Transition of basic protein during spermatogenesis of *Fenneropenaeus chinensis* (Osbeck, 1765)

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Abstract According to the ultrastructural characteristic observation of the developing male germ cells, spermatogenesis of the crustacean shrimp, *Fenneropenaeus chinensis*, is classified into spermatogonia, primary spermatocytes, secondary spermatocyte, four stages of spermatids, and mature sperm. The basic protein transition during its spermatogenesis is studied by transmission electron microscopy of ammoniacal

silver reaction and immunoelectron microscopical distribution of acetylated histone H4. The results show that basic protein synthesized in cytoplasm of spermatogonia is transferred into the nucleus with deposition on new duplicated DNA. In the spermatocyte stage, some nuclear basic protein combined with RNP is transferred into the cytoplasm and is involved in forming the cytoplasmic vesicle clumps. In the early spermatid, most of the basic protein synthesized in the new spermatid cytoplasm is transferred into the nucleus, and the chromatin condensed gradually, and the rest is shifted into the pre-acrosomal vacuole. In the middle spermatid, the nuclear basic protein linked with DNA is acetylated and transferred into the proacrosomal vacuole and assembled into the acrosomal blastema. At the late spermatid, almost all of the basic protein in the nucleus has been removed into the acrosome. During the stage from late spermatid to mature sperm, some de novo basic proteins synthesized in the cytoplasm belt transfer into the nucleus without a membrane and almost all deposit in the periphery to form a supercoating. The remnant histone H4 accompanied by chromatin fibers is acetylated in the center of the nucleus, leading to relaxed DNA and activated genes making the nucleus non-condensed.

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Introduction

The morphology and structure of decapoda crustacean sperm are different from that of the general zoogenic sperm. Though they vary from species to species, all share the same characteristics such as without flagella, are non-motile, and without a condensed nucleus. In the past a few years, investigators have studied this type of sperm from different angles, such as spermatogenesis (Du et al. 1987; Wang et al. 1997, 1998; Zhao et al. 1997; Yang et al. 1998; Kang et al. 2000; Kang and Wang 2000; Poljaroen et al. 2010), acrosomal reaction (Wu et al. 2002), and oocyte activation (Wu et al. 1999), etc. However, the research on basic proteins during spermatogenesis of the decapoda crustacean has been limited to only a few species. Also, in many previous studies on spermiogenesis of decapods crustaceans, it was suggested that, for the most part, the nucleus loses histones in the most advanced stages of spermiogenesis, leaving the mature sperm DNA without associated basic proteins (Chevaillier 1966, 1967, 1968; Vaughn and Locy 1968; Langreth 1969; Vaughn and Hinsch 1972; Vaughn and Thomson 1972; Kleve et al. 1980; Wang et al. 1996; Kang et al. 2001).

It was previously believed that there is no basic protein in the mature sperm of most decapoda crustaceans. However, to understand how the sperm DNA is packaged and protected within them, what role the DNA is playing and why the nucleus presents at a non-condensed state, etc., it is interesting to investigate the decapoda crustacean using advanced EM technology. In fact, the remnant of modified histones in human and some higher animal sperm nuclei (Hecht et al. 2009) also poses the question as to why there is no basic protein in most decapoda crustacean sperm's nucleus. In this context, we choose the decapoda crustacean, the Chinese shrimp *Fenneropenaeus chinensis* as the subject to investigate in these issues.

Based on our observation of the development of acrosome during spermiogenesis (Ge et al. 2008a, b) and the membrane complex in spermatogenesis of *F. chinensis* (Kang et al. 2008), we classify its spermatogenesis into spermatogonia, primary spermatocyte, secondary spermatocyte, four stages of spermatid, and mature sperm, according to the ultrastructural characteristics of the developing male germ cells. We observe the histone transition during its spermatogenesis with transmission electron microscopy (TEM) of

ammoniacal silver reaction (ASR) and further study the immunoelectron microscopical distribution of acetylated histone H4 during its spermatogenesis, trying to present the transition characteristics of basic protein during spermatogenesis of *F. chinensis* and attempt to correlate these findings with the non-condensed sperm nucleus.

Materials and methods

Transmission electron microscopy (TEM)

Live adult male *F. chinensis* shrimp (from Shilihai Prawn Farm in Tanghai, Hebei, China) were dissected quickly at 0–4°C in order to obtain the spermary and vas deferens samples of 0.5 mm³. The samples were treated with 2.5% glutaraldehyde at 0–4°C in 0.1 mol l⁻¹ sodium cacodylate buffer solution (pH 7.2) for 2 h and then rinsed using 0.1 mol l⁻¹ sodium cacodylate buffer solution, followed by treatment with 1% osmium tetroxide in the same buffer solution for 1 h and then rinsed again with 0.1 mol l⁻¹ buffer solution. The samples were dehydrated by using alcohol and acetone and then embedded in Epon812 for polymerization at 60°C for 48 h. Ultra-thin (60–90 nm) slices were obtained by using an ULTRACUT E (701704) cutter and mounted on a 300-mesh Ni TEM grid and counterstained with lead citrate and uranyl acetate prior to TEM observation (JEM-100SX, operated at 75 kV).

Transmission electron microscopy (TEM) of ammoniacal silver reaction (ASR)

Another group of live male *F. chinensis* shrimp (from Shilihai Prawn Farm in Tanghai, Hebei, China) were dissected quickly at 0–4°C in order to obtain the spermary and vas deferens samples of 0.5 mm³. The samples were treated with 2.5% glutaraldehyde and 2% polyformaldehyde at 4°C in 0.1 mol l⁻¹ sodium cacodylate buffer solution (pH 7.2) for 2 h and thoroughly washed in 0.1 mol l⁻¹ sodium cacodylate buffer solution and then distilled water for about 20 min separately before immersion in the ammoniacal silver solution (AS). The latter was prepared just before use by the gradual addition of 10% silver nitrate solution to concentrated ammonium hydroxide until a

slight turbidity persisted. Samples immersed in the AS were left still for 5 min. Another thorough washing in distilled water for 30 min followed the AS treatment. The samples were then placed in 3% formalin solution for about 5 min, at which point a yellow-to-brown color change was observed. They were washed again in distilled water and dehydrated immediately by using an increasing concentration of ethyl alcohol, acetone, and then finally embedded in Epon812 for polymerization at 60°C for 48 h. Ultra-thin TEM samples (60–90 nm) were cut with a diamond knife on an ULTRACUT E (701704) cutter, and then placed on a 300-mesh Ni TEM grid. These samples were stained with uranyl acetate prior to observation (JEM-100SX TEM, operated at 75 kV). The samples used as negative control were treated with 0.25 mol l⁻¹ HCl four times (each time for 40 min) at 4°C before first fixation with 2.5% glutaraldehyde and 2% polyformaldehyde in 0.1 mol l⁻¹ sodium cacodylate buffer solution (pH 7.2), and the subsequent process was the same as for the experiment group.

Immunoelectron microscopical distribution of acetylated histone H4

Another group of live adult male *F. chinensis* shrimp (from Shilihai Prawn Farm in Tanghai, Hebei, China) was also dissected quickly at 0–4°C in order to obtain the spermary and vas deferens samples of 0.5 mm³. The samples were treated with 0.5% glutaraldehyde and 4% polyformaldehyde at 4°C in 0.1 mol l⁻¹ sodium cacodylate buffer solution (pH 7.2) for 2 h, then rinsed using 0.1 mol l⁻¹ sodium cacodylate buffer solution, followed by incubation with 0.1 mol l⁻¹ fresh glycine fluid (diluted in 0.1 mol l⁻¹ sodium cacodylate buffer solution) for 10 min and then rinsed again with 0.1 mol l⁻¹ buffer solution. The samples were dehydrated by using alcohol and acetone, then embedded in Epon812 for polymerization at 60°C for 48 h. Ultra-thin (60–90 nm) slice was obtained by using an ULTRACUT E (701704) cutter and the sample was mounted on a 300-mesh gold TEM grid.

The sample was etched at room temperature with water-saturated sodium periodic acid for 40 min and then incubated at 90°C in 0.05 mol l⁻¹ Tris–HCl. After natural cooling at room temperature, the sample was incubated with 2% goat serum (diluted in 0.01%

sodium cacodylate buffer solution) for 30 min to seal the non-specific antigen, followed by incubation with rabbit polyclonal anti-acetylated H4 (Anti-Histone H4, Acetylated (2–19) Rabbit pAb, from Calbiotech (Germany)). The sample was then diluted 1:20 in fresh 0.5% goat serum and 0.01 mol l⁻¹ sodium cacodylate buffer solution) overnight at 0–4°C, rinsed using 0.1 mol l⁻¹ sodium cacodylate buffer solution six times for a total of 1 h, and then incubated with sheep anti-rabbit secondary antibody conjugated to 10 nm colloidal gold (from Boaosan, Beijing). The sample was then diluted 1:5 in fresh 0.5% goat serum and 0.01 mol l⁻¹ sodium cacodylate buffer solution at room temperature for 2 h, after being rinsed using 0.1 mol l⁻¹ sodium cacodylate buffer solution and distilled water, stained with uranyl acetate for 20 min prior to observation (JEM-100SX TEM, operated at 75 kV). In the negative control, the primary antibody (rabbit polyclonal anti-acetylated H4) was replaced by the sodium cacodylate buffer solution, and the other process is the same as for the experiment group.

Results

Ultrastructural characteristics of the developing male germ cells

According to the shape, characteristic features, and chromatin organization, the male germ cells of *F. chinensis* can be classified into spermatogonia, primary spermatocytes, secondary spermatocyte, four stages of spermatid, and mature sperm.

Spermatogonia

A spermatogonium (Sg) located at the periphery of the seminiferous tubules is an oval-shaped cell about 10–12.3 µm in macroaxis, and 7.5–10 µm in brachy-axis with a large centrally located nucleus containing mostly euchromatin and the slight heterochromatin scatters in one pole of the nucleus. The cytoplasm contains abundant ribosomes and mitochondria. Especially at the same pole along with the nucleus some mitochondrial vesicles can be seen. The nuclear pore is extremely distinct at this stage (Fig. 1a, b).

Fig. 1 TEM images **a–d** exhibiting the ultrastructural characteristic of the *F. chinensis* germ cell from Spermatogonium to secondary spermatocyte. **a, b** Spermatogonia, of about 10–12.3 μm in macroaxis, and 7.5–10 μm in brachyaxis, and the nuclear pore (np) can be seen clearly at this time. **c** Primary spermatocyte, spherical cells about 8–11 μm . The prominent nucleolus, the synaptonemal complexes, the membrane vesicles, and annular structure are present in the nucleus. Abundant ribosomes mainly cohering to the endocyttoplasmic reticulum, some mitochondria, as well as rare Golgi complexes exist in the

cytoplasm. **d** Secondary spermatocyte, about 9.5–10.8 μm in macroaxis and 6.5–8.5 μm in brachyaxis, with chromatin exhibiting condensed block or cord-like structures. There are still some membrane complexes in the nucleus at this stage. The scale bar is 1 μm and the letters are defined as follows: as annular structure, cm cytomembrane, ERv endocyttoplasmic reticulum vesicles, G Golgi's apparatus, M mitochondrion, mv membrane vesicle, N nucleus, nm nuclear membrane, np nuclear pore, and Nu nucleolus

Primary spermatocytes

Primary spermatocytes are spherical cells about 8–11 μm , with moderately basophilic nuclei and patchy heterochromatin scattered throughout. The prominent nucleolus and the synaptonemal complexes can be seen. Membrane vesicles and annular structure as we have described before (Kang et al. 2008) are present in the nucleus. The cytoplasm contains abundant ribosomes with which most cohered to the endocyttoplasmic reticulum, some mitochondria, as well as rare Golgi complexes (Fig. 1c).

Secondary spermatocytes (SSc)

The first meiotic division of a primary spermatocyte generates two secondary spermatocytes. The duration of this stage of spermatogenesis is relatively short and

the secondary spermatocytes are seldom observed in histological sections. The size of a secondary spermatocyte is about 9.5–10.8 μm in macroaxis, and 6.5–8.5 μm in brachyaxis, and its chromatin exhibits condensed block or cord-like structures that are aggregated together. There are still some membrane complexes in the nucleus at this stage (Fig. 1d).

Spermatids (St)

During spermiogenesis, spermatids derived from SSc differentiate into spermatozoa. The basophilic nucleus contains a varying ratio of heterochromatin to euchromatin during spermiogenesis, and the four stages can be classified mainly based on the different degree of chromatin condensation and the cell's morphology.

Shortly after meiosis the new round spermatid is about 3.5–5.0 μm , with a spherical nucleus and

exhibiting many endocyttoplasmic reticulum vesicles and very few mitochondria vesicles in the cytoplasm. Its double nuclear membrane wraps up the uniformly distributed granular chromatin. Some of the consecutive spermatids share the same cytomembranes (Fig. 2a).

The early stage spermatid shows that chromatin is gradually condensed into interconnecting thick cords and evenly distributed throughout the nucleus (Fig. 2b). In this stage, the endocyttoplasmic reticulum vesicles close to the nucleus in cytoplasm fuse together into rough endocyttoplasmic reticulum around the nucleus. Along with the polarization of the nucleus, the endocyttoplasmic reticulum vesicles, away from the nucleus in the cytoplasm, are assembled to the end opposite of the nucleus and diffused into proacrosomal vacuole where the acrosomal

blastema shows a de novo-type transformation and grows gradually.

The middle spermatids with sharing cytomembrane separate with each other and show that chromatin is decondensed into fine grains scattered throughout the nucleus (Fig. 2c, d). The rough endoplasmic reticulum around the nucleus is fragmented, and with cytosome growing downwards, some of them become smooth endoplasmic reticulum or fold and wrap into a series of tiny vesicles spreading into the tight cytoplasm belt. The nuclear membrane is dissolved gradually, leading to the disappearance of the plasmalemma and organelles. At the same time, the acrosomal vacuole condenses and shrinks and the acrosomal blastema is enlarged.

In the late spermatid, chromatin is decondensed into fine fibers and presents a flocculent structure within

Fig. 2 a–d TEM images showing the morphologic transformation of the germ cell from the new round spermatid to the middle spermatid. **a** The new round spermatid, about 3.5–5.0 μm in diameter, containing a spherical nucleus and exhibiting many endocytoplasmic reticulum vesicles and very few mitochondria vesicles in the cytoplasm. It can be seen that some of the consecutive spermatids share the same cytomembrane. **b** The early spermatide; the chromatin is gradually condensed into interconnecting thick cords, and with the polarization of the nucleus, the endocytoplasmic reticulum vesicles, away from the nucleus in the cytoplasm, are assembled to the end opposite to the nucleus and diffused into proacrosomal

vacuole within which the acrosomal blastema shows a de novo-type transformation and grows gradually. **c**, **d** The middle spermatid, which shows that chromatin is decondensed into fine grains scattered throughout the nucleus, and the middle spermatids sharing cytomembrane separates gradually with each other. At the same time, the acrosomal vacuole condenses and shrinks, and the acrosomal blastema is enlarged. The scale bar is 1 μm and the letters are defined as follows: *ab* acrosomal blastema, *av* acrosomal vesicle, *ER* endocytoplasmic reticulum, *ERv* endocytoplasmic reticulum vesicles, *N* nucleus, *nm* nuclear membrane, *pav* proacrosomal vacuole, *rER* rough endocytoplasmic reticulum, and *scm* sharing cytomembrane

which exists a distinct nucleolus containing fibrillar center (FC), granular component (GC), and a dense fibrillar component (DFC), and the lamellar cytoplasm wraps up the karyoplasma zone (Fig. 3b, c). Opposite and close to the nucleus, the acrosome is generated. Under the acrosomal cap at both sides of the nucleus, rings of convoluted membrane punches, mitochondria, annular lamina bodies that wind from endocyttoplasmic reticulum, fragments of smooth endocyttoplasmic reticulum, nuclear membrane and some nuclear particles constitute the membrane complex that originated from the cytoplasm. This membrane complex constantly releases many vesicles and particulate materials into the acrosomal area (Fig. 3a, b).

Spermatozoa (Sz)

With the formation of the typical spike on an apical body via further differentiation, mature sperm, about

3.4 μm long, is created (Fig. 3d). The non-condensed nucleus surrounded by a thin cytoplasmic rim is filled with chromatin fibers, and the membrane complex at both sides of the nucleus under the acrosomal cap still can be seen (Fig. 3d, e).

Transmission electron microscopy of ammoniacal silver reaction

TEM observation results of the ASR for basic proteins present a deposit of discrete electron-opaque particles located within the spermatogenic cell in a different stage of the spermatogenesis of *F. chinensis*.

ASR particles in spermatogonia

In cytoplasm, there is a large amount of fine ASR particles, about 10–30 nm in diameter, and most of which cohere on rough endoplasmic reticulum

Fig. 3 a–e TEM images exhibiting the ultrastructural characteristic of the *F. chinensis* germ cell from the late spermatid to the mature sperm. a–c The late spermatids, being characterized by the fact that chromatin is decondensed into fine fibers and presents a flocculent structure within which exist a distinct nucleolus and the lamellar cytoplasm wraps up the karyoplasma zone. When acrosome is generated, under the acrosomal cap at both sides of nucleus, rings of convoluted membrane punches, mitochondria, annular lamina bodies that wind from endocytoplasmic reticulum, fragments of smooth endocytoplasmic reticulum, nuclear membrane and some nuclear particles constitute the membrane complex that

originated from cytoplasm. a Magnification of the membrane complex in b. c Magnification of the acrosome and the nucleolus in b. d, e The mature sperm, which is about 3.4 μm long and the typical spike on an apical body is formed. The non-condensed nucleus surrounded by a thin cytoplasmic rim is filled with chromatin fibers and the membrane complex at both sides of the nucleus under the acrosomal cap still can be seen. The scale bar is 1 μm and the letters are defined as follows: ab acrosomal blastema, av acrosomal vacuole, ER endocytoplasmic reticulum, M mitochondria, mc membrane complex, mp rings of convoluted membrane punches, N nucleus, Nu nucleolus, and s spike

(Fig. 4a). Furthermore, some of these cytoplasmic ASR particles are transferred into the nucleus through the nuclear pores (Fig. 4b). In the nucleus, the ASR particles, about 30–50 nm in diameter, accumulate within the perinuclear heterochromatic region and are distributed as internal clumps in the chromatic region (Fig. 4a, b).

ASR particles in primary spermatocytes

A small quantity of ASR particles, about 20–30 nm in diameter, spreads within the heterochromatic region in the periphery of the nucleus. The nucleolus is apparent, and ASR particles, about 20 nm in diameter, appear primarily associated with the perinucleolar

Fig. 4 TEM of ASR images (a–f) show the changing process of basic proteins from the stages of spermatogonium to secondary spermatocyte of *F. chinensis*. **a, b** Spermatogonia, the distribution of the fine ASR particles in the cytoplasm with most of them cohered on rough endoplasmic reticulum, the ASR particle transportation from cytoplasm to the nucleus through the nuclear pores, as well as accumulation of ASR particles in the nucleus within the perinuclear heterochromatic region, indicating that basic proteins synthesized in the cytoplasm are transferred into the nucleus and deposited on the newly duplicated DNA at this stage. **c–e** Primary spermatocyte. The nucleolus is apparent and the ASR particles of about 20 nm in diameter are only present within the fibrillar center

(FC) and the granular component (GC), but not in the dense fibrillar component (DFC), showing that the nucleolus is active and may generate a lot of RNPs combined with basic proteins. ASR particles represent the RNPs, which are probably transferred into the cytoplasm through the nuclear pores and are involved in the formation of the cytoplasmic vesicle clumps (VC). **f** Secondary spermatocyte. The ASR particles of about 20 nm in diameter are still visible within the heterochromatic region of the nucleus, and there are less in the cytoplasm than observed in the former stage. The scale bar is 1 μ m and the letters are defined as follows: cm cytomembrane, N nucleus, nm nuclear membrane, np nuclear pore, Nu nucleolus, and vc vesicle clump

heterochromatin and some with the nucleolus, within which the ASR particles exist namely within the fibrillar center (FC) and the granular component (GC), but not in the dense fibrillar component (DFC) (Fig. 4c). Some ASR particles of about 20 nm in diameter in the nucleus are transferred into the cytoplasm through the nuclear pores (Fig. 4d).

In the cytoplasm, the fine ASR particles of about 20 nm in diameter and derived from the nucleus are involved in the formation of the cytoplasmic vesicle clumps (VC). In the VC, the ASR particles decrease in size from the periphery to the central zone, from 20 to 5 nm in diameter (Fig. 4d, e).

ASR particles in the second spermatocyte

The ASR particles of about 20–30 nm in diameter still exist within the heterochromatic region of the nucleus. In the cytoplasm, there is a reduced number of particles of about 20 nm in diameter than that observed in the former stage (Fig. 4f).

ASR particles in the new round spermatid

In the new spermatid cytoplasm, the smaller (but uniform) ASR particles of about 20–30 nm in diameter are associated with ribosomes, and these particles spread in the non-vesicle region located within the endoplasmic reticulum zone (Fig. 5a). Some cytoplasmic ASR particles of about 20–30 nm in diameter move into the nucleus through the vague nuclear pores (Fig. 5a). In the nucleus, these particles assemble regularly into a strap or cluster shape that is similar to heterochromatin clumps without a distinct heterochromatic region, which simultaneously leads to an increase in the size of the ASR particles of about 30–60 nm in diameter.

ASR particles in the early spermatid

Along with the polarization, the nucleus shrinks and the chromatin condenses, and the coarse ASR particles of about 50–70 nm in diameter are spread in the chromosome masses (Fig. 5b, c).

At the cytoplasm belt, the fine ASR particles of about 20–30 nm in diameter stick onto the endoplasmic reticulum around the nucleus. Under the proacrosomal vacuole at both sides of the nucleus, a small

quantity of ASR particles of about 20 nm in diameter still exist in the cytoplasm region directly under the proacrosomal vacuole (Fig. 5b, c).

Within the proacrosomal vacuole far from the nucleus, in the acrosomal blastema characterized by a high electron density, we can see some smaller ASR particles of about 5–10 nm in diameter, as shown in Fig. 5b and c.

ASR particles in the middle spermatid

At this stage, with condensed chromosome disaggregating into fine grains, ASR particles of about 20–50 nm in diameter spread across the whole nucleus. In the regions close to the acrosomal vesicle, the ASR particles of about 40–50 nm in diameter appear to be larger in diameter and higher in density than those in the center of the nucleus of about 20–30 nm in diameter (Fig. 5d–f).

Under the acrosomal vesicle at both sides of the nucleus, a small number of ASR particles of about 10–20 nm in diameter still exist, close to the acrosomal vesicle, and the endoplasmic reticulum around the nucleus is fragmented, and becomes tiny, smooth, and without ASR particles attached (Fig. 5d–f).

With the acrosomal vesicle shrinking and the acrosomal blastema enlarging, the acrosomal vesicle consists of many fine ASR particles of about 20 nm in diameter considered to come from the nucleus. These small ASR particles are assembled in the acrosomal blastema, resulting in a gradual increase in the electron density of the acrosomal blastema (Fig. 5d–f).

ASR particles in the late spermatid

Where the chromosome forms a flocculent structure, no ASR particles are observed in most of the nucleus region opposite to the acrosome. But in the nuclear region close to the acrosome, there is still a small quantity of fine ASR particles of about 10–20 nm in diameter, which seem to have a tendency to be transferred into the acrosome directly or via the cytoplasm belt (Fig. 6a, b).

While the membrane complex is formed under the acrosome cap at both sides of the nucleus, some small ASR particles of about 10–20 nm in diameter are distributed in the vesicles of the membrane complex. These ASR particles may be transferred

Fig. 5 TEM of ASR images (a–f) exhibit the changing process of basic proteins during the stage from new spermatid to the middle spermatid of *F. chinensis*. **a** The new round spermatid. The ASR particles of about 20–30 nm in diameter associated with ribosomes are spread in the non-vesicle region of the cytoplasm, showing that there are basic proteins newly synthesized. Some of these basic proteins appear to move into the nucleus through the vague nuclear pores and deposit on the DNA, making the ASR particles assemble regularly into a strap or cluster shape in the nucleus similar to the heterochromatin clumps without a distinct heterochromatic region. **b, c** The early spermatid, along with the polarization, the nucleus shrinks and the chromatin condenses, the coarse ASR particles of about 50–70 nm in diameter spread are in the chromosome masses. The fine ASR particles of about 20–30 nm in diameter stick onto the endoplasmic reticulum around the nucleus show that there are still some basic proteins newly synthesized which may be

used for the chromatin condensation. In addition, because there is no basic protein removal from the nucleus to the proacrosomal vacuole at this stage, the small ASR particles in the proacrosomal vacuole and the acrosomal blastema indicating the presence of basic proteins which are considered to be synthesized in and then transferred from the cytoplasm region. **d–f** During the middle spermatid stage, the condensed chromosome disaggregates into fine grains. ASR particles of about 20–50 nm in diameter are spread across the whole nucleus, indicating the initiation of the basic proteins removal from the nucleus to the acrosomal vesicle, resulting in a gradual increase in the electron density of the acrosomal blastema. The scale bar is 1 μ m and the letters are defined as follows: *ab* acrosomal blastema, *av* acrosomal vacuole, *ER* endocytosplasmic reticulum, *N* nucleus, *nm* nuclear membrane, *pav* proacrosomal vacuole, and *v* vesicle

into the acrosome in the end. The ASR particles in the acrosome, which come from the nucleus and the membrane complex, continue to assemble into the acrosomal blastema (Fig. 6a). At the end of this

stage, in the late spermatid, there are few ASR particles in the nucleus and hardly any ASR particles in the acrosome and acrosomal blastema (Fig. 6b).

Fig. 6 TEM of ASR images. **a–c** The changing process of basic proteins during the stage from late spermatid to mature sperm of *F. chinensis*. **d** The negative control. **a, b** The late spermatid, with the chromosomes forming a flocculent structure. There is almost no ASR particles observed in most of the nucleus region, especially in that opposite to the acrosome, showing that almost all of the basic proteins in the nucleus have been removed to the acrosome until this stage. At the end of this stage, however, there are few ASR particles in the nucleus and hardly any ASR particles in the acrosome and acrosomal blastema, suggesting that the basic proteins arrived in the acrosomal blastema can be changed into other materials. **c** The mature sperm in vas deferens. There are no ASR particles in the

entire acrosome, and a large quantity of ASR particles with relatively large diameter of up to 40–60 nm scatters in the periphery of the round nucleus, which shows that these basic proteins in the nucleus must be newly synthesized from the end of the late spermatid to mature sperm stage, resulting in the replacement of nuclear basic proteins. **d** The negative control of mature sperm in vas deferens. As the sample is pre-extracted thoroughly by HCl before the first fixation, the basic proteins and some other membrane materials are extracted, resulting in absent ASR particles and the damage of sperm membrane. The scale bar is 1 μm and the letters are defined as follows: *ab* acrosomal blastema, *av* acrosomal vacuole, *mc* membrane complex, *N* nucleus, and *s* spike

ASR particles in the mature sperm of vas deferens

In the mature sperm, a large quantity of ASR particles with a relatively large diameter of up to 40–60 nm scatters in the periphery of the round nucleus. Under the acrosomal cap in the non-vesicle region of the membrane complex within the cytoplasm belt, the ASR particles are similar to those observed in the nucleus. At this stage, the mature sperm exhibits the typically differentiated spikes, and there are no ASR particles in the whole acrosome (Fig. 6c). Figure 6d is the negative control. In the mature sperm of HCl pre-extracted samples, there are no ASR particles, and the sperm membrane shows a certain degree of damage.

Immunoelectron microscopical distribution of acetylated histone H4

In spermatogonia, gold particles are on behalf of the anti-acetyl-histone H4 antibodies distributed in the cytoplasm, as well as in the nucleus. They are located in the cytoplasmic non-vesicle region scattered with many ribosomes, and in the periphery of the heterochromatic region of the nucleus (Fig. 7a). Some labeling was observed in the periphery of the heterochromatic region of the primary spermatocyte nucleus and there were no particles in the nuclear membrane vesicle and almost no labeling in the cytoplasm (Fig. 7b, c). As for the secondary spermatocyte, there is almost no labeling in either the nucleus or cytoplasm

Fig. 7 Immunogold electron microscopy of ultrathin-sections of *F. chinensis* spermary incubated with (2–19) rabbit anti-acetylated histone H4 pAb. **a** Spermatogonium. Gold particles in the cytoplasmic non-vesicle region show that the newly synthesized basic proteins are acetylated first, then some of these basic proteins are transferred into the nucleus and deposited on the newly duplicated DNA, resulting in the gold particle distribution in the periphery of the heterochromatic region. **b, c** Primary spermatocyte. Only a little labeling was observed in the periphery of the heterochromatic region of the nucleus, showing that before the heterochromatin gene is

promoted to active euchromatin gene, histone H4 combined with DNA is first acetylated. No labeling in the nuclear membrane vesicle shows that the acetylated histone H4 may not be transferred by membrane vesicles. **d** Secondary spermatocyte. There is almost no labeling in either the nucleus or the cytoplasm and no particle exchange can be seen between the nucleus and cytoplasm, suggesting that the metabolism in second spermatocyte is inactive. The scale bar is 1 μ m and the letters are defined as follows: *cm* cytomembrane, *mv* membrane vesicle, *N* nucleus, and *nm* nuclear membrane

(Fig. 7d). In the new spermatid, some gold particles are randomly present in the cytoplasmic non-vesicle region and some are distributed in the nucleus, and almost all at periphery of the heterochromatic region (Fig. 8a). In the early spermatid, characterized by nucleus polarization and chromatin condensation, there are almost no gold particles scattered in the proacrosomal vacuole and the nucleus (Fig. 8b). In the middle spermatid state, the condensed chromosome disaggregates into fine grains indicated by spreading of the gold particles across the whole nucleus. Under the acrosomal vesicle at both sides of the nucleus, several particles are localized within the membrane complex (Fig. 8c). With the acrosomal vesicle shrinking and the acrosomal blastema enlarging, the gold particles derived from the nucleus move into the acrosomal vesicle and assemble to the acrosomal

blastema (Fig. 8c). When the chromosomes form a flocculent structure, the late spermatid stage is attained (Fig. 8d). At this time, there is no labeling in most of the nuclear region opposite to the acrosome. But in the nuclear region close to the acrosome, some gold particles can still be seen, which in the end will be transferred to the acrosome directly or via the cytoplasm belt. Under the acrosomal cap at both sides of the nucleus, particles which in later stages will be transferred into the acrosome appear in the vesicles of membrane complex. The gold particles in the acrosome derived from the nucleus and those transferred from the membrane complex continue to assemble into the acrosomal blastema (Fig. 8d). In the late spermatid, however, the gold particles in the acrosome are fewer than those in the middle spermatid. Though the gold particles derived from the nucleus in the

Fig. 8 Immunogold electron microscopy of ultrathin-sections of *F. chinensis* spermary and vas deferens incubated with (2–19) rabbit anti-acetylated histone H4 pAb. **a** The new round spermatid, labeled by an antibody in cytoplasmic non-vesicle region and almost all at the periphery of the heterochromatic region in the nucleus, showing that the newly synthesized histone H4 in the cytoplasm is acetylated first, then transferred into the nucleus through the nuclear pores, and deposited onto DNA. **b** The early spermatid. There is no labeling in the proacrosomal vacuole or the nucleus, indicating that the genes are inactive and there is still no basic protein removal at this time. **c** The middle spermatid; 10-nm gold particles spread across the whole nucleus and in the acrosomal vesicle, as well as the acrosomal blastema, showing that histone H4 starts to be removed at this stage, and before its removal, it is acetylated first to decrease the affinity with the DNA as a prelude. **d** Late spermatid. There is no labeling in most of the nuclear region

opposite to the acrosome, and the gold particles in the acrosome and the acrosomal blastema are reduced to that at the middle spermatid stage, suggesting that acetylated H4 will be deacetylated when it arrives at the acrosomal blastema. **e** The mature sperm. Gold particles accompanied by the chromatin fibers are randomly scatter in the nucleus, and there is no labeling in the cytoplasmic belt and the acrosome, showing that there are still some remnants of histone H4 in the nucleus, and its acetylated form may make the DNA relaxed and the genes activated, resulting in a non-condensed nucleus. **f** The negative control of mature sperm. No labeling shows that there is no false-positive, and that the result in the test of immunoelectron microscopical distribution of acetylated histone H4 is correct. The scale bar is 1 μ m and the letters are defined as follows: *ab* acrosomal blastema, *av* acrosomal vacuole, *mc* membrane complex, *N* nucleus, *nm* nuclear membrane, *pav* proacrosomal vacuole, *v* vesicle, and *s* spike

acrosome still assemble into the acrosomal blastema from the middle to the late spermatid stage, it is impossible to observe the change in the number of gold particles in the acrosomal blastema (Fig. 8b–d). In mature sperm, a lot of gold particles accompanied by the chromatin fibers randomly scatter in the nucleus, and there is no labelling in the cytoplasmic belt and the acrosome (Fig. 8e). Figure 8f is the negative control, and there are no gold particles within the mature sperm.

Discussion

Using TEM to study the ultra-fine structure of the male germ cell of *F. chinensis* during spermatogenesis, we have found, in the early spermatogenesis (spermatogonia, primary spermatocytes, and secondary spermatocyte), that the cellular stages and their morphological characteristics are not different from those of other invertebrates and vertebrates, except for the membrane vesicle and annular structure in the nucleus, which we have described before (Kang et al. 2008). The cellular differentiation before and during the meiotic process is quite conserved (Roosen 1969; Sobhon et al. 2001; Manochantr et al. 2003).

However, during spermiogenesis, the spermatids show many differences from those of other invertebrates and vertebrates. In contrast to the increasingly condensed chromatin in spermatids of most other invertebrates and vertebrates, the chromatin of this prawn was condensed first in the early spermatid (Fig. 2b), then disaggregated to form fine particles in the middle spermatid (Fig. 2c, d) and became flocules subsequently in the late spermatid stage (Fig. 3b, c). In the mature sperm, the chromatin fibers become totally decondensed (Fig. 3d, e), which may be the adaptation to the mode of fertilization and sperm transport to the eggs.

In this prawn, as well as in some other decapod crustaceans, the sperms are packaged in a mucin-rich spermatophore that is inserted and adheres to the thelycum within the female's abdomen and near the opening of the oviducts, so the sperm does not need to travel by swimming a long distance in the female reproductive tract, such as in cases of other animals with internal fertilization or in an aqueous medium, as in cases of animals with external fertilization.

As a result, they share the same characteristics, such as having no flagella, being non-motile, and having no condensed nucleus. The nucleus with decondensed chromatin could be advantageous to the species, because once the sperm fertilizes an egg its nucleus could fuse immediately with the egg nucleus.

To clarify if there is any basic protein in the non-condensed mature sperm nucleus of *F. chinensis*, and how these basic protein still exist and in what manner is it combined with the chromatin, we observed transition characteristics of basic protein during its spermatogenesis using TEM of ASR and immunoelectron microscopical distribution of acetylated histone H4. The ammoniacal silver method is a worthy technique, however, great care must be taken while using pure chemical reagents and clean glassware in order to avoid unnecessary contaminations and damage to the samples. Ammoniacal silver solutions should be prepared immediately before use. During the TEM observation, the deposit, presumably a complex of silver particles 10–20 nm in diameter, which occurs individually or as aggregates of up to 50–70 nm in diameter, shows the fine structural localization of basic protein, and the 10-nm colloidal gold particles present the profiles of acetylated histone H4.

Basic protein in spermatogonia

In the stage of spermatogonia, the existence of cytoplasmic ASR particles of about 10–30 nm in diameter (Fig. 4a, b) and gold particles (Fig. 7a) shows that there is newly synthesized basic protein on the ribosomes at this time, and these basic proteins may be transferred into the nucleus and deposited on the newly duplicated DNA to complete its packaging.

During chromatin replication and assembly, newly synthesized H4 in cytoplasm is acetylated at lysines 5 and 12 prior to deposition onto the nascent DNA (Chicoine et al. 1986; Sobel et al. 1995). The “Lys5/Lys12” acetylation pattern of newly synthesized H4 is highly conserved and has been found in organisms as diverse as protozoa (*Tetrahymena*), *Drosophila*, and humans (Sobel et al. 1995). The core-acetylated histone H4 is rapidly imported into the nucleus by members of the karyopherin (Kap)/importin family (Blackwell et al. 2007). As the newly synthesized histone H4 is reversibly acetylated, it becomes

deacetylated after being organized in nucleosomes (Ruiz-Carrillo et al. 1975; Jackson et al. 1976; Sealy and Chalkley 1979; Cousens and Alberts 1982).

Basic protein in primary spermatocyte

The ASR deposit is associated with the heterochromatin in the periphery of the nucleus and perinucleolar areas (Fig. 4c–e). Precisely, the ASR particles of about 20 nm in diameter exist in the fibrillar center (FC) and the granular component (GC) of the nucleolus, which shows that the ribonucleoprotein (RNP) combined with the basic protein is synthesized in the nucleolus (Fig. 4c). As some basic protein may be derived from the active genes, and most of the ribonucleoprotein (RNP) is basic protein. So, at this time the ASR particles on behalf of the RNP are in close contact with basic protein derived from DNA. It can be seen from Fig. 4d that the RNP particles of about 20 nm in diameter are transferred into the cytoplasm through the nuclear pores. The gold particles are only located in the periphery of the heterochromatin in the nucleus, and without any gold particles in the euchromatin region (Fig. 7b, c), showing that before the heterochromatin gene is promoted to active euchromatin gene, histone H4 combined with DNA is first acetylated to recruit the multiple regulatory factors that can change chromatin states for transcription regulation (Choi et al. 2007). The acetylation of histones localized in chromatin regions rich in actively transcribing genes, which precedes transcription, alters nucleosome and chromatin structure, decondensing the chromatin fiber and making the nucleosomal DNA accessible to transcription factors (Davie and Hendzel 1994).

Spermatogenesis is characterized by phase-specific expression of many genes exclusively expressed in the spermatogenic cells. Most of the spermatogenesis-associated genes such as cyclins, proto-oncogenes, azoospermia factor (AZF) genes, cytoskeleton genes, heat shock genes, nucleoprotein transition genes, centrin genes, and apoptosis genes, etc., are expressed in primary spermatocyte (Ge et al. 2008a, b). The mRNA of these genes are transferred into cytoplasm.

Membrane vesicles in the nucleus play an essential role in materials transportation from the nucleus to the cytoplasm (Kang et al. 2008). No labeling in the nuclear membrane vesicle (Fig. 7b, c) shows that the acetylated histone H4, and other modified basic

protein, may not be transferred by membrane vesicles.

In the cytoplasm, the RNP particles from the nucleus are involved in the formation of the cytoplasm vesicle clumps (VC), in which the silver particles become smaller and smaller from the periphery to the central zone (from 20 to 5 nm in diameter) showing that the RNP particles have been digested but are preserved in the VC (Fig. 4d, e). Some gold particles in the cytoplasm accompanied by endoplasmic reticulum cisterna show that there is still newly synthesized acetylated histone H4 on the ribosomes (Fig. 7b, c).

Basic protein in second spermatocyte

The ASR particles of about 20 nm in diameter (Fig. 4f) and gold particles (Fig. 7d) presented in the nucleus and cytoplasm are both fewer than in previous stages, and there is no particle exchange seen between the nucleus and cytoplasm, which suggests that the metabolism in the second spermatocyte is inactive.

Basic protein in the new spermatid

The fine cytoplasmic ASR particles of about 20–30 nm in diameter (Fig. 5a) and gold particles (Fig. 8a) spread in the non-vesicle region and especially within the endoplasmic reticulum zone (Figs. 5a, 8a), indicate that a large quantity of basic proteins has been synthesized in the cytoplasm of the new spermatid, and the newly synthesized histone H4 is acetylated first. The newly synthesized basic proteins will be transferred into the nucleus through the nuclear pores, and deposited onto the DNA, making the ASR particles present a heterochromatin outline. It implies that the chromatin has started to condense at this time (Fig. 5a).

From gold particles distributed in the periphery of the heterochromatin in the nucleus (Fig. 8a), we propose that some acetylated histone H4 is derived from cytoplasm and will be deposited onto DNA to make the chromatin condensed.

Basic protein in the early spermatid

In the early spermatid, along with the polarization of the nucleus, the heterochromatin increases gradually

with ASR particles deposited on (Fig. 5b, c). The amount of the deposit is increased and somewhat coarser in particle size. With further condensation of chromatin into heterochromatin, some region of the heterochromatin is heavily covered with ASR particles, which are up to 50–70 nm in diameter. The smaller ASR particles of about 20–30 nm in diameter binding on the endoplasmic reticulum around the nucleus show that there is still a little basic protein synthesized in the cytoplasm belt and some of which are transferred into the nucleus, resulting in gradual chromatin condensation.

No gold labeling in the nucleus at this stage (Fig. 8b) indicates that there is no acetylated H4, showing that the genes are inactive. From Fig. 5b and c, we can see the small ASR particles of about 20 nm in diameter in the cytoplasm region close to the proacrosomal vacuole. These small ASR particles are transferred into the proacrosomal vacuole first and then assemble into the acrosomal blastema. This suggests that some basic proteins newly synthesized in the cytoplasm are transferred into the proacrosomal vacuole and involved in forming the acrosomal blastema at this stage, in agreement with results from other authors (Bloch 1966; Vaughn and Thomson 1972; Wang et al. 1996; Kang et al. 2001).

Basic protein in the middle spermatid

In the middle spermatid, the chromosomes disassemble into fine grains. The gold particles spread all over the nucleus (Fig. 8c), showing that histone H4 is acetylated first before its removal. It might be that H4 hyperacetylation is the first step towards histone removal (Braun 2001; Meistrich et al. 1992). From Fig. 5d–f, we can see that ASR particles of about 20–50 nm in diameter in the nucleus increase in density, in particular, in the region very close to the acrosomal vesicle. The larger size is probably due to some ionic attraction and coalescence of smaller silver particles to form aggregates in the process of the particle removal. It shows that the chromosome is disaggregated and the basic protein combined with DNA is being transferred into the acrosomal vesicle. Before being transferred, the basic protein concentrates first in the nuclear periphery close to the acrosomal vesicle.

The small ASR particles of about 20 nm in diameter and gold particles in the acrosomal vesicle seem to be

derived from the nucleus and to assemble into the acrosomal blastema (Figs. 5d–f, 8c). This suggests that the basic proteins from the nucleus are involved in the formation of the acrosomal blastema. In the acrosomal blastema, the ASR particles derived from the nucleus (represented by gold particles) show histone H4 removal indicated by its acetylation at this stage (Fig. 8c).

In addition, in the cytoplasm belt, there are only some smooth tiny fragments of endoplasmic reticulum without ASR particles, suggesting that there are no basic proteins synthesized at this time. But under the acrosomal vesicle at both sides of the nucleus in some cytoplasmic regions close to the acrosomal vesicle, a small number of ASR particles of about 10–20 nm in diameter, several gold particles can be seen (Figs. 5d–f, 8c), inferring that these basic proteins, containing acetylated H4, should come from the nucleus.

Basic protein in the late spermatid

In the late spermatid, the ASR and gold particle afflux show that the basic protein is keeping on being transferred from the nucleus to the acrosomal vesicle, which make the nucleus contain almost no basic protein (Figs. 6a–b, 8d).

Basic proteins in the nucleus and those synthesized in the cytoplasm have been transferred into the acrosomal vesicle and then assembled into the acrosomal blastema from the stage of early to the late spermatid. In the late spermatid, there are almost no ASR / gold particles left in the acrosome, and there is no increase of them in the acrosomal blastema. This implies that the basic proteins transferred into the acrosome will be converted into other materials with assembling into the acrosomal blastema.

Basic protein in the mature sperm of vas deferens

The existence of nuclear ASR particles of about 40–60 nm in diameter (Fig. 6c) and gold particles (Fig. 8e) show that there are still basic proteins in the nucleus of mature sperm, which is different to Liu's results (Liu 2006). The profiles of the ASR particle distribution show that almost all basic proteins are confined in the periphery of the nucleus and form a supercoating of the nucleus without membrane, and the gold particles show that acetylated H4, which may

not be detected using TEM of ASR, spreads in the whole mature sperm nucleus.

In the negative control, there are no ASR particles in the sperm (Fig. 6d) because this sample has been treated with 0.25 mol l^{-1} HCl before its first fixation. HCl makes the sperm membrane damaged and the sperm basic proteins extracted, resulting in no ASR particles in the sperm for the negative control. So the ASR particles in the test group are on behalf of the basic proteins. As there is no labeling in the negative control of immunoelectron microscope test in Fig. 8 f, meaning that there are no false-positives, and the test result is correct and dependable.

The acetylation of N-terminal lysine residues in core histones is a very specific phenomenon (Thorne et al. 1990) that has been implicated in three distinct cellular processes. The first is the regulation of gene expression. The second is the deposition of free histones onto newly synthesized DNA. The third is the displacement of histones by transition proteins and protamines during vertebrate spermatogenesis (Bruce et al. 2002). At the mature sperm stage, there is no new DNA synthesized, and the distribution profiles of ASR (gold) particles show that there is no basic protein transfer to acrosome at this time as well. So the acetylation of histone H4 in the central region of the nucleus (together with chromatin fiber), indicates that the gene is active and the DNA is in a loose state, making the mature sperm nucleus non-condensed. The non-condensed sperm nucleus may facilitate fertilization in crustaceans agreeing with Kurtz and Poljaroen's reports (Kurtz et al. 2008, 2009; Poljaroen 2010).

The existence of ASR particles in the membrane complex reveals that during the phase from the late spermatid to the mature sperm, the rough endoplasmic reticulum emerges and basic proteins are synthesized in the cytoplasm. Consequently, the basic proteins may move into the nucleus. There are no ASR particles observed in the whole acrosome, indicating that there are no basic proteins in the acrosome at this time. This shows that the basic proteins synthesized in the membrane complex at this stage are completely transferred into the nucleus.

The switch from a nucleosome—based to a protamine-based chromatin structure is a characteristic feature of sperm maturation in many vertebrates including humans. First, testis-specific linker histones appear (Yan et al. 2003; Martianov et al. 2005; Tanaka

et al. 2005; Catena et al. 2006), and then histones are replaced by transition proteins (major types: TP1 and TP2), which in turn are replaced by protamines, leading to condensed chromatin with a doughnut structure (Braun 2001; Sassone-Corsi 2002; Kimmins and Sassone-Corsi 2005). However, the histone-to-protamine exchange process is incomplete, with a small percentage (5–15%) of the genome bound to modified nucleosomes at the periphery of the nucleus (Tanphaichitr et al. 1978; Wykes and Krawetz 2003; Armand and Jamie 2006). It is not simply randomly distributed remnants of histones but significantly enriched at many loci and very important for embryo development. This includes genes of key embryonic transcription factors and signaling pathway proteins (Hammoud et al. 2009a, b). During the spermiogenesis of *F. chinensis*, at the late spermatid stage, almost all basic protein in the nucleus has been removed, and in the mature sperm's nuclear periphery exist a lot of basic protein, signifying that these basic proteins are newly synthesized and displace the somatic histones. As for the acetylated histone H4 spread in the mature sperm nucleus, we suppose it is the remnant of inefficient basic protein replacement, which is not acetylated during early spermiogenesis and cannot be removed, as well as cannot be not detected using TEM of ASR. Whereas the replaced basic protein is within the centre of the sperm nucleus of many vertebrates, including human, it is in the nuclear periphery of *F. chinensis* sperm. In addition, whereas the remnant histone is in the nuclear periphery of many vertebrates, including human, it is in the centre of the nucleus of *F. chinensis* sperm. This may be determined by the sperm structure and adapted to the mode of fertilization, respectively.

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