE 2 with the concentration of MeOH 10%. Least duration and the lowest mobility of post-thaw sperm motility were observed from sperm frozen at GFSE 4 with the concentration of MeOH 5% (14±4 s and 2.33±1.52%; P<0.05).

Effects of Extenders and Concentrations of MeOH on Quality Post-Thawed Sperms after 5 Days: Maximum motility duration and the upmost mobility of post-thawed sperms after 5 days was related in the treatments with extender GFSE 2 and the concentration of MeOH 10% (22.66±3.78 s and 13 ±2.64%; Table 3), While the minimum duration motility of post-thawed sperms was observed in the treatments with extender GFSE 4 and the concentration of MeOH 10% (7±2 s). Least mobility of post-thaw sperm motility were observed from sperm frozen at GFSE 4 with the concentration of MeOH 5% (1.66±1.15%; P<0.05).

DISCUSSION

Most extenders in the present study were used successfully in freezing Cyprinidae semen. GFSE 2 with the concentration of MeOH 10% was the most suitable combination of extender for cryopreservation of Goldfish sperm based on the presence of highest post-thaw sperm motility in comparison with other combinations. This may be due to a synergistic effect resulting in superior performance after freezing. Extenders GFSE 2 consists of NaCl and sucrose where Sucrose functioned as a non permeating cryoprotectant that may protect the external part of the sperm cell [15] or stabilize liposomal membranes during freezing [16]. Cryopreserved sperm is a reliable procedure for the maintenance of fish species because of its relative easiness for freezing and storage. Methanol as an internal cryoprotectant significantly improved motility, progressive motility and its duration of

cryopreserved sperm. Methanol was employed as successful internal cryoprotectant in other Cypriniforms *Hypophthalmichtys molitrix* [17]; *Tor khudree* [18]; *Tinca tinca* [19]; *Cyprinus carpio* [20]; *Danio*[21]. Sugars have been used extensively as supplements to extenders to improve sperm motility during cryopreservation of sperm in several fish species such as African catfish (*Clarias gariepinus*) [22, 23] and various sturgeon species [12, 14]. Optimal extender for freezing of fish sperm varies among fish species. Saline solutions are widely used for cryopreservation of Cyprinidae species [24, 25]. However, saline solutions have also been reported to be less efficient extenders in comparison to saccharides or artificial seminal plasma for cryopreservation of sperm in African catfish (*C. gariepinus*) [23], Common carp (*C. carpio*) [20]. In this experiment, post-thawed sperms with MeOH concentration of 10% has the highest mobility and motility duration and this was contrast to the results obtained by Shigueki Yasui *et al*. [26]. These researchers have Announced that the maximum motility and motility duration of post-thawed sperm of *Misgurnus anguillicaudatus* was with MeOH concentration of 15%. [27] reported that the most suitable cryoprotectant for sperm cryopreservation of Java Barb (*Barbonymus gonionotus*), was with MeOH 10%.

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 upmost mobility and the motility duration of post-thawed sperms were observed from sperm frozen at extender GFSE 2 with the concentration of MeOH 10%.

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