Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Document heading doi:10.12980/JCLM.2.2014C1254

Preliminary studies on the cryopreservation of spermatozoa in the fresh water fish common carp (*Cyprinus carpio* L.)

Kuppusamy Umaa Rani*, Natesan Munuswamy

Unit of Aquaculture and Cryobiology, Department of Zoology, University of Madras, Guindy Campus, Chennai-600 025, India

PEER REVIEW

Peer reviewer

Dr. M. Pratheeba, Assistant Professor, Department of Biotechnology, Sri Sankara Arts and Science College, Kancheepuram, India.
Tel: 917708710222
E-mail: pssakthi@gmail.com

Comments

This is a valuable research work in which authors have demonstrated toxicity study, viability, motility and morphology. The experiment was conducted both control and cryopreserved sperm.
Details on Page 185

ABSTRACT

Objective: To investigate the effects of various extenders containing different cryoprotectants on post-thaw viability, motility and scanning electron microscopic study of frozen spermatozoa from carp.

Methods: After screening a variety of cryoprotectants and extenders, a protocol for the cryopreservation has been achieved using extender and cryoprotectants like dimethylacetamide 5%-20% at appropriate dilution ratio (1:10). For all experimental tests, the motility and viability percentage of spermatozoa were examined after storage of 5 d at 4 °C.

Results: The maximum motility of 75% has been observed with 10% dimethyl sulfoxide. Scanning electron microscopic studies on normal and cryopreserved spermatozoa showed prominent head, middle piece and different segments of flagellum. There were no significant deformities noticed on the surface topography of cryopreserved spermatozoa.

Conclusions: Thus the results clearly documented that cryoprotectant 10% dimethyl sulfoxide, affords better cryopreservation at 4 °C for the spermatozoa of *Cyprinus carpio*.

KEYWORDS

Cryopreservation, Dimethylacetamide, Viability, Motility, *Cyprinus carpio*

1. Introduction

Common carp [*Cyprinus carpio* (*C. carpio*)] is an economically important fish species cultured mostly in Asia and Europe. Global production of farmed common carp was about 6.14% (3 172 488 tonnes) of the total world aquaculture production[1]. Artificial insemination can be applied to increase the production to meet this demand due to exploitation of natural stocks. Cryopreservation is a valuable technique to assist in the genetic improvement of cultured stocks as well as providing a continuous supply of good quality sperm for artificial insemination. Successful storage of fish sperm in liquid nitrogen has been reported for more than 200 species[2], but the protocol varies with species. Extender composition, cryoprotectant concentration, and freezing method are known to affect cryopreservation success[3,4]. Cryopreservation of common carp sperm using saline extenders and dimethyl sulfoxide (DMSO) or dimethylacetamide (DMA) produced variable results on post-thaw sperm motility and/or fertilization and hatching success[5-7]. However, evaluation of the various cryopreservation methods is hindered by the different extenders, cryoprotectants and freezing methods that have been used.

In contrast, damaging effects to carp sperm by equilibration with DMSO were observed[8], and other comparative DMSO studies showed a better suitability of methanol and glycerol and DMA for freezing of carp sperm[9,10]. The usefulness of the latter cryoprotectants for cryopreservation of fish sperm was first reported for

*Corresponding author: Kuppusamy Umaa Rani, Unit of Aquaculture and Cryobiology, Department of Zoology, University of Madras, Guindy Campus, Chennai-600 025, India.
E-mail: umaa.krani@gmail.com
Foundation Project: Supported by DST, Government of India (Grant No. SR/SO/As-03/2003) and UGC-DRS-II.

Article history:
Received 2 Jan 2014
Received in revised form 9 Jan, 2nd revised form 12 Jan, 3rd revised form 14 Jan 2014
Accepted 15 Feb 2014
Available online 28 Mar 2014

rainbow trout semen by McNiven *et al*[11]. Since then, DMA has been applied with varying success in cryopreserving sperm of several fish species (*e.g. Oncorhynchus masou formosanus*, *Perca flavescens*, *Esox masquiongy*, *Silurus glanis*, *Salvelinus alpinus*, *Clarias gariepinus*). The milt dilution ratio has been shown to have a strong effect on post-thaw motility and fertility[12]. For cryopreservation of salmonid semen, the dilution ratio of 1:1 to 1:3 was proved to be the best and it was recommended by Legendre and Billard[13] and Babiak *et al*[10].

Chilled storage of fish sperm could be achieved by storage in undiluted and diluted form. These approaches are easy to perform without requiring expensive equipment or specific training, and allow prolonging sperm viability for weeks[14,15]. Storage of undiluted semen at low temperature (0-4 °C) has been reported to result in decreased fertilization capacity after short time storage in some of cultured species[14,16]. Chilled storage of sperm has been successfully reported in striped bass *Morone saxatilis* (Walbaum)[17], channel catfish *Ictalurus punctatus* and several species salmonids[18-20].

The main purpose of this study was to investigate the effects of various extenders containing different cryoprotectants on post-thaw viability, motility and scanning electron microscopic study of frozen spermatozoa from carp.

2. Materials and methods

2.1. Brood stock selection

Common carp is one of the most important freshwater fish species in aquaculture. They are generally known to spawn once a year during monsoon months. Increased rainfall, low water temperature and adequate nutrition are believed to trigger final gonad maturation. It is rather a cumulative effect of environmental interactions that influence breeding. For the present study, sexually mature males [(100.00±1.73) g of body weight and (12.50±0.33) cm total length] were chosen randomly. Males were examined to determine the sexual maturity by the presence of semen in the genital papilla after a light pressure of the abdomen.

2.2. Gamete collection

Sperm was collected by gently hand-stripping. Semen was collected from 5 anesthetized (0.1 g/L MS 222) males by manual abdominal stripping 12 h after a single injection of 2 mg/kg of carp pituitary extract (CPE) at 20-22 °C water temperature. Eggs were collected by hand stripping 10-12 h after a double injection of 3.5 mg/kg of CPE. The first injection, 10% (0.35 mg/kg) CPE was given 10 h before the second (3.15 mg/kg). For sperm collection, the urogenital papilla's of mature male fishes were carefully dried and sperm was hand-stripped directly into test tubes. Following sperm collection, the tubes containing sperm were placed in a styrofoam box containing crushed ice (4 °C). Contamination of sperm with water, urine or faeces was carefully avoided. Sperm was transported to the laboratory within 15 min. For collection of eggs from commom carp, females were wiped dry, stripped by gentle abdominal massage and the eggs from each female were collected in a dry metal bowl. Eggs were checked visually and only those lots of homogenous shape, colour and size were used in the fertilization experiments.

2.3. Cryopreservation

Common carp sperm were collected from nine males in separate plastic containers for cell culture with sperm:air volume ratios of 1:10 to 1:30 and were stored under aerobic conditions at 4 °C for 2-4 h. Sperm quality was checked for the percentage of sperm motility from video records. Only sperm samples showing more than 40% motility were used for cryopreservation. Sperm were diluted 1:5 (sperm:extender) in a Kurokura-1 (16) extender (128.4 mmol/L NaCl, 2.7 mmol/L KCl, 1.4 mmol/L $CaCl_2$, 2.4 mmol/L $NaHCO_3$) and equilibrated for 40 min at 4 °C. Fifteen percent pure DMA was added and every milliliter of mixture was transferred to a 2 mL cryotube, and then the cryotubes were directly transferred to a preprogrammed Planer Kryo 10 series III at 20 °C and cooled from 20 to -20 °C at a rate of 5 °C/min and then from -20 to -40 °C at a rate of 10 °C/min , held for 5 min at -40 °C, and finally transferred into liquid N_2. The spermatozoa were thawed in a water bath at 35 °C for 110 seconds.

2.4. Scanning electron microscopic studies

Evaluation of the effect of storage time on sperm morphology showed that as semen storage increased, the quality of semen decreased. Freshly collected milt was fixed overnight in 4% glutaraldehyde. The tissues were rinsed in orthophosphoric acid and then washed in different grades of ethanol series (30%, 50%, 70% and 100%). The sample was sputter coated with gold (Denbon sputter coater Derk II) and viewed under a scanning electron microscope and selective fields were magnified to capture the images of spermatozoa at different magnifications[21].

2.5. Statistical analysis

Data obtained from the present study were subjected to standard statistical analysis. All data were expressed as mean±SD. Correlation between motility (%) and viability was assessed by Karl Pearson's method of correlation[22].

3. Results

3.1. Morphology of spermatozoa

The spermatozoa of common carp consists of rounded head, cylindrical midpiece and an elongated flagellum. Only the head of the spermatozoa is visible under light microscope (20×). Therefore scanning electron microscopic studies were conducted to analyze the entire structure of spermatozoa. The head, midpiece and tail of spermatozoa were clearly visible under scanning electron microscope (Table 1).

Table 1
Morphometric analysis of spermatozoa of *C. carpio*.

Parameters	Measurements (μm)
Length of spermatozoa	23.00±1.15
Head length	1.77±0.53
Head width	1.47±0.57
Midpiece length	0.59±0.45
Midpiece width	0.59±0.45
Flagellum length	21.30±0.17

Mean±SD of 3 replicates.

3.2. Semen quality

The spermatological properties of the semen collected from the adult male common carp weighing about (100±2) g are shown in Table 2. Semen volume was rather variable and ranged from 0.1-1.0 mL and mean volume was (0.8±0.2) mL. The density of sperm in fresh sample was 250-400×10^9/mL. The pH of freshly collected milt ranged from (7.0±2.0) and the osmolality of the seminal plasma was about 280-320 mOsmol/kg.

Table 2
Spermatological parameters of common carp semen.

Semen Characteristics	Semen volume (mL)	Spermatozoa density (×10^9 /mL)	Semen pH
Mean±SD	0.8±0.2	360.00±9.24	7.2±1.4
Range	0.1-1.0	250-400	6.2-8.2

3.3. Selection of extender

The osmolality of four prepared extenders, namely Cortland medium, fish Ringer solution, Hanks balanced salt solution (HBSS) and Mounibs medium, were screened using a cryo-osmometer. The osmolality of HBSS mimicked the osmolality of the seminal plasma, therefore HBSS was analysed to be a suitable extender for cryopreserving carp sperm.

3.4. Cryoprotectant toxicity

Toxicity assessment was performed using various cryoprotectants such as DMSO, glycerol, ethane 1,2-diol, propane 1,2-diol and methanol at varying concentrations (10%, 15% and 20%). The viability was calculated using eosin-nigrosin stain based on the dye exclusion principle. Poor survival rates (35%) was recorded for samples preserved using 20% DMSO. Moderate survivability (61.5%, 62.3%, 60.8%) was recorded with glycerol (10% and 20%) and DMSO 15%. Maximum viability (75.8%) was observed with samples cryopreserved using 10% DMSO. Cryoprotectants that yielded low survivability were excluded and the low molecular weight cryoprotectants showing moderate and maximum survivability *i.e.*, DMSO and glycerol were used for further cryopreservation studies under liquid nitrogen (Figure 1).

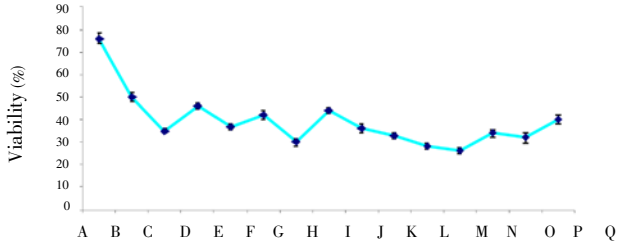

Figure 1. Cryoprotectant toxicity assessment of spermatozoa.
A: 10% DMSO, B: 15% DMSO, C: 20% DMSO, D: 10% Glycerol, E: 15% Glycerol, F: 20% Glycerol, G: 10% Ethanediol, H: 15% Ethanediol, I: 20% Ethanediol, J: 10% Propanediol, K: 15% Propanediol, L: 20% Propanediol, M: 10% Methanol, N: 15% Methanol, O: 20% Methanol.

3.5. Cryopreservation

The cryoprotectants DMSO and glycerol which yielded maximum survivability were used for cryopreserving the semen samples. The conventional method of freezing was adapted for preservation. The post thaw survivability was determined using eosin-nigrosin staining and the survivability was expressed as the percentage of unstained sperm. The effect of cryoprotectants (DMSO and glycerol) on the post-thaw motility and viability was analysed. The samples preserved with DMSO 10% yielded maximum post thaw motility and viability.

3.6. Post thaw motility

The percentage of motility of spermatozoa was used as a parameter to appraise its viability. Thus from the results obtained, samples cryopreserved in DMSO 10% showed higher motility (78.00±1.18) compared to glycerol 10% (30.00±1.03). Hence DMSO 10% was assessed as the best cryoprotectant for carp spermatozoa.

3.7. Post thaw viability

Viability of common carp sperm varied in different percentages of DMSO and glycerol. DMSO 10% showed maximum viability (75.8%) compared to control and other cryoprotectants. A significant correlation was observed between motility and viability.

3.8. Ultrastructure of spermatozoa

The spermatozoa morphology as revealed by scanning electron microscopic is shown in Figure 2. The spermatozoa of carp is uniflagellate having a circular head and a cylindrical midpiece. The stored milt showed clumping and irregularity in head morphology when compared to fresh milt as shown in Figure 2.

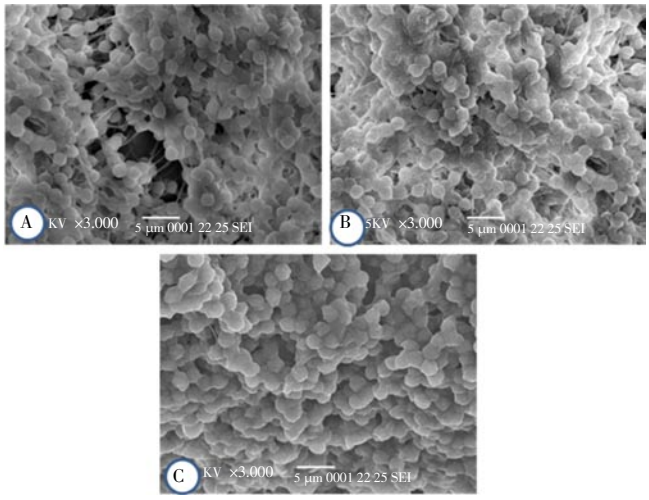

Figure 2. Scanning electron micrographs showing the morphology of spermatozoa in *C. carpio*.

A: Spermatozoa (S) (control) showing no change or deformation in spermatozoan morphology, B: Post-thaw spermatozoa (10% DMSO) showing normal surface topography, C: Post- thaw spermatozoa (10% glycerol) showing abnormal surface topography. Note the spermatozoa are distinct with prominent head (H) and long flagella (F) and no clumping of spermatozoa in DMSO (10%).

4. Discussion

The present study describes an attempt to cryopreserve the semen of *C. carpio* for a short period of time. Short-term storage of semen at 4 °C is mostly applied to overcome asynchrony in maturation and difficulties in gamete transportation. In commercial fish production, the evaluation of semen quality is of interest to increase the efficiency of artificial fertilization[23]. In *C. carpio*, the mean semen volume was similar to the results reported by Akcay *et al*.[24] but different from those of Bozkurt and Secer[25]. The difference may be due to differences in feeding conditions and regime, environmental factors or spawning time.

The osmolality of seminal plasma was used as a controlling point to develop extenders for semen of many fish species and reversibly suppresses the spermatozoa activation. Osmolality of any extenders used for artificial propagation of fish seminal plasma is generally adjusted by the use of Na^+ and K^+ levels. Sperm quiescence in undiluted semen occurs roughly in the range of 270-300 mOsmol/kg. The seminal plasma osmolality in the European eel has been reported to be in the range of 325-330 mOsmol/kg and extenders with this range of values helped in reversibly suppressing the motility of spermatozoa[26]. The sperm count of fishes varies widely with different species. The highest sperm count was estimated in bluefin tuna, *Thunnus thynnus* at 50-60 000 million per milliliter[27]. In carp, the average sperm count is 250-400 million per milliliter[28]. Similar results of $(360.00\pm9.24)\times10^9$/mL were obtained in the present study, for the milt samples obtained from the experimental animal *C. carpio*.

In the present study, the mean spermatozoa motility was analyzed to be (78.00±1.16) in 10% DMSO when compared to control (100%) and higher than the motility (%) observed in glycerol 10% (30.00±1.03), which was similar to the findings of Bozkurt and Secer[25] but differed from those of Akcay *et al*[24]. Results regarding post-activation motility and duration of movement are also supported by the studies of Jayaprakas and Lal[29]. Both properties decreased with time but the proportion of motile cells decreased faster in fresh semen samples than in activated ones. Similar results were reported by Lahnsteiner *et al*.[30], Lubzens *et al*.[31] and Bozkurt *et al*[25]. Various activating media have been used to induce sperm motility and evaluate sperm quality. Most commonly used media are distilled water, 0.3% NaCl and 1% $NaHCO_3$[32]. Generally the motility rate of frozen-thawed spermatozoa is very high in marine fish species compared to fresh water species as proved by Lahnsteiner *et al*[30].

Selecting a suitable extender is a key factor in successful cryopreservation of fish semen. In this work, four extenders (Cortland medium, fish Ringer solution, HBSS and Mounibs medium) which had been used in cryopreserving semen of fresh water fish were tested for cryopreservation of *C. carpio* sperms. Cortland and HBSS gave best post-thaw motility followed by Ringer and Mounibs natural medium, when 15% DMSO was used as cryoprotectant in the cryopreservation of red seabream as reported by Liu *et al*[22]. Mounibs modified medium and Mounibs medium have been used successfully by other researchers in cryopreservation of sperm of some marine fish species, such as Atlantic cod[33], turbot[34] and sea bass[35].

The cryoprotectants tested for the present study were DMSO, glycerol, ethane diol, propane diol, ethanol and methanol which have been widely used in cryopreservation of fish semen and embryos[36]. In cryopreservation of mirror carp semen and of Brazilian fish species, Akcay *et al*.[24] found that semen frozen with DMSO had the highest post thaw motility (55%). Freezing with 15% DMSO resulted in 69% fertilization against a fresh sperm control of 83%. In most of the studies, DMSO at concentrations 5%-15% was the most effective[32]. DMSO has the characteristic of small molecular weight and a high rate to permeate into the cells of various fish species including Atlantic croaker[37], Yellowfin seabrem[38], and rainbow trout[39]. DMSO and

glycerol are the most commonly used cryoprotectants for cryopreservation of marine fish sperm[40]. In most studies, DMSO provides the best results, probably due to its fast penetration into spermatozoa and its interaction with the phospholipids of the sperm membrane[40]. However, DMSO is toxic at high concentrations, the motility duration of frozen-thawed barramundi (*Lates calcarifer*) spermatozoa was reduced when the DMSO concentration was higher than 5% and also in the black grouper, the sperm motility was decreased at a concentration of 30%[41]. Glycerol can be less efficient, possibly due to its toxic effects, osmotic stress and reduced speed to penetrate into the spermatozoa[14]. In addition, the efficiency of glycerol for sperm cryopreservation may be species specific[40]. Glycerol as a suitable cryoprotectant for whitefish, a salmonid, eventhough glycerol has been reported to be toxic to salmon and trout spermatozoa[19]. They further explained that this exception was connected with differences in seminal plasma composition in different fish, because the concentration of glycerol in the seminal plasma of whitefish is 22 times higher than in rainbow trout. Scanning electron microscopic observations of common carp spermatozoa stored at 4 °C for 5 d showed altered morphological variations compared to freshly collected milt. The scanning electron micrograph clearly showed distorted head morphology and clumping[21].

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Financial assistance from DST, Government of India (Grant No. SR/SO/As-03/2003) and UGC-DRS-II are gratefully acknowledged.

Comments

Background

Cryopreservation plays an important role in the preserving of endangered species. Changing life style in human becomes impotency and causes abnormality in the sperm. Therefore there is a need for cryopreservation of sperm all over the world.

Research frontiers

The present research work depicts toxicity of various cryoprotectants, viability motility and morphology of both control and cryopreserved spermatozoa of fish.

Related reports

Toxicity study was conducted and the worst cryoprotectant was removed and the best cryoprotectants used for the cryopreservation studies

Innovations and breakthroughs

Cryopreservation is one of the most emerging field in biotechnology for which the fish protocol is equal to humans. In the present study, authors have demonstrated the toxicity study of various cryoprotectants, viability, motility and scanning electron micrographs of selected cryoprotectants.

Applications

From the literature survey, it has been found that it is very important to humans those who are working in software field. This scientific study supports and suggests the use of this fish is same commonly for humans.

Peer review

This is a valuable research work in which authors have demonstrated toxicity study, viability, motility and morphology. The experiment was conducted both control and cryopreserved sperm.

References

[1] Food and Agriculture Organization of the United Nations. FAO yearbook. Fishery and aquaculture statistics, 2006. Rome: Food and Agriculture Organization of the United Nations; 2008. [Online] Available form: ftp://ftp.fao.org/docrep/fao/011/i0400t/i0400t.pdf. [Accessed on 15th August, 2013]

[2] Brown GG, Brown LD. Cryopreservation of sperm of striped bass and white bass. In: Tiersch TR, Mazik PM, ediors. *Cryopreservation in aquatic species*. Baton Rouge: World Aquaculture Society; 2000, p. 130-137.

[3] Sansone G, Fabbrocini A, Ieropoli S, Langellotti A, Occidente M, Matassino D. Effects of extender composition, cooling rate, and freezing on the motility of sea bass (*Dicentrarchus labrax* L.) spermatozoa after thawing. *Cryobiology* 2002; **44**: 229-239.

[4] DeGraaf JD, Berlinsky DL. Cryogenic and refrigerated storage of rainbow smelt *Osmerus mordax* spermatozoa. *J World Aquac Soc* 2004; **35**: 209-216.

[5] Lahnsteiner F, Berger B, Horvath A, Urbanyi B, Weismann T. Cryopreservation of spermatozoa in cyprinid fishes. *Theriogenology* 2000; **54**: 1477-1498.

[6] Linhart O, Rodina M, Cosson J. Cryopreservation of sperm in common carp *Cyprinus carpio*: sperm motility and hatching success of embryos. *Cryobiology* 2000; **41**: 241-250.

[7] Warnecke D, Pluta HJ. Motility and fertilizing capacity of frozen/thawed common carp (*Cyprinus carpio* L.) sperm using dimethyl-acetamide as the main cryoprotectant. *Aquaculture*

2003; **215**: 167-185.

[8] Lubzens E, Daube N, Pekarsky I, Magnus Y, Cohen A, Yusefovich F, et al. Carp (*Cyprinus carpio* L.) spermatozoa cryobanks—strategies in research and application. *Aquaculture* 1997; **155**: 13-30.

[9] Lakra WS, Krishna G. Preliminary trials for cryopreservation of spermatozoa of selected carps and catfishes. *Indian J Anim Sci* 1997; **67**: 90-92.

[10] Babiak I, Glogowski J, Brzuska E, Szumiec J, Adamek J. Cryopreservation of common carp, *Cyprinus carpio* L. *Aquac Res* 1997; **28**: 567-571.

[11] McNiven MA, Gallant RK, Richardson GF. Dimethyl-acetamide as a cryoprotectant for rainbow trout spermatozoa. *Theriogenology* 1993; **40**: 943-948.

[12] Brown GG, Mims SD. Cryopreservation of paddlefish *Polyodon spethula* milt. *J World Aquac Soc* 1999; **30**(2): 245-249.

[13] Legendre M, Billard R. Cryopreservation of rainbow trout sperm by deep-freezing. *Reprod Nutr Dev* 1980; **20**: 1859-1868.

[14] Stoss J. Fish gamete preservation and spermatozoan physiology. In: Hoar WS, Randall DJ, Donaldson EM, editors. *Fish physiology*.Vol. 9, part B. San Diego: Academic Press; 1983, p. 305-350.

[15] Rana KJ. Preservation of gametes. In: Bromage NR, Roberts RJ, editors. *Broodstock management and egg and larval quality*. Cambridge: Cambridge University Press; 1995, p. 53-75.

[16] Lahnsteiner F, Weissman T, Patzner RA. Aging processes of rainbow trout semen during storage. *Prog Fish-Cult* 1997; **59**: 272-279.

[17] He S, Woods LC 3rd. Effects of glycine and alanine on short-term storage and cryopreservation of striped bass (*Morone saxatilis*) spermatozoa. *Cryobiology* 2003; **46**: 17-25.

[18] Christensen JM, Tiersch TR. Refrigerated storage of channel catfish sperm. *J World Aquac Soc* 1996; **27**: 340-346.

[19] Scott AP, Baynes SM. A review of the biology, handling and storage of salmonid spermatozoa. *J Fish Biol* 1980; **17**: 707-739.

[20] Erdahl AW, Graham EF. Fertility of teleost semen as affected by dilution and storage in a seminal plasma-mimicking medium. *Aquaculture* 1987; **60**: 311-321.

[21] Mattei X. Spermiogenèse comparée des poisons. Comp. In: Baccetti B, editor. *Comparative spermatology*. New York: Academic Press; 1970, p. 57-69. French.

[22] Liu QH, Li J, Zhang SC, Ding FH, Xu XZ, Xiao ZZ, et al. An efficient methodology for cryopreservation of spermatozoa of red seabream (*Pagrus major*) with 2-mL cryovials. *Aquac Soc* 2006; **37**: 289-297.

[23] Bozkurt Y. Relationship between body condition and spermatological properties in scaly carp (*Cyprinus carpio*) semen. *J Anim Vet Adv* 2006; **5**(5): 412-414.

[24] Akçay E, Bozkurt Y, Seçer S, Tekin N. Cryopreservation of mirror carp semen. *Turk J Vet Anim Sci* 2004; **28**(4): 837-843.

[25] Bozkurt Y, Secer S. Effect of short-term preservation of mirror carp (*Cyprinus carpio*) semen on motility, fertilization and hatching rates. *Israeli J Aquacult-Bamid* 2005; **57**(3): 207-212.

[26] Ohta H, Izawa T. Diluent for cool storage of the Japanese eel (*Anguilla japonica*) spermatozoa. *Aquaculture* 1996; **142**: 107-118.

[27] Doi M, Hoshino T, Taki Y, Ogasawara Y. Activity of the sperm of the bluefin tuna (*Thunnus thynnus*), under fresh and preserved conditions. *Bull Jpn Soc Sci Fish* 1982; **48**: 495-498.

[28] Routray P, Verma DK, Sarkar SK, Sarangi N. Recent advances in carp seed production and milt cryopreservation. *Fish Physiol Biochem* 2007; **33**: 413-427.

[29] Jayprakas V, Lal TS. Factors affecting the motility and short-term storage of spermatozoa of the Indian major carps (*Labeo rohita* and *Cirrhinus mrigala*). *J Aquac Trop* 1996; **11**: 67-68.

[30] Lahnsteiner F, Berger B, Weismann T, Patzner R. The influence of various cryoprotectants on semen quality of the rainbow trout (*Oncorhynchus mykiss*) before and after cryopreservation. *J Appl Ichthyol* 1996; **12**: 99-106.

[31] Lubzens E, Daube N, Pekarsky I, Magnus Y, Cohen A, Yusefovich F, et al. Carp (*Cyprinus carpio* L.) spermatozoa cryobanks-strategies in research and application. *Aquaculture* 1997; **155**: 13- 30.

[32] Viveiros AT, Godinho HP. Sperm quality and cryopreservation of Brazilian freshwater fish species: a review. *Fish Physiol Biochem* 2009; **35**: 137-150.

[33] Mounib MS, Hwang PC, Idler DR. Cryogenic preservation of Atlantic cod (*Gadus morhua*) sperm. *J Fish Res Board Can* 1968; **25**: 2623-2632.

[34] Dreanno C, Suquet M, Quemener L, Cosson J, Fierville F, Normant Y, et al. Cryopreservation of turbot (*Scophthalmus maximus*) spermatozoa. *Theriogenology* 1997; **48**: 589-603.

[35] Fauvel C, Suquet M, Dreanno C, Zonno V, Menu B. Cryopreservation of sea bass (*Dicentrarchus labrax*) spermatozoa in experimental and production simulating conditions. *Aquat Living Resour* 1998; **11**(6): 387-394.

[36] Ji XS, Chen SL, Tian YS, Yu GC, Sha ZX, Xu MY, et al. Cryopreservation of sea perch (*Lateolabrax japonicus*) spermatozoa and feasibility for production-scale fertilization. *Aquaculture* 2004; **241**: 517-528.

[37] Gwo JC, Strawn K, Longnecker MT, Arnold CR. Cryopreservation of Atlantic croaker spermatozoa. *Aquaculture* 1991; **94**: 355-375.

[38] Gwo JC. Cryopreservation of yellowfin seabream (*Acanthopagrus latus*) spermatozoa (Teleost, Perciforms, Sparidae). *Theriogenology* 1994; **41**: 989-1004.

[39] Conget P, Fernández M, Herrera G, Minguell JJ. Cryopreservation of rainbow trout (Oncorhynchus mykiss) spermatozoa using programmable freezing. *Aquaculture* 1996; **143**: 319-329.

[40] Suquet M, Dreanno C, Fauvel C, Cosson J, Billard R. Cryopreservation of sperm in marine fish. *Aquac Res* 2000; **31**(3): 231-243.

[41] Gwo JC. Cryopreservation of black grouper (*Epinephelus malabaricus*) spermatozoa. *Theriogenology* 1993; **39**: 1331-1342.