

Spermiogenesis and Taxonomical Values of Sperm Ultrastructures in Male *Crassostrea ariakensis* (Fujita & Wakiya, 1929) (Pteriomorpha: Ostreidae) in the Estuary of the Seomjin River, Korea

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ABSTRACT : Characteristics of the developmental stages of spermatids during spermiogenesis and phylogenetic classification of the species using sperm ultrastructures in male *Crassostrea ariakensis* were investigated by transmission electron microscope observations. The morphology of the spermatozoon of this species has a primitive type and is similar to those of Ostreidae. Ultrastructures of mature sperms are composed of broad, modified cap-shaped acrosomal vesicle and an axial rod in subacrosomal materials on an oval nucleus, four spherical mitochondria in the sperm midpiece, and satellite fibres which appear near the distal centriole. The axoneme of the sperm tail shows a 9+2 structure. Accordingly, the ultrastructural characteristics of mature sperm of *C. ariakensis* resemble to those of other investigated ostreids in Ostreidae in the subclass Pteriomorpha. In this study, particularly, two transverse bands (stripes) appear at the anterior region of the acrosomal vesicle of this species, unlike two or three transverse bands (stripes) in *C. gigas*. It is assumed that differences in this acrosomal substructure are associated with the inability of fertilization between the genus *Crassostrea* and other genus species in Ostreidae. Therefore, we can use sperm ultrastructures and morphologies in the resolution of taxonomic relationships within the Ostreidae in the subclass Pteriomorpha. These spermatozoa, which contain several ultrastructures such as acrosomal vesicle, an axial rod in the sperm head part and four mitochondria and satellite fibres in the sperm midpiece, belong to the family Ostreidae in the subclass Pteriomorpha.

Key words : *Crassostrea ariakensis*, Spermiogenesis, Germ cell, Sperm ultrastructure

INTRODUCTION

The oyster, *C. ariakensis* (Fujita & Wakiya, 1929) is distributed along the estuary of the Seomjin River and Wando, Jeollanam-do, Korea. More specifically, in Korea, this species is mainly found at the mud including silty and

clay around the rocks in the intertidal and subtidal zones. However, in connection with the recent sharp reduction in its standing stock by reckless overcatching and environmental pollutions, it has been noted as a regional target organism that should be managed for the conservation of natural living resources. Particularly, studies on repro-

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ductive strategy of this species are supposed to be one of the most important tasks for the propagation of natural resources.

To date, ten oyster species in Ostreidae in the subclass Pteriomorpha were reported in Korea. Regarding reproduction of *C. ariakensis*, there are only one report such as spermiogenesis of spermatids and the ultrastructures of spermatozoa (Kim, 2001). Recently, sperm ultrastructures have been used as a tool in assessing taxonomic problems and phylogenetic relationships in the Metazoa through the use of spermiocladistic analysis (Jamieson, 1987, 1991). Recently, sperm ultrastructure has been used successfully as an aid in the examination of the phylogeny of bivalvia (Popham, 1979; Bernard & Hodgson, 1985; Healy, 1983; 1989; Healy & Lester, 1991). Daniels et al. (1971) suggested that comparative ultrastructure of oyster spermatozoa might be taxonomically useful because morphological differences between taxa could be found.

Although spermatogenesis and mature sperm ultrastructures have been described in some Ostreidae species in the subclass Pteriomorpha (Daniels et al., 1971; Popham, 1974, 1979; Healy and Lester, 1991; Sousa and Oliveria, 1994; Eckelbarger and Davis, 1996; Kim, 2001; Kim *et al.*, 2010), little information is available on spermiogenesis and mature sperm ultrastructures associated with taxonomic problems and phylogenetic relationships of the oyster in the Seomjin River, *C. ariakensis*.

Above all, it is very important to clarify some differences between ultrastructures of mature spermatozoon associated with the nucleus, acrosomal vesicle and the number of mitochondria. In addition, appearance of an axial rod (substructure) and the satellite fibers should be confirmed. The purpose of the present study is to clarify the some ultrastructural characteristics of spermatids during spermiogenesis, and also to confirm phylogenetic relationships of the oysters using the sperm ultrastructures of *C. ariakensis* and other closely related oyster species within

Ostreidae in the subclass Pteriomorpha. Therefore, it needs to clarify the appearance of the number of transverse bands (as the substructure) on the acrosomal vesicles of mature sperm of this species.

MATERIALS AND METHODS

1. Sampling

The specimens of *C. ariakensis*, were collected monthly in the intertidal and subtidal zones at the estuary of the Seomjin River, Chollanam-do, Korea, for one year from January to December, 2007. A total of 144 male oysters were used for transmission electron microscope observations.

2. Transmission electron microscope observation

For transmission electron microscope observations, excised pieces of the gonads were cut into small pieces and fixed immediately in 2.5% paraformaldehyde-glutaraldehyde in 0.1 M phosphate buffer solution (pH 7.4) for 2 hours at 4°C. After prefixation, the specimens were washed several times in the buffer solution and then postfixed in a 1% osmium tetroxide solution in 0.2 M phosphate buffer (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 Epon-embedded specimens were cut with glass knives on a Sorvall MT-2 microtome and LKB ultramicrotome at a thickness of about 80-100 nm. 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-II (80-KV) electron microscope.

RESULTS

1. Ultrastructural changes in spermatids during spermiogenesis and characteristics of sperm ultrastructures

The general ultrastructures of the testis of *C. ariakensis* is similar to those of other bivalve species. The processes of spermiogenesis of this species appear to be similar to species of other families in the subclass Pteriomorpha. However, compared with those of the families in the subclass Pteriomorpha, there are some differences in sperm ultrastructures: a few transverse bands on the anterior region (top) of the acrosomal vesicle and an axial rod, which is embedded in a coarsely granular matrix (subacrosomal materials), is detected in the spermatid and spermatozoon stages.

1) Spermatid stage

Spermiogenesis occurs in the spermatids. For convenience, spermatid stage could be classified arbitrarily into two stages: the early and late stages. In the early stage of spermiogenesis, the nuclei (about 2.7-2.8 μm diameter) of spermatids (3.6-4.1 μm) is spherical and occupies the center of the cell. contain heterochromatin materials in the nucleus. During spermiogenesis, the external morphology of the spermatid nucleus gradually changes during the differentiation of the spermatids. Consequently, the morphologies of the spermatid nuclei are laterally widened, and one or a few granules were formed by the Golgi complex in the cytoplasm of the spermatid (Fig. 1A). And a few granules formed a large proacrosomal vesicle (Fig. 1B), a proacrosomal vesicle migrated to anterior end of the spermatid (Fig. 1C), where they coalesce to form a single electron-dense proacrosomal vesicle (Fig. 1D). A single acrosomal vesicle locates at the anterior pole of the spermatids (Fig. 1E).

In the late stage of spermiogenesis, the acrosomal vesicle is initially oval in shape but gradually assumes modified cap-like form with a slightly pointed anterior nuclear fossa and a sharply invaginated posterior nuclear fossa. At this time, the acrosomal vesicle and an extensive deposit of subacrosomal (extravesicular) materials. At this time, the satellite fibres appeared near the distal centriole under the nucleus and

spherical mitochondria (Fig. 1F). Two components of the acrosomal vesicle can be recognized: the processes of acrosomal vesicle formation are very complex. The acrosomal vesicle is membrane bound, consequently, become modified cap-like form by various morphological changes. The acrosomal vesicle is gradually changed to oval in shape and the anterior nuclear fossa of laterally wide nucleus is deeply invaginated, and then the space is occupied by subacrosomal materials. Anteriorly oval nucleus is deeply invaginated, however, posterior nuclear fossa is not deeper than that of anterior nuclear fossa. A longitudinal flagellum is connected to the distal centriole near two large spherical mitochondria in the sperm midpiece. The satellite fiber is located near the distal centriole under the nucleus and spherical mitochondria (Fig. 1G).

2) Spermatozoon stage

The morphology of the spermatozoon of *C. ariakensis* has a primitive type. And the head part of the sperm is about 2.02 μm long, and the sperm is approximately 48-50 μm long including a wider nucleus (1.65 μm width) than the length of the nucleus (0.94 μm long), and also a finely granular subacrosomal materials are present in the anterior invagination region (anterior nuclear fossa) of the nucleus. The acrosome is modified cap-like form. Two centrioles (proximal and distal centrioles) and large two spherical mitochondria are found in the midpiece of the sperm. The proximal centriole lies at 90° to the sperm longitudinal axis or the distal centriole near the basal invagination of the nucleus (posterior nuclear fossa). The distal centriole lies parallel to the sperm longitudinal axis and forms the point of origin of flagellar axoneme. Two transverse banding appeared at the anterior region of the acrosomal vesicle. An acrosomal vesicle (0.64 μm long and 0.27 μm width) is composed of electron dense opaque materials on the nucleus is composed of the acrosomal vesicle (being composed of the modified cap-shaped basal rings) and

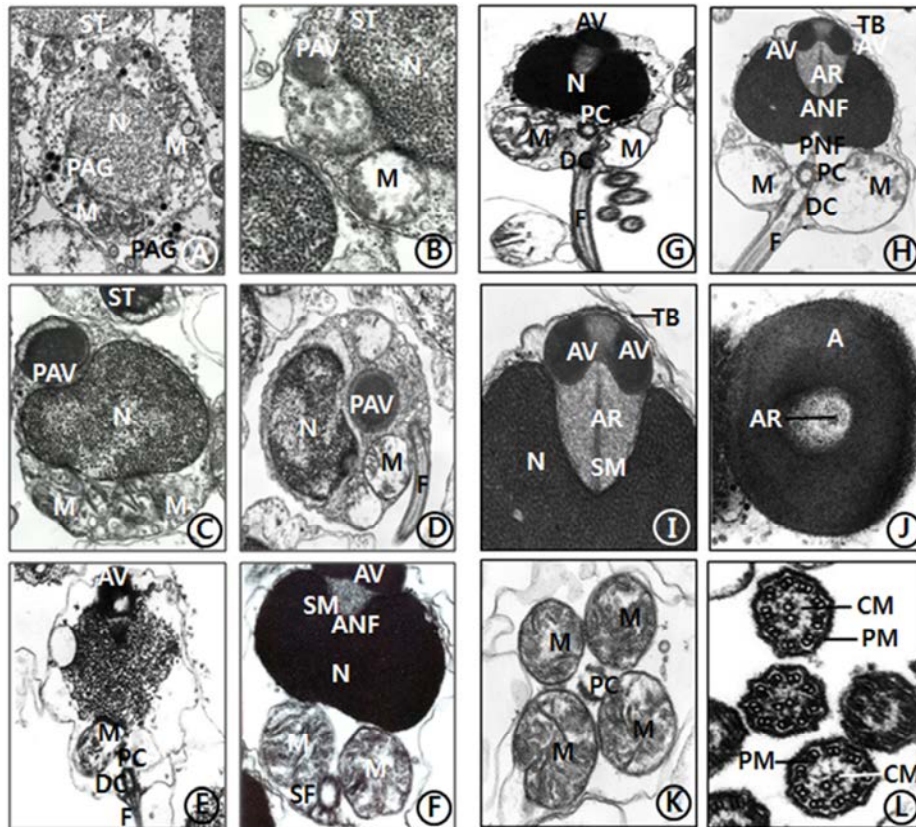


Fig. 1. Transmission electron micrographs of spermiogenesis and mature spermatozoon in male *Crassostrea ariakensis* (A-L). A, A spermatid (ST) in the early stage of spermiogenesis. Note several proacrosomal granules (PAG) just before the nucleus (N); B, C, The processes of the formation of the proacrosomal vesicles (PAV) of spermatids (ST). Note proacrosomal vesicles (PAV) just before the nucleus (N) and two spherical mitochondria (M) in the midpiece of sperm; D, The middle stage of spermiogenesis, formations of proacrosomal vesicle (PAV), the midpiece of sperm and a flagellum. Note proacrosomal vesicle (PAV) on the nucleus (N) and mitochondria (M) and a flagellum (F); E, The formations of the acrosomal vesicle (AV) and the sperm midpiece. Note the acrosomal vesicle (AV), proximal centriole (PC), distal centriole (DC) surrounded with spherical mitochondria (M), and a flagellum (F); F, A spermatid in the late stage of spermiogenesis. Note appearance of the acrosomal vesicle, subacrosomal materials (SM) in the invagination region of the nucleus (N) and formation of the sperm midpiece showing a satellite fibre (SF) near the distal centriole and spherical mitochondria (M); G, A spermatid in the late stage of spermiogenesis. Note an acrosomal vesicle (AV), an axial rod (AR) in the subacrosomal materials (SM) on the nucleus (N) and the proximal centriole (PC) and distal centrioles (DC) surrounded with mitochondria (M) near the satellite fibre (SF) in the sperm midpiece and a flagellum (F); H, A completed spermatozoon. Note transverse bandings (TB) on the anterior part of acrosomal vesicle (AV), an axial rod (AR); I, Completed acrosomal vesicle (AV) and the appearance of the axial rod and sperm midpiece. Note two (or three) transverse bandings (TB) on the anterior part of the acrosomal vesicle and an axial rod in the subacrosomal materials between the acrosomal vesicle (AV) and the nucleus (N), proximal centriole (PC) and distal centriole (DC) and flagellum (F); J, Cross sectioned acrosome (A) and nucleus. Note the acrosome (A) and an axial rod (AR) in the subacrosomal granular materials; K, Cross sectioned sperm midpiece. Note the proximal centriole (PC) surrounded with four spherical mitochondria (M); L, Cross sectioned flagella of sperms. Note an axoneme showing a 9+2 structure containing a pair of single doublet central microtubules (CM) and nine pairs of peripheral microtubules (PM).

subacrosomal materials which comprises an axial rod embedded in a coarsely granular matrix. In particular, an axial rod, which is embedded in a coarsely granular matrix (subacrosomal materials), is found between the acrosomal vesicle and the nucleus (Figs. 1H, 1I, 1J), as have seen in most species of Ostreidae.

In this stage, an acrosomal vesicle shows high electron dense opaque at the acrosomal membrane. In particular, two transverse bands (stripes) are present in the anterior region of acrosomal vesicle. At this time, two centrioles (proximal centriole and distal centriole) and two spherical mitochondria are located in the midpiece of the sperm. A long flagellum, which is occurred from the distal centriole, is located in the tail part of the sperm (Fig. 1H), as seen in *C. gigas* (Ostreidae) of the subclass Pteriomorpha.

Anteriorly oval nucleus (0.94 μm long and 1.65 μm width) is deeply invaginated, and then the space is occupied by subacrosomal materials. Posteriorly, the nuclear contents are highly electron dense and granular in texture. In the cross sectioned acrosomal vesicle, an axial rod is present in the subacrosomal granular materials (Figs. 1I, 1J). Posterior to the nucleus is the midpiece. This region consists of four spherical mitochondria surrounding a pair of triplet substructure centrioles. The cristae of each mitochondrion are randomly arranged (Fig. 1K). A flagellum is composed of a 9+2 substructure axoneme enclosed by the plasma membrane: that is, nine peripheral doublets (microtubules) surrounding a central pair microtubules (Fig. 1L and enclosed by the plasma membrane and measures approximately 42-45 μm).

DISCUSSION

1. Taxonomical values of sperm morphologies and ultrastructures

Commonly, the morphology of the spermatozoon of bivalve species has a primitive type and is similar to those

of other bivalves. In genal, the sizes of the sperm nuclei of bivalves prove unuseful in taxonomic analysis, because the morphological characteristics of sperm nuclei are irregular and vary among the species in a family (Healy, 1995; Kim et al., 2010).

Early investigations of bivalve sperm ultrastructure demonstrated the taxonomic value of comparative studies (Galtsoff & Phillipott, 1960; Franzen, 1956; Popham, 1979), and such studies are widely used in taxonomic analyses (Hodgson & Bernard, 1986; Healy, 1988, 1995, 1996). The primitive sperm show sufficient structural variability that they are useful in taxonomic studies (Kim et al., 2010). In general, morphology of ostreid sperm shows broad, modified cap like acrosome, extensive subacrosomal deposit materials (the appearance of an axial rod), oval nucleus, four pericentriolar mitochondria (Healy & Lester, 1991; Kim et al., 2010).

In this study, as seen in the morphology of spermatozoon of *C. gigas*, mature spermatozoa of *C. ariakensis* consist of broad, modified cap-shaped acrosomal vesicle, an axial rod embedded in a granular matrix (subacrosomal materials), an oval nucleus showing deeply invaginated anteriorly, two triplet substructure centrioles surrounded by four spherical mitochondria.

Results of the present study support the suggestion of Daniels et al. (1971) that sperm ultrastructure may be of taxonomic use in the Ostreidae. Therefore, compared with most structures and morphologies, spermatozoa of *C. ariakensis* closely resemble those of other investigated ostreids (Daniels et al., 1971; Osanai & Kyojuzuka, 1982; Healy & Lester, 1991).

In this study, the acrosomal contents have a relatively uniform electron density. In case of Ostreidae species unlikely other family of bivalves, the nucleus of the spermatid become wider than long, and a finely granular subacrosomal materials are present in the anterior invagination region of the nucleus. It is one of special characteristics of spermatozoa

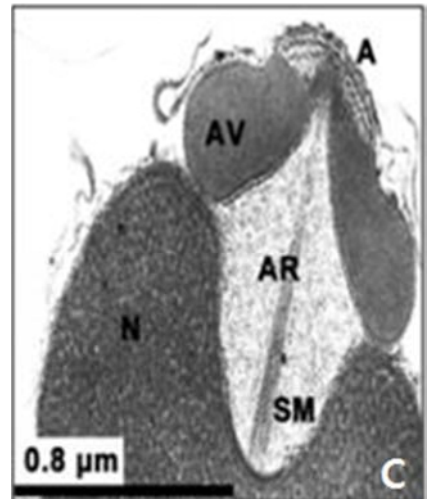
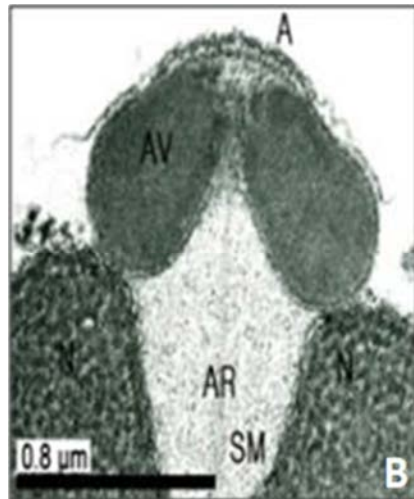
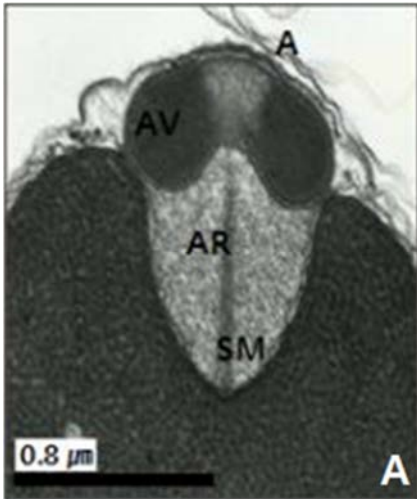


Fig. 2A. Electron photo- micrographs of the sperm ultrastructures of *Crassostrea ariakensis* in Ostreidae in the subclass Pteriomorpha.

The number of transverse bands (stripes) on the anterior region of right and left basal rings of the acrosomal vesicle: Two transverse bands (stripes).

Appearance of the axial rod (AR): exists in subacrosomal materials (SM) in the anterior nuclear invagination on the sperm nucleus (N) in Ostreidae.

Appearance of the satellite fibres: exists near the distal centriole and mitochondria in Ostreidae. Therefore, it belongs to the subclass Pteriomorpha. (Present, 2014)

Fig. 2B. Electron photo- micrographs of the sperm ultrastructures of *Crassostrea nipponica* in Ostreidae in the subclass Pteriomorpha.

The number of transverse bands (stripes) on the anterior region of right and left basal rings of the acrosomal vesicle: Two transverse bands (stripes).

Appearance of the axial rod (AR): Existed in subacrosomal materials (SM) in the anterior nuclear invagination on the sperm nucleus (N) in Ostreidae.

Appearance of the satellite fibres: exists near the distal centriole and mitochondria in Ostreidae. Therefore, it belongs to the subclass Pteriomorpha. (Kim, 2001)

Fig. 2C. Electron photo- micrographs of the sperm ultrastructures of *Crassostrea gigas* in Ostreidae in the subclass Pteriomorpha.

The number of transverse bands (stripes) on the anterior region of right and left basal rings of the acrosomal vesicle: Two or three transverse bands.

Appearance of the axial rod (AR): Existed in subacrosomal materials (SM) in the anterior nuclear invagination on the sperm nucleus (N) in Ostreidae.

Appearance of the satellite fibres: exists near the distal centriole and mitochondria in Ostreidae. Therefore, It belongs to the subclass Pteriomorpha. (Kim et al., 2010)

of Ostreidae species.

Regarding a central filamentous axial rod, in this study, the subacrosomal matter contains a central filamentous axial rod composed of antero-posterior-oriented filaments as seen in the family Ostreidae species of the subclass Pteriomorpha.

The taxonomic value of sperm morphology at the level of species or genus in the Ostreidae will be determined only through a broad, comparative study. In this study, the

anterior region of the acrosomal vesicle consisted of alternating electron dense and electron lucent bands. In particular, the acrosomal membrane in the acrosomal vesicle exhibited high electron dense opaque region. while two or three bands are present in the anterior region of the acrosomal vesicle which was exhibited electron-lucent whorls. A similar whorled substructure might also occur in acrosomes of *C. virginica*, through the micrograph presented by Daniels et al. (1971) were not detailed enough to

confirm this. In this study, two transverse bandings (stripes) appeared in *C. ariakensis*. However, two or three transverse banding appeared at the anterior region of the acrosomal vesicle in *C. gigas* (Kim et al., 2010; Kang et al., 2012). In addition, Healy & Lester (1991) reported that three to four horizontal bandings appeared at the anterior region of the acrosomal vesicle in *S. commecials* as a feature of the genus *Saccostrea*.

Hodgson & Bernard (1986) and Healy (1989) stated that different subclasses of bivalves each have unique acrosomal morphologies, and the number of mitochondria in the sperm midpiece tends to be stale within any family or superfamily varying from a maximum of 14 in the mytilord *Modiolus difficilis* (Drozdov & Reunov, 1986) to a minimum of 4 (common to many bivalve families) (Healy, 1989, 1995).

In this study, the number of mitochondria at the midpiece of the spermatozoon are four, and satellite fibres are found, as seen in *C. gigas* (Kim et al., 2010) and in *C. nipponica* (Kim, 2001). Judging from the resuts on the ultrastructure of mature spermatozoon, it is supposed that this species belongs to family Ostreidae in the subclass Pteriomorphia because the spermatozoon of this specis has special structural charateristics of the acrosomal vesicle containing an axial rod appeared between the acrosomal vesicle and the anterior nucleus fossa, and satellite fibres near the distal centriole and spherical mitochondria.

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