Appearance of the Incorporation Cone and Extrusion of the Second Polar Body in Hamster Eggs

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Abstract: *Fertilized hamster eggs were recovered from the ampulla of the oviduct at various times after in vivo insemination. To examine the distribution of the egg cortical actin and the position of nuclei from freshly ovulated unfertilized eggs to the 2-cell stage, they were double stained with Rhodamine-phalloidin and Hoechst 33342. In the freshly ovulated hamster eggs arrested at metaphase-II of meiosis, the egg surface above the meiotic spindle was devoid of microvilli and had instead conical projections. This area showed strong fluorescence, indicating that the actin was aggregated. As the sperm head was incorporated and began to decondense in the ooplasm, an incorporation cone was formed at the site of sperm entry. The cone consisted of relatively long, conical projections which closely resembled those of freshly ovulated eggs, and also showed strong fluorescence. This cone lasted while the sperm nucleus was decondensing and diminished at the time when the male pronucleus was formed. The results obtained with regard to the formation of the incorporation cone were comparable with those of mouse and rat eggs. Moreover, as to the elimination of second polar body, it was found that the body underwent two constrictions, resulting in a gourd-like body.*

Key words: *Cortical actin, Incorporation cone, Surface change, Second polar body, Hamster eggs.*

In marine invertebrates, it is well known that a cone of cytoplasm termed the 'fertilization cone' forms in association with the fertilizing sperm [1, 2]. The invertebrate cone forms at the time of sperm-egg interaction and may function in sperm incorporation [3]. On the other hand, subsequent to entry of the spermatozoa into the mammalian egg, a bulge appears at the site of the sperm entry. This protuberance has been termed the incorporation cone or the fertilization cone [4, 5].

Although a small cone has been reported in rabbit [6], rat [7–11], mouse [5, 12–14] and human [15], there are a few detailed studies on hamster eggs [16, 17]. In this paper, we report the appearance and structure of the cone at the sperm-egg interaction of the hamster. We also describe the formation and the extrusion of the second polar body.

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Materials and Methods

Eggs recovery at various stages of maturation division and 2-cell development

Mature male and female golden hamsters were housed in a room with controlled temperature (22 to 25°C) and with an 11 hours light cycle (7:00 to 18:00). The females were induced to superovulate by injecton of hormones. The females were injected with 30 IU of pregnant mare serum gonadotorophin (PMSG) on the morning of day 1 of the estrous cycle, and then with 25 IU of human chorionic gonadotrophin (hCG) on the evening of Day 3. Ovulated unfertilized eggs were obtained from the females, 13 to 15 hr after hCG injection, and were recovered by flushing them from the ampulla of the oviducts with Dulbecco's phosphate-buffered saline with 0.1% bovine serum albumin (PBS with BSA), pH 7.4. In order to obtain fertilized eggs *in vivo*, dry sperm was squeezed out after one cauda epididymis was punctured in several places with forceps. A sperm suspension was prepared in a watch glass in 2 ml of a 0.85% NaCl solution. Ten to 12 hr after the injection of hCG, the uterine horns were exposed under ether anesthesia and a 0.2–0.3 ml sperm suspension was injected into each horn of the uteri by means of a tuberculin syringe with a No. 26 gauge needle [18]. The females were sacrificed 5–25 hr later and their oviducts were isolated in PBS with BSA. The fertilized eggs were recovered by the above mentioned method. The

eggs surrounded by cumulus cells were treated with 0.1% Hyaluronidase (Type II, Sigma) to disperse the cumulus cells. They were thoroughly washed in fresh PBS with BSA, and the zona pellucida was removed from the eggs with 0.1% Trypsin. The eggs were again thoroughly washed with fresh medium. All the procedures were performed at room temperature, about 18–23°C. The ovulated unfertilized and fertilized zona-free eggs thus obtained were prepared immediately for studies with fluorescence microscopy and scanning electron microscopy as described below.

Rhodamine-phalloidin and Hoechst 33342 staining

To examine the distribution of the polymerized actin and the position of the egg nucleus at various stages of maturation division or sperm nucleus, oocytes or eggs were fixed with 7% formalin in a stabilization solution (30 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 10 mM Pipes, pH 6.9) for 30–60 min, rinsed in a stabilization solution containing 0.1 mg/ml Saponin (Merk) for 30–60 min and then treated with 165 ng/ml Rhodamine-phalloidin (Rh-phalloidin) for 60 min. Rh-phalloidin (Molecular Probes, Inc. Or) was diluted from the stock solution (3 mg/ml in ethanol) in a stabilization solution immediately before use according to the procedure described by Shimizu [19]. After a thorough washing in stabilization solution for more than 30 min, the eggs were immersed in Hoechst 33342 containing 100 μg/ml in a stabilization solution for 30 min. In order to observe double stained eggs, they were placed on a glass slide beneath a coverslip supported by vaseline spots, and the coverslip was slightly compressed with a needle. Eggs double stained with Rh-phalloidine and Hoechst 33342 were examined and photographed in different focal planes on the same specimen, with illumination at 546 nm and 480 nm, respectively.

Study by electron microscopy

Zona-free oocytes and eggs were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 2 hr or overnight. After fixation, they were transferred to glass pieces, 5 × 5 mm, coated with a poly-L-lysine [18]. The glass pieces with attached ova were dehydrated in a graded acetone series and dried in a Hitachi PCP-2 critical point drying apparatus. Specimens were coated with Pt+Pd in a Hitachi E-102 sputter coater and examined with a Hitachi S-2300 SEM.

Results

Fluorescent microscopy

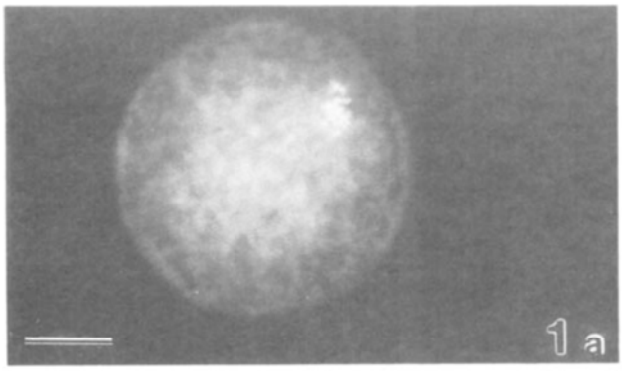
A total of 190 eggs recovered at different times after the artificial insemination *in vivo* were studied. All fertilized eggs obtained were monospermic. When freshly ovulated, unfertilized eggs recovered at 0 hr after the insemination were studied with fluorescent microscopy, and cortical fluorescence was revealed strongly on the egg surface overlying the second meiotic metaphase (Figs. 1a and b).

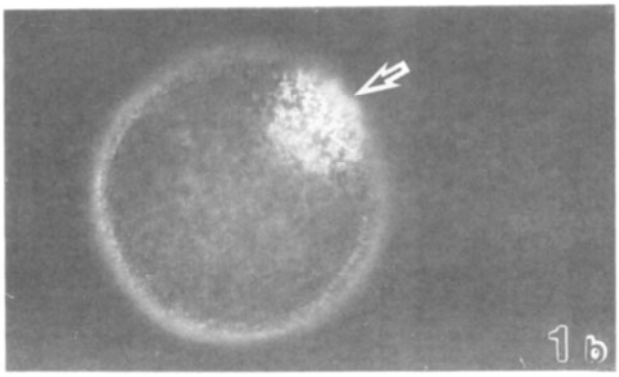
In the eggs recovered at 5–6 hr after the insemination *in vivo*, two cases were observed. While a spermatozoon was attached and/or fused to the egg plasma membrane, the resumption of the second meiotic division had not yet occurred (Figs. 3a and b). When the sperm head was incorporated into the ooplasm and had begun to decondense in the ooplasm, the eggs attained telophase-II (Figs. 4a and 5a). Moreover, it was found that a circular surface overlying where the sperm head was commencing its decondensation in the ooplasm showed stronger fluorescence than other areas of the egg cortex (Figs. 4b and 5b). This circular surface seems certain to be an 'incorporation cone', and also, the second polar body being extruded showed strong fluorescence (Figs. 4b and 6b). The strong circular fluorescent area, the incorporation cone, was maintained while the sperm head continued its decondensation (Fig. 6a).

In the eggs recovered at 8–9 hr after the insemination, it was observed that the male pronucleus was formed near the female one (Fig. 7a), and both its strong cortical fluorescence and the incorporation cone diminished. Also, the egg cortical fluorescence returned again to equal distribution (Fig. 7b). All eggs recovered at 23–25 hr after the insemination reached the 2-cell stage. The surface of each blastomere showed uniform cortical fluorescence (Figs. 8a and b).

Scanning electron microscopy

In the freshly ovulated, unfertilized eggs, it was always found that a part of the egg surface was devoid of microvilli and had long conical projections (Fig. 2). It is well known that this area remains on the rest of the egg surface after the first polar body has extruded. On the eggs recovered at 7–8 hr after the insemination, it was found that the sperm head was completely incorporated into the ooplasm, but the entire length of the sperm tail still projected freely over the egg surface (Figs. 9 and 10), or partially attached in a wave-like fashion to the egg surface (Fig. 11). In the same stage, the second polar body was eliminated from the area where the first polar body had been extruded (Figs. 10 and 11). Moreover, it was observed that an area, 10 to 15 μm in diameter with long conical projections developed at the





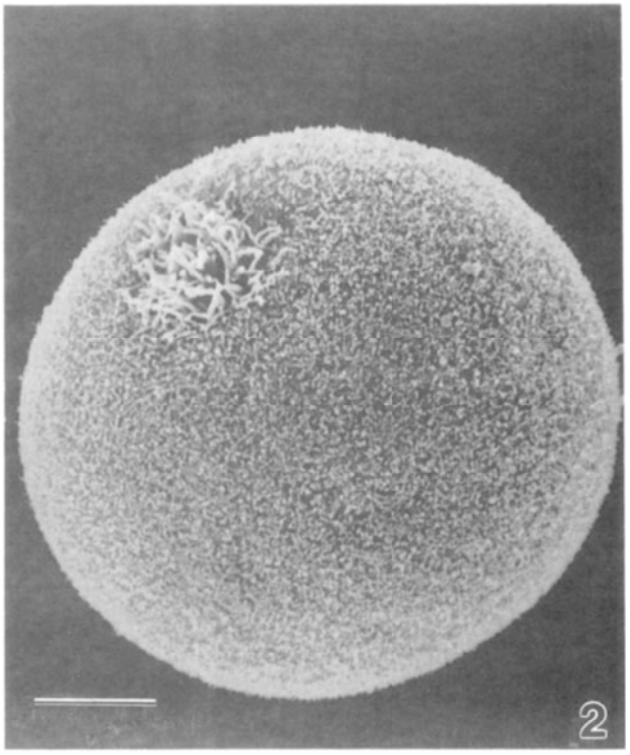


Fig. 1. A freshly ovulated, unfertilized egg double stained for chromosomes and actin. Note the chromosomes of metaphase-II (a) and strong fluorescence of the egg cortex (arrow) overlying the chromosomes (b). Bar represents 20 μm.

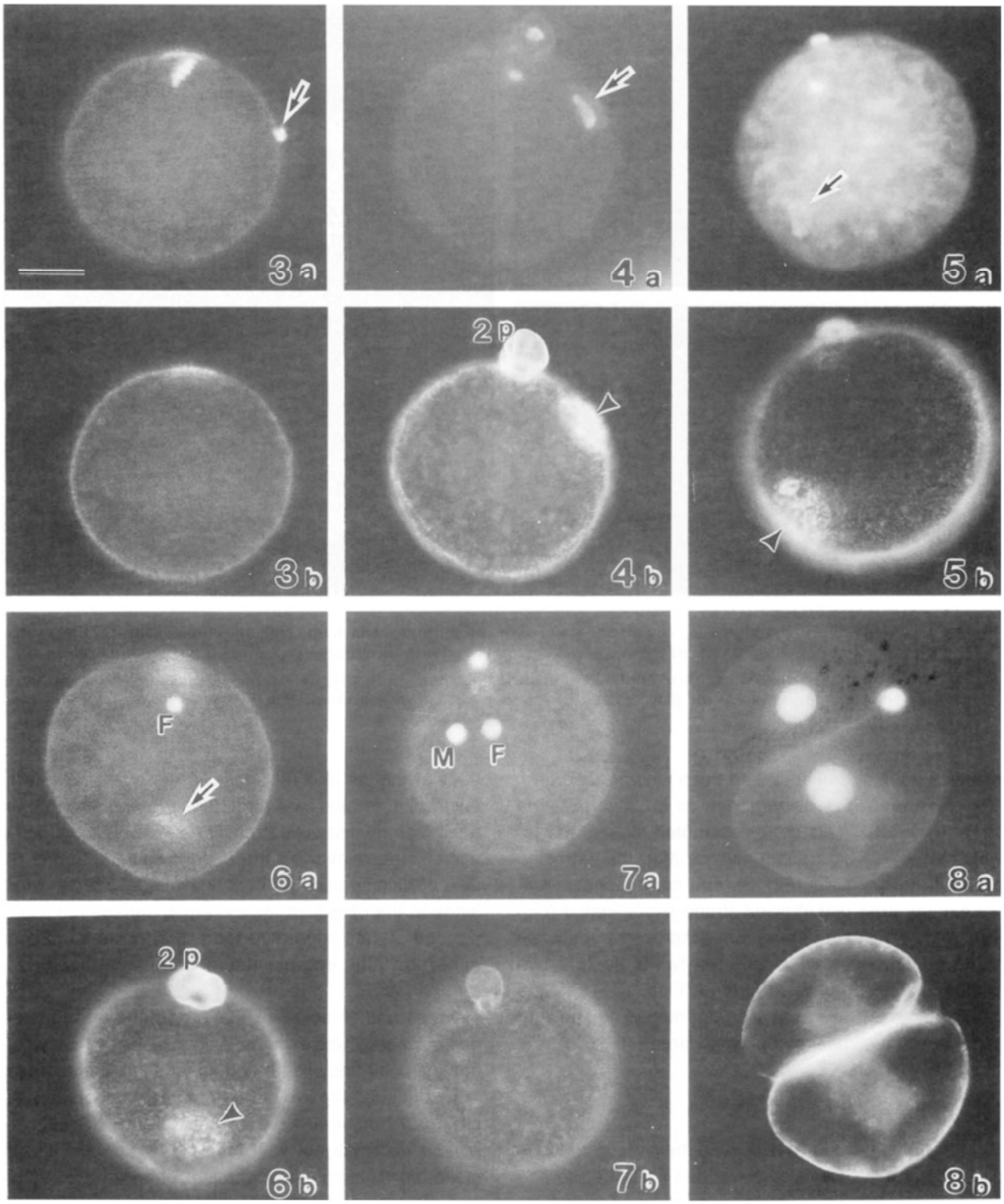
Fig. 2. SEM of an unfertilized egg recovered 13h after hCG injection. Note a region on the egg surface with a cluster of cytoplasmic projections at the place where the first polar body was extruded. Bar represents 10 μm.

site where the sperm head had been incorporated (Fig. 12). This area may be regarded as an 'incorporation cone' (Fig. 12). This cone resembled closely that of which remained on the egg surface after the extrusion of the first polar body (Fig. 2), and corresponded with the circular area that showed strong fluorescence (Fig. 1b). It was observed that the second polar body while being eliminated was constricted twice, so that two bulges, like a gourd, were formed (Figs. 13–15). The small bulge of the gourd was connected to the egg surface with a slender cytoplasmic strand (Fig. 15).

Discussion

In this paper, we have described the surface changes on hamster ova recovered at various times before and after insemination *in vivo*, using an actin-specific fluorescent probe, Rh-phalloidin and SEM. Freshly ovulated hamster eggs arrested at metaphase-II, possessed an area which was devoid of microvilli and had instead long conical projections. This area remains on the egg surface after the first polar body has extruded [18, 20–23]. In mouse and rat eggs, on the contrary, a large microvilli-free area was formed on the egg after the extrusion of the first polar body [24–27]. This area, rich in microfilament, overlies the meiotic spindle, and is devoid of binding sites for concanavalin A, a lectin which binds to the carbohydrate moiety of surface molecules [28, 29]. It has been reported that attachment of spermatozoa to the egg plasma membrane in this area is rare [20, 28].

Hirao and Hiraoka [18] have reported that the second polar body was extruded a short distance from where the first polar body had emerged (cf. Figs. 9 and 10, ref. 18), and that the region where the first polar body emerged remained as a circular area with relatively sparse microvilli but with long conical projections instead, and that this area was almost unchanged before and after the fertilization. However, in this study, we observed that the extrusion of the second polar body occurred in the same region where the first polar body had emerged. This fact strongly suggests that the amplification of the egg surface contributes to supplying a part of the membrane covering the second polar body,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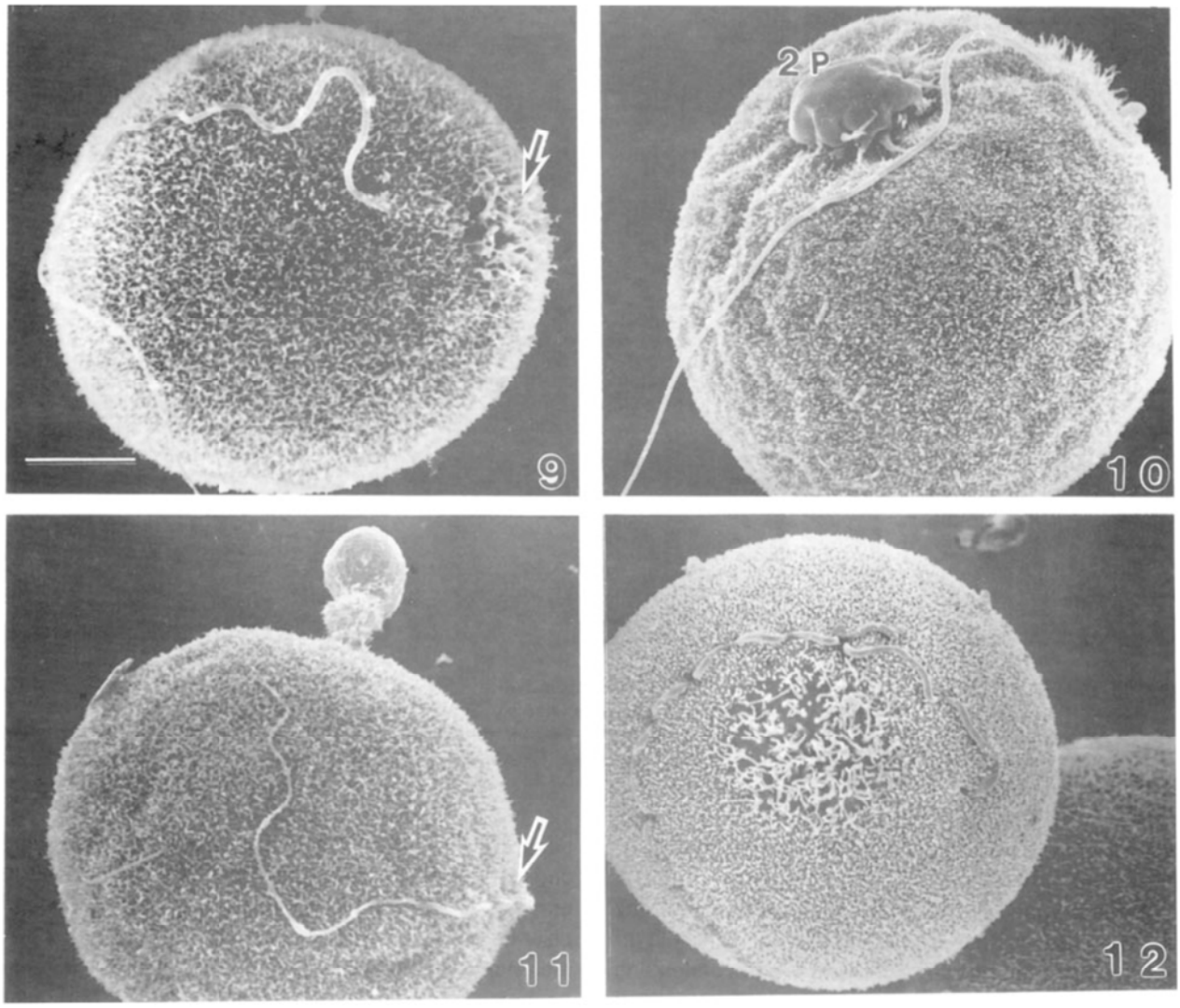


Figs. 9–12. SEM of fertilized eggs recovered 7–9 hr after the insemination. Fig. 9: note that the incorporation cone with long conical projections (arrow) is seen at the site of sperm entry. Fig. 10: shows the extrusion of the second polar body (2p) and the long sperm tail projecting freely over the egg surface. Fig. 11: note the entire length of the sperm tail partially attached to the egg surface (arrow). Fig. 12: shows the polar view of the incorporation cone well developed, and the sperm tail attached in a wave-like fashion to the egg surface. Bar represents 10 μm.

Fig. 3. Egg recovered 5–6 hr after the insemination *in vivo*. a: showing a sperm head (arrow) on the egg surface. b: showing strong fluorescence of the egg cortex, particularly the area where metaphase-II chromosomes are located. Bar represents 20 μm.

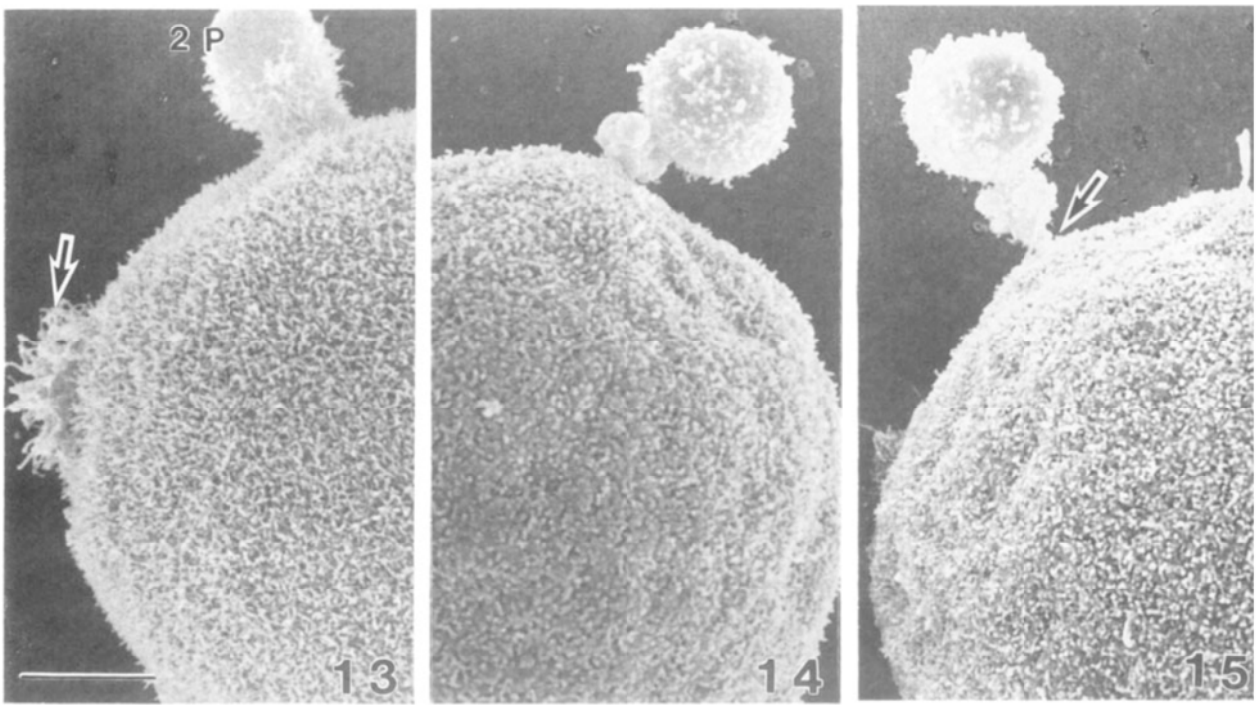
Fig. 4. Egg recovered 5–6 hr after insemination *in vivo*. a: showing telophase-II and decondensing sperm head (arrow). b: showing the second polar body being eliminated (2p), and the incorporation cone with strong cortical fluorescence (arrowhead).

Fig. 5. A fertilized egg, showing telophase-II and decondensing sperm head (arrows) (a), and incorporation cone (arrowhead) with strong circular fluorescence (b).

Fig. 6. A fertilized egg recovered 7–8 hr after the insemination. a: note the female pronucleus (F) and decondensing sperm head (arrow). b: showing the second polar body (2p) and a circular area, incorporation cone (arrowhead) with strong fluorescence.

Fig. 7. A fertilized egg recovered 9–10 hr after the insemination. a: showing the female pronucleus (F) and male pronucleus (M). b: note the uniform distribution of the cortical fluorescence.

Fig. 8. 2-cell egg recovered 20–23 hr after the insemination. Note large nucleus in each cell and that of the second polar body (a), and the uniform cortical fluorescence (b).



Figs. 13–15. SEM of fertilized eggs recovered 9–10 hr after the insemination. Fig. 13: shows the second polar body (2p) being eliminated and a side view of the incorporation cone (arrow). Fig. 14: note that the second polar body underwent double constrictions, resulting in a gourd-like body. Fig. 15: note that the small bulge of the gourd connects to the egg surface with a slender cytoplasmic strand (arrow). Bar represents 10 μm.

instead of synthesizing new membrane materials [23, 30].

We also found that a new circular area with many long cytoplasmic protrusions consisted of a thick layer of actin in the egg cortex overlying the decondensing sperm head in the ooplasm. In mouse and rat eggs, it has been reported that an unique change in the actin distribution occurs after fertilization, and that this change is related to the formation of the incorporation cone at the site of sperm entry [9, 14, 29]. It is clear, in hamster, that the incorporation cone is much alike to the pattern of the cortical actin, but different in the distribution of microvilli compared with those of the mouse and the rat. This cone lasted about 3–4 hr and disappeared at the time when the male pronucleus was formed near the female one. We speculate that while this cone lasted cortical actin was required for the restoration of the disturbances of microvilli and the egg cortex at the site of sperm entry and not for the cone formation at the time of sperm-egg interaction. Also, changes in the actin distribution must be closely related to the rotation of meiotic apparatus, and to the formation and the extrusion of the second polar body. The forming second polar body was protruded as a bulge almost free of the microvilli which showed strong fluorescence. This bulge underwent constrictions twice between the outer large one and the base of the egg surface, so that the second polar body eventually formed as a gourd-like body. Such morphological changes might be regarded as an indispensable movement for which there isn't a particular area on the egg surface after the extrusion of the second polar body. The distribution of actin in the egg cortex, in fact, returned to uniformity in the two-cell embryo. However, further work is needed to research the extrusion of the second polar body in detail and to define its role in connection with cortical actin during the maturation division and the sperm penetration.

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