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Milt Properties and Spermatozoa Structure of Filefish(*Thamnaconus modestus*)

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말쥐치(*Thamnaconus modestus*) 정액의 특성과 정자의 미세구조

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ABSTRACT : The milt properties of filefish(*Thamnaconus modestus*) included physical properties of sperm and biochemical properties of seminal plasma. The physical properties of milt were 0.3±0.1 mL · $fish^{-1}$ in sperm volume, 2.6±0.1×10^7 spermatozoa · mL^{-1} in sperm concentration and 73.3±6.7 in spermatocrit. The biochemical properties of seminal plasma contained 9.8±0.9 mmol · L^{-1} potassium, 164.0±4.0 mmol · L^{-1} sodium, 151.0±1.2 mmol · L^{-1} chloride, 14.9±0.6 mg · dL^{-1} calcium, 7.2±0.1 mg · dL^{-1} magnesium, 1.0 mg · dL^{-1} glucose, 0.1 g · dL^{-1} total protein and 1.0 mg · dL^{-1} total lipid. The osmolality and pH of seminal plasma were 322.8±2.8 mOsmol · kg^{-1} and 7.7±0.1, respectively. The spermatozoon of filefish consisted of three parts: head without acrosome, mid-piece with five mitochondria and flagellum with "9+2" pattern. The head of spermatozoon in longitudinal section was horseshoe-shaped, and 1.3~1.6 μm long and 1.0~1.3 μm wide.

Key words : Filefish, *Thamnaconus modestus*, Milt properties, Seminal plasma, Spermatozoa, Structure.

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±0.1×10^7 spermatozoa · mL^{-1}, 73.3±6.7였다. 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 mOsmol · kg^{-1}, 7.7±0.1였다. 투과형 전자현미경으로 미세구조를 관찰한 결과, 정자는 첨체가 없는 머리, 5개의 미토콘드리아로 이루어진 중편부 및 "9+2"의 미세소관 편모를 가진 꼬리로 구성되어 있었다. 세로로 절단한 정자 머리는 장경 1.3~1.6 μm, 단경 1.0~1.3 μm로 말편자 모양을 하고 있었다.

INTRODUCTION

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). Study on seminal plasma parameters is 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

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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 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 polyspermy and cryopreservation techniques(Billard 1983; Billard et al., 1995). However, there is little information available on milt properties and structure of spermatozoa in filefish *Thamnaconus modestus* until now. Therefore, the objectives of this research were (1) to determine milt properties and (2) to find out the structure of spermatozoa in filefish.

MATERIALS AND METHODS

1. Fish and Milt Collection

All experiments were carried out at the National Fisheries Research and Development Institute, Korea, during the spawning season of filefish. For milt collection, five mature males(TL=28.6±1.4 cm, BW=321.0±9.0 g) were used in this study. They were kept into a spawning tank(2 m^3) with flow-through seawater(32 psu) at a flow rate of 0.2 $L \cdot s^{-1}$ with water temperature of 15.0～16.5℃ and with aeration, and were fed once a day with the commercial feed(Ecolife 16 F N°4.5, Biomar Co., France) during experimental period.

Spermiation was induced by single intramuscular injection of human chorionic gonadotropin(hCG)(Daesung Microbiological Lab. Co. Ltd., Korea) with dose of 100 $IU \cdot kg^{-1}$ 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℃) 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.

2. Milt Properties

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 (×400) 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).

To analyze biochemical properties in 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 Fuji Dri-Chem 3500 (Fujifilm Co. Ltd, Japan). The pH and osmolality of seminal plasma were determined by pH meter(Istek, Korea) and omsometer(Wescor Inc., USA), respectively.

Mean±standard error(SE) was calculated for each milt characteristic using triplicate samples for at least 5 males.

3. Structure of Spermatozoa

The structure of spermatozoa was studied using transmission electron microscopy. The fresh milt was fixed for 3 hours in 2.5% glutaraldehyde solution in 0.1 M phosphate buffer solution(PBS, pH 7.2) at 4℃, washed in the same buffer and immersed for 2 h in 1.0% osmium tetroxide(OsO_4) in the PBS at 20℃. 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 the 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(JEM-1230,

Japan and JEOL 1010, JEOL Ltd., Japan).

RESULTS

1. Milt Properties

Milt properties were divided into physical properties of milt and biochemical properties of the seminal plasma. The physical properties of milt were presented in Table 1.

The biochemical properties of the seminal plasma were given in Table 2. The biochemical properties of seminal plasma contained potassium, sodium, chloride, calcium, magnesium, glucose, total protein, total lipid, pH and osmolality.

2. Structure of Spermatozoa

The spermatozoa of filefish consisted of three distinct parts: head without acrosome, mid-piece and flagellum. The fresh spermatozoa morphology was illustrated in Fig. 1.

Table 1. Physical properties in milt of filefish

Property	Value
Milt volume(mL · fish^{-1})	0.3±0.1
Sperm concentration(spz×10^{7} · mL^{-1})	2.6±0.1
Spermatocrit	73.3±6.7

Table 2. Biochemical properties in seminal plasma of filefish

Component	Value
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(mOsmol · kg^{-1})	322.8±2.8

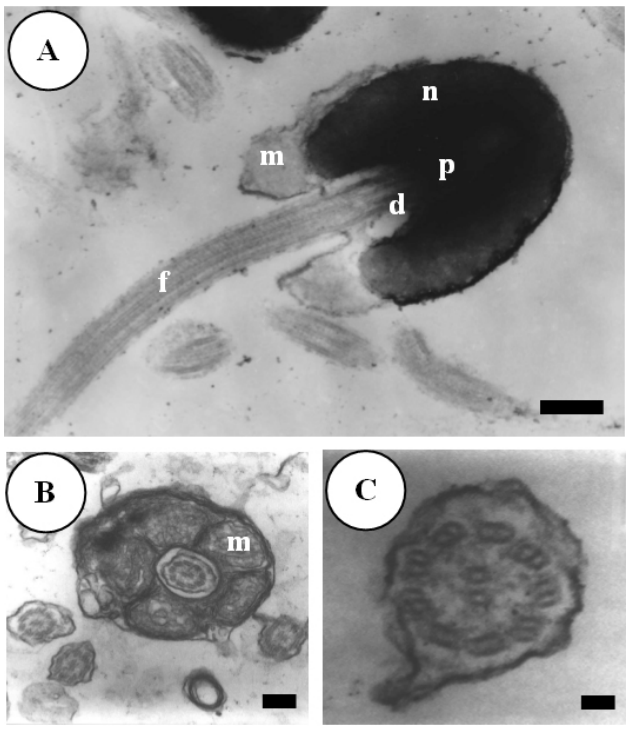

Fig. 1. Photographs of structure of spermatozoa in filefish by transmission electron microscope. A: longitudinal section of fresh spermatozoon. B: cross section of mid-piece with five mitochondria. C: cross section of flagellum with "9+2" pattern. d: distal centriole, f: flagellum, m: mitochondrion, n: nucleus, p: proximal centriole(bar=0.2 μm).

1) 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. 1A). 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.

2) 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. 1B). 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.

3) 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. 1C).

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. The concentration was lower than flatfishes, marbled sole *Limanda yokohamae* 3.6±1.4×10^{10} spz · mL^{-1}, brown sole *L. herzensteini* 1.5±0.6×10^{10} spz · mL^{-1}, starry flounder *Platichthys stellatus* 0.9±0.3×10^{10} spz · mL^{-1}, olive flounder *Paralichthys olivaceus* 1.6±0.6×10^{10} spz · mL^{-1}(Chang and Chang, 2002). It also was lower than grey mullet *Mugil cephalus*, black porgy, river puffer *Takifugu obscurus*, bream *Abramis brama* and captive *Brycon siebenthala*: 1.1±0.4×10^{10} spz · mL^{-1}(Chang et al., 1999a), 2.3±1.3×10^{10} spz · mL^{-1}(Chang et al., 1995), 1.1±0.3×10^{10} spz · mL^{-1}(Chang et al., 1999b), 11.7± 4.3×10^{9} spz · mL^{-1}(Glogowski et al., 1999) and 13.9± 4.0×10^{9} spz · mL^{-1}(Cruz-Casallas et al., 2005), respectively. Spermatocrit of filefish was higher than those 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 the spermatocrit was lower than in black porgy 97.4±2.1(Lim and Chang, 1996), grey mullet 96.7±2.6(Chang et al., 1999a), 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 *Platichthys 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 *Salmo salar*.

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 · dL^{-1}, chub *Leuciscus cephalus* 8.9 mg · dL^{-1}, vimba *Vimba vimba* 6.1 mg · 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., 1999a). 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 spawning 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 hypo-osomolality 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).

Filefish spermatozoa were very similar to spermatozoa in other teleost fishes in their structure. Absence of an acrosome in the filefish spermatozoa was clear compared with 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*; 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). Nevertheless, it was known that the spermatozoon head of filefish was horseshoe-shaped one in this study. Chang and Chang(2002) reported that the 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. On the contrary, it was revealed that the cross section of mid-piece of spermatozoa in filefish had five mitochondria. In addition, the 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. However, the flagellum in spermatozoa of Atlantic eel had “9+0” pattern(Jamiesion, 1991).

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