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Ultrastructure of Germ Cells, Cyst Epithelial Cells and Interstitial Cells during Spermatogenesis of the Stone Flounder, *Kareius bicoloratus*

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Ultrastructure of germ cells, the cyst epithelial cells and interstitial cells during spermatogenesis of the stone flounder, *Kareius bicoloratus* (Pleuronectidae) sampled on the west coast of Korea were investigated by electron microscopic observations. In the primary spermatocyte, the synaptonemal complexes appear in the zygotene stage of the prophase during maturation division. In the growing testis, especially, the interstitial cells (Leydig cells) appear near the primary, secondary spermatocytes and spermatids. Well-developed interstitial cells (steroid hormone secreting cells) which are located in the interlobular space in growing testis have three morphological characteristics of a vesicular nucleus, mitochondria with tubular cristae and smooth endoplasmic reticulum. During spermatogenesis, the primary and secondary spermatocytes attach to the cyst epithelial cell (Sertoli cell) having an elongated ovoid or triangular nucleus and several mitochondria in the cytoplasm. In the growing testis, lipid droplets, the mitochondrial rosettes and glycogen particles appear in the cytoplasm of the cyst epithelial cells near the secondary spermatocytes and spermatids. Particularly, the mitochondria, endoplasmic reticulum, little lipid droplets and the large amount of glycogen particles are present in the cytoplasm of the cyst epithelial cell in the late growing testis. In the late stage of spermiogenesis, the proximal centriole is joined to the nuclear envelope, the distal centriole forms the basal body of the flagellum and gives rise to the axial filament of the flagellum. No acrosome of the sperm is formed as seen in other teleost fish. The head of the spermatozoon is approximately 3 μm in length and its tail is about 30 μm in length. The axoneme of the tail flagellum of the spermatozoon consists of nine outer doublet microtubules at the periphery and two centrial singlet microtubules at the center. The spermatozoon of this species has two axonemal lateral fins. Especially, the cyst epithelial cells which located near groups of gametes in the various stages, show three functions: nutrition, phagocytosis and steroidogenesis. Especially, the nuclei of cyst epithelial cells in the recovery stage of the testicular developmental stages appear to be irregular in shape after spermiation. Of three functions of the cyst epithelial cell, several characteristics of phagocytosis are showed in the cytoplasm of the cyst epithelial cells in the recovery stage of the testicular developmental stages. At this stage, therefore, it is assumed that the cyst epithelial cells are involved in degeneration and resorption of undischarged germ cells after spermiation.

Key words : Ultrastructure, *Kareius bicoloratus*, germ cells, cyst epithelial cells, interstitial cells, spematogenesis

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Introduction

The stone flounder, *Kareius bicoloratus* is distributed along the coasts of Korea, China, Taiwan and Japan (Kim and Kang, 1993). More specifically, in Korea, this species is found especially in the subtidal zone of the southern and western parts of Korea and commercially useful winter spawning fish. Recently, as the standing stock of this species gradually decreased due to reckless overharvesting it has been noted as a target organism that should be produced artificially and managed by the reasonable fishing regime. But, recently, artificial breeding productions of this species have been succesfully carried out in Korea.

So far, as to the Korean and Japanese stone flounder there have been many previous studies on aspects of reproduction, including egg and larval development (Kim, 1982; Kim and Kim, 1989; Jun *et al*., 2002), growth and maturity (Mori *et al*., 1986), on aspects of ecology, including growth and feeding (Uehara and Shimizu, 1999; Choi, 2000), early life history (Minami, 1984; Moon, 1997), age and growth (Salgado-Ugarte, 1991; Uehara and Shimizu, 1996), on morphology including larval morphology (Han and Kim, 1997), on aquaculture including seedling production technical development (Jun *et al*., 1999, 2000, 2001) of the species. Though reproductive ecology of this species have been investigated by some authors, there are still gaps regarding the reproductive biology of this fish. It is necessary that reproductive mechanism and functions of various cells associated with spermatogenesis should be understood for this fish as a winter spawning fish.

The spermatogenic cyst epithelial cells of the testes in lower vertebrates such as Pisces and Amphibia are believed to be the homologue of the Sertoli cell in higher vertebrates (Loft, 1968; Hoar, 1969). Chung and Lee (1994) reported that in the spottybelly greenling, *Hexagrammos agrammus* the cyst epithelial cells (Sertoli cell) in the testicular lobules, exhibited a close association with the developing and degenerating spermatids and the function of the cyst epithelial cells in this fish was similar to those of the Sertoli cells in higher vertebrates. In addition to the function of the cyst epithelial cell (Sertoli cell), Gresik *et al.* (1973) described that the function of the interstitial cells (Leydig cell), which are distributed in the interlobular space in the testes of teleost, is quite similar to those of the Leydig cells in adult mammalian testes. Regarding these cells associated with reproductive mechanism during spermatogenesis, however, structures of the cyst epithelial cell and interstitial cell in accordance with germ cell development have not yet been clarified. Therefore, spermatogenesis associated with comparative development of the spermatogenetic cyst epithelial cell (Sertoli cell) and the interstitial cell (Leydig cell), that related to meiosis and steroid secretion, should be investigated in detail. The main aim of the present study is to understand ultrastructures and functions of the cyst epithelial cells and interstitial cells associated with spermatogenesis by electron microscopic observations.

Materials and Methods

Samples of the stone flounder, *Kareius bicoloratus* were collected monthly by trawl net at the vicinity of the subtidal zone of the coastal waters between Eochungdo, Chollabuk-do and Gyeongryelbiyeoldo, Taean-Gun, Chungnam-do, Korea, from January to December, 1998. For electron microscope observations, excised pieces of testes were cut into small pieces and fixed immediately in 2.5% glutaraldehyde-2% paraformaldehyde (0.1 M cacodylate buffer, pH 7.4) 2 hours at 4°C. After prefixation, the specimens were washed several times in the buffer solution and then postfixed in 1% osmium tetroxide solution in 0.2 M phosphate buffer solution (pH 7.4) for 1 hour at 4°C. Specimens then were dehydrated in increasing concentrations of ethanol, cleared in propylene oxide and embedded in an Epon-Araldite mixture. Ultrathin sections of a Epon-embedded specimens were cut with glass knives on a Sorvall MT-2 microtome and a LKB ultramicrotome at a thickness of about 800～1,000 Å. Tissue sections were mounted on collodion-coated copper grids, doubly stained with uranyl acetate followed by lead citrate and observed with a JEM 100 CX-2 (80 kv) electron microscope.

Results

1. Electron microscope observations of germ cell development during spermatogensis

Spermatogenesis occurs in the testicular lobules of the testis and can be divided into four successive stages: (1) spermatogonial (2) spermatocyte (3) spermatid (4) spermatozoon stages.

1) Spermatogonial stage

The spermatogonia, which appear near the elongated cyst epithelial cell (Sertoli cell), is ovoid or spherical in shape and is about 7～8 μm in length. The nucleus measuring approximately 4 μm contains a large nucleolus and electron dense chromatin in the interphase, or heterochromatin are scattered throughout a nuclear matrix during mitotic division. Several mitochondria and a lot of glycongen particles are in the cytoplasm (Fig. 1A).

2) Spermatocyte stage

(1) Primary spermatocytes

The spermatogonia develop into the primary spermatocytes. The nucleus of the primary spermatocytes undergoes complex morphological changes during the early meiotic prophase. At this stage, electron-dense synaptonemal complexes appear in the zygotene stage of the prophase during the first maturation division. In the cytoplasm of the primary spermatocyte, several mitochondria and the vesicles are present. At this stage, the primary spermatocytes attach to the cyst epithelial cell having an elongated or triangular nucleus (Fig. 1B).

In the prophase of the first maturation division, the clusters of the interstitial cell (Leydig cells) in the interlobular space are easily distinguishable from the connective tissue composing the wall of the lobule. These cells having each vesicular nucleus are surrounded with the fibroblasts in intercellular spaces and are generally round to oval in shape. In the growing testis, especially, the interstitial cells contain ovoid or spherical mitochondria with tubular cristae. And smooth endoplasmic reticula and several vesicles are present in the cytoplasm (Fig. 1C).

(2) Secondary spermatocytes

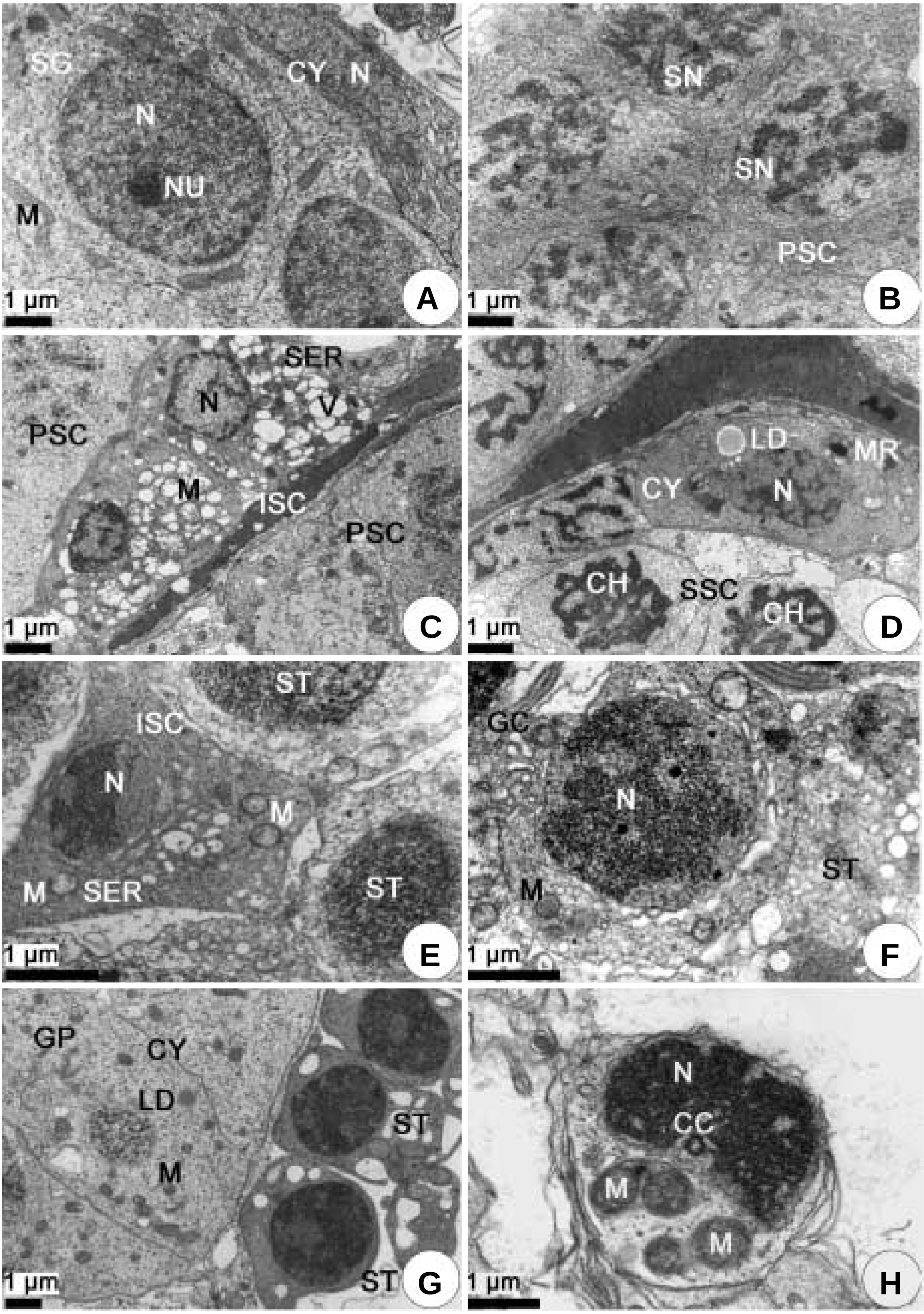
Primary spermatocytes develop into secondary spermatocytes by the first maturation division. At this time, the interstitial cells are involved in the formation of the secondary spermatocytes by the first maturation division. The size of the secondary spermatocyte is about 5～6 μm. Its nucleus has a variable shape and the nuclear envelope is not easily observed. Highly electron-dense chromatin and chromosomes are showed in most of the space in the nucleus, its cytoplasm contains some mitochondria and vacuoles near the cyst epithelial cells (Sertoli cells) which contain an elongated or triangular nucleus and the mitochondrial rosettes, several mitochondria and lipid droplets in the cytoplasm (Fig. 1D).

3) Spermatid stage

Secondary spermatocytes develop into spermatids by the second maturation division. At this time, the interstitial cell is present near the spermatids. Several spherical mitochondria and smooth endoplasmic reticulum are present in the cytoplasm of the interstitial cell (Fig. 1E). The size of the spermatid is about 4～5 μm. Its nucleus is oval and about 2.5 μm in diameter, at this time, condensation of the heterochromatin masses occur along the nuclear envelope. In the early stage of differentiation of the spermatid, the morphology of the spermatid changes gradually, spherical mitochondria and centrosome move to a position just behind the nucleus of the spermatid (Fig. 1F). In the middle stage of differentiation of the spermatid, the morphology of the cyst epithelial cell nucleus is an elongated or triangular types in shape. A number of spermatids during spermiogenesis attach to the cyst epithelial cell (Sertoli cell). At this time, some lipid droplets and mitochondria in the cytoplasm of the cyst epithelial cell are observed near numerous spermatids during spermiogenesis (Fig. 1G). At this time, the neck piece contained a centriolar complex, which are composed of centriole and lamellated body seen as a curved cap and a centriole, appear at the basal pole and move to an orthogonal position (Fig. 1H). In the late stage of differentiation of the spermatid development, two centrioloes appear: the proximal centriole is joined to the nuclear envelope, the distal centriole forms the basal body of the flagellum and gives rise to the axial filament of the flagellum. The flagellum occur from basal body. As nuclear elongation proceeds the spermatid is coat off with the cytoplasm. No acrosome is formed as seen in those of other teleost fishes. The condensed chromatin of the sperm head is covered by unit membrane (Fig. 1I, J).

4) Spermatozoon stage

The head of a completed spermatozoon is approximately 3 μm in length and its tail is about 30 μm in length. The axoneme of the tail flagellum of the spermatozoon consists of nine outer doublet microtubules at the periphery and two centrial singlet microtubules at the center. The sper-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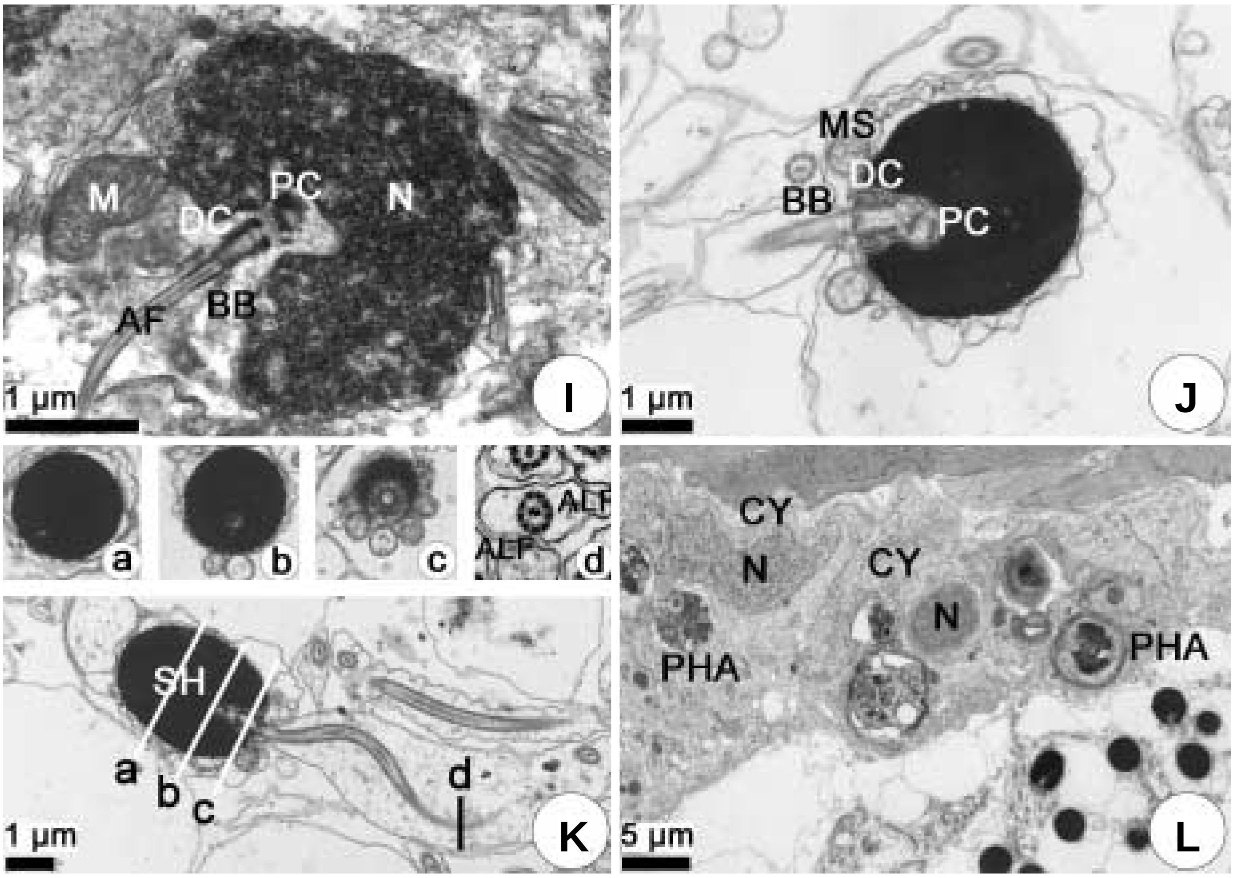

Fig. 1. Electron micrographs of germ cells, cyst epithelial cells and interstitial cells during spermatogenesis in *Kareius bicoloratus* (A ~ L). A, the spermatogonia (SG) near the cyst epithelial cell (CY) in the testicular lobule. Note a large nucleus (N) with a nucleolus (NU) and several mitochondria (M) in the cytoplasm of the spermatogonia and an elongated nucleus (N) of the cyst epithelial cell (CY); B, the primary spermatocytes. Note synaptonemal complexes (SN) in the zygotene stage of the prophase in the nucleus of the primary spermatocytes; C, the interstitial cells near the primary spermatocytes (PSC). Note a vesicular nucleus (N), oval or rod-shaped mitochondria (M), smooth endoplasmic reticulum (SER) and vacuoles in the interstitial cell (ISC); D, the secondary spermatocytes (SSC) near the cyst epithelial cell. Note chromosomes (CH) in the metaphase during meiosis and the elongated nucleus and the mitochondrial rosettes in the cyst epithelial cell; E, an interstial cell (ISC) near the spermatids (ST). Note electron dense chromatin in the nucleus (N) and several mitochondria, smooth endoplasmic reticulum (SER) in the cytoplasm of the interstitial cell near the spermatids; F, the spermatid during the early stage of spermiogenesis. Note high electron dense nucleus (N) and spherical mitochondria (M), the Golgi complex (GC) in the cytoplasm of the spermatid; G, the spermatids (ST) attaching to the cyst epithelial cell (CY). Note several spermatids during spermiogenesis around the cyst epithelial cell having lipid droplets (LD) and glycogen particles (GP) in the cytoplasm; H, a spermatid during the middle stage of spermiogenesis. Note centriolar complex (CC) attaching to the nucleus (N) and the mitochondria (M) of the spermatid; I, the spermatid during the late stage of spermiogenesis. Note proximal centriole (PC) and the distal centriole (DC) forming the basal body (BB) and the axial filament (AF) of the flagellum; J, a sperm during the late stage of spermiogenesis. Note proximal centriole (PC) and the distal centriole (DC) forming the basal body (BB) and mitochondrial sheath (MS) in the middle piece; K, a completed spermatozoon and cross sectioned parts of a spermatozoon; a, cross section of the sperm head; b, cross section of the part between sperm head (SH) and the middle piece; c, cross section of the middle piece; d, cross section of the tail flagellum (9+2 structure) and two axonemal lateral fins (ALF); L, phagosomes in the cytoplasm of the cyst epithelial cells and degenerating spermatids. Note irregular nucleus (N) and phagosomes (PHA) by phagocytosis in the cytoplasm of the cyst epithelial cell (CY) near degenerating spermatids after spermiation.

matozoon of this species has two axonemal lateral fins (Fig. 1K-a ~ d). After spermiation, the nuclei of cyst epithelial cells appears to be irregular in shape and several characteristics of phagocytosis in the cytoplasm of the cyst epithelial cells are showed near undischarged degenerating spermatozoa or spermatids (Fig. 1L).

Discussion

In our study, the mitochondrial rosettes appeared in the cytoplasm of the cyst epithelial cell near the primary and secondary spermatocytes in the early growing testis of *Kareius bicoloratus*. In general, functions of the mitochondrial rosette were reported by some authors: they appeared in the early growing oocyte in *Carassius auratus* (Gupta and Yamamoto, 1971) and *Agrammus agrammus* (Chung and Lee, 1985) and played an important role in propagation of the mitochondria.

Therefore, it is assumed that the mitochondrial rosettes is involved in propagation of the mitochondria in the cytoplasm of the cyst epithelial cellduring spermatogenesis.

Recently, there are some reports regarding the fine structure of the cyst epithelial cells (Sertoli cell) having capacities for several functions. Gresik *et al.* (1973) described that the cyst epithelial cell has three functions of nutrition, phagocytosis and steroidogenesis. Chung and Lee (1994) stated that in the *Hexagrammos agrammus*, the mitochodria, endoplasmic reticulum, a little lipid droplets (as an evidence of steroidogenesis) and the large amount of glycogen particles were present in the cytoplasm of the cyst epithelial cell in the mature stage, particularly, glycogen particles seem to be involved in the nutrition of spermatids during spermiogenesis. Gresik *et al.* (1973) stated that in the cyst lumen of *Oryzias latipes*, active phagocytosis was recognized by occurrence of vacuoles in degenerating spermatids, residual cytoplasm of spermatids, residual bodies. Vaupel (1929) considered the cyst epithelial cells of guppy to be involved in phagocytosis. In our study, similar phenomenona can be found in the lumen of cyst epithelial cell in the testis of *K. bicoloratus*. After spermiation, it is assumed that the cyst epitheial cells are involved in degeneration and resorption of undischarged germ cells because of occurrence of phagosome in spermatids.

Morphological characteristics of the interstitial cells (Leydig cell), which were located in the interlobular space in the testis of *K. bicoloratus*, were similar to those of *H. otakii* and other fish (Chung *et al.*, 1997). In general, the interstitial cells of the fish have three morphological characteristics of a vesicular nucleus, mitochondria with tubular cristae and smooth endoplasmic reticulum (Follenius, 1968).

A vesicular nucleus was seen in the steroid secreting cells of teleosts (Follenius and Porte, 1960; Follenius, 1964; Chung and Lee, 1994). Large oval or the rod shaped mitochondria with tubular cristae were very common in steroidogenic cells (Belt and pease, 1956; Christensen and Gillim, 1969; Chung and Lee, 1994). If the intersitial cells were filled with smooth endoplasmic reticulum being present in a tubular form, it is a characteristic of the cell which metabolize steroids (Christensen and Gillim, 1969; Fawcett *et al.*, 1969).

Thus, in our study, three characteristics mentioned above could be found easily in the interstitial cells in growing and maturing testes. Our electron microscopy showed an absence of lipid concentrations and no lipid droplets were observed as seen in testicular steroidogenic cells of the guppy (Follenius and Porte, 1960) and spottybelly greenling (Chung and Lee, 1994).

The axonemal lateral fins on the flagellum of spermatozoon were presented in most of externally fertilizing sperms except for Ostariophysi (Characiform, Cypriniform, Siluriform clade) (Jamieson, 1989). However, in case of most internally fertilizing spermatozoa, the axonemal lateral fins are absent (Jamieson, 1989; Lee, 1995; Chung *et al.*, 1997). In our study, *K. bicoloratus* belongs to externally fertilizing sperm and has two lateral fins. Therefore, our result coincides with Jamieson's report.

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돌가자미 *Kareius bicoloratus*의 정자형성과정 중 생식세포, Cyst 상피세포 및 간질세포의 미세구조

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한국 서해안에서 채집한 돌가자미 *Kareius bicoloatus*의 정자형성과정 중 생식세포, Cyst 상피세포 및 간질세포들의 미세구조를 전자현미경 관찰에 의해 조사하였다. 제1차 정모세포에서 연접사복합체 (synaptonemal complex)들이 성숙분열 중 전기의 쌍사기에 출현하였다. 특히, 감수분열을 위한 테스토스테론 호르몬을 분비하는 세포로 알려진 간질세포 (Leydig cell)들은 제1차 정모세포 및 정세포 가까이에서 뚜렷하게 출현하였다. 포상핵을 가지는 이들 간질세포들은 소엽 간 간질조직 내의 섬유모세포들에 의해 둘러싸여 출현하였다. 특히, 이들 세포들은 타원형 또는 구상의 미토콘드리아들을 가지는데, 미토콘드리아들은 관상 크리스테 (cristae)를 갖는 것이 특징이다. 또한, 활면소포체와 여러 개의 공포들도 세포질 내에서 출현하였다.

성장 · 발달 중인 정소 내에서 정소소엽 간 간질조직에서 나타나는 잘 발달된 간질세포들(스테로이드호르몬분비세포)은 포상의 핵과 관상 크리스테를 갖는 미토콘드리아를 가지며, 활면소체를 갖는 형태적 특징을 보였다. 정자형성과정 중, 제1차, 제2차 정모세포들은 cyst 상피세포 (Sertoli cell)들에 인접하거나 부착하여 나타났다. cyst 상피세포들의 핵은 길게 신장된 타원형 또는 삼각형의 핵을 가지며 세포질 내에 여러 개의 미토콘드리아가 출현하였다. 성장 · 발달 중인 정소 내에서 제2차 정모세포들과 정세포 가까이에 위치한 cyst 상피세포의 세포질 내에는 지방적, 미토콘드리아 로제트 (mitochondeial rosettes)들과 글리코겐 입자들이 나타났다. 특히, 후기 성장 중인 정소 내에서 cyst 상피세포의 세포질 내에는 미토콘드리아, 소포체, 작은 지방적 및 다량의 글리코겐입자들이 출현하였다.

정세포 발달 (정자변태) 후기에 기부중심립이 핵막에 부착하며, 원위중심립은 편모의 기저체를 형성하고, 편모축사를 형성하였다. 정자 첨체는 다른 경골어류에서와 같이 첨체를 형성하지 않았다.

정자 두부의 길이는 대략 3 μm 정도이며, 미부길이는 약 30 μm 정도이다. 정자 미부편모의 축사 (axoneme)는 주변에 9쌍의 주변이중미세관 (nine outer doublet microtubule)이 있고 중앙에 두 개의 중앙미세관 (two centrial singlet microtubules)으로 구성되어 9+2구조를 나타내었다. 본 종의 정자는 체외수정 종들이 갖는 두 개의 axonemal lateral fin을 가진다.

본 연구에서 여러 단계의 생식세포들 가까이에서 출현하는 cyst 상피세포 (Sertoli cell)들은 영양공급, 식세포작용, 스테로이드형성의 3가지 기능을 나타내었다. 특히, 정소 발달단계 중 퇴화 · 회복기에 출현하는 cyst 상피세포들의 핵은 배정 후에는 모양이 불규칙하게 나타나며, 식세포작용을 나타내는 여러 특징들이 cyst 상피세포의 세포질 내에서 나타나고 있어, cyst 상피세포들은 배정 후 방출되지 않은 생식세포들의 퇴화 · 흡수에 관여하는 것으로 추정된다.