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Thesis for the Degree of Master of Science

Milt Properties and Sperm Cryopreservation in Filefish (*Thamnaconus modestus* ünther)

by

Le Minh Hoang

Department of UR Interdisciplinary Program of Fisheries and Oceanography, The Graduate School,

Pukyong National University

February 2008

Milt Properties and Sperm Cryopreservation in Filefish (*Thamnaconus modestus* Günther)

말쥐치 (*Thamnaconus modestus* Günther) 정액 성상과 정자의 냉동보존

Advisor: Young Jin Chang

by

Le Minh Hoang

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

In the Department of UR Interdisciplinary Program of Fisheries and Oceanography,

The Graduate School,

Pukyong National University

February 2008

Milt Properties and Sperm Cryopreservation in Filefish (*Thamnaconus modestus* Günther)

A Dissertation

by

Le Minh Hoang

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Le Minh Hoang 의 이학석사 학위논문을 인준함

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요 약

말쥐치(*Thamnaconus modestus*) 정액의 물리화학적 특성과 정자의 미세구조에 대하여 분석하였다. 정액량과 정자농도, spermatocrit는 각각 0.3±0.1 mL·fish^{-1}, 2.6±0.1x10^{7} spermatozoa·mL^{-1}, 73.3±6.7 였다. 정장의 화학적 조성에 있어서 potassium 9.8±0.9 mmol·L^{-1}, sodium 164.0±4.0 mmol·L^{-1}, chloride 151.0±1.2 mmol·L^{-1}, calcium 14.9±0.6 mg·dL^{-1}, magnesium 7.2±0.1 mg·dL^{-1}, glucose 1.0 mg·dL^{-1}, 총단백질 0.1 g·dL^{-1}, 총지질 1.0 mg·dL^{-1} 였으며, 삼투질농도와 pH는 각각 322.8±2.8 mmol·kg^{-1}, 7.7±0.1 였다.

본 연구의 목적은 말쥐치 정액 냉동보존시 적정 희석액과 동해방지제를 찾는 것이다. 이 종의 정액 냉동보존은 실험기간에 평가하였다. 4 개의 희석액, 2 종류의 인공정장(ASP), glucose 0.3

M 그리고 해수어의 Ringer's 요해를 사용하였다. 두 개의 동해방지제 dimethyl sulfoxide (DMSO)와 methanol로 테스트 했다. 정액은 동해방지제 농도 5%, 10%, 15% 그리고 20%가 포함된 첨가제에 1:3 비율로 희석하고, 5 분간 액체질소 증기로 평형시티 후 액체질소에 냉동하였다. 정액을 10% DMSO을 포함된 ASP1 희석액과 15% DMSO가 포함된 ASP2 로 냉동한 냉동/해동 정액에서 높은 운동성 40.5±2.8%과 수영속도 81.5±4.1 $\mu m \cdot s^{-1}$를 각각 관찰되었다. 이 연구에서 동해방지제 DMSO가 포함된 ASP 희석액이 다른 것에 비해 좋다는 것을 발견했다.

투과형 전자현미경으로 미세구조를 관찰한 결과, 정자는 첨체가 없는 머리와 5 개의 미토콘드리아로 이루어진 중편부 및 "9+2"의 미세소관 편모인 꼬리로 구성되어 있었다. 세로로 절단한 정자 머리는 장축 1.3-1.6 μm, 단축 1.0-1.3 μm 로 말편자 모양을 하고 있었다.

Milt Properties and Sperm Cryopreservation in Filefish (*Thamnaconus modestus* Günther)

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Abstract

Milt properties and sperm cryopreservation in filefish *Thamnaconus modestus* were estimated. The milt properties of filefish included physical properties of milt and biochemical properties of seminal plasma. The physical properties of milt revealed 0.3±0.1 $mL \cdot fish^{-1}$ in sperm volume, $2.6 \pm 0.1 \times 10^{7}$ $spermatozoa \cdot mL^{-1}$ in sperm concentration and 73.3±6.7 in spermatocrit. The biochemical properties of seminal plasma contained 9.8±0.9 $mmol \cdot L^{-1}$ potassium, 164.0±4.0 $mmol \cdot L^{-1}$ sodium, 151.0±1.2 $mmol \cdot L^{-1}$ chloride, 14.9±0.6 $mg \cdot dL^{-1}$ calcium, 7.2±0.1 $mg \cdot dL^{-1}$ magnesium, 1.0 $mg \cdot dL^{-1}$ glucose, 0.1 $g \cdot dL^{-1}$ total protein and 1.0 $mg \cdot dL^{-1}$ total lipid, and had an osmolality of 322.8±2.8 $mmol \cdot kg^{-1}$, and pH of 7.7±0.1. The sperm cryopreservation in this species was estimated during of experiments. Two kinds of artificial seminal plasma (ASP1, ASP2), 0.3 M glucose and marine fish Ringer's solution as diluent and dimethyl sulfoxide (DMSO) and methanol as cryoprotectant were used for sperm cryopreservation in flilefish.

Sperm was diluted at the ratio of 1 to 3 with extenders containing cryoprotectants with concentration of 5%, 10%, 15% and 20%, and frozen at liquid nitrogen after equilibration for 5 min at liquid nitrogen vapor. The highest motility (40.5±2.8%) and swimming speed (81.5±4.1 $\mu m \cdot s^{-1}$) in frozen/thawed sperm were observed when sperm was frozen in ASP1 diluent containing 10% DMSO and in ASP2 containing 15% DMSO, respectively. It was found that the ASP diluent containing DMSO cryoprotectant was better than the others in this study. Under the transmission electron microscope, the spermatozoon of filefish consisted of three distinct parts, head without acrosome, mid-piece with five mitochondria and flagellum with “9+2” pattern. The head of spermatozoon was horseshoe-shaped in longitudinal section with 1.3-1.6 μm long and 1.0-1.3 μm wide. There was a small alteration of shape in cryopreserved spermatozoa compared with fresh spermatozoa.

1. Introduction

The filefish (*Thamnaconus modestus* Günther, 1877) is a small member of the family Monacanthidae that inhabiting at the reef-associated in the seawater. This species was distributed in the Northwest Pacific Ocean: Hokkaido, Japan to the Ryukyu Islands, also known from the East to the South China seas (Masuda *et al.*, 1984). In addition, Park (1985) reported that the filefish was found in Korea during its spawning season from April to June in the southern areas and May to June in the eastern areas. However, the annual yield of filefish has decreased gradually. Hence, this species becomes an ideal for conservation and aquaculture.

The reproductive ability of males has been evaluated by milt properties in land animals. In fish, however, there has been a little information on milt properties to judge reproductive ability. There were several studies to describe milt properties which can influence quality of milt, such as sperm concentration, sperm motility and the composition of the seminal plasma (Hwang and Idler, 1969; Piironen and Hyvärinen, 1983). Studies on seminal plasma parameters are crucial for two reasons. First, it can be use for understanding the basic biochemical process occurring during the maturation of sperm in the male reproductive accessory organ (Miura and Miura, 2003), the spontaneous motility of sperm in the sperm duct (Cosson, 2004) and the initiation of sperm motility after release into the external environment (Cosson *et al*., 1999). Second, it also is useful for evaluation of the inter- and intra-specific aspects of reproductive ability (Alavi and Cosson, 2005; Alavi and Cosson, 2006). In addition, the knowledge of milt properties is also essential for making artificial seminal plasma that is a new diluent for fish sperm preservation.

The structure of spermatozoa, on the other hand, has been studied in 280 species (Mattei, 1991). Study of the structure of fish spermatozoa (spz) provides information for understanding their

reproductive biology, taxonomic and evolutionary relationships at family level (Jamieson, 1991; Mattei, 1988, 1991), as well as for optimizing artificial reproduction, preventing of polyspermy problems and development of cryopreservation techniques (Billard 1983; Billard *et al*., 1995). However, there is no information available on the milt properties and structure of spermatozoa in filefish *Thamnaconus modestus* until now.

Since Blaxter (1953) used frozen sperm to hybridize spring and fall spawning herring, fish sperm cryopreservation has been well established for many years and has been used in more than 200 fish species (Figiel and Tiersch, 1997; Tiersch, 2000). However, only a limited number of reports exist on successful cryopreservation of shellfish sperm (Gwo, 2000; Wayman and Tiersch, 2000; Chao and Liao, 2001). Until now, there is no data to define optimum sperm cryopreservation procedure in the case of filefish *Thamnaconus modestus*.

Sperm cryopreservation is considered as a valuable technique for the artificial reproduction and genetic improvement as well as for biological conservation programs (Chao and Liao, 2001). The profits of fish sperm preservation are determined as solving the unbalanced sex ratio and the asynchronous eggs and sperm discharge time between females and males during spawning season. Moreover, it has advantage in transportation of sperm, easy selective breeding or hybridization as well as preservation of decreasing indigenous species. Filefish sperm volume limits experimental replication and the numbers of treatments possible without pooling of samples. This hinders research, especially if many experimental variables are evaluated. The small sample volume necessitates identification of optimal sperm-to-egg ratios to maximize fertilization potential and places greater emphasis on increasing and maintaining sperm viability after thawing. Development of sperm cryopreservation techniques for filefish is hindered by its small sperm volumes.

The objectives of this research were (1) to determine milt properties and (2) to find out the best diluent and cryoprotectant for sperm long-term preservation of filefish *Thamnaconus modestus*. The success of each sperm cryopreservation method was evaluated by sperm motility parameters (swimming speed and motility) of frozen/thawed sperm.

2. Materials and methods

2.1. Fish and milt collection

All experiments were carried out at National Fisheries Research and Development Institute, Korea, during spawning season of filefish. For milt collection, five mature males with the total length and body weight were measured and the result was presented in Table 1. They were kept into a spawning tank (2 m^3) with flow-through seawater at a flow rate of 0.2 $L \cdot s^{-1}$ with aeration, and were fed once a day with the commercial feed (Ecolife 16 F N°4.5, Biomar Co., France) during experimental period. In addition, environmental conditions of rearing tank were determined and given in Table 2. The shape of filefish was illustrated in Fig. 1.

Table 1. Total length and body weight of male for milt collection in filefish

Number of fish	Body weight (g)	Total length (cm)
	Mean±SE	Mean±SE
5	321.0±9.0	28.6±1.4

Fig. 1. The morphological shape of filefish.

Spermiation was induced by single intramuscular injection of human chorionic gonadotropin (hCG) (Daesung Microbiological Lab. Co. Ltd., Korea) with dose of 100 IU·kg^{-1} fish weight. The males were anaesthetized in 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 kept in the ice (4^{o}C) until use. The quality of milt collected is subject to change with contaminations by feces and urine of fish or environmental water. The urinary bladder of fish was gently emptied and the genital area was wiped with paper towel before the milt was stripped by hand.

Table 2. Environmental conditions during experimental period

Factor	Range
Water temperature (^{o}C)	15.0~16.5
Salinity (psu)	32~33
pH	7~8
DO (ppm)	6~7

All experiments were summarized by a diagram and illustrated in Fig. 2.

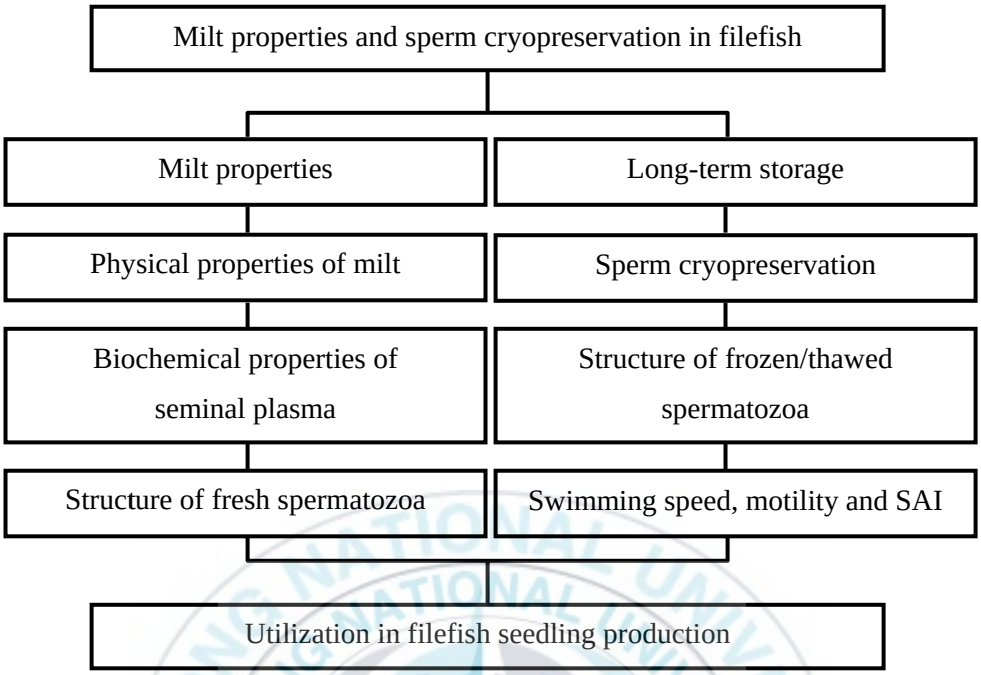

Fig. 2. Diagram of experiments in milt properties and sperm cryopreservation.

2.2. Evaluation of sperm activity

2.2.1. Motility, swimming speed and SAI of sperm

The motility of fresh sperm from each male was determined immediately after collection. The percentage of sperm exhibiting rapid, vigorous and forward movement was classified under a microscope by diluting the sperm into artificial seawater (ASW; NaCl 27 g, KCl 0.5 g, $CaCl_2$ 1.2 g, $MgCl_2$ 4.6 g, $NaHCO_3$ 0.5 g in liter of distilled water (DW)) at the ratio of 1 to 1000. Sperm samples with total motility more than 90% were pooled and used in this study.

In order to evaluate frozen/thawed sperm viability of each cryopreservation condition, motility, sperm activity index (SAI) and swimming speed of frozen/thawed sperm were

estimated. To measure motility and swimming speed of frozen/thawed sperm, prediluted sperm were diluted in ASW at the ratio of 1 to 50 (1 μL prediluted 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 X200 magnification under the microscope (Axioskop 2 plus Zeiss, Germany) setting video camera (Carl Zeiss, Germany) and video timer (VTG-55B, Germany) to connect with video recorder and player (Samsung VHS, SV-G1000, Korea). A sample was observed three times under the microscope and the time for one was 1.5 minutes. The frozen/thawed sperm heads were 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 the percentage of motile and immotile sperm. The degree of motility 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 3. The swimming speed of frozen/thawed sperm was determined by the distance of moving sperm for 1 second.

Table 3. 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

2.2.2. Physical properties of milt

The volume of the collected milt was measured in 1.5 mL Eppendorf tube. Spermatozoa concentration was counted with a hemocytometer chamber under a microscope (x400) after dilution with 2% eosin solution. Spermatocrit was determined after centrifugation at 15,000 rpm for 10 min in 75 mm capillary tubes (Bouck and Jacobson, 1976).

2.2.3. Biochemical properties of the seminal plasma

To analysis the biochemical properties of seminal plasma, 1 mL of milt was poured in the Eppendorf tube and put into centrifugal machine at 15,000 rpm for 10 minutes. The supernatants were frozen and stored in the refrigerator for 3 days until analysis. The biochemical components in seminal plasma were determined by VITROS DTII Chemistry System. The pH and osmolality of seminal plasma were determined by pH meter (istek, Korea) and omsometer (Wescor Inc., USA), respectively.

2.2.4. Structure of spermatozoa

The structure of fresh and cryopreserved spermatozoa was studied using transmission electron microscopy. The fresh milt or cryopreserved sperm were fixed for 3 hours in 2.5% glutaraldehyde solution in 0.1 M phosphate buffer solution (PBS, pH 7.2) at 4^{o}C, washed in the same buffer and immersed for 2 h in 1.0% osmium tetroxide (OsO_4) in the PBS at 20^{o}C . The samples were washed again with the PBS, then serially dehydrate with ethanol from 50% to 100% and embedded in Epon 812. Hardened blocks were sectioned at 0.5-0.7 μm-thick and sections mounted on copper grids. These were post-stained with 2% uranylacetate in 50% ethanol and lead citrate solution. Finally, the grids were examined and photographed using transmission electron microscope (TEM) (JEM-1230, Japan and JEOL 1010, JEOL Ltd., Japan).

2.3. Sperm cryopreservation

2.3.1. Procedure of sperm cryopreservation

For each experiment, collected milt was diluted at the ratio of 1 to 3 with ice-cold diluent containing cryoprotectant. The diluted milt was sucked 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. For thawing, the straws were immersed into the waterbath (30^{o}C) after 20 seconds and frozen/thawed sperm viability was immediately estimated. The procedure of experiments for sperm cryopreservation in filefish was illustrated in Fig. 3.

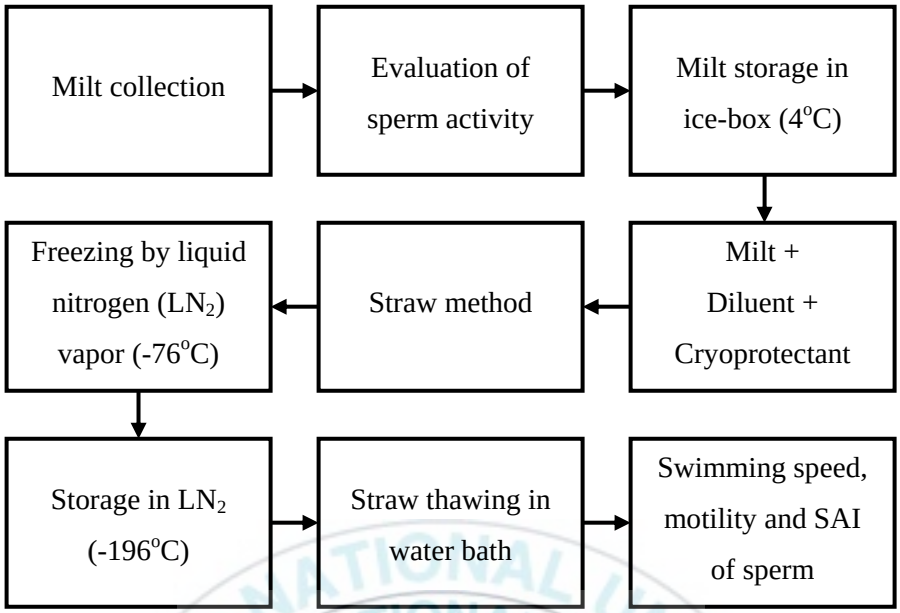

Fig. 3. Procedure of the experiments for sperm cryopreservation in filefish.

2.3.2. Sperm cryopreservation in different kinds of diluents and cryoprotectants

The sperm cryopreservation in filefish was estimated during of experiments. Two kinds of artificial seminal plasma (ASP1, ASP2), 0.3 M glucose and marine fish Ringer's solution (MFRS) as diluent and dimethyl sulfoxide (DMSO) and methanol as cryoprotectant were used for sperm cryopreservation in this species. The constituents of diluents were given in Table 4. The concentration of cryoprotectatnt was 5, 10, 15, and 20%. The diluted milt was frozen as above-mentioned method.

Table 4. Constituents of diluents used in the experiments

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 ($mmol \cdot kg^{-1}$)	335.0	337.2	444.0	389.0

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

2.4. Statistical analysis

Mean±standard error (SE) were calculated for each milt characteristic using triplicate samples for at least 5 males. Statistical evaluation was performed by one-way ANOVA in SPSS version 16.0. Means were separated using Tukey's multiple range test and differences were considered to be significant at $P<0.05$.

3. Results

3.1. Milt properties

Milt properties were divided into physical properties of milt and biochemical properties of the seminal plasma.

3.1.1. Physical properties of milt

The physical properties of filefish milt involved sperm volume, sperm concentration and spermatocrit. The values of them were presented in Table 5.

Table 5. Physical properties of milt in filefish

Property	Value Mean±SE
Sperm volume ($mL \cdot fish^{-1}$)	0.3±0.1
Sperm concentration (spz $x10^{7} \cdot mL^{-1}$)	2.6±0.1
Spermatocrit	73.3±6.7

3.1.2. Biochemical properties of the seminal plasma

The biochemical properties of seminal plasma contained potassium, sodium, chloride, calcium, magnesium, glucose, total protein, total lipid, pH and osmolality and the results of it were given in Table 6.

Table 6. Biochemical properties of seminal plasma

Component	Value Mean±SE
Potassium (mmol·L^{-1})	9.8±0.9
Sodium (mmol·L^{-1})	164.0±4.0
Chloride (mmol·L^{-1})	151.0±1.2
Calcium (mg·dL^{-1})	14.9±0.6
Magnesium (mg·dL^{-1})	7.2±0.1
Glucose (mg·dL^{-1})	1.0
Total protein (g·dL^{-1})	0.1
Total lipid (mg·dL^{-1})	1.0
pH	7.7±0.1
Osmolality (mmol·kg^{-1})	322.8±2.8

3.1.3. Structure of fresh spermatozoa

The spermatozoon of filefish consisted of three distinct parts: head without acrosome, mid-piece and flagellum. The structure of fresh spermatozoa was illustrated in Fig. 4.

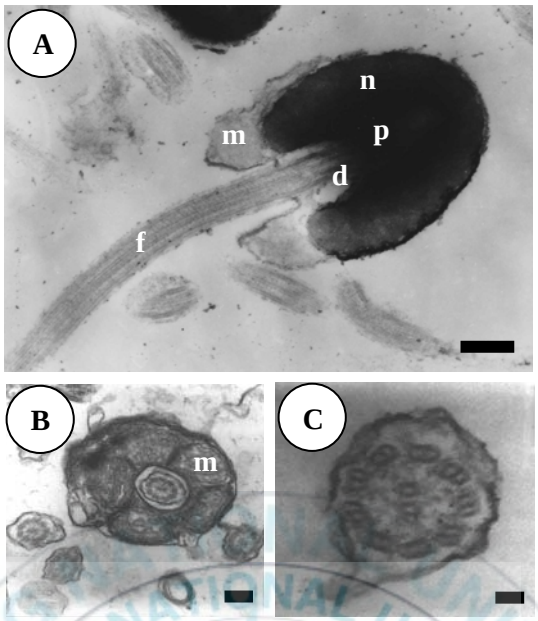

Fig. 4. Photographs of structure of spermatozoa in filefish by transmission electron microscope. A: longitudinal section of fresh spermatozoon. B: cross section of mid-piece with five mitochondria. C: cross section of flagellum with “9+2” pattern. d: distal centriole, f: flagellum, m: mitochondrion, n: nucleus, p: proximal centriole (bar= 0.2 μm).

Head: In the head, there was a round nucleus covered by a thin layer of cytoplasm. The nucleus, deeply penetrated by implantation fossa, is approximately horseshoe-shaped in longitudinal section (Fig. 4A). It is 1.3-1.6 μm long and 1.0-1.3 μm wide. The chromatin is electron dense and compact though with some indication of approximation of large masses between which several lacunae are usually present. The profile of the nucleus at its envelope has corresponding indentations but is otherwise smooth.

Mid-piece: A small number of large, sparsely cristate, and irregular mutually adpressed mitochondria is grouped in a single layer around the cytoplasm canal. In longitudinal section, the

cytoplasm collar continues as a short spur-like prolongation behind the mitochondria on each side. There were five round mitochondria surrounding the mid-piece sleeve (Fig. 4B). The nucleus has invagination, where the proximal centriole and distal centriole were located. The proximal centriole was perpendicular to the distal centriole which connected with flagellum.

Centrioles and flagellum: The two centrioles lie within the anterior half of the deep implantation fossa. The proximal centriole is unusual not only in being in the same axis as the basal body but also in having its longitudinal axis similarly oriented, it is near but not at the anterior limit of nuclear fossa. At least one, apparently the distal centriole consists of 9 triplets. The “9+2” flagellum has long lateral fins and the plane of which is slightly tilted relative to that of the two central singlets (Fig. 4C).

3.2. Sperm cryopreservation

3.2.1. Structure of cryopreserved spermatozoa

When milt of filefish was cryopreserved with appropriate condition where was observed the highest sperm motility. In this study, milt were diluted in ASP1 containing 10% DMSO, almost spermatozoa were had no visible ultrastructure changes after freeze-thaw compared with fresh spermatozoa. The morphological alteration of a small number of spermatozoa, however, were observed and illustrated in Fig. 5.

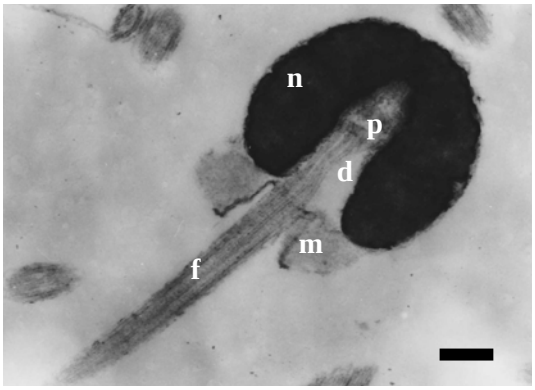

Fig. 5. Photographs of structure of cryopreserved spermatozoon in filefish by transmission electron microscope. d: distal centriole, f: flagellum, m: mitochondrion, n: nucleus, p: proximal centriole (bar = 0.2 μm).

3.2.2. Sperm cryopreservation in different kinds of diluents and cryoprotectants

Sperm cryopreservation in different kinds of diluents (ASP1, ASP2, glucose and MFRS) containing different kinds of cryoprotectants (DMSO and methanol) was estimated and the results were illustrated in Figs. 6, 7, 8 and 9.

In ASP1 diluent, the highest 40.5±2.8% motility of frozen/thawed sperm was obtained from 10% DMSO and no survival was observed in the 15% and 20% methanol. Although 10% DMSO resulted in high sperm motility, increasing the DMSO concentration to 15% and 20% reduced motility to 34.5±2.4% and 29.7±2.5%, respectively. In addition, the sperm were diluted in this diluent containing methanol with concentration of 5% to obtain 40.2±3.1% in motility but rising the concentration of methanol to 10%, 15% and 20% decreased motility to 26.2±2.1% and no survival, respectively. The highest 58.8±4.9 $\mu m \cdot s^{-1}$ swimming speed of frozen/thawed was observed in the 5% methanol and no swimming was obtained in the 15% and 20% methanol. The

SAI, in general, was in direct proportional to the motility and swimming speed. Although the motility in the 10% and 15% DMSO was different, the SAI was not different (Fig. 6).

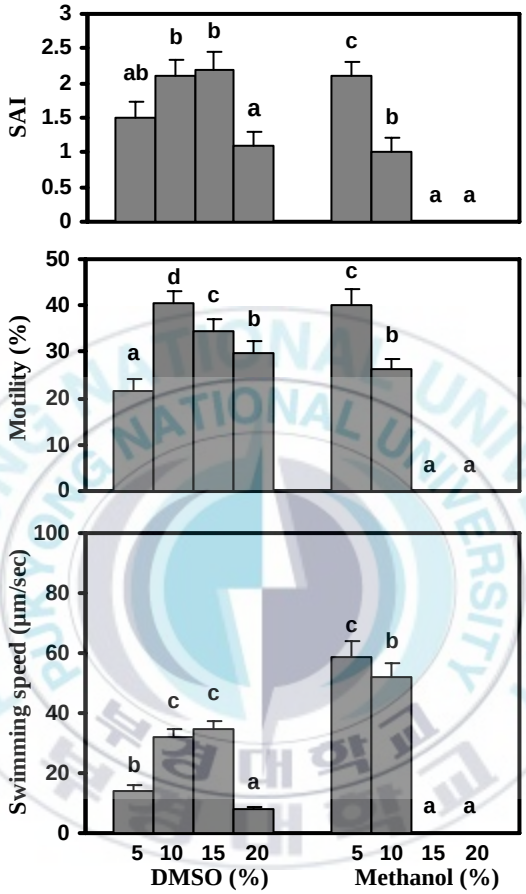

Fig. 6. Swimming speed, motility and sperm activity index (SAI) of frozen/thawed sperm in the first artificial seminal plasma (ASP1) diluent. Different letters indicate significant differences between different concentrations ($P<0.05$).

In Fig. 7, the result of frozen/thawed sperm motility was obtained the highest 34.9±3.1% in ASP2 containing 15% DMSO and the lowest 7.6±1.8% in this diluent containing 20% methanol. Similarly, minimum 12.1±1.8 μm·s^{-1} and maximum 81.5±4.1 μm·s^{-1} swimming speed of frozen/thawed sperm were observed in 20% methanol and 15% DMSO, respectively. The SAI, in this diluent, was directly proportional to the motility and swimming speed.

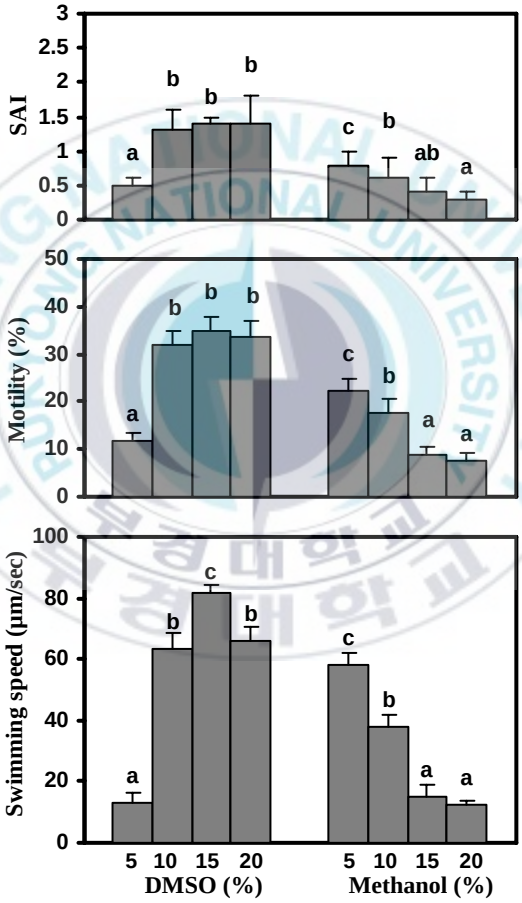

Fig. 7. Swimming speed, motility and sperm activity index (SAI) of frozen/thawed sperm in the second artificial seminal plasma (ASP2) diluent. Different letters indicate significant differences between different concentrations (P<0.05).

The highest 30.0±3.0% and no survival motility of frozen/thawed sperm were observed when sperm was cryopreserved in MFRS containing 20% DMSO and 5% methanol, respectively. There was a correlation between the swimming speed, the motility and the SAI of frozen/thawed sperm (Fig. 8).

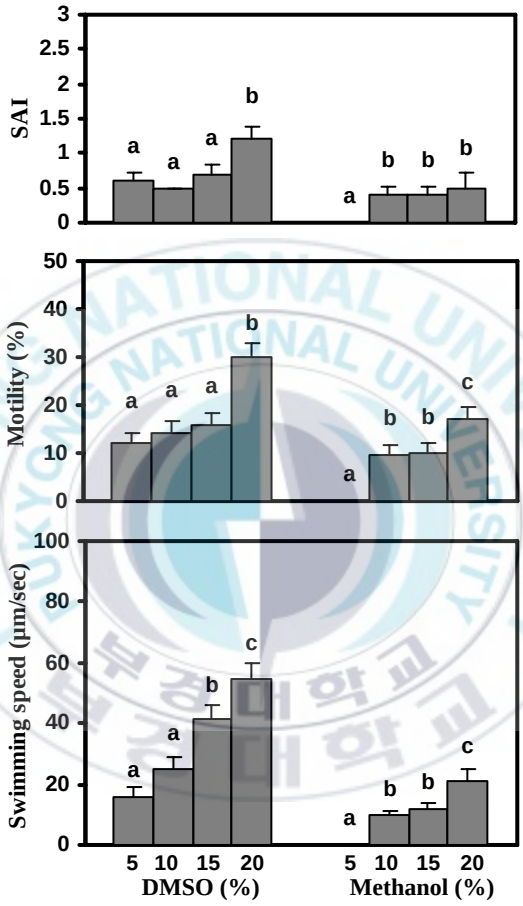

Fig. 8. Swimming speed, motility and sperm activity index (SAI) of frozen/thawed sperm in the marine fish Ringer's solution (MFRS) diluent. Different letters indicate significant differences between different concentrations ($P<0.05$).

The motility of frozen/thawed sperm was ranged from immotile and 34.9±3.6% in 0.3 M glucose containing (15% and 20%) methanol and 20% DMSO, respectively. In addition, the swimming speed and the SAI of frozen/thawed sperm were similarly observed as motility (Fig. 9).

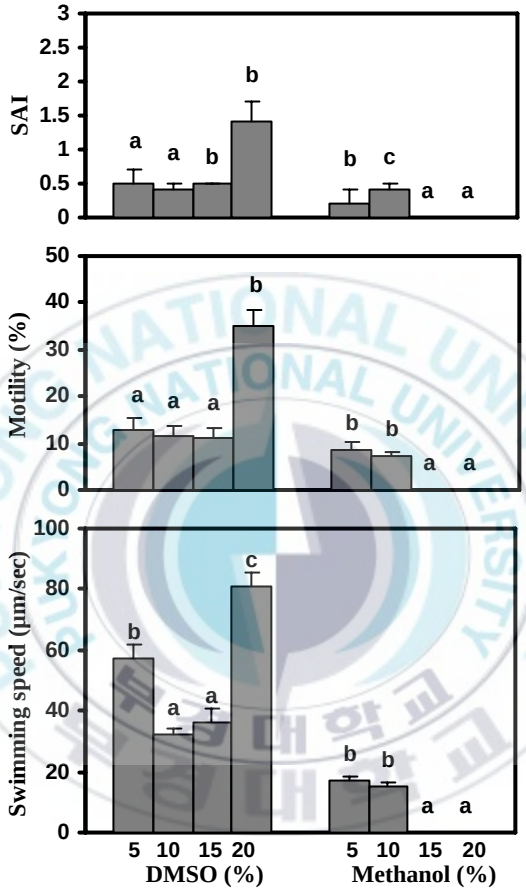

Fig. 9. Swimming speed, motility and sperm activity (SAI) of frozen/thawed sperm in 0.3 M glucose diluent. Different letters indicate significant differences between different concentrations ($P<0.05$).

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

4. Discussion

The volume of collected milt per filefish during spawning season was lower than that 1.97 mL·fish^{-1} from black porgy *Acanthopagrus schlegeli* (Chang *et al*., 1995). The present study also showed that the filefish produce sperm with a very low sperm concentration compared with other fish species. For flatfishes, marbled sole *Limanda yokohamae* 3.6±1.4x10^{10} spz·mL^{-1}, brown sole *L. herzensteini* 1.5±0.6x10^{10} spz·mL^{-1}, starry flounder *Platichthys stellatus* 0.9±0.3x10^{10} spz·mL^{-1}, olive flounder *Paralichthys olivaceus* 1.6± 0.6x10^{10} spz·mL^{-1} (Chang and Chang, 2002), for grey mullet *Mugil cephalus* 1.1±0.4x10^{10} spz·mL^{-1} (Chang *et al*., 1999b), for black porgy 2.3±1.3x10^{10} spz·mL^{-1} (Chang *et al*., 1995), for river puffer *Takifugu obscurus* 1.1±0.3x10^{10} spz·mL^{-1} (Chang *et al*., 1999d), for bream *Abramis brama* 11.7±4.3x10^{9} spz·mL^{-1} (Glogowski *et al*., 1999) and for captive *Brycon siebenthalae* 13.9±4.0x10^{9} spz·mL^{-1} (Cruz-Casallas *et al*., 2005) have been reported. Spermatocrit of filefish in the present study was higher than that of brown sole 63.2±16.9, starry flounder 51.6±15.6, olive flounder 60.2±16.6 (Chang and Chang, 2002) and captive 41.5±10.8 (Cruz-Casallas *et al*., 2005), respectively. But it was lower than in black porgy 97.4±2.1 (Lim and Chang, 1996), grey mullet 96.7±2.6 (Chang *et al*., 1999b), marbled sole 91.8±7.4 (Chang and Chang, 2002). These differences could be related to many factors, such as the age and weight of male fish (Suquet *et al*., 1994; 1998; Chang, 1997), ecology and spawning behavior of broodstock (Piironen and Hyvärinen, 1983) and sampling period and method (Suquet *et al*., 1994).

Sodium, potassium, chloride and calcium are believed to exert their effects on the sperm by maintaining their osmotic balances. The concentration of potassium of filefish was higher than 4.0±1.0 mmol·L^{-1} in marbled sole and 7.5±3.2 mmol·L^{-1} in starry flounder. But it was lower than 10.8±4.1 mmol·L^{-1} in brown sole and 20.8±9.0 mmol·L^{-1} in olive flounder (Chang and Chang, 2002). In

marine fish, potassium has no inhibitory effects on sperm motility in flounder *Platichtys flesus* and summer whiting *Sillago ciliate* (Goodall *et al*., 1989), while the potassium may conduct the motility in freshwater fish (Billard and Cosson, 1992). Several studies showed significant correlations between the mineral content and the osmolality of the seminal plasma (see review by Alavi and Cosson, 2006). Furthermore, Piironen (1985) concluded that the close correlation between the concentration of calcium and magnesium, and the sperm concentration points to a regulatory role for the ionic fractions of these minerals during spermiation of Atlantic salmon.

The glucose concentration showed that it was a very low compared with four flatfish species (Chang and Chang, 2002). In addition, it was lower than those of bleak *Alburnus alburnus* (2.2 $mg \cdot dL^{-1}$), chub *Leuciscus cephalus* (8.9 $mg \cdot dL^{-1}$), vimba *Vimba vimba* (6.1 $mg \cdot dL^{-1}$) (Lahnsteiner *et al*., 1994). Lahnsteiner *et al*. (1994) reported that spermatozoa of cyprinid fishes were restricted to glucose for energy resource. Thus, the value of the glucose concentration may supply important energy resource for sperm motility and survival in filefish. Fructose has been known to be the primary source of energy in the milt of mammals (Kruger *et al*., 1984). So, it is necessary to study about utilizing fructose as resource of energy in filefish. The protein concentration in the present study was lower than that (0.6 ± 0.1 $g \cdot dL^{-1}$) reported for jundiá *Rhamdia quelen* (Borges *et al*., 2005). In addition, the protein content is significantly different from that of non-salmonid fish (Piironen and Hyvärinen, 1983; Lahnsteiner *et al*., 1995, 1996), which points to species-specific differences that may reflect differences in spermatozoa metabolism between fish species (Lahnsteiner *et al*., 1998) Although the origin and functions of proteins in fish seminal plasma are not completely known, this may indicate that part of the proteins present in seminal plasma originates from disrupted spermatozoa (Kowalski *et al*., 2003). On the other hand, Lahnsteiner *et al*. (2004)

reported that some proteins of seminal plasma were shown to have a key role in the motility of sperm cells.

The pH value of seminal plasma in filefish was higher than 7.3±0.1 in black porgy (Chang *et al*., 1995), but lower than 7.8±0.2 in Asian catfish *Clarias macrocephalus* (Tan-Fermin *et al*., 1999), 7.7±0.3 in marbled sole, 8.1±0.3 in brown sole, 7.6±0.4 in starry flounder, 7.9±0.2 in olive flounder (Chang and Chang, 2002), 8.7±0.1 in jundiá (Borges *et al*., 2005), 7.8±0.1 in grey mullet (Chang *et al*., 1999b). Wang and Crim (1997) reported that pH of seminal plasma in the beginning of the spermiation period was lower than in the middle and near the end of spawning season in ocean pout *Macrozoarces americanus*. In addition, pH of seminal plasma exhibited the best motility. Thus, it is likely that the pH of seminal plasma in filefish used in this study may have some conditions which maintain highly spermatozoa viability during spermiation period. In contrast to pH, the osmolality of seminal plasma was higher in filefish than in Cyprinidae. It is worth remarking that the osmolality was higher than in marine than in freshwater fish seminal plasma (Alavi and Cosson, 2006). The lower osmolality of seminal plasma might be caused partly by higher hydration in the testis (Piironen, 1985) because of the hypoosomolality in fresh water. It is clear that sperm motility is included by hypo- and hyper-osmotic pressure in freshwater and marine fishes, respectively (Billard *et al*., 1993, Suquet *et al*., 1994).

The high variance of frozen/thawed sperm quality has been reported in different fish species. 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 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 (Chao et al., 1987; Gwo et al., 1991; Chen et al., 1993; Palmer et al., 1993). In the present study, four extenders (ASP1, ASP2, MFRS and 0.3 M glucose) were examined for sperm cryopreservation in filefish. 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 (Chao et al., 1975; Chang et al., 1999a) and river puffer (Chang et al., 1999c), the MFRS was found to be good diluent. The ASP, in the case of the filefish, was good diluent. The motility and swimming speed 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 (Alderson and MacNeil, 1984; Bergeron et al., 2002; Lahnsteiner et al., 1997; McNiven et al., 1993; Yao et al., 2000; Young et al., 1992; Glogowski 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 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 sperm motility of 40.5±2.8% was achieved with sperm frozen/thawed 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.

Filefish spermatozoa were very similar to spermatozoa in other teleost fishes in their structure. Absence of an acrosome in the filefish spermatozoa is clearly and its presence in sturgeon *Acipenser stellatus.* The head of filefish spermatozoa was small (1-1.3 μm) but other teleostean fishes spermatozoa (2-4 μm). Different shapes of spermatozoa head occur in teleostean fishes with external fertilization: ball-shaped spermatozoa head in northern pike *Esox lucius*; big spermatozoa one in silver carp *Hypophtalmichthys molitrix*; ovoid-shaped one in cardinal fish *Apgon imberbis*; kidney-like one in Mediterranean rainbow wrasse *Coris julis*; banana-shaped one in Atlantic eel *Anguilla anguilla* (Jamieson, 1991). In this study, it was known that the filefish spermatozoa head was horseshoe-shaped one. Chang and Chang (2002) reported that cross section of mid-piece had eight mitochondria in marbled sole, seven mitochondria in brown sole and starry flounder, and six mitochondria in olive flounder. It was revealed that cross section of mid-piece of spermatozoa in filefish had five mitochondria. In addition, cross section of flagellum in filefish spermatozoa had “9+2” pattern. This result was the same with four flatfish species (Chang and Chang, 2002) and other fish species but it was different compared “9+0” pattern in spermatozoa of Atlantic eel (Jamiesion, 1991).

After freezing and thawing, there was a small alteration of spermatozoa shape compared with fresh spermatozoa in this research. The changes might adversely affect the function of mitochondria and tails, thus reducing the sperm flagellate movement. Similar changes have also been reported in

frozen/thawed sperm of the Atlantic croaker *Micropogonias undulatus* (Gwo and Armold, 1992), the founder *Paralichthys olivaceus* (Zhang et al., 2003), the grayling *Thymallus thymallus* (Lahnsteiner et al., 1992) and ocean pout *Macrozoarces americanus* (Yao et al., 2000). Milt properties and sperm cryopreservation appear to be useful and reliable technique for conservation of gene resources in the filefish. The procedure according the present study was shown in Fig. 10.

Fig. 10. Procedure of milt properties and sperm cryopreservation in filefish.

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