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Diluents and Cryoprotectants for Cryopreservation of Filefish *Thamnaconus modestus* Sperm

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The present study aimed to find the best diluent and cryoprotectant for sperm cryopreservation of filefish *Thamnaconus modestus*. Two kinds of artificial seminal plasma (ASP1, ASP2), 0.3 M glucose and marine fish Ringer's solution (MFRS) were employed as diluent. Dimethyl sulfoxide (DMSO) and methanol as cryoprotectant were selected for sperm cryopreservation. Sperm was diluted at the ratio of 1:3 with diluents containing cryoprotectants and adjusted for final concentration at 5%, 10%, 15% and 20%. Mixed milt was frozen at liquid nitrogen vapor after equilibration for 5 min. The highest motility (40.5±2.8%) and swimming speed (81.5±4.1 μm/s) of frozen/thawed sperm were observed in ASP1 diluent containing 10% DMSO and in ASP2 containing 15% DMSO, respectively. Results showed that cryopreservation with ASP as diluent and DMSO as cryoprotectant could be adopted for long term storage of filefish sperm.

Keywords: *Thamnaconus modestus*, Filefish, Cryopreservation, Diluent, Cryoprotectant

Introduction

Sperm cryopreservation is a common technique for the artificial reproduction and genetic improvement as well as for biological conservation programs (Chao and Liao, 2001). Fish sperm preservation is advantageous to solve the unbalanced sex ratio, the asynchronous eggs and sperm discharge time between females and males during spawning season. Moreover, it has advantage in transportation of sperm, facilitates selective breeding or hybridization as well as sperm preservation of decreasing indigenous species. For sperm preservation, diluents and cryoprotectants are commonly used. As common diluents, marine fish Ringer's solution (MFRS) and 0.3 M glucose have been successfully used for the sperm preservation of various fish (Kusuda, 2004).

Since Blaxter (1953) has been used frozen sperm to hybridize spring and fall spawning herring, the technology for fish sperm cryopreservation has been well established for many years and applied for more than 200 fish species (Figiel and Tiersch, 1997; Tiersch, 2000; Chao and Liao, 2001). Fish sperm cryopreservation has been investigated mainly in salmonids (Cabrita et al., 1998; Drokin et al., 1998; Labbe and Maisse, 1996) and some other fresh-water fish species (Aoki et al., 1997; Chao et al., 1987; Lubzens et al., 1997). And then, it is actively studied in marine fish species (Chao and Liao, 2001; Kusuda, 2004).

Filefish *Thamnaconus modestus* is one of the important marine commercial fish in Korea. However, the information on reproductive biology and sperm cryopreservation of filefish remains unknown.

The aim of this study was to find out a new diluent and cryoprotectant for cryopreservation of the filefish sperm. The effects of diluent and cryoprotectant on cryopreservation were assessed in terms of sperm activity parameters: sperm activity index (SAI), movable sperm ratio (MSR) and sperm swimming speed (SSS).

Materials and Methods

Fish and milt collection

For milt collection, five maturing males (TL=28.6±1.4 cm, BW=321.0±9.0 g) were used during the spawning season of the filefish. Fish were held in a spawning tank (2 m^3) with flow-through seawater (32 psu) at a flow rate of 0.2 L/s under simulated natural photoperiod at 15.0-16.5 ºC. Fish were fed once a day with the commercial feed (Suhyup Feed Co. Ltd., Uiryeong, Korea) during experimental period. Spermiation was induced by intramuscular injection of human chorionic gonadotropin (HCG) (Daesung Microbiological Lab. Co. Ltd., Korea) at the dose of 100 IU/kg fish body weight.

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They were anaesthetized with 100 ppm of ethyl 3-aminobenzoate methanesulfonate salt (MS-222, Sigma, USA) before milt collection. Milt was collected individually by serial waves of abdominal pressure, and then stored in the ice (4°C) until use. The feces and urine or environmental water was gently emptied and the genital area was wiped with paper towel before the milt collection was stripped by hand.

Evaluation of sperm activity

The sperm motility from each male was determined immediately after collection. The percentage of sperm exhibiting rapid, vigorous and forward movement was classified under a microscope (Axioskop 2 plus Zeiss, GÖttingen, Germany) by diluting the sperm into artificial seawater (NaCl 27 g, KCl 0.5 g, $CaCl_2$ 1.2 g, $MgCl_2$ 4.6 g, $NaHCO_3$ 0.5 g in a liter of distilled water). Sperm samples with total motility more than 90% were pooled and used for cryopreservation.

In order to evaluate frozen/thawed sperm activity under different cryopreservation condition, SAI, MSR and SSS of frozen/thawed sperm were estimated. To measure motility of frozen/thawed sperm, sperm were diluted in ASW at the ratio of 1 to 50 (1 μL sperm to 49 μL ASW). From here, 1 μL was put into slide glass (Teflon Printed Glass Slide; 21 wells; diameter of each well, 4 mm; Funakoshi Co., Japan) without cover slide. And then, frozen/thawed sperm activity was observed immediately at ×200 magnification under the microscope setting video camera (Carl Zeiss, Germany) and video timer (VTG-55B, Germany) to connect with video recorder and player (Samsung VHS, SV-G1000, Korea). Each sample was observed three times under the microscope and the time for one was 1.5 minutes. The motility of frozen/thawed sperm was recorded by video tape at the same time with video timer. To analysis the frozen/thawed sperm activity, the recorded tape was played at the video recorder and player with monitor where the sperm activity were observed and estimated. The motility of frozen/thawed sperm was evaluated by SAI, MSR and SSS. SAI was estimated using a scale of arbitrary index from I to IV, and the percentage of sperm corresponding to each index was recorded. At this time, the scores of 3, 2, 1 and 0 were allowed for the index of I, II, III and IV, respectively. SAI was calculated following formula using the scores and the percentage of sperm corresponding to each index. The numerical index for the evaluation of SAI was presented in Table 1. The SSS of frozen/thawed sperm was determined by the distance of moving sperm for 1 second.

Sperm cryopreservation

Sperm cryopreservation in filefish was estimated through experiments. Four diluents, two kinds of artificial seminal plasma (ASP1, ASP2), marine fish Ringer's solution (MFRS) and 0.3 M glucose, were used. The compositions of diluents were given in Table 2. Two cryoprotectants, dimethyl sulfoxide (DMSO) and methanol were tested. Milt was diluted at a ratio of 1 to 3 with diluents containing cryprotectants with final concentration of 5%, 10%, 15% and 20%. The diluted milt was taken into 500 μL straws and placed on a tray in liquid nitrogen (LN_2) vapor at 3.5 cm above the surface of LN_2 in a styrofoam box covered by lid. After a freezing period of 5 minutes, the straws were transferred into LN_2 and stored for 90 days. The straws were thawed in a waterbath (30°C) after 20 seconds. And then, frozen/thawed sperm viability was immediately estimated.

Statistical analysis

Data were expressed as mean±standard error (SE). Statistical evaluation was performed by one-way ANOVA using SPSS version 16.0. Means were separated using Tukey's multiple range test and differences were considered to be significant at $P<0.05$.

Results

The highest MSR of 40.5±2.8% was obtained when sperm was cryopreserved with ASP1 diluent in 10% DMSO, and no survival was observed in the 15% and 20% methanol. However, increasing the DMSO concentration to 15% and 20% reduced to 34.5±2.4% and 29.7±2.5%, respectively. In addition, MSR of the sperm diluted with 5% obtained 40.2±3.1%, but rising the concentration of methanol to 10%, 15% and 20% decreased to 26.2±2.1% and 0%, respectively. The highest SSS (58.8±4.9 μm/s) of frozen/thawed was observed in the 5% methanol but no swimming was in the 15% and 20% methanol. The SAI, in general, was in direct ratio to the MSR and the SSS. Although the MSRs in the 10% and 15% DMSO were different, the SAIs were not different (Fig. 1).

Table 1. Numerical index for the evaluation of sperm activity index (SAI)

Index	Score	Motility characteristics
I	3	Sperm display forward movement rapidly
II	2	Sperm display forward movement slowly
III	1	Sperm display vibrating movement moderately
IV	0	Immotile sperm

SAI=score x % motile sperm/100.

Table 2. Constituents of diluents used for sperm cryopreservation of filefish *Thamnaconus modestus*

Constituent	ASP1	ASP2	MFRS	0.3 M glucose
KCl (g/L DW)	0.77	0.3	0.60	-
NaCl (g/L DW)	9.92	9.0	13.50	-
$CaCl_2$ (g/L DW)	0.13	0.13	0.35	-
$MgCl_2$ (g/L DW)	0.05	0.05	0.02	-
$NaHCO_3$ (g/L DW)	-	0.5	0.03	-
Glucose (g/L DW)	0.01	0.01	-	54.06
pH	7.6	7.8	7.7	6.8
Osmolality (mmol·kg^{-1})	335.0	337.2	444.0	389.0

ASP: artificial seminal plasma, DW: distilled water, MFRS: marine fish Ringer's solution.

In Fig. 2, the MSR of frozen/thawed sperm was obtained the highest value (34.9±3.1%) in ASP2 containing 15% DMSO and the lowest value (7.6±1.8%) in this diluent containing 20% methanol. Similarly, the minimum (12.1±1.8 μm/s) and the maximum (81.5±4.1 μm/s) SSS of frozen/thawed sperm were observed in 20% methanol and 15% DMSO, respectively. The SAI, in this diluent, was directly proportional to the MSR and the SSS.

The highest MSR (30.0±3.0%) and immobile sperm were observed when sperm was cryopreserved in MFRS containing 20% DMSO and 5% methanol, respectively. There was a correlation between the SSS, the MSR and the SAI of frozen/thawed sperm (Fig. 3).

The MSR of frozen/thawed sperm was ranged from 0% (in 15% and 20% methanol) to 34.9±3.6% of 0.3 M glucose in 20% DMSO, respectively. In addition, the tendency of SSS

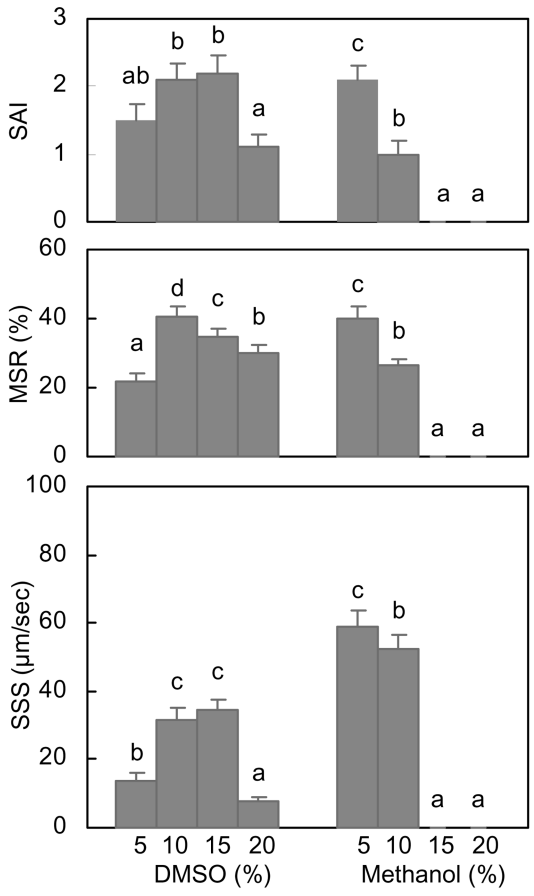

Fig. 1. Sperm swimming speed (SSS), movable sperm ratio (MSR) and sperm activity index (SAI) of frozen/thawed filefish *Thamnaconus modestus* sperm in the first artificial seminal plasma (ASP1) diluent. Different letters indicate significant differences between different concentrations (P<0.05).

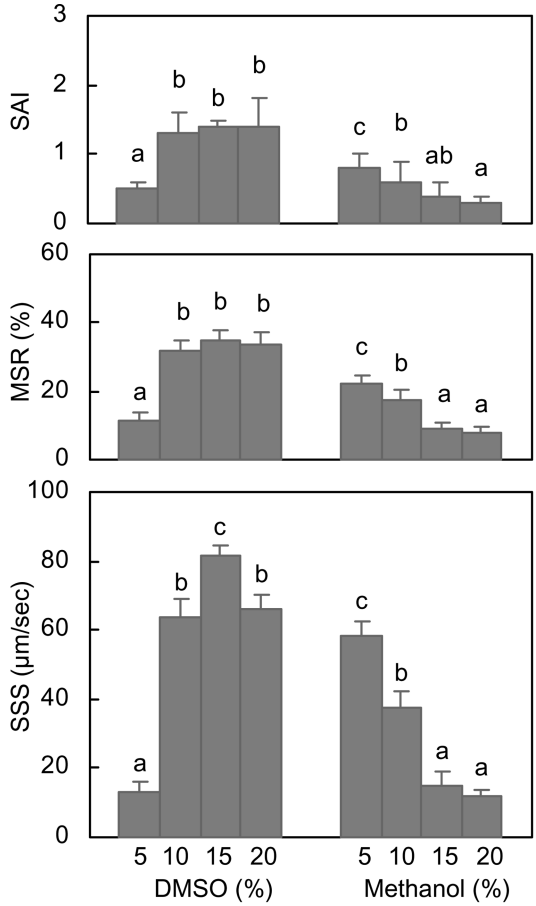

Fig. 2. Sperm swimming speed (SSS), movable sperm ratio (MSR) and sperm activity index (SAI) of frozen/thawed filefish *Thamnaconus modestus* sperm in the second artificial seminal plasma (ASP2) diluent. Different letters indicate significant differences between different concentrations (P<0.05).

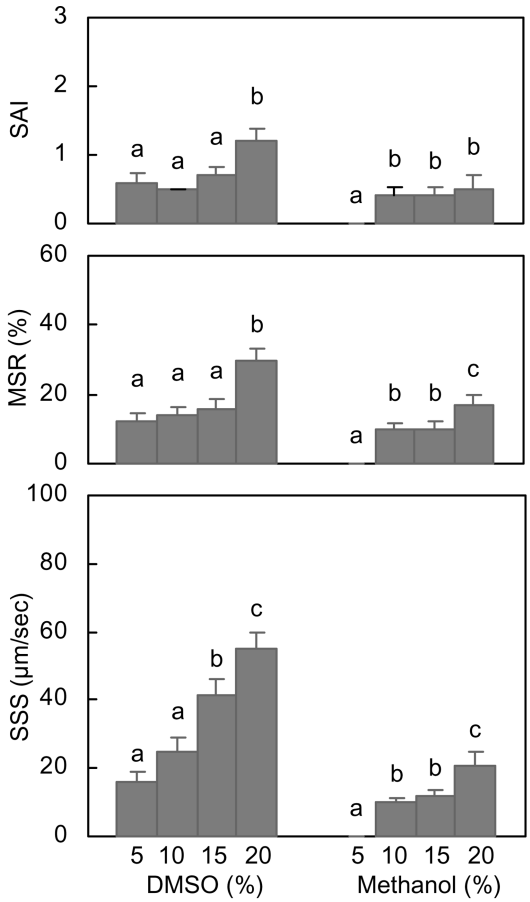

Fig. 3. Sperm swimming speed (SSS), movable sperm ratio (MSR) and sperm activity index (SAI) of frozen/thawed filefish *Thamnaconus modestus* sperm in the marine fish Ringer's solution (MFRS) diluent. Different letters indicate significant differences between different concentrations (P<0.05).

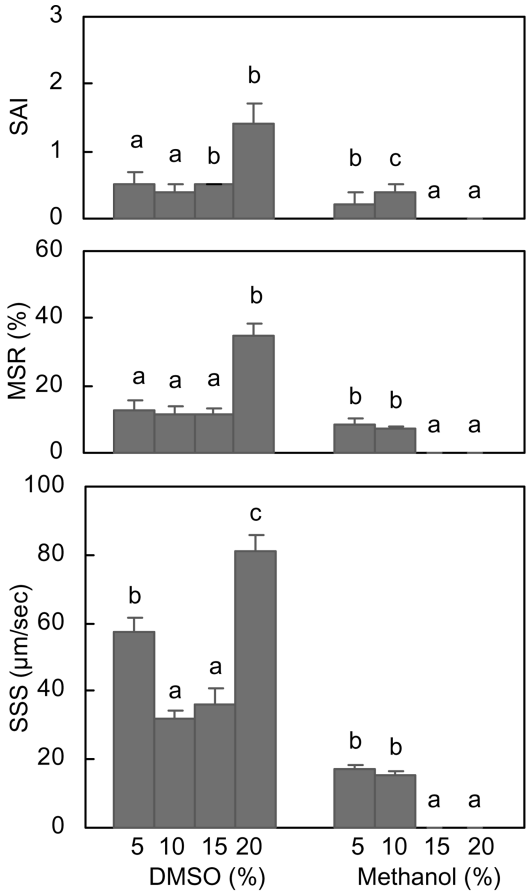

Fig. 4. Sperm swimming speed (SSS), movable sperm ratio (MSR) and sperm activity (SAI) of frozen/thawed filefish *Thamnaconus modestus* sperm in 0.3 M glucose diluent. Different letters indicate significant differences between different concentrations (P<0.05).

and the SAI of frozen/thawed sperm were similar to that of MSR (Fig. 4).

Discussion

The high variance of frozen/thawed sperm quality has been reported in different fish species. Post-thaw sperm viability and motility in milt cryopreservation mainly depends on the prevention of cryo-injury during freezing and thawing. Successful fish sperm cryopreservation can be achieved by optimizing the primary condition, the diluent, the cyoprotectant, the equilibrium time, the freezing rate, the storage procedure and the thawing rate (Chang, 1997; Jamiesion, 1991).

The first process for cryopreservation of fish sperm is that milt is diluted with diluent containing cryoprotectant. The diluent plays an important role as the regulator on osmotic pressure, pH and ion component etc. It must maintain sperm alive but immotile prior to freezing. Various diluents have been used successfully for cryopreservation of fish sperm (Gwo et al., 1991; Chen et al., 1993). In the present study, four extenders, ASP1, ASP2, MFRS and 0.3 M glucose, were examined for sperm cryopreservation in filefish. Kurokura and Hirano (1980) reported that marine fish sperm did not require diluent of complex compostion, and Suquet et al. (2000) also reported that most diluents used in marine fish were saline or sugar solutions. The 0.3 M glucose was good diluent in Atlantic salmon *Salmo salar* (Stoss and Refstie, 1983) sperm cryopreservation. In addition, in the sperm cryopreservation of grey mullet *Mugil cephalus* (Chao et al., 1975; Chang et al., 1999b) and river puffer *Takifugu obscurus* (Chang et al., 1999a), the MFRS was found to be good diluent. The ASP, in the case of the filefish, was good diluent in this study. The MSR and SSS of frozen/thawed sperm declined if the constituent of KCl and NaCl decreased even in the same ASP. Therefore, the KCl and NaCl were the main components in the filefish sperm cryopreservation.

The cryoprotectants such as DMSO, glycerol, methanol and propylene glycol are the most commonly cryoprotectants used in fish sperm cryopreservation (McNiven et al., 1993; Bergeron et al., 2002). These cryoprotectants protect sperm cells from damage during the process of freezing and thawing. The effectiveness of each cryoprotectant, however, varies with different fish species. For example, DMSO and glycerol were equally effective but methanol and propylene glycol were ineffective for *Sillago ciliata* sperm (Young et al., 1992), while 10% methanol yielded higher sperm motility and fertility than DMSO and glycerol for salmonid fish sperm (Lahnsteiner et al., 1997). Lim et al. (2007) reported that DMSO was a better cryoprotectant than methanol for cryopreservation of starry flounder *Platichthys stellatus* sperm. In this study, DMSO and methanol were tested for the cryopreservation of filefish sperm. The motility of the frozen/thawed sperm was decreased in the two cryoprotectants, while the highest motility was achieved with 10% DMSO. It would therefore appear that DMSO is the most suitable cryoprotectant for the filefish sperm cryopreservation. Although the MSR of 40.5±2.8% was achieved in ASP1 diluent containing 10% DMSO, but it was still lower than that 90% of fresh sperm. Methanol was the best cryoprotectant for the sperm cryopreservation of cyprinid species such as zebrafish *Danio rerio* (Harvey et al., 1982) and other groups of fishes such as salmonids (Lahnsteiner et al., 1997), catfishes (Steyn, 1993; Tiersch et al., 1994) and tilapia *Sarotherodon mossambicus* (Harvey, 1983). In the case of filefish, however, DMSO was better cryoprotectant than methanol.

In conclusion, the highest MSR of frozen/thawed filefish sperm was observed when it was frozen in ASP1 diluent containing 10% DMSO. The highest SSS of frozen/thawed sperm, however, was obtained in ASP2 diluent containing 15% DMSO.

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