Applied Microscopy
Korean Society of Microscopy

pISSN 2287-5123 · eISSN 2287-4445
http://dx.doi.org/10.9729/AM.2014.44.1.1

Spermatogenesis of Siamese Fighting Fish, *Betta splendens*, Osphronemidae, Teleostei

Sung Ha Lim, Yeong Kyeong Koh, Byung Soo Chang[1], Dong Heui Kim*

Department of Environmental Medical Biology, Yonsei University Wonju College of Medicine, Wonju 220-701, Korea
[1]Department of Cosmetology, Hanseo University, Seosan 356-706, Korea

*Correspondence to:
Kim DH,
Tel: +82-33-741-0332
Fax: +82-33-732-4446
E-mail: fish7963@yonsei.ac.kr

Received February 28, 2014
Revised March 11, 2014
Accepted March 11, 2014

The spermatogenesis of Siamese fighting fish, *Betta splendens*, belongs to Osphronemidae was investigated by light and electron microscopic observations. In primary spermatocyte stage, the nucleus was comparatively large ellipsoidal, and mitochondria showed a marked development in cytoplasm. In secondary spermatocyte stage, the germ cells were smaller than that of primary spermatocytes. The nucleus was a spherical shape and intercellular space was formed between germ cells. In spermatid stage, the early spermatids were not much different from a secondary spermatocyte. But, the chromatin condensation was occurred from the outside to the inside. The nucleus was more condensed. Intracellular space was larger than early spermatid. The mitochondria were rearranged in a middle piece, and occupied about half of the head part in early sperm. In sperm stage, the head of mature sperm was a spherical shape and had no acrosome. The flagellum was showed the typical 9+2 array of microtubules. Also, the tail of sperm had no lateral fins and outer coarse fibers. These ultrastructural characteristics can be used in classification of species.

Key Words: *Betta splendens*, Spermatogenesis, Ultrastructure, Siamese fighting fish

INTRODUCTION

Taxonomically, *Betta splendens*, which is generally known as Siamese fighting fish belongs to the Osphronemidae. The common name 'fighting fish' came from their specific behaviors. When two males encounter each other in the same living place, typical pattern of aggressive behavior is occurred (Figler, 1972; Lobb & McCain, 1976). The labyrinth organ, which is situated on either side of the head in the gill cavity directly above the gills, allows *Betta splendens* to have ability to breathe atmospheric air (Bailey & Sandford, 1998).

Generally, spermatogenesis of teleost varies depending on species and inhabited environments. The spermatogenesis of fish has been studied in some species, *Liza aurata* (Bruslé, 1981), *Salmo gairdnen* (Billard, 1984), *Acanthopagrus schlegeli* (Gwo & Gwo, 1993), *Boleophthalmus pectinirostris* (Chung, 2008), Atlantic cod, *Gadus morhua L.* (Rebours & Ottesen, 2013) and *Epinephelus bruneus* (Kim et al., 2013).

There are various studies about *Betta splendens* in relation to their aggressive behavioral responses (Thompson & Strum, 1965), intrasexual communications (Doutrelant et al., 2001), sex reversal (Pandian & Sheela, 1995), and development (Mabee & Trendler, 1996). Also, there is a study focusing on changes of testis followed by aging under light microscope (LM) (Woodhead, 1974). But there are few studies and limited information about spermatogenesis in ultrastructural approach of *Betta splendens*. Moreover, to increase understandings about hormonal effects regarding to their patterns of behaviors (Kirankumar & Pandian, 2002), it is necessary to investigate spermatogenesis of *Betta splendens*. Especially, in contrast to sperms of genus *salmo* and genus *Oncorhynchus* (Alfert, 1956) which spawn demersal eggs, settled under the water, sperms of *Betta splendens* spawn pelagic eggs which float on the water. The differences between each sperm spawning demersal and pelagic eggs, respectively, are expected regarding to spermatogenesis and

ultrastructures of sperm including head shape of the sperm, arrangement of mitochondria in the middle piece, cross section of flagellum, and existences of acrosome and lateral fin. Therefore, the spermatogenesis of *Betta splendens*, belongs to Osphronemidae were investigated by light and electron microscopic observations.

MATERIALS AND METHODS

Fish Keeping

The Siamese fighting fish, *Betta splendens* males used in this study were purchased from SanHo Aquarium (Wonju, Korea). They were separated and single fish were placed in 300 mL beaker within a glass aquarium (50×40×40 cm). The tap water used for keeping was treated with Fritz-guard (Fritz Co., Ltd., USA) to remove chlorine, and its temperature and pH were maintained at 28°C±1°C and 7.0±0.5, respectively. Biological filtration was performed using a sponge filter (Brilliant Sponge Filter; Tetra Co., Ltd., Germany), and excrement settled to the bottom of the beaker was eliminated by exchange one-quarter of the water each day. An artificial light was illuminated for ten hours per day to simulate a daytime environment, and frozen bloodworms (Blood Worms; Hikari Sales USA Inc., USA) was provided as food two times per day, at 9 a.m. and 5 p.m.

Light Microscopy

For LM observations, testes were fixed in 10% neutral buffered formaline for 24 hours at 4°C. After washing, the specimens were dehydrated in increasing concentrations of ethanol, cleared in xylene, and embedded in paraplast (Media paraplast embed; Polysciences Inc., USA). The paraffin block were cut by microtome (Reichert UltraCut E; Reichert-Jung, USA), stained with H&E and observed through a LM (Olympus CH2; Olympus Corp., Japan).

Electron Microscopy

For transmission electron microscope (TEM) observation, testis was fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 2 hours at 4°C. After prefixation, the specimens were washed twice in the same buffer solution and then postfixed in 1% osmium tetroxide solution in 0.1 mol/L phosphate buffer solution (pH 7.4) for 2 hours at room temperature. Specimens were dehydrated in increasing concentrations of ethanol, cleared in propylene oxide, and embedded in an Epon mixture (PolyBed 812 kit; Polysciences Inc., USA). Ultrathin sections of Epon-embedded specimens were taken with an Ultracut E (Reichert-Jung, Austria) ultramicrotome at a thickness of about 60~70 nm. Tissue sections were mounted onto collodion-coated copper grids, double stained with uranyl acetate followed by lead citrate, and observed with a TEM (JEM 1200EX-II; JEOL, Japan) at 80 kV.

RESULTS

Structure and Morphology of the Testis

A pair of deltoid testis of Siamese fighting fish, *Betta splendens*, with its white color, was located between air bladder and intestine. The size of testis was major axis 2.16 mm,

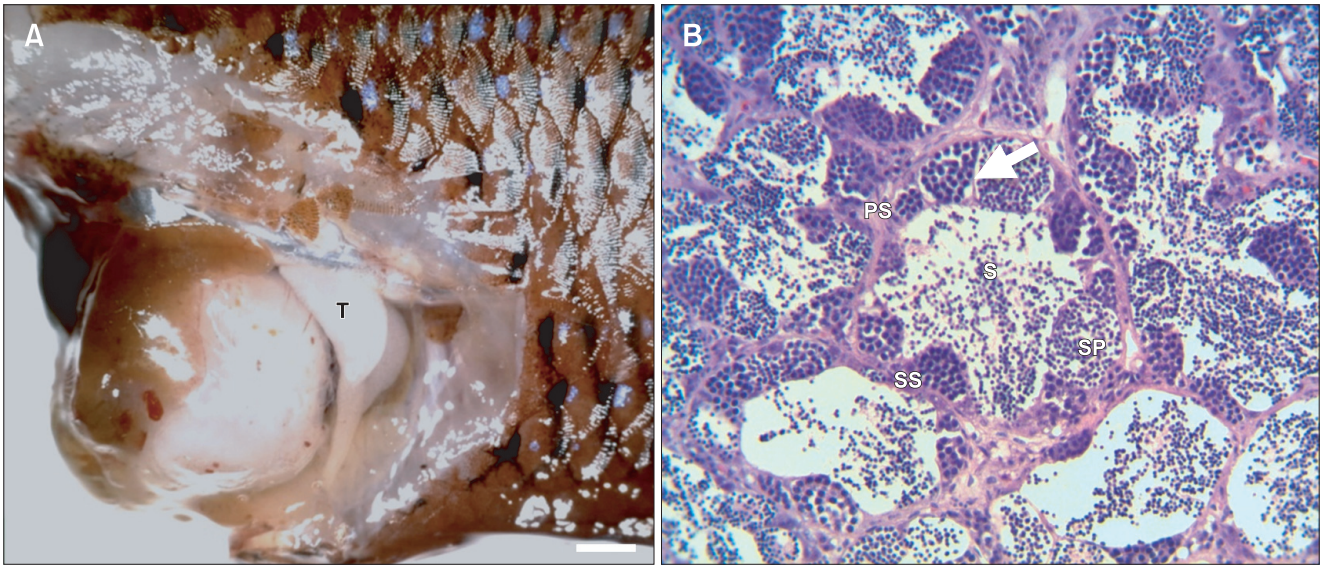

Fig. 1. The light micrograph of testis in *Betta splendens*. (A) The testis (T) was located between air bladder and intestine (scale bar=1 mm). (B) Testis section with H&E stain (×400). Various germ cells were observed in the testicular cyst (arrow). PS, primary spermatocyte; S, sperm; SS, secondary spermatocyte; SP, spermatid.

minor axis 1.11 mm (Fig. 1A). The testis of *Betta splendens* contained numerous testicular cysts, and spermatogenesis was synchronized in these testicular cysts. The size of nucleus had a tendency to getting smaller according to their development and the mature sperms were scattered in testicular cyst. The tail of sperm was filamentous and pink under LM (Fig. 1B).

Electron Microscopic Observations of Spermatogenesis

The spermatogenesis occurs in the testicular cyst, and can be classified into four stages: (1) primary spermatocyte, (2) secondary spermatocyte, (3) spermatid, and (4) sperm stages.

In primary spermatocyte stage, one or two primary spermatocytes were appeared in testicular cyst. The nucleus was comparatively large ellipsoidal, and mitochondria showed a marked development in cytoplasm (Fig. 2A). The primary spermatocytes in the pachytene stage of prophase and Sertoli cells were found in testis. The size of primary spermatocytes was smaller according to their development (Fig. 2B). The Leydig cells were located between testicular cysts, and smooth endoplasmic reticulum, Golgi complex, and mitochondria were well-developed (Fig. 2C). In secondary spermatocyte stage, the secondary spermatocytes were smaller than that of primary spermatocytes. The nucleus was a spherical shape and intercellular space was formed between germ cells (Fig. 2D).

In spermatid stage, the early spermatids were not much different from a secondary spermatocyte. But, germ cells got smaller and the chromatin of spermatid was highly condensed according to their development. The chromatin condensation was occurred from the outside to the inside. In some spermatids, the flagella were started to be formed or already formed. The mitochondria were moved to tail (Fig. 3A). Developed as a result, the nucleus of spermatid was leaned to one side. The nucleus was more condensed.

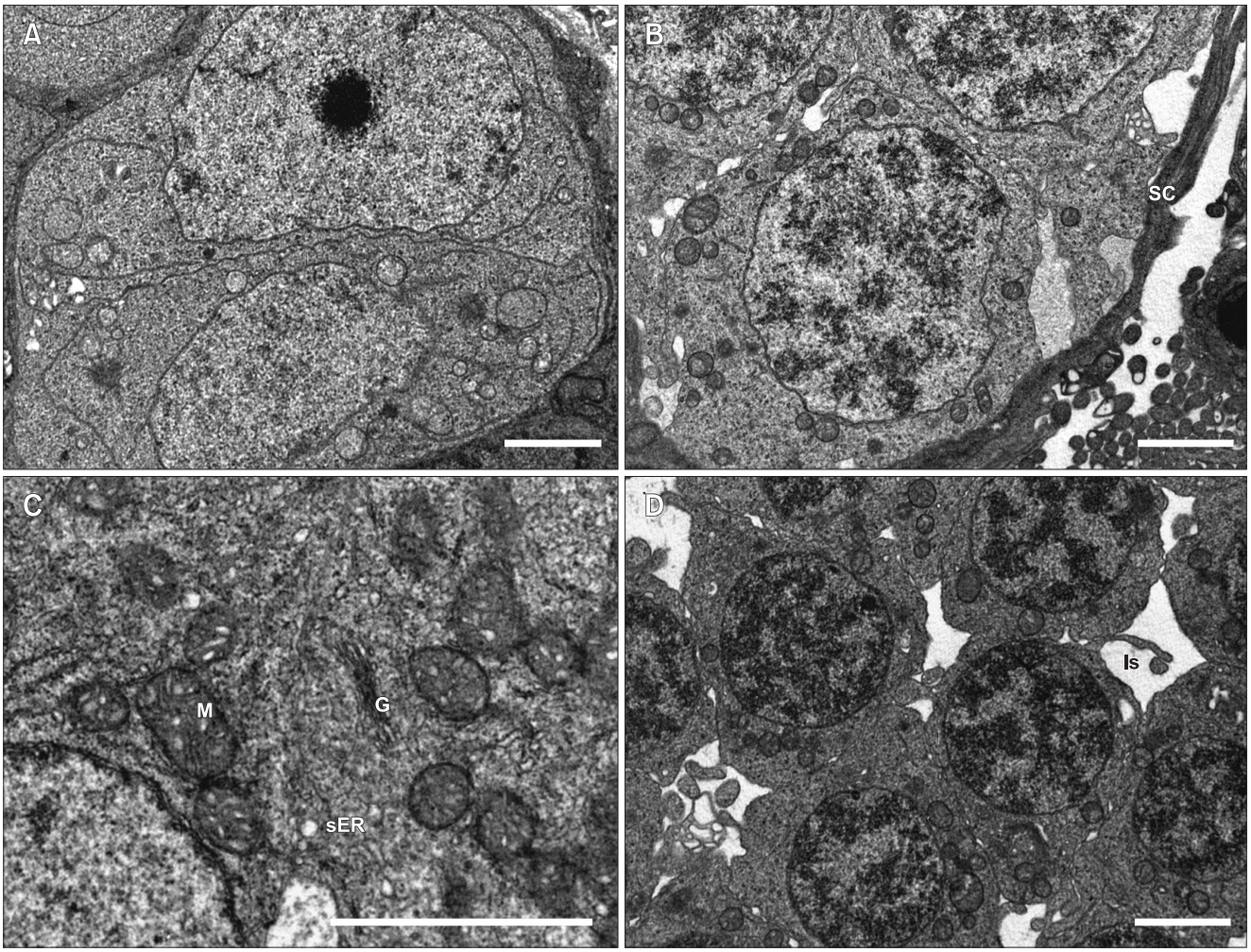

Fig. 2. The transmission electron micrograph of spermatocytes (scale bars=2 μm). (A) The primary spermatocytes. (B) The Sertoli cell (SC) and the primary spermatocytes in the pachytene stage of prophase. (C) Leydig cell. (D) Secondary spermatocytes. M, mitochondria; sER, smooth endoplasmic reticulum; G, Golgi complex; Is, intercellular space.

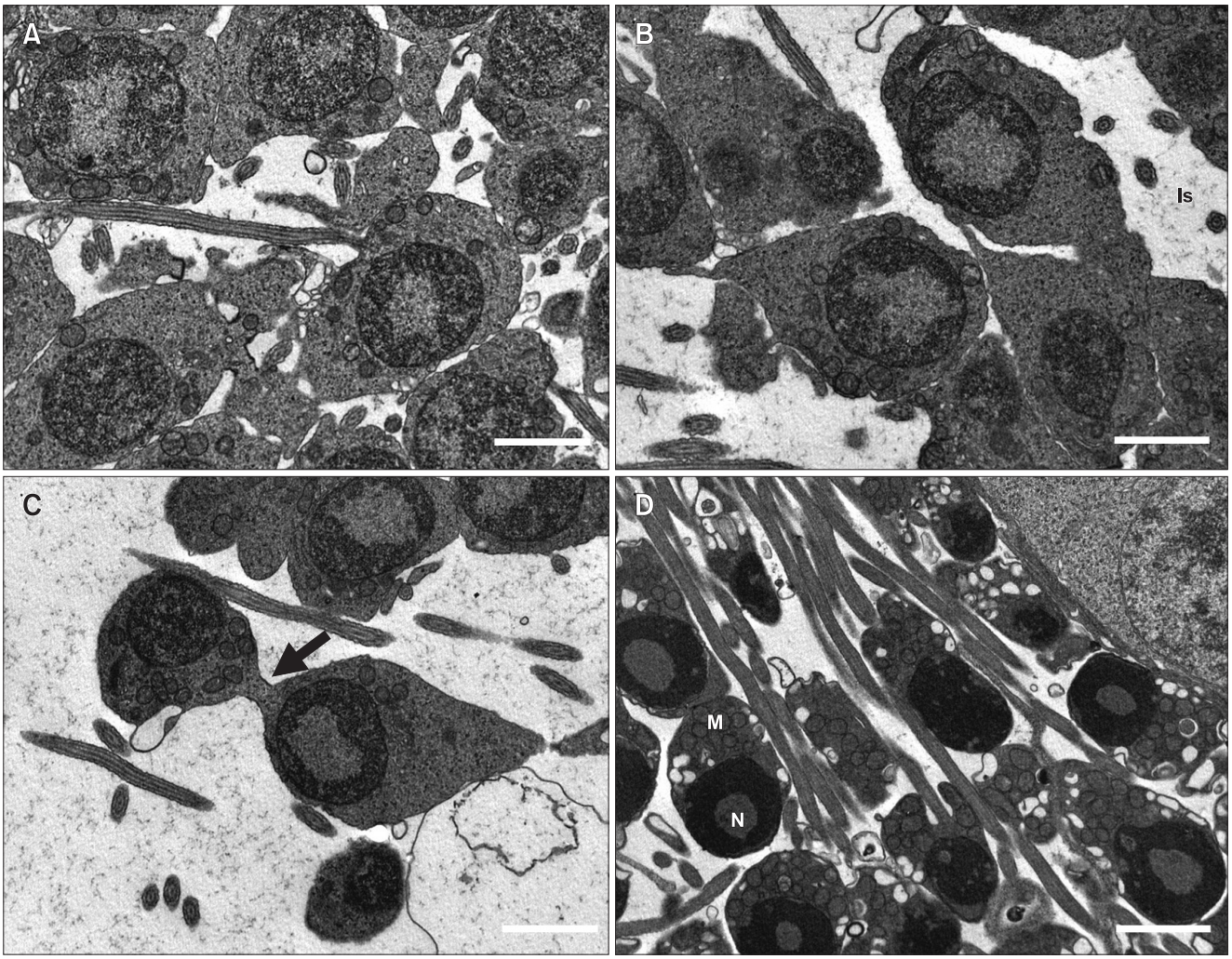

Fig. 3. The transmission electron micrograph of spermatids (scale bars=2 μm). (A) The early spermatid during the spermiogenesis. (B) The late spermatid. (C) The cytoplasmic bridge (arrow) was observed in between spermatid and spermatid. (D) The early sperm. Is, intercellular space; M, mitochondria; N, nucleus.

Intracellular space was larger than early spermatid (Fig. 3B). The cytoplasmic bridge was found in through the spermatids (Fig. 3C). Nucleus of sperm was more condensed, and the mitochondria were rearranged in a middle piece, and occupied about half of the head part in early sperm. But, the center of nucleus had low electron density (Fig. 3D).

In sperm stage, the sperm was formed by loss of cytoplasm. The head of mature sperm was a spherical shape and had no acrosome. The mitochondria were 9 to 13 mitochondria in middle piece under longitudinal section. The flagellum was showed the typical 9+2 array of microtubules. Also, the tail of sperm had not lateral fins and outer coarse fibers (Fig. 4A). Longitudinal section of sperm tail showed morphology of general tail. But, there was no lateral fin (Fig. 4B).

DISCUSSION

In fish, spermatogenesis occurs in cysts which are formed when a single spermatogonium is surrounded by Sertoli cells that envelope a developing germ cell clone derived from one spermatogonial stem cell (Grier, 1981). Leydig cells have three major morphological characteristics, a vesicular nucleus, mitochondria with tubular cristae, and a number of smooth endoplasmic reticulum (Chung, 2008). In our study, that of *Betta splendens* had the similar tendency too. The testes contained numerous testicular cysts, and spermatogenesis was synchronized in these testicular cysts such as general teleost.

The development of testis in fishes is closely related with photo period, developments of testis and testicular cyst were inhibited, and increased apoptosis under the continuous light condition. These results related with decrease of testosterone

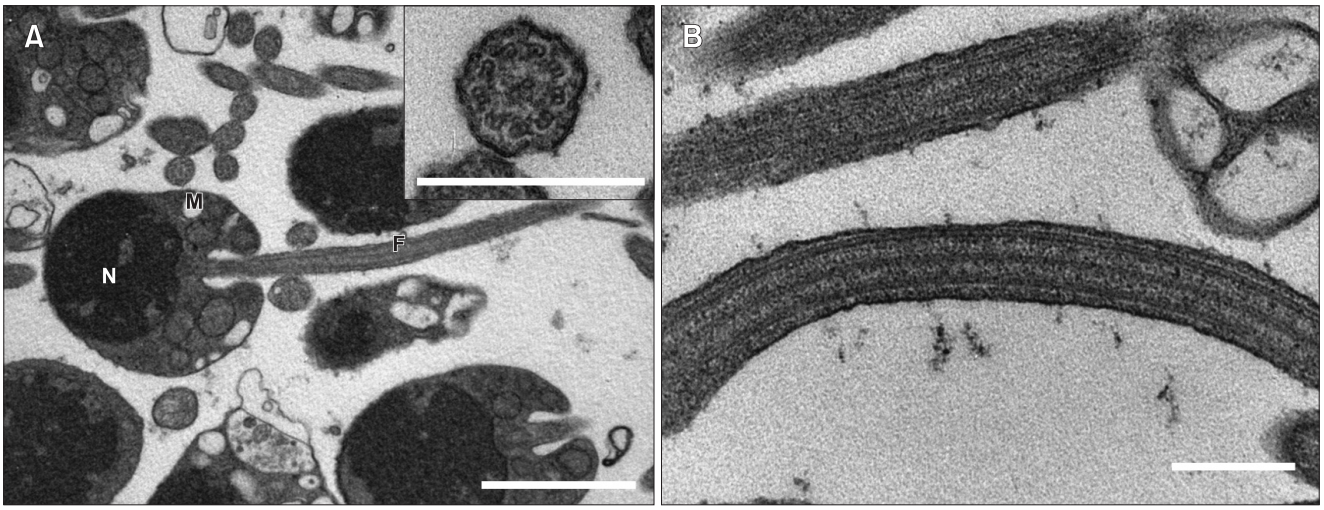

Fig. 4. The transmission electron micrograph of sperm. (A) The longitudinal section of matured sperm (scale bar=2 μm). The inset shows 9+2 pattern of microtubule in cross-sectioned flagellum (scale bar=500 nm). (B) Longitudinal section of sperm tail (scale bar=500 nm). N, nucleus; M, mitochondria; F, flagella.

(Almeida et al., 2009; Bayarri et al., 2009).

In fishes inhabiting temperate climate regions, spermatogenesis occurs at spring increasing water temperature (Kim et al., 2009, 2010). But, the spermatogenesis of tropical fishes occurs at any time of the year (Kim et al., 2003a; Lee et al., 2009). The *Betta splendens* inhabits a small pond, river or drain in Thailand and Cambodia, tropical area. This species spawn pelagic eggs which float on the water (Bailey & Sandford, 1998). So we look forward to differences of spermatogenesis or sperm morphology with general fishes, but could not find out any differences except nuclear condensation pattern in spermatogenesis. The nuclear condensation of germ cell occurred all over in most teleosts. But, chromatin condensation was occurred from the outside to the inside in *Betta splendens*.

In general, spermatogenesis of teleost may be summarized in increase of the number of germ cells, decreases of the size of germ cell, nuclear condensation, mitochondria rearrangement, and flagella formation. Also, developmental stages are classified in a different way according to the researchers. In this study, the spermatogenesis of *Betta splendens* was classified into four stages, primary spermatocyte, secondary spermatocyte, spermatid, and sperm stages. But, Woodhead (1974) classified into six stages, primary spermatogonia, secondary spermatogonia, primary spermatocyte, secondary spermatocyte, spermatid, and spermatozoa under LM. Also, Kim et al. (2003b) classified into four stages, spermatogonium stage, spermatocyte stage, spermatid stage, and spermatozoon stage in olive flounder, *Paralichthys olivaceus*.

Spermiogenesis involves preparatory morphological events followed by conspicuous modifications such as intracellular movements including diplosome and mitochondrion migration, spermatid lengthening, nuclear rotation and structural changes including dense chromatin granules, increase in size of mitochondria, and loss of cytoplasm (Bruslé, 1981). In our study, there were some empty testicular cysts among the testicular cysts. It was supposed to be formed cavity by phagocytosis of residual body after ejaculation.

Ultrastructural studies of fish spermatozoa have shown that characters obtained from sperm ultrastructure and the process of spermiogenesis may be phylogenetically analyzed, thus providing important data for the elucidation of relationship patterns among several fish groups (Mattei, 1991).

In general, the head part of sperm in teleost has no acrosome. So egg envelope has single micropyle, sperm entry site, in the area of the animal pole (Joo & Kim, 2013). The sperm head varies in shape. There are bullet shape (Jones & Butler, 1988), crescent (Fishelson et al., 2007), spherical (Lee et al., 2009; Kim et al., 2010), and long cone shape (Kim et al., 2003a).

In most teleost, the flagellum or sperm tail shows the typical 9+2 array of microtubules. But, microtubule pattern of *Anguilla australis* is a 9+0 structure (Todd, 1976). The sperm tail of threespine stickleback have a lateral fin (Deung et al., 1999), In the case of *Xiphophorus maculatus*, ovoviviparous fish, the sperm has a loop-like structure at the end of a tail (Kim et al., 2003a). Also, *Coreoleuciscus splendidus* has 7 outer coarse fibers at the outside of the axoneme (Kim et al., 2009). In *Betta splendens*, the head of mature sperm was a spherical shape and had no acrosome. The flagellum was showed the typical 9+2 array of microtubules. Also, the tail of sperm had no lateral fins and outer coarse fibers. These ultrastructural characteristics can be used in classification of species.

There were some empty testicular cysts among the testicular

cysts. It was supposed to be formed cavity by phagocytosis of residual body after ejaculation.

CONCLUSIONS

The spermatogenesis and sperm structure of Siamese fighting fish, *Betta splendens*, belongs to Osphronemidae was investigated by light and electron microscopic observations. The spermatogenesis occurs in the testicular cyst, and can be classified into four stages: (1) primary spermatocyte, (2) secondary spermatocyte, (3) spermatid, and (4) sperm stages. The spermatogenesis of *Betta splendens* can be summarized in increase of the number of germ cells, decreases of the size of germ cell, nuclear condensation, mitochondria rearrangement, and flagella formation. The head of mature sperm was a spherical shape and had no acrosome. The flagellum was showed the typical 9+2 array of microtubules. Also, the tail of sperm had no lateral fins and outer coarse fibers. These ultrastructural characteristics can be used in classification of species.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

REFERENCES

Alfert M (1956) Chemical differentiation of nuclear proteins during spermatogenesis in the salmon. *J. Biophys. Biochem. Cytol.* **2**, 109-114.

Almeida F F, Taranger G L, Norberg B, Karlsen O, Bogerd J, and Schulz R W (2009) Photoperid-Modulated testis matureation in Atlantic cod (*Gadus morhua*, L.). *Biol. Reprod.* **80**, 631-640.

Bailey M and Sandford G (1998) *The New Guide to Aquarium Fish: A Comprehensive and Authoritative Guide to Tropical Freshwater, Brackish, and Marine Fishes*. pp. 70-77, (Smithmark Publishers, New York).

Bayarri M J, Zanuy S, Yilmaz O, and Carrillo M (2009) Effects of continuous light on the reproductive system of European sea bass gauged by alterations of circadian variations during their first reproductive cycle. *Chronobiol*. **26**, 184-199.

Billard R (1984) Ultrastructural changes in the spermatogonia and spermatocytes of Poecilia reticulata during spermatogenesis. *Cell Tissue Res*. **237**, 219-226.

Bruslé S (1981) Ultrastructure of spermiogenesis in Liza aurata risso, 1810 (Teleostei, Mugilidae). *Cell Tissue Res.* **217**, 415-424.

Chung E Y (2008) Ultrastructure of germ cells, the Leydig cells, and Sertoli cells during spermatogenesis in *Boleophthalmus pectinirostris* (Teleostei, Perciformes, Gobiidae). *Tissue Cell* **40**, 195-205.

Deung Y K, Kim D H, and Reu D S (1999) Ultrastructure of gametes in the threespine stickleback, *Gasterosteus aculeatus aculeatus. Korean J. Electron Microsc.* **29**, 177-178.

Doutrelant C, McGregor P K, and Oliveira R F (2001) The effect of an audience on intrasexual communication in male Siamese fighting fish, *Betta splendens. Behav. Ecol.* **12**, 283-286.

Figler M H (1972) The relation between eliciting stimulus strength and habituation of the threat display in male Siamese fighting fish, *Betta splendens. Behaviour* **42**, 63-96.

Fishelson L, Gon O, Holdengreber V, and Delarea Y (2007) Comparative spermatogenesis, spermatocytogenesis, and spermatozeugmata formation in males of viviparous species of clinid fishes (Teleostei: Clinidae, *Blennioidei*). *Anat. Rec*. **290**, 311-323.

Grier H J (1981) Cellular organization of the testis and spermatogenesis in fishes. *Am. Zool.* **21**, 345-357.

Gwo J C and Gwo H H (1993) Spermatogenesis in the black porgy, *Acanthopagrus schlegeli* (Teleostei: Perciformes: Sparidae). *Mol. Reprod. Dev*. **36**, 75-83.

Jones P R and Butler R D (1988) Spermatozoon ultrastructure of *Platichthys flesus. J. Ultrastruct. Mol. Struct. Res*. **98**, 71-82.

Joo K B and Kim D H (2013) Comparative ultrastructures of the fertilized egg envelopes in *Danio rerio* and *Danio rerio* var. *frankei*, Cyprinidae, Teleostei. *Appl. Microsc.* **43**, 14-20.

Kim D H, Chang B S, Kim W J, Lee M S, Teng Y C, and Lee K J (2010) The Spermatogenesis of Chinese minnow, Leuciscinae, Teleostei. *Korean J. Microsc.* **40**, 1-8.

Kim D H, Lee K J, Kim S, and Teng Y C (2009) The Spermatogenesis of *Coreoleuciscus splendidus*, Cyprinidae, Teleostei. *Korean J. Microsc.* **39**, 227-236.

Kim D H, Reu D S, and Deung Y K (2003a) An ultrastructural study on the spermatogenesis of *Xiphophorus maculatus. Korean J. Electron Microsc.* **33**, 267-274.

Kim J W, Kim B S, Choi C Y, and Lee J S (2003b) Ultrastructural study on the development of male germ cell of the olive flounder, *Paralichthys olivaceus* (Teleostei: Pleuronectidae). *Korean J. Electron Microsc.* **33**, 243-250.

Kim S H, Lee C H, Song Y B, Hur S W, Kim H B, and Lee Y D (2013) Ultrastructure of late spermatids and spermatozoa during spermiogenesis in longtooth grouper *Epinephelus bruneus* from Jeju, Korea. *Tissue Cell* **45**, 261-268.

Kirankumar S and Pandian T J (2002) Effect on growth and reproduction of hormone immersed and masculinized fighting fish *Betta splendens. J. Exp. Zool*. **293**, 606-616.

Lee K J, Chang B S, Teng Y C, Kim S, Song M S, Joo K B, and Kim D H (2009) The Spermatogenesis of *Cichlasoma managuensis,* Cichlidae, Teleost. *Korean J. Microsc.* **39**, 219-226.

Lobb M L and McCain G (1976) Proceedurally related differences in the aggressive behavior of *Betta splendens* (Regan). *Animal Learning & Behavior* **4**, 367-373.

Mabee P M and Trendler T A (1996) Development of the cranium and paired fins in *Betta splendens* (Teleostei: Percomorpha): intraspecific variation and interspecific comparisons. *J. Morphol*. **227**, 249-287.

Mattei X (1991) Spermatozoon ultrastructure and its systematic implications in fishes. *Can. J. Zool*. **69**, 3038-3055.

Pandian T and Sheela S (1995) Hormonal induction of sex reversal in fish. *Aquaculture* **138**, 1-22.

Rebours C and Ottesen O H (2013) Ultrastructure of spermatozoa of the Atlantic cod *Gadus morhua* L. observed by scanning and transmission electron microscopy. *Anim. Reprod. Sci*. **139**, 155-161.

Thompson T and Strum T (1965) Classical conditioning of aggressive display in Siamese fighting fish. *J. Exp. Anal. Behav*. **8**, 397-403.

Todd P R (1976) Ultrastructure of the spermatozoa and spermiogenesis in New Zealand freshwater eels (Anguillidae). *Cell Tissue Res*. **171**, 221-232.

Woodhead A D (1974) Ageing changes in the Siamese fighting fish, *Betta splendens*. I. The testis. *Exp. Gerontol*. **9**, 75-81.