Electron Microscopic Observations on Sperm Penetration in Cytochalasin-treated Eggs of the Rose Bitterling

Tadayuki Ohta*, Mitsutaka Yoshida, and Shintaro Kato
Department of Biological Science, Aichi University of Education, Kariya City 448–8542, Japan

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ABSTRACT. The effects of cytochalasin B (CB), which acts on microfilaments, on sperm penetration into eggs of a teleost fish were investigated ultrastructurally. Eggs from the rose bitterling, Rhodeus ocellatus ocellatus, were pre-treated in physiological saline containing CB and inseminated in water also containing CB. Microvilli at the sperm entry site (SES) under the micropyle disappeared or shortened in length following CB treatment. However, a spermatozoon attached to and fused with the SES of CB-treated eggs. The spermatozoon present in a swollen mass (SM) remained at the egg surface even after membrane fusion and did not enter the cortex. It was unclear whether or not sperm movement from the cortex to the inner cytoplasm is CB-sensitive. The SM formed and plugged the micropyle. CB did not inhibit cortical alveolus breakdown. Based on the present experiments with fish eggs, it is concluded that CB inhibits sperm movement from the egg surface to the cortex, but not sperm attachment (binding), membrane fusion and SM formation during the process of sperm penetration.

The spermatozoon of a teleost fish is devoid of acrosomal structures. On the other hand, the egg is enveloped by an egg membrane (chorion) which has a micropyle at the animal pole. The egg plasma membrane just beneath the micropyle, through which a spermatozoon penetrates, is exposed directly to the exterior. A fertilizing spermatozoon is therefore able to attach to the egg plasma membrane without making a hole in the chorion. In eggs of the rose bitterling, attachment and subsequent fusion of a fertilizing spermatozoon with the egg plasma membrane results in formation of a swollen mass (SM) containing the naked spermatozoon after the first (14, 15).

Cytochalasin B (CB) acts on microfilament function by inhibiting actin polymerization. We previously investigated the effects of CB on SM formation (16) and found that SM formation was not inhibited by CB treatment. These preliminary observations of CB-treated eggs showed that sperm penetration seemed to occur, but that penetration of the sperm flagellum was slow in contrast to that seen in non-treated eggs. However, superficial observations by scanning electron microscopy (SEM) cannot clarify the details of penetration. In many animals, sperm penetration into CB-treated eggs has been inhibited (3, 5, 11, 12, 17, 21). However, it is unclear which steps in the process of sperm penetration are CB-sensitive. The present report was conducted to investigate the CB-sensitive steps in the process of the sperm penetration of fish eggs, using scanning and transmission (TEM) electron microscopy, as well as fluorescence microscopy.

MATERIALS AND METHODS
Adult rose bitters (Rhodeus ocellatus ocellatus) were kept in aerated aquariums together with freshwater bivalves, Anodonta woodiana at 18–23°C (water temperature). During breeding seasons spermatozoa and eggs were obtained from males displaying nuptial colors and females with a lengthened ovipositor, respectively.

To observe sperm penetration, unfertilized eggs were transferred from physiological saline into tap water (water that had been boiled then cooled to 25°C, pH adjusted to 7.2 by N/10 HCl), and a sperm suspension was added (5–10 μl sperm in 5 ml water). At selected time intervals (0–20 min) after the beginning of insemination (BI), eggs were fixed for about 4 h with cold fixative (1.3% glutaraldehyde, 1.6% paraformaldehyde, 0.02 M s-collidin buffer, 0.08 M phosphate buffer, pH 7.2) containing 3% sucrose.

Cytochalasin B (CB, Sigma Chemical Co., St. Louis, MO) was dissolved in dimethylsulfoxide (DMSO, 0.5% or less final concentration at use) and diluted with physiological sa-
line or water to make stock solutions which were 80 μg/ml CB-4% DMSO in physiological saline or water. The stock solutions were refrigerated at about 4°C. Just before use, they were diluted in physiological saline or water to final concentrations of 3–10 μg/ml. For observations of sperm penetration into CB-treated eggs, unfertilized eggs that had been treated for 5 min in 10 μg/ml CB-physiological saline were inseminated in 5 ml of water containing the same concentration of CB. At selected time intervals (0–20 min), eggs were fixed for about 4 h with the same fixative described above.

After the fixed eggs were washed in 5% sucrose-0.1 M phosphate buffer overnight at 4°C, they were postfixed in 1% OsO₄-0.1 M phosphate buffer containing 3% sucrose for 1.5 h at the same temperature. The eggs were dehydrated in a graded series of alcohol solutions, immersed finally in isopropyl acetate and dried in a critical point dryer (Hitachi, HCP-2, Hitachi) with liquid CO₂. Specimens coated with gold were examined with a JEOL, JEM-2000FX electron microscope equipped with a scanning attachment (JEOL, Tokyo).

For TEM, eggs treated with 10 μg/ml CB and 20 min post-insemination were fixed in the same fixatives. They were then dehydrated in a graded series of alcohol and acetone solu-

Figs. 1–4. Morphology of a sperm entry site (SES) in cytochalasin B (CB)-treated eggs.

Fig. 1. Morphology of a SES just beneath the micropyle of a control (CB non-treated) egg. A tuft of many microvilli (MV) is present at the SES. x 9,000.

Fig. 2. A SES in an egg treated with 3 μg/ml CB (dechorionated for observations). Microvilli of the SES (arrows) are reduced in number and in length. x 6,000.

Fig. 3. A SES in an egg treated with 7 μg/ml CB (dechorionated). Microvilli have almost disappeared in many eggs. x 6,000.

Fig. 4. A SES in an egg treated with 10 μg/ml CB (dechorionated). Microvilli have disappeared as in eggs treated with 7 μg/ml CB. x 6,000.
Sperm Penetration in CB-treated Egg

tions before they were embedded in Quetol 812 (Nisshin EM Co., Tokyo). Sections cut with glass knives were stained with uranyl acetate and lead citrate and observed with the above described electron microscope.

For fluorescence microscopy, eggs incubated for 5 min in 10 µg/ml CB-physiological saline were transferred into water containing sperm and the same concentration of CB. After 1, 5, 10 and 20 min, they were fixed at least 18 h with the above fixative containing 3% sucrose. After the eggs were washed in 0.1 M phosphate buffer, they were stained for 2 h with Hoechst 33342 (Sigma Chemical Co. St. Louis, MO) at a concentration of 10 µg/ml to reveal nuclei or chromosomes of the fertilizing spermatozoon and egg. To eliminate spermatozoa attached to the chorions, eggs were dechorionated with fine forceps while they were being washed in 0.1 M phosphate buffer containing 5% sucrose. Regions in and around the sperm entry site (SES) of the dechorionated eggs were observed and photographed with Fuji-color 1600 film through a

Figs. 5-8. Sperm penetration in control eggs.

Fig. 5. An egg fixed 1 min after the beginning of insemination (BI). A fertilizing spermatozoon is attached at the SES. A flagellum (F) is still present outside of the egg. × 6,000.

Fig. 6. An egg fixed 5 min after BI. A swollen mass (SM) has formed and plugs the micropyle. × 6,000.

Fig. 7. An egg fixed 10 min after BI. × 6,000.

Fig. 8. An egg fixed 20 min after BI. The whole spermatozoon has penetrated the egg. × 6,000.

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fluorescence microscope (Olympus, BH2-RFCA, Tokyo) under conditions of U excitation.

RESULTS

Morphological changes in sperm entry site of cytochalasin B-treated eggs

A micropyle was present in the chorion at the animal pole of each egg. A tuft of microvilli was located at the SES just beneath the micropyle (Fig. 1). The membranes of the microvilli were the first site a fertilizing spermatozoon reaches. The morphology of the SES in most eggs did not change noticeably when the eggs were placed in physiological saline for 30 min (see 16). In contrast, treating the eggs with CB (3–10 μg/ml)-physiological saline for 5 min induced the microvilli to decrease in number or shorten in length, depending on the concentration of CB (Figs. 2–4).

Figs. 9–12. Sperm penetration in CB-treated eggs.

Fig. 9. An egg fixed 1 min after BI. A spermatozoon is observed at the SES. Sometimes a spermatozoon with a swollen head was observed. × 6,000.

Fig. 10. An egg fixed 5 min after BI. A flagellum is visible outside of the egg. × 6,000.

Fig. 11. An egg fixed 10 min after BI. The area of and around the SES in a dechorionated egg. A shortened flagellum was often visible. CB did not inhibit cortical alveolus breakdown. × 1,500.

Fig. 12. An egg fixed 20 min after BI. × 5,000.
The process of sperm penetration in controls (cytochalasin B non-treated eggs)
A fertilizing spermatozoon attached initially to microvilli of the SES immediately after insemination in water. In eggs 1 min after BI, a fertilizing spermatozoon attached to the SES was observed (Fig. 5). The long sperm flagellum was observed outside of the egg. Sperm-egg membrane fusion had begun within this time (see 14). In eggs fixed 5 min after BI, a SM (diameter, about 3.1 ± 0.7 μm, means ± S.E., n = 25) was observed (Fig. 6). The size of the SM was about 3.2 ± 0.3 μm (n = 25) at 10 min after BI (Fig. 7). Sperm flagella were observed in about 2% (1/42) of the eggs. Twenty minutes after BI, an almost spherical or slightly transformed SM (about 3.5 ± 1.3 μm, n = 36) plugging the micropyle was observed (Fig. 8). No sperm flagellum (0/24) was visible in superficial observations and the spermatozoon had entered the inner cytoplasm.

The process of sperm penetration of eggs in the presence of cytochalasin B
After 5 min of incubation in 10 μg/ml CB-physiological saline, unfertilized eggs were transferred from physiological saline to water containing the same concentration of CB. Sperm were added simultaneously. In some eggs observations of the sperm head and SM, or sometimes the sperm flagellum were impaired by substances covering the micropyle.

In about 73% (58/80) of the eggs at 1 min after BI, a fertilizing spermatozoon was observed in the micropyle (Fig. 9). The spermatozoon that adhered to the SES showed no notable difference in external appearance other than a slight swelling of the head in comparison with that fertilizing a control egg. Five minutes after BI, flagella were observed superficially in about 42% of the eggs (27/64, Fig. 10). The diameter of the SM or sperm head was about 3.5 ± 0.96 μm (n = 61). Ten minutes after BI, short sperm flagella were observed in about 18% of the eggs (12/68). Dechorionation revealed the egg surface under and around the micropyle. The eggs formed a large SM and did not show noticeable changes in their surfaces over the area devoid of cortical alveoli (Fig. 11). Twenty minutes after BI, sperm flagella were visible in about 13% of the eggs (5/40). The SM was present (Fig. 12) and the diameter was about 3.8 ± 0.97 μm (n = 24).

Figs. 13–14. Transmission electron micrographs of CB-treated eggs 20–30 min after BI.
Fig. 13. An ultrathin section of the micropyle of an egg fixed 20 min after BI. The sperm membrane has fused with the membrane of the SES. The sperm nucleus (N) and mitochondrion (M) are present in a SM. The fertilizing spermatozoon has not moved from the SM to the cortex. CH: chorion. × 15,000.
Fig. 14. An egg fixed 30 min after BI. The egg cortical alveoli have broken down. Components of cortical alveoli were present in a perivitelline space (PS). × 4,800.
Figs. 15–18. Fluorescent micrographs of sperm nuclei and egg chromosomes in control eggs. × 250.

Fig. 15. One minute after BI. A sperm nucleus (s of A) and egg chromosomes (e of B) are distinguishable.

Fig. 16. Five minutes after BI. A, a sperm nucleus in focus. B, egg chromosomes in focus.

Fig. 17. Ten minutes after BI. Three fluorescent spots are visible. A, a sperm nucleus in focus. B, a mass of egg or polar body chromosomes in focus.

Fig. 18. Twenty minutes after BI. The sperm nucleus in focus (A) seems to have decondensed slightly. B, two masses (arrows) of chromosomes (near nuclei of egg and polar body) in focus.

Although SEM observation seemed to indicate the sperm had penetrated the egg, TEM observations were required to verify whether or not complete penetration had occurred in the CB-treated eggs. Observations were conducted on sections through the micropyle in eggs fixed 20 min after BI. TEM confirmed sperm-egg membrane fusion and revealed that the fertilizing spermatozoon remained in the SM (Fig. 13).

CB treatment did not prevent cortical alveolus breakdown (CABD) by the eggs (Fig. 14). Part of the cytoplasm in and around the SES adhered to the chorion and separated from the rest of the egg cytoplasm upon CABD in control eggs. However, in CB-treated eggs 30 min after BI, this separation was not observed. Although CABD occurred in CB-treated eggs, changes in the cortical layer did not appear to be the same as those of control eggs. In addition, the form of the eggs was different from that of control eggs; the eggs flattened, especially at the vegetal pole.

Light microscopic observations revealed that no eggs inseminated in CB developed further (127/127). Even if they were washed in water without CB 10 min after BI, all eggs failed to cleave when incubated in this water (37/37).

Observation of sperm penetration in eggs by fluorescence microscopy

The sperm nucleus and egg chromosomes were stained clearly with Hoechst (Figs. 15A–18B). One and five minutes after BI, a sperm head was visible at the SES in all control eggs (Figs. 15A and 16A). Egg chromosomes were present at a distance from the SES (Figs. 15B and 16B). Ten minutes after BI the sperm nucleus at the SES had decondensed a little and the egg chromosomes were dividing (Fig. 17A, B). Twenty minutes after BI, decondensation of the sperm nucleus had advanced further (Fig. 18A). Two masses of chromosomes (near nuclei) of egg and polar body were present close to each other (Fig. 18B, arrows).

Similar observations were carried out with CB-treated eggs. In about 20% of the eggs observed 1 to 20 min after BI, a fertilizing spermatozoon was not observable at the SES (Table I). One minute after BI, a condensed sperm nucleus and egg chromosomes were observed (Fig. 19). Five and ten minutes after BI, two separated masses of chromosomes and a sperm nucleus were present (Figs. 20 and 21). Three fluorescent masses were present in eggs 20 min after BI (Fig. 22). In about 70–94% of the eggs examined 1–20 min after BI the sperm nucleus fluoresced at the SES, although the fluorescence was visible only in the cortical cytoplasm, not the inner cytoplasm (Table I).
Figs. 19–22. Fluorescent micrographs of sperm nuclei and egg chromosomes in CB-treated eggs. × 250.

**Fig. 19.** One minute after BI. A sperm nucleus(s) is visible at a SES. A mass of chromosomes(e) is present near the sperm nucleus. The appearance does not differ from that of control eggs.

**Fig. 20.** Five minutes after BI. Two fluorescent spots, a sperm nucleus, egg chromosomes are visible.

**Fig. 21.** Ten minutes after BI. Three fluorescent spots, a sperm nucleus, two masses (arrows) of egg and polar body chromosomes are visible.

**Fig. 22.** Twenty minutes after BI. The sperm nucleus (arrow) seems to remain at the position of the SES.

**DISCUSSION**

The following effects of CB on eggs and fertilization were observed: (1) microvilli at the SES just beneath the micropyle shortened or disappeared, depending on the concentration of CB; (2) cortical alveolus (granule) breakdown was not inhibited; (3) a SM was formed at the SES in inseminated eggs; (4) the process of sperm penetration into eggs differed from that into non-treated eggs; (5) sperm attachment (binding) and sperm-egg membrane fusion were not CB-sensitive in most eggs; but (6) movement of the naked sperm from the SM into the cortex was impaired.

In the carp and the rose bitterling, a fertilizing spermatozoon attaches first to the membrane of the microvilli at the SES just beneath a micropyle (9, 15). Membrane fusion between sperm heads and egg microvilli has begun within 10 sec postinsemination (14). After membrane fusion, the SES transforms into a SM which during early stages contains the naked sperm fated to move into the cortex. The SM enlarges increasingly until it plugs the micropyle, blocking possible penetration of excess spermatozoa following the fertilizing spermatozoon (15). With the lapse of time, the naked spermatozoon moves to the cortex and then the inner cytoplasm, and its nucleus soon transforms into a sperm pronucleus.

The morphology of the SES just beneath the micropyle varies among different species of teleosts (2, 6, 8, 9, 10, 15). In cyprinid fishes, the SES has many microvilli in a circle coinciding with the inside diameter of the micropylar aperture (6, 10, 15). The form of egg microvilli is maintained by filamentous actin at the SES in fish (7) and by an amorphous filamentous material, which is organized into microfilaments at fertilization, in the sea urchin (13). CB, a fungal metabolite, acts on cytoplasmic microfilaments by inhibiting actin polymerization (1, 4, 20). CB treatment inhibits microvillar elongation (13), fertilization cone formation and sperm incorporation in sea urchin (3, 12, 18), *Urechinus carpio* (5), *Spatula* (12) and starfish (11) eggs. In the zebrafish, however, fertilization cone formation was not inhibited, but sperm incorporation was (21). Treatment with CB resulted in reduction or disappearance of microvilli at the SES and SM formation in the rose bitterling (16) as well as in the zebrafish (21). Therefore, it is concluded that microfilaments function in maintenance of microvilli shape but do not participate actively in SM formation in rose bitterling eggs.

The processes of sperm penetration in the present species are divided into the following steps: (1) sperm attachment (binding) to the egg surface at the SES, (2) membrane fusion between sperm and eggs, (3) move-

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<th>Table 1. Sperm nuclei at the SES.</th>
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ment of the naked sperm into the cortex and then the inner cytoplasm, and (4) movement of the sperm pronucleus and its fusion with the egg pronucleus. There have been few reports so far on sperm-egg membrane fusion in CB-treated eggs. In the sea urchin, *Litocellina variegate*, sperm-egg binding and membrane fusion occur in the presence of cytochalasins (17). In the mouse oocyte, sperm head incorporation is not cytochalasin-sensitive, but sperm tail incorporation is sensitive (19). In the present SEM and TEM observations on CB-treated eggs, although sperm binding and membrane fusion occurred in many eggs, the fertilizing spermatozoon did not move to the cortex, remaining instead at the egg surface. Longo (13) reported that CB treatment after incorporation (membrane fusion) inhibited sperm movement from the fertilization cone into the egg cortex. These findings suggest that egg microfilaments may play an important role in sperm movement from the SM to the cortex. Wolenski and Hart (21) investigated the effects of CB on fertilization of zebrafish eggs and reported that a sperm cell failed to enter the cytoplasm in eggs fertilized after preincubation in CB. Simerly et al. (19) observed that penetration of the sperm tail was inhibited by CB treatment of mouse oocytes. In the present observations as well, a sperm tail was observed at the surface in many eggs until later stages after insemination compared with the controls. It is uncertain whether or not steps of (3) or (4) were CB-sensitive in the present observations. Moreover, the fate of the sperm tail in CB-treated eggs remains unresolved.

CB does not inhibit the cortical reaction in the sea urchin (3, 17) or in fish (21). This suggests that microfilaments do not participate in membrane fusion between the egg plasma membranes and the membranes of the cortical alveoli or in exocytosis of the contents. CB-treated eggs do not change form, while control fertilized eggs change to a globular form after CABD. Microfilaments seem to have a significant role in the maintenance of egg form after CABD.

REFERENCES


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