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RESEARCH ARTICLE

Cortical reaction and solicitation mechanism in *Hemibarbus labeo* ovum

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Abstract There are 1 to 4 rows and five types of cortical alveoli in the cortex of the pallas (*Hemibarbus labeo*) egg. From the outer to the inner of the cortex, the diameter of the cortical alveoli decreases gradually. We found a new structure named solicitation speckle at the low latitude of the animal pole and near the micropylar apparatus between the ovum envelope and cell membrane in the zygote of fish. The solicitation speckle similar to type I cortical alveoli was very purple in H.E chromosome, and it had no obvious boundary with the ovum envelope and membrane. Moreover, no membrane around the solicitation speckle in TEM was found. In SEM, the solicitation speckle looked flocculent. During cortical reaction, the solicitation speckle played a very important function in arousing cortical reaction. Thirty-five seconds after fertilization, cortical alveoli began to break down near the low latitude of the animal pole. At the same time, the same thing happened near the micropylar apparatus before cortical reaction. Both starting points encountered and healed up at the vestibule of the micropylar apparatus. The cortical reaction that happened near the low latitude of the animal pole was another new pattern. The cortical reaction was divided into four parts that included latent period, developmental period, climactic period and declining period. In the latent period, no cortical alveoli were released. In the developmental period, a few cortical alveoli were released outside the cortex. In the climactic period, several cortical alveoli were inosculated into a big vesicle and released intensely. In the declining period, the type V cortical alveoli and the other remnant cortical alveoli were released. Five minutes after fertilization, cortical alveoli were released entirely in the animal pole. Five minutes after fertilization, all of the remnant cortical alveoli were released. This leads us to conclude that cortical reaction is induced by type I cortical alveoli, and the solicitation speckle is a volcanic chain reaction under water or the other lower osmotic pressure of fluids. The outer cortical reaction can accelerate the inner cortical reaction. While cortical alveoli releases in batches, the cell plasma membrane is reorganized over and over. No cortical alveoli were found below the micropylar tube where sperm enters the ovum, which suggests that the cortical reaction prevents polyspermy.

Keywords *Hemibarbus labeo*, cortical alveoli, cortical reaction, solicitation speckle, solicitation mechanism

1 Introduction

Many accumulated metabolites, including acidic mucopolysaccharide and acidic phosphatase, are positive reaction in PAS at the cortical alveoli mature ovum in fishes. There are differences in the bulk and inclusion of the cortical alveoli at different sites in the mature ovum (Brummett and Dumont, 1981). Once stimulated by spermatozoa or by physical and chemical factors, the ovum will activate and the cortical alveoli in the ovum will be released into the ovum lacuna through exocytosis, which is also named cortical reaction. There, Ca^{2+} is required and redundant membrane is released in the cytoplasm during cortical reaction (Fluck, 1991; Gillot, 1991). The spermatozoa will be agglutinated by the inclusion of the cortical alveoli (Kudo and Sato, 1985), which chokes back polyspermy. At the same time, the cortical reaction forms a fertilization cone, which holds back polyspermy too. The starting time and the climactic time during cortical reaction are different in fishes, and before cortical reaction is over, the cytomembrane that is destroyed will be restored during cortical reaction (Brummett and Dumont, 1981; Hong, 1994; Gao, 1995). Generally speaking, the site where the cortical reaction starts is located at the animal pole, and then rapidly moves to the vegetal pole. But, it is the opposite with *Rhooeus ocellatus* where cortical reaction starts at the vegetal pole (Ohta, 1990). There is no cortical reaction below the micropylar apparatus

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of *Fundulus heteroclitus* (Brummett and Dumont, 1979) and *Cyprinus carpio* var. singuonensis (Hong, 1994). We observed the cortical reaction in the fertilized eggs, *Hemibarbus labeo*, using histological sections, TEM and SEM in detail, and found that the cortical reaction of *Hemibarbus labeo* starts at the low latitude of the animal pole. At the same time, we found a new structure named solicitation speckle that is closely linked with cortical reaction in the fertilized eggs of *Hemibarbus labeo*.

2 Materials and methods

The reproductive population of *Hemibarbus labeo* was collected in dounstream of Fujiang, He Chuan, Chong Qing on March 16–25, 2001. In order to collect enough fertilized eggs, the vigorous fishes were artificial induced for spawning. We mingled the eggs and sperm in a beaker without water, and then put the admixture into water and mixed around lightly in 17ºC. Some of the fertilized eggs were fixed, and the others were incubated. After careful statistics, the fecundation rate was 97.1%, and the hatching rate was 85.4%.

2.1 Histological section

Different period oosperms and mature ovum were fixed with Simth's and Bouin's solution. After 24 h, all the specimen were shifted into 70% alcohol. We discerned the micropylar apparatus or animal pole and cut them under an anatomical lens with a sharp blade. After dehydration by 95% *n*-butyl alcohol twice and then by 100% twice, the preparations were embedded in paraffin. Seriate histological sections were made to 5−7μm, after dyeing with H.E, and we took photographs with a Nikon microscope.

2.2 Transmission electron microscope

Oosperms during different developmental periods and mature ovum were fixed in 0.1 mol/L phosphate buffer containing 2.5% glutaraldehyde for 12 h and then were washed thrice in 0.1 mol/L phosphate buffer before post-fixation in 1% osmium tetroxide. After dehydration through an ascending series of ethanol solutions and replacement with acetone, the preparations were embedded in epoxy resin 650. Ultrathin sections were cut and collected on grids, and stained with uranyl acetate and lead citrate, and then viewed in transmission electron microscope (H-600 and JEM-2000EX).

2.3 Scanning electron microscope

Oosperms during different developmental periods and mature ovum were fixed with in 0.1 mol/L sodium cacodylate buffer containing 2.5% glutaraldehyde for 12 h and then were washed thrice in 0.1 mol/L sodium cacodylate buffer. We discerned micropylar apparatus or animal pole and cut them under an anatomical lens with a sharp blade. After getting rid of the envelope and plasma membrane and dehydrated by a graded alcohol series of 70, 85 and 95% ethanol, we changed to 100% ethanol twice. After mounting on scanning electron microscope stubs, according to different requirements, the specimen were fixed using glass needles. The mounted specimens were sputter-coated with gold and examined with a SEM (Hitachi-S-250).

3 Results

3.1 Morphology of cortical alveoli in mature egg

There were 1 to 4 rows of cortical alveoli enwrapped by unilaminar biomembrane in the cortex of the pallas, *Hemibarbus labeo* egg. The diameter of the cortical alveoli away from the micropylar apparatus was bigger. The layers of cortical alveoli were more, and it was easier to dye with H.E than near the micropylar apparatus. The average diameter of the cortical alveoli was 14.667 μm, but the diameter was 5.357 μm near the micropylar apparatus. There was no cortical alveoli near the micropylar tube.

There are five types of cortical alveoli in the cortex of the egg according to their location, magnitude and inclusion. Type I, distributed at low latitude of the animal polar, cohered to the cell membrane, and the diameter was less than 1.920 μm. Its amount was a few. The granules of the inclusion were very big, and the inclusion very dark in H.E (Plate I. 4). In TEM, most of the type I have broken (plate II. 17). Type II resembled type I, but the average diameter was 4.123 μm (Plate I. 3). There were 1 to 2 rows of alveoli belonging to type III close to the cell membrane, and the average diameter of Type III was 18.333 μm. There were many type III, and the alveoli was light-colored in H.E (Plate I. 3,4,5). There were 2 to 3 rows of alveoli belonging to Type IV far from the cell membrane. The average diameter of Type IV was 24.510 μm. There were many of this type, and the alveoli was also light-colored in H.E (Plate I. 4,5). Type V was in sight of the yolk, but a few were located on the cell membrane. The number was few, and there were some big granules in type V (Plate I. 5). A new structure named solicitation speckle was found at the low latitude of the animal pole near the micropylar apparatus, and it lay between the ovum envelope and the cell membrane in *Hemibarbus labeo* ovum. The solicitation speckle similar to type I of the cortical alveoli was very purple in H.E (Plate I. 1,2), and it had an obvious boundary with the ovum envelope and cell membrane. Moreover, no membrane around the solicitation speckle was found in TEM (Plate II. 18). In SEM, the solicitation speckle looks flocculent (Plate II. 24).

In the ovum, which had the envelope removed, there were bushy microvilli on the plasma membrane in SEM (Plate II. 25), and cortical alveoli of different sizes arranged

tightly below the cell membrane (Plate II. 25). With the envelope removed, with cortical alveoli avulsion, the alveolate cortical alveoli appeared, and the inclusion in cortical alveoli was spherical or flocculent (Plate II. 26). Scanning the inner plasma membrane, the abundant cortical alveoli below the inner plasma membrane was alveolate too, and some of them approached the plasma membrane, even a few inosculated with the plasma membrane (Plate II. 27). No cortical alveoli and microvilli on the plasma membrane was found below the micropylar tube (Plate II. 24).

3.2 Release of cortical alveoli during fertilization

3.2.1 Observations in histology

Oosperms during the different developmental periods were made into seriate paraffin sections with H.E staining. Thirty-five seconds after fertilization, the type II cortical alveoli near the low latitude of the animal pole began to break down, and the inclusion of the cortical alveoli was released into the egg envelope and cell membrane immediately. It became purple speckled (Plate I. 6). At the same time, cortical reaction occurred near the vestibule of the micropylar apparatus and became purple speckled too (Plate I. 8). The purple speckle was one of the markers of cortical reaction occurring in H.E. Forty seconds after fertilization, the cortical alveoli near the type II of the cortical alveoli broke down (Plate I. 7). In some specimen, ovum lacuna occurred near the cortical alveoli near the low latitude of the animal pole (Plate I. 9). Fifty seconds after fertilization, a great deal of cortical alveoli broke down, with the purple inclusion being released, and the ovum lacuna stretching on all sides. The membrane of the cracked cortical alveoli and the cell membrane could be reorganized, and they formed a new plasma membrane that was dyed darkly. A little inclusion out of the new membrane was adsorbed, and there was an abundance of cortical alveoli that kept orbicular inside the new membrane. Fifty-five seconds after fertilization, the cortical reaction in the climactic period was approaching, and it extended from the low latitude of the animal pole to the high latitude (micropylar apparatus) (Plate I. 10,11). Near the vestibule of the micropylar apparatus, cortical alveoli kept on breaking down, and became more and more purple speckled. Sixty seconds after fertilization, cortical reaction kept on extending to the high latitude of the animal pole, and the ovum lacuna was enlarged. Along with the cortical reaction progress, the ovum lacuna occurred near the micropylar apparatus. Seventy seconds after fertilization, when cortical reaction kept on extending to the high latitude of the animal pole, the climax of the cortical reaction was approaching near the micropylar apparatus. Except in the micropylar tube, a bigger ovum lacuna appeared around the micropylar apparatus where some purple inclusion existed. However, the outspread of the cortical reaction was restricted to the sites near the vestibule of the micropylar apparatus. Eighty seconds after fertilization, the first stage of the cortical reaction was finished, and most outboard cortical alveoli were broken down. Later, the new cell membrane was restored perfectly (Plate I. 12). However, the new membrane was rough, and there were 1 to 3 rows of cortical alveoli most of which inosculated together inside the new membrane. Subsequently, the inclusion was released from the cortical alveoli diffusing rapidly in the ovum lacuna. At 120 and 150 seconds after fertilization, cortical reaction kept on extending around all sides. But, the ovum lacunae that first occurred in the low latitude of the animal pole and vestibule of the micropylar apparatus did not get through. Most of the cortical alveoli near the micropylar apparatus broke down, and cortical reaction was basically over. Micropylar apparatus was markedly elevated (Plate I. 13). No cortical reaction was found below the micropylar tube where sperm entered. At 180 seconds after fertilization, cortical reaction was entirely over near the micropylar apparatus, but the contrary case was in the other region. On the one hand, the cortical reaction proceeded in the region where cortical alveoli did not break down. On the other hand, the climax of cortical reaction stepped into a subordinate phase in the region where cortical alveoli have broken down. It was the brief process of the subordinate phase: several cortical alveoli inside the new cell membrane got together and inosculated into a big vesicle. Later, the vesicle inosculated with the new membrane and released inclusion of the vesicle. Consequently, there appeared 78.100 μm lacuna on the surface of the egg (Plate I. 14). The cortical reaction of the subordinate phase occurred at the same time in different regions. Five minutes after fertilization, the subordinate phase of the climactic period was entirely over. Most of the cortical alveoli were released inside the new cell membrane that formed again. There were only type V cortical alveoli, the others were remnant cortical alveoli and chipping of yolk (Plate I. 15). Thus, the climax was entirely over. Micropylar apparatus was markedly elevated entirely, and the arcual cell membrane below the micropylar apparatus regularly restored (Plate I. 16). Eight minutes after fertilization, cortical reaction was entirely over, and then the blastoderm formed and the membrane of the ovum was elevated.

3.2.2 Observations in TEM

Ten seconds after fertilization, the outside of the cortical alveoli began to inosculate each other, and was about to form a big vesicle (Plate II. 19). Forty seconds after fertilization, type II cortical alveoli broke down. Along with the cortical alveoli released, microvillus and some of the cell membrane moved into the ovum lacuna (Plate II. 21). The plasma membrane of the cortical alveoli and the cell membrane regrouped and formed a new plasma membrane (cell membrane) at the base of the cortical alveoli. The external surface area of the new membrane was rough, and adsorbed some granules. Inside the new membrane, cortical alveoli began to inosculate each other (Plate II. 20). The process was as follows: the contiguous cortical alveoli moved to the cell membrane and was close with each other. Because of cortical alveoli extrusion, the liquid component of the cytomatrix

flowed to other regions, and the organelles, such as lysosome and mitochondrion, got together along the cortical alveoli. Later, the membrane of the organelle merged with the membrane of the cortical alveoli. Because of cortical alveoli extrusion, the membrane of the cortical alveoli was laniated, and the contiguous cortical alveoli inosculated each other and formed a big interconnected vesicle. Once the vestibule broke down, it indicated that several cortical alveoli were released at the same time. Eighty seconds after fertilization, the outer cortical alveoli broke down in the proper orders, and the ovum lacuna was formed. Outspread of solicitation speckle could result in forming the ovum lacuna. In the regions where cortical reaction did not occur, the diffusing granules in solicitation speckle accelerated to form the ovum lacuna, and separated the ovum vestibule from the cell membrane too. At 110 seconds after fertilization, the outer cortical alveoli broke down entirely, and the new plasma membrane was smooth and looked like the coherent base of the cortical alveoli. Most of the membrane was lost during cortical reaction, and the lost membrane was regenerated with cortical alveoli. Inside the new cell membrane, plentiful organelles, such as lysosome, mitochondrion and endoplasmic reticulum (Plate II. 22) were concentrated. At 240 seconds after fertilization, cortical reaction in the climactic period came near to its end, and there was only the type V cortical alveoli that would be released in the ovum. The lost plasma membrane was never filtered into the fecundation envelope and was never concerned with the fecundation envelope too.

3.2.3 Observations in SEM

Forty seconds after fertilization, cortical reaction happened and released flocculent inclusion. The remnant membrane of the cortical alveoli could be observed easily (Plate II. 28). The flocculent inclusion was the other marker that cortical reaction happened in SEM. Sixty seconds after fertilization, cortical reaction expanded to all the sides. There was an obvious boundary between the region where cortical reaction appeared and the region where cortical reaction did not (Plate II. 29). In the region where no cortical reaction appeared, the plasma membrane was kept whole, and there were a lot of bushy microvilli. The region where cortical reaction occurred was very dark and we could see nothing. There was only a flocculent inclusion on the boundary. At 100 seconds after fertilization, cortical reaction expended in most of the ovum. There was some inclusion to be released from the cortical alveoli in the internal surface area of the primary envelope (Plate II. 31). The internal surface area of the micropylar apparatus was very smooth and clean, and there was no inclusion around the internal surface area of the micropylar apparatus, which suggested that no cortical reaction occurred near the micropylar apparatus (Plate II. 30). At 240 seconds after fertilization, the subordinate phase of the climactic period was entirely over in most of the ovum. The remnant cortical alveoli pierced into the new cell membrane where there was sparse microvilli (Plate II. 32). The other new plasma membrane that was fully restored was tender and had some bushy microvilli (Plate II. 33). Sparse microvilli appeared on the plasma membrane below the micropylar apparatus, which had no microvilli in the mature ovum (Plate II. 34).

4 Discussion

4.1 Stages of cortical reaction

Numerous studies show that there is a starting time and persistent climax in cortical reaction in fishes. To study it expediently, we divide cortical reaction into four parts including the latent period, developmental period, climactic period and declining period. Similar to *Fundulus heteroclitus* (Brummett and Dumont, 1981), cortical alveoli in *Hemibarbus labeo* distributes along a gradient, and different styles of cortical alveoli are released in turn. The boundary between the latent period and the developmental period is the initial point when a few type I cortical alveoli are released during cortical reaction. There is no obvious boundary between the developmental period and the climactic period, but the type I and type II cortical alveoli are released during the developmental period. By contrast, the type III and type IV cortical alveoli are released like a bull at a gate during the climactic period. Only type V cortical alveoli and the other residual cortical alveoli are released during the declining period. Similar to *Carassius auratus* (Gao, 1995), different cortical alveoli are released at different periods in *Hemibarbus labeo* because the inclusions of the cortical alveoli are different.

Latent period appears before cortical alveoli are released. No cortical alveoli are released, and no ovum lacuna appears in the latent period. Only the trend moved forward, and some cortical alveoli began to inosculate each other for preparing for release. The length of the latent period is directly affected by water. If there is no water around the mature ovum, there is a longer effective fertilization time. For instance, if there is no water at 20ºC–27ºC, there is more than 1–1.5 h effective time to impregnate in the mature ovum of *Ctenopharyngodon idellus* (Liu, 1993). According to this, we can speculate that the latent period is more than 1–1.5 h in *Ctenopharyngodon idellus*. With sperm entering the ovum, the latent period shortens sharply. In general, it will last 1 s to 2–3 min. After cortical alveoli breaks down, the ovum lacuna forms and the developmental period starts. The latent period is 10 s in *Carassius auratus* (Gao, 1996), 30 s in *Brachydanio rerio* (Wolenski and Hart, 1987), and 30 s in *Cyprinus carpio* (Kudo and Sato, 1985). The latent period is 35 s in *Hemibarbus labeo*. To retain the ovum for a long time, people often adopt dry fertilization to protract the latent period to facilitate enough contact between the ovum and the sperm. Different investigators have different cognition for *Carassius auratus* (Zhang, 1993; Gao, 1996), which could be associated with different experiment conditions such as temperature. Furthermore, the time that

the ovum lacuna appears is more different in viviperception and fixed preparation. The former is 3 min after fertilization (He, 1999), and the latter is 40 s after fertilization in this study in *Hemibarbus labeo*.

After cortical alveoli breaks down, the ovum lacuna is formed, and the developmental period starts. The ovum lacuna that appears between the cell membrane and the ovum envelope is not a result of the ovum envelope prolongation but from cortical alveoli release and ovum collapse. It is a typical feature that a single or a few cortical alveoli are released during the developmental period. The number of the released cortical alveoli is initially few, and forms an amaranthine speckle in H.E. So, the amaranthine speckle and the ovum lacuna are the markers that indicate that cortical reaction has entered the developmental period. The developmental period in *Hemibarbus labeo* is 35−55 s after fertilization.

The typical features of the climactic period are as follows: several cortical alveoli inside the new plasma membrane merge and inosculate into a big vesicle. Later, the vesicle inosculates with the new plasma membrane and releases inclusion of the vesicle. In *Hemibarbus labeo*, the peripheric cortical alveoli of about 1 to 3 rows inosculate into a big vesicle and are released, and then the inboard cortical alveoli of about 1 to 3 rows inosculate into a big vesicle and are released too. So, the climactic period can be divided into two phases. At the first stage, the peripheric cortical alveoli are released. At the second stage, the inboard cortical alveoli are released. After the inboard cortical alveoli are released, only a few cortical alveoli are left behind the ovum. According to this criterion, the climactic period lasts out about 4 min in *Hemibarbus labeo*, 2 min in *Carassius auratus* (Gao, 1996), and 1−3min in *Brachydanio rerio* (Hart and Yu, 1980).

The type V cortical alveoli and the other remnant cortical alveoli are released unceasingly. Eight minutes after fertilization, cortical reaction is entirely over, and then the blastoderm barely forms.

4.2 Starting point of cortical reaction

Generally speaking, cortical reaction begins from the site where the sperm enters the ovum in fish and moves rapidly from the animal pole to the vegetal pole; it is the reverse in *Rhooeus ocellatus* where the cortical reaction starts at the vegetal pole and then moves toward the animal pole (Ohta, 1990). Moreover, there is no cortical alveoli below the sunk micropylar apparatus of *Fundulus heteroclitus* (Brummett and Dumont, 1979), *Cyprinus carpio* var. singuonensis (Hong, 1994). According to the photographs of allotetraploid hybrids of red crucian carp fertilization (Li, 2002), no cortical alveoli appears below the micropylar apparatus. During fertilization, while making tracks of the region near the micropylar tube where sperm enters the ovum, we did not detect cortical reaction in *Hemibarbus labeo* in H.E and SEM. The cortical reaction happens near the low latitude of the animal pole and near the vestibule of the micropylar apparatus. Later, the reaction stretches immediately on all sides from the two starting points. Finally, both starting points encounter and heal up at the vestibule of the micropylar apparatus. This is a new pattern where cortical reaction happens near the low latitude of the animal pole following *Rhooeus ocellatus*. No cortical reaction happens near the micropylar tube in *Hemibarbus labeo*, which suggests that it is restricted that cortical reaction chokes back polyspermy in some fish.

There are no cortical alveoli near the micropylar tube below the deep sunken micropylar apparatus, and there is no resistance when sperm enters the ovum. Cortical reaction results in the ovum to excite and shrink intensely. For instance, the ovum dwindles 1/4 in size in *Carassius auratus* during fertilization (Zhu, 1960). If no cortical alveoli appear near the micropylar tube, the sperm's expulsion from the ovum during the intense cortical reaction is avoidable. Thus, because there is no cortical reaction near the micropylar tube below the deep sunken micropylar apparatus, the sperm can efficiently enter the ovum quickly and successfully in *Cyprinus carpio* var. singuonensis (Gao, 1996) and *Hemibarbus labeo*. However, entering the ovum quickly results in polyspermy, which is very harmful to the oosperm. This leads to a dependence on other mechanisms to insure monospermy.

4.3 Solicitation speckle and the released cortical alveoli hastening the progress of cortical reaction

Only when there is water, even without sperm around the fish's ovum, can the ovum be excited (Liu, 1966). For instance, the excited ovum is apart from the sperm entering the ovum of *Brachydanio rerio* (Wolenski and Hart, 1987). So long as water exists, the ovum can be excited by some physical and chemical factors (Zhu, 1960). Ca^{2+} or glycoprotein in the ovum envelope can place a premium on cortical reaction. Because of the hypoosmotic substance, such as acidic mucopolysaccharide in the inclusion of cortical alveoli during fertilization, once the surface area of the plasma membrane (a semipermeable membrane), is increased with water, the cortical reaction will happen. Both the released cortical alveoli and the solicitation speckle between the plasma membrane and the ovum envelope can hasten the progress of cortical reaction, because both of them have hypoosmotic substances that is purple in H.E.

Type I cortical alveoli solicitation induces cortical reaction Type I cortical alveoli are the nearest to the plasma membrane, which is close to the osmotic ovum envelope, and it is fuscous which means that there is more hypoosmotic substance. During the latent period, most of them break down and releases hypoosmotic substance. Apparently, it is favorable for water moving in, which makes the surface area of the cell membrane increase and accelerates cortical reaction. Cortical alveoli break down in the ovum during

rigorous dry fecundation before meeting water in *Hemibarbus labeo*, which suggests that the mature ovum looks calm before meeting water or inseminate. But, intricate changes are happening inside it. A similar thing also happens in *Clupea pallasi* (Huang and Yan, 1997).

The solicitation speckle induces cortical reaction The solicitation speckle lies between the plasma membrane and the ovum envelope, and it is similar with the inclusion in type I cortical alveoli in H.E and TEM. What is more important is that there is no membrane around it. With water going through the ovum envelope, the granules diffuse in the solicitation speckle, which makes the surface area of the plasma membrane with water also increase and accelerate cortical reaction.

Cortical reaction solicitation during the climactic period The cortical alveoli are released largely during the climactic period, and it is the solicitation mechanism during the climactic period: with type I cortical alveoli released and the speckle solicitation, water goes through the ovum envelope and reaches the plasma membrane, and the surface area of the cell membrane with water enlarges, which is helpful in releasing the cortical alveoli. At the same time, many cortical alveoli inosculate into big vesicles. Once any part of the vesicle inosculates with the plasma membrane, the vesicle is released, that is, many cortical alveoli are released at the same time, and the surface area enlarges intensely and more cortical alveoli are released until the climactic period finishes. Therefore, we can draw the conclusion that cortical reaction is induced by type I cortical alveoli and the solicitation speckle, and is a volcanic chain reaction under water or the other lower osmotic pressure of fluids. In *Carassius auratus* during fertilization, the reaction can be observed under the naked eye (Zhu, 1960).

4.4 Cell membrane repair goes with cortical reaction

With cortical alveoli released in batches, the plasma membrane is continually destroyed and continually restored. Before cortical alveoli are released, the region below the micropylar apparatus shows bushy microvilli on the plasma membrane. During the developmental period, single cortical alveoli inosculate with the cell membrane and forms an inlaid membrane that will break down later, and the outer inlaid membrane will release into the ovum lacuna. The new cell membrane that just formed is rough and has no microvilli on it. During the climactic period, with large numbers of cortical alveoli released, the new cell membrane is destroyed again, and more inlaid membranes are released into the ovum lacuna. When the climactic period is over, the plasma membrane is restored. The new restored cell membrane is tender, and some microvilli are present on it in SEM. During the declining period, a few remnant cortical alveoli pierce into the new plasma membrane on there is sparse microvilli. When the cortical reaction finishes, sparse microvilli appear on the cell membrane below the micropylar apparatus, which have no microvilli in the mature ovum. The inlaid membrane in *Hemibarbus labeo*, *Cyprinus carpio* var. singuonensis (Hong, 1994) and *Brachydanio rerio* (Hart and Yu, 1980) is temporary. Some are released into the ovum lacuna, and the others are drawn back to the cytoplasm. Later research made clear that cell membrane repair is selective in *Brachydanio rerio* (Hart and Collins, 1991). The inclusion released from the cortical alveoli contact directly with the fecundation envelope, but the plasma membrane never filters into the fecundation envelope and is never concerned with the fecundation envelope in TEM.

4.5 Fecundation envelope near the micropylar apparatus is elevated at the soonest

After the cortical reaction happens, three reasons can be clarified that the fecundation envelope near the micropylar apparatus is elevated at the soonest in *Hemibarbus labeo*. First, the micropylar apparatus is funnelform and so there is more surface area to accommodate more cortical alveoli in the cytoplasm below the micropylar apparatus. Second, the cortical reaction lasts for a shorter time near the micropylar apparatus and more cortical alveoli are released in unit time. The more cortical alveoli are released, the higher the osmotic pressure into the ovum lacuna is, then the more water enters. Finally, the fecundation envelope near the micropylar apparatus is elevated at the soonest and forms a crescent pool named by Li in histological section (Li, 2002). Third, the micropylar apparatus is funnelform and it is easy to part from the cell membrane. Elevating the fecundation envelope rapidly makes the micropylar tube depart from the plasma membrane rapidly, which is useful for reducing polyspermy.

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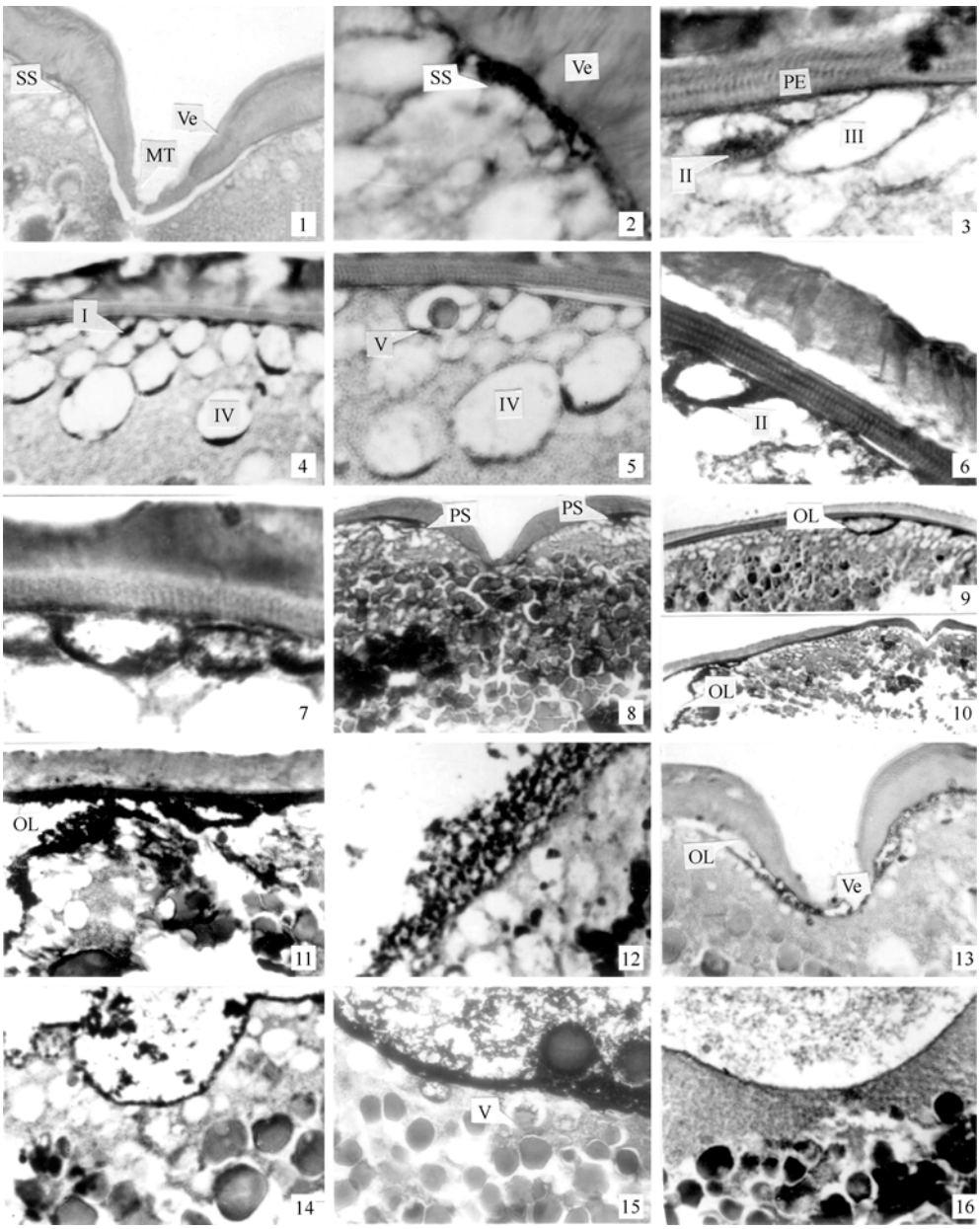
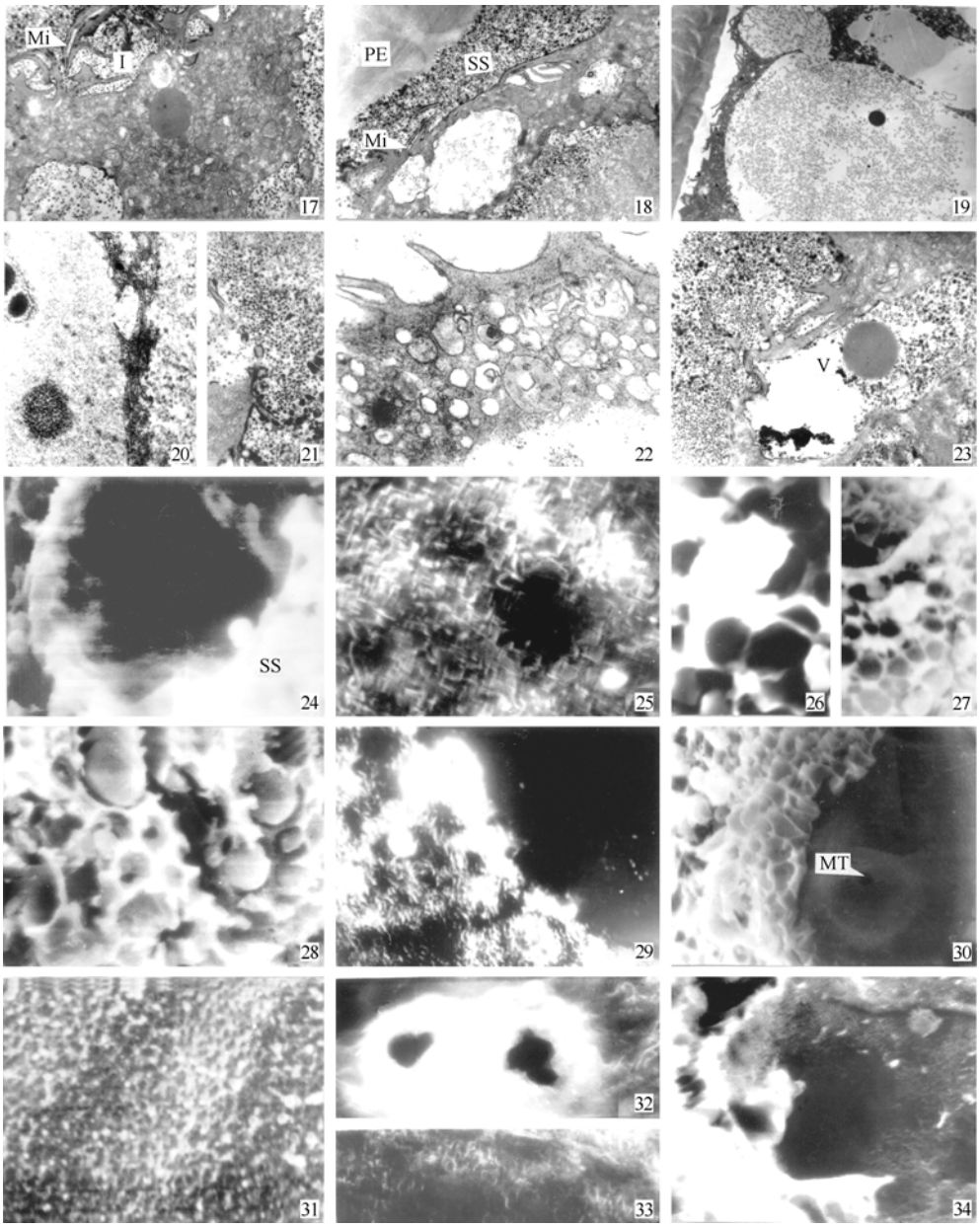


1: Showing solicitation speckle (SS) near vestibule (Ve) and micropylar tube (MT). H.E, ×600; 2: Magnification of Fig. 1, showing solicitation speckle (SS). H.E, ×2000; 3: Showing primary envelope (PE), secondary envelope (SE) and typeII,III of cortical alveoli. H.E, ×1400; 4. showing type III and IV of cortical alveoli. H.E, ×800; 5: Showing type IV and V of cortical alveoli. H.E, ×1200; 6: The type II of cortical alveoli breakdowns near low latitude of the animal pole. H.E, ×1200; 7: The cortical alveoli breakdowns near the type II of cortical alveoli. H.E, ×1400; 8: Purple speckle (PS) cortical reaction forms near micropylar apparatus of vestibule. H.E, ×240; 9: Ovum lacuna (OL) comes into being near cortical alveoli near low latitude of animal pole. H.E, ×200; 10: Showing micropylar apparatus (MA) and ovum lacuna (OL). H.E, ×96; 11: Magnification of fig.10, showing ovum lacuna (OL) H.E, ×480; 12: Cortical reaction of the first climactic period finishes. H.E, ×560; 13: Ovum lacuna (OL)comes into being near micropylar apparatus. H.E, ×480; 14: Cortical reaction of the second climactic period begins. H.E, ×560; 15: Showing the rudimental V of cortical alveoli. H.E, ×480; 16: Cortical reaction near micropylar apparatus finishes. H.E, ×480

Plate I



17: Showing type I of cortical alveoli and microvilli (Mi). TEM, ×15000; 18: Showing primary envelope (PE), solicitation speckle (SS) and microvilli (Mi). TEM, ×20000; 19: The outer cortical alveoli begin to syncretize. TEM, ×4500; 20: The inner cortical alveoli begin to syncretize. TEM, ×15000; 21: The outer cortical alveoli breakdowns. TEM, ×44000; 22: Cortical reaction of the first climactic period finishes. TEM, ×10000; .23: The rudimental type V of cortical alveoli. 24. No cortical alveoli below plasma membrane near micropylar apparatus. SEM, ×1500; 25: There have bushy microvilli on the plasma membrane SEM, ×3000; 26: There have abundant cortical alveoli below plasma membrane SEM, ×1500; 27: There have abundant cortical alveoli below the inner plasma membrane. SEM, ×1500; 28: Cortical reaction happens. SEM, ×1500; 29: Cortical reaction expands. SEM, ×1500; 30: There have something that cortical alveoli release in the inner primary envelope, showing micropylar tube(MT). SEM, ×6000; 31: There have nothing that cortical alveoli release in the inner surface of micropylar apparatus. SEM, ×1500; 32: The remnant cortical alveoli pierce into the new cell membrane on which have sparse microvilli. SEM, ×3000; 33: There have relatively bushy microvilli on the new plasma membrane that is restored fully. SEM, ×3000; 34: Sparse microvilli appear on the plasma membrane below micropylar apparatus. SEM, ×1500

Plate II