韓國家畜繁殖學會誌 19(3) : 205~216(1995)
Korean J. Animal Reprod.

Microtubule and Microfilament Dynamics in Porcine Oocytes during Meiotic Maturation, Fertilization and Parthenogenesis

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돼지 난자의 성숙, 수정 및 단위발생시 Microtubule과 Microfilament의 움직임

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요 약

Microtubules와 microfilaments는 포유동물 난자의 주요한 세포 구조물들로, 이들은 난자의 성숙, 수정 및 배발달시 핵질의 이동과 세포질 분열에 직접 관여하는 것으로 알려져 왔다. 난자내 세포구조물의 정확한 움직임은 정상적인 배 발달을 위해 필수적이다. Microtubules는 α, β- tubulin이 서로 연결되어 이루어져 있으며, 수정시 웅성 · 자성전핵 움직임과 세포분열시, 유사 및 감수분열시 그 역할을 한다. 생쥐를 제외한 대부분의 동물에서 microtubules의 역할은 수정시 정자가 centrosome을 난자내로 이전하여 sperm aster를 형성함으로써 시작된다고 보고되고 있다. 따라서 정자의 도움없이 배발달이 일어나는 단위발생시 microtubules의 형성은 연구들 사이에 흥미로운 연구대상이 되고 있다. 한편 microfilaments는 세포분열시 세포질을 분할하는 기계적인 역할을 하는 것으로 알려져 있으며, 최근 생쥐 난자에서는 정자의 난자내 융합과 웅성 및 자성 전핵의 이동에 관여한다고 보고되고 있다. 포유동물 난자의 체외성숙, 체외수정을 유도할 때 여러가지 비정상적인 핵움직임과 세포분열이 관찰되어지고, 낮은 배발달율이 보고되고 있는데, 최근 연구자들은 세포구조물, 즉 microtubules와 microfilaments의 비정상적인 역할에서 기인한다고 보고 있다. 따라서 포유동물 난자의 성숙 · 수정 및 단위발생시 세포구조물의 움직임과 역할 및 상호관계에 대한 정확한 이해는 체외수정율 및 배발달 향상에 중요한 기초 자료로 이용되리라고 본다.

(key word : microtubules, microfilaments, porcine ova, maturation, fertilization, parthenogenesis)

I. INTRODUCTION

Microtubules and microfilaments are major cytoskeletal components in mammalian ova and provide the framework for chromosomal movement and cellular division. Extensive changes of cytoskeletal organization occur during maturation and fertilization. The changes in cytoskeletons are essential for the normal meiotic maturation and the formation of the biparental diploid genome of the embryo. Disturbance of the cytoskeletal organization during *in vitro* maturation and fertilization can result in abnor-

mal gamete development and early embryonic death (Kim et al., 1996a&c).

Ⅱ. MATRUATION

During meiotic maturation in mammalian oocytes, considerable chromosomal and cytoplasmic changes occur including germinal vesicle breakdown (GVBD), chromosomal condensation, polar body extrusion, and the formation of the meiotic spindle. These structural changes are associated with changes in the organization of microtubules and microfilaments during specific phases of the cell cycle. Microtubules, homologous polymers of α-, β- and γ-tubulin, are dynamic and intrinsically polar filaments. The organization of microtubules is controlled by centrosomes located at the spindle poles and at kinetochores on chromosomes (Le Guen and Crozet, 1989). In the mouse, cell cycle transition after GVBD is accompanied by extensive reorganization of the microtubule network (Kubiak et al., 1992, Verlhac et al., 1994). However, the change in microtubule assembling during meiosis of mouse oocytes may not be a typical for that in other mammalian species. Unfertilized mouse oocytes contain numerous centrosomal foci in addition to spindle associated centrosomes. Following fertilization the centrosomal foci are attracted to the surfaces of both male and female pronuclei and are involved in pronuclear movement and in the formation of the mitotic spindle (Schatten et al., 1985; Maro et al., 1985). In contrast, in most mammals functional microtubules appear to be developed from the centrosome introduced by the sperm (Yllera-Frandez et al., 1992, Le Guen and Crozet, 1989; Long et al., 1993, Breed et al., 1994). Although cytoplasmic microtubules are not observed in most mammalian oocytes, treatment with taxol, a drug that promotes microtubule assembly, results in the formation of subcortical asters that nucleate microtubules in rabbit (Yllera-Ferandez et al., 1992) and sheep oocytes (Le Guen and Crozet, 1989). Le Guen and Crozet (1989) suggested that some mammalian oocytes possess centrosomal (or microtubule organization center, MTOC) material scattered in the cytoplasm. The distribution of microfilaments has also been studied in the mammalian oocytes. In matured mouse (Maro et al., 1984) and rat (Zernickka-Goetz et al., 1993) oocytes, microfilaments are located mainly in the cell cortex overlying the meiotic spindle. This domain rich in microfilaments seems to be responsible for the maintenance of the meiotic spindle and chromosomes in a peripheral position (Webb et al., 1986).

Very recently, Kim et al. (1996a) studied microtubule and microfilament dynamics in pig oocytes during meiotic maturation. The study has focused on the intergrated organization between cytoskeletal components and chromatin during maturation. Fig. 1. and 2. showed immunofluorescence localization of microtubules and microfilaments in porcine oocytes matured *in vivo* and *in vitro*. The study has demonstrated that both microtubule and microfilament dynamics are integrated and interact with chromosomal changes during oocyte maturation. The condensed chromatin may recruit cytoplasmic material dispersed in the cytoplasm after GVBD and evoke microtubule assembly, which is necessary for meiosis and maintenance of the metaphase plate. Microfilaments are involved in chromosomal movement to a peripheral position after GVBD which may be important for continued embryonic development after fertilization. The precise mechanism whereby chromosomes induce microtubule and microfilament organization remains unknown.

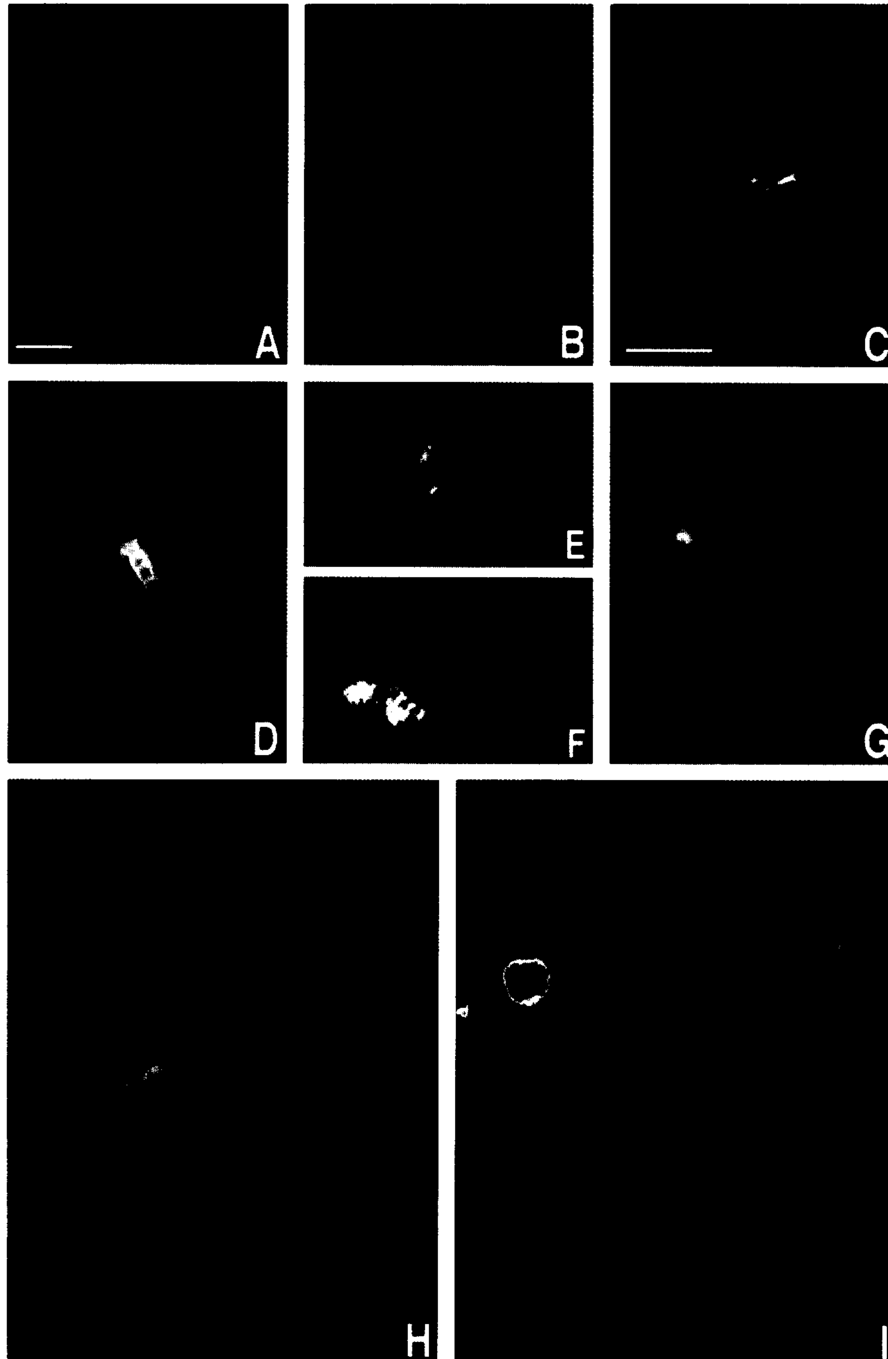


Fig. 1. Immunofluorescence localization of microtubules in porcine oocytes matured *in vivo* (C & H) and *in vitro* (A, B, D, F, G & I). Green, microtubules; red, chromatin; yellow, area of microtubules and DNA overlapping. Bar= 25 μm. A. At germinal vesicle stage, microtubules were not detected. B. After GVBD, microtubules were produced near the condensed

chromatin. C. During the prometaphase stage microtubule asters were observed with each group of chromatin. D. The aster elongated and encompassed the chromatin. At metaphase I, microtubules were only seen in the meiotic spindle. E. In telophase, the chromosomes divided in a state of metaphase. The microtubules were around chromatin. F. In some cases microtubules were found in a midbody (arrow). G. Metaphase plate and polar body at metaphase Ⅱ stage. H. Microtubules were detected only in the spindle. I. Taxol induced numerous cytoplasmic microtubules. The meiotic spindle became bigger and broader (arrow) and the polar body also induced microtubules (arrow head).

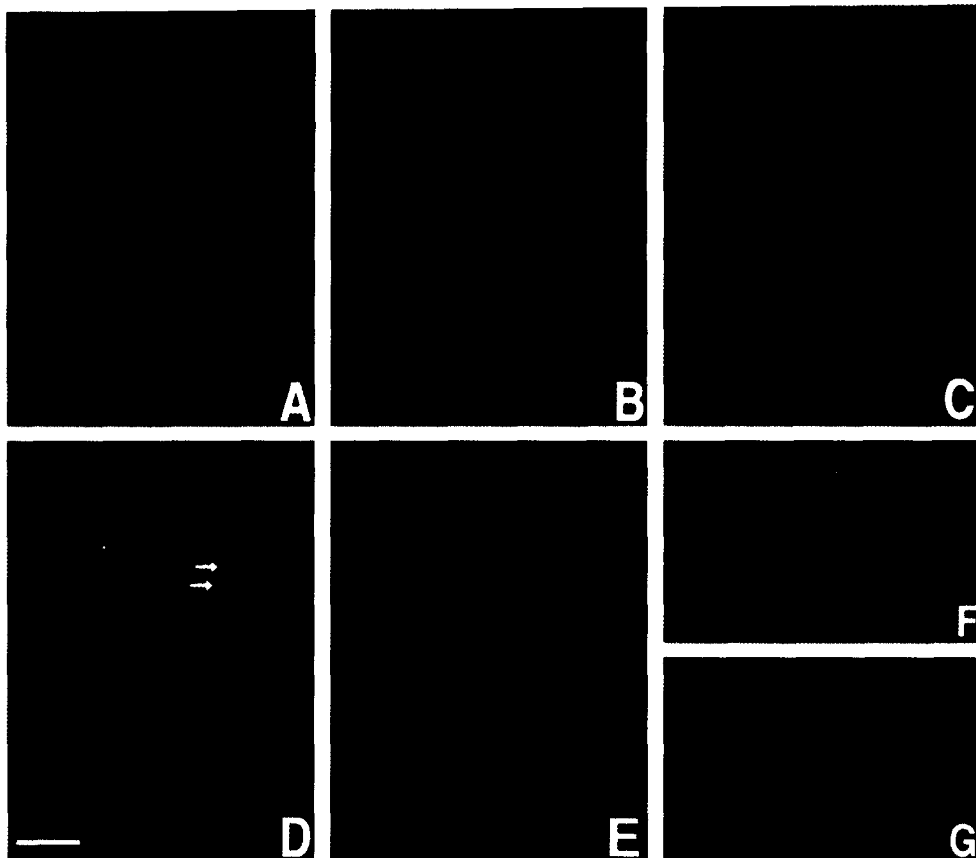


Fig. 2. Immunofluorescence localization of microfilaments in porcine oocytes matured *in vivo* (C & E) and *in vitro* (A, B, D, F & G). Blue, microtubules; red, chromatin; Bar= 25 μm. A. At germinal vesicle stage, microfilaments were observed around the cell cortex and as a disarrayed formation in the cytoplasm. B. After GVBD, the microfilaments were concentrated to the chromatin. C. At metaphase I, a thick and a thin microfilaments area existed in the egg cortex. Chromosomes were located in the microfilament rich domain. During anaphase to telophase chromatins were still connected the microfilament rich domain. D. At metaphase Ⅱ, the chromatins were located at the thick microfilament area. F, G. Abnormal patterns of microfilament organization and chromosome location in oocytes matured

***in vitro*. In some cases (F) chromosomes moved to the center. In other cases (G) an abnormal shape of microfilaments was observed.**

Ⅲ. FERTILIZATION

Observations made during fertilization in bovine oocytes showed that an aster of microtubules is seen adjacent to the incoporated sperm head (Navara et al., 1994). This sperm aster enlarges during sperm decondensation and extends into the total cytoplasm at the time of pronuclear apposition. Such a distribution suggests that microtubules derived from the sperm aster are involved in the movement of both pronuclei by pulling them toward the center of the cell. This observation is consistent with the findings in most animals (Schatten, 1994). In the mouse, however, the centrosome is organized from the centrosomal foci which preexisted in the cytoplasm of the unfertilized oocyte (Maro et al., 1985). Szollosi and Hunter (1973) have studied ultrastructural aspects of fertilization in the pig by electron microscopy. They observed the appearance of clusters of electron-dense, filamentous materials in the cytoplasm and in the absence of a centriole associated with the incorporated sperm, suggesting a maternal origin of centrosomes in porcine zygotes. However, Kim et al. (1994) have observed, unequivocally, a sperm aster immediately after sperm penetration. Further, multiple sperm asters form in polyspermic oocytes after *in vitro* fertilization providing additional evidence that the sperm centrosome organizes microtubules in the pig.

Although paternal inheritance of a functional centrosome has been suggested in most animals, it is still controversial whether the sperm itself contributes the centrosome. Since cell divisions are successful after parthenogenesis, the oocytes must contain sufficient maternal materials to organized a bipolar mitotic apparatus. This has led to the hypothesis that the sperm introduces a strong attractant for recruiting centrosomal material stored in the oocyte (Steans and Kirschner, 1994). Kim et al. (1996a) treatment of unfertilized pig oocytes with taxol, a drug that promotes microtubule assembly, induced numerous microtubule foci (400~500), but taxol did not induce microtubules in fertilized eggs when treated with taxol(Kim et al., 1996e). This result suggests that the sperm aster may be produced by collecting centrosomal material (γ-tubulin) which preexisted in the cytoplasm. Fig. 3. shows microtubule organization in pig oocytes during fertilization.

The distribution of microfilaments has also been studied in mammalian ova. In mature mouse (Maro et al., 1984) and rat (Zernicka-Goetz et al., 1993) oocytes, microfilaments are located mainly in the cell cortex overlying the meiotic spindle. This domain, rich in microfilaments, seems to be responsible for the maintenance of the meiotic spindle and chromosomes in a peripheral position (Webb et al., 1986). Maro et al. (1984) found that following fertilization or parthenogenetic activation of mouse oocytes, this domain disappears and microfilaments are concentrated around the pronuclei. Recent study (Kim et al., 1996b) demonstrated that, in mature pig oocytes, two domains (a thick and a thin microfilament area) exist in the oocyte cortex. Chromosomes were located in the thick microfilament domain of the cortex, which may be important for polar body extrusion and normal embryonic development after fertilization. Recent data in our lab also showed changes of microfilament distribution during the cell cycle (Kim, Chung, Day, unpublishied data). How-

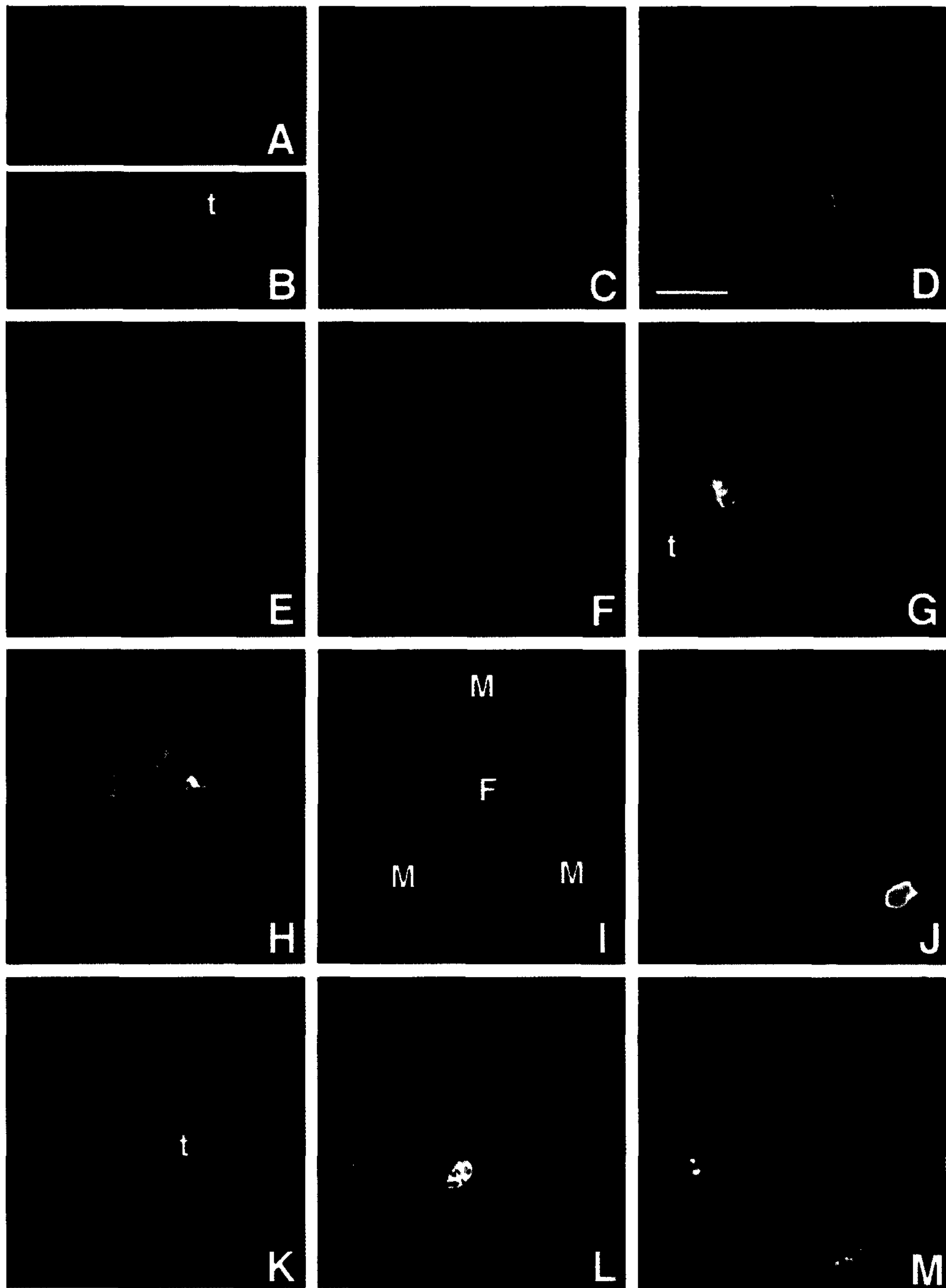


Fig. 3. Laser scanning confocal microscopic image of microtubules and chromatin in the porcine oocyte during fertilization. Green, microtubules; red, chromatin, Bar = 20 μm. t, sperm tail A. Microtubules are seen only in the meiotic spindle in the metaphase Ⅱ stage oocyte. B. Shortly after sperm penetration, microtubules are found in association with the incorpor-

ated sperm head and C. female chromatin emitted the second polar body and forms a midbody. D. Sperm aster enlarges and no microtubules are observed in the female chromain. E. At the time of pronuclear apposition, microtubules fill the whole cytoplasm. F. After male and female chromatin union, the microtubule matrix is less detectable. G. The eccentric mitotic metaphase spindle has tightly focused anastral poles and the chromosomes are aligned on the spindle equator. H. By telophase, astral microtubules are prominent and nuclei are reconstituted. I. During polyspermy, multiple sperm asters are observed in associated with each male chromatin (M). No microtubules are observed in the female chromatin (F). J. Taxol induces numerous cytoplasmic microtubules in the matured, unfertilized oocyte. K. After sperm penetration, the number of cytoplasmic microtubule foci decrease in the cytoplasm. L. Taxol treatment does not induce cytoplasmic microtubules but does induce larger sperm asters in the dispermy zygote. M. At the 4-cell stage, microtubules appears contiguously in the cytoplasm of daughter blastomere.

ever, very limited information is available on this subject for species other than the mouse.

Ⅳ. PARTHENOGENESIS

In mammals, parthenogenesis is the extraordinary process in which the oocyte initiates cell division without paternal contribution. Study of parthenogenesis has contributed considerably to the understanding of many aspects of early embryonic development. Parthenogenetic activation can be induced by a variety of stimuli such as electrical shock, Ca^{2+}, inositol triphosphate, heat, alcohol, cycloheximide, puromycin, ets. In the mouse, parthenogenetic embryos are capable of development through the preimplantation period and progress to the somite stages of development following implantation (Kubiac, 1989). In general, parthenotes in mouse and rabbit have well developed embryonic tissue but poorly develped extraembryonic membranes. Activation of porcine (Funahashi et al., 1994; Kim et al., 1996b) has been studied. A relatively high number of pig oocytes (70 to 80%) formed pronuclei after activation. However, considerably fewer activated eggs developed to morulae or blastocysts as compared to *in vitro* fertilized eggs (Funahashi et al., 1996). Since paternal inheritance of a functional centrosome has been suggested for most animals, it is possible that impaired development of parthenotes may be the result of the absence of fertilizing sperm centriole. The cell cycle in the parthenote occurs on schedule; the maternal chromosomes condense at mitosis and decondense during the next interphase. In the absence of a reproducing centrosome, Matia (1984) described as 'apolar' or 'nonpolar' mitosis in which the single cell undergoes repeated rounds of division attempts, but can only form monoasters each cycle. Curiously, parthenogenetic rabbit blastocysts display centrioles, structures not normally observed in early development in this species (Szollosi and Ozil, 1991). Recent studies in the bovine parthenogenetic oocytes (Navara et al., 1994) showed disarrayed microtubules in the cytoplasm and some microtubules extended from the remnants of the second meiotic spindle. These parthenotes then formed bipolar spindles and divided normally. These results suggest that mammalian oocytes are able to form a functional centrosome in lieu of any contribution by the sperm. Fig. 4. showed microtubule organization during parthenogenesis in porcine oocytes (Kim et al., 1996c).

Microfilament organization after activation has been studied in mouse and rat oocytes (Ma-

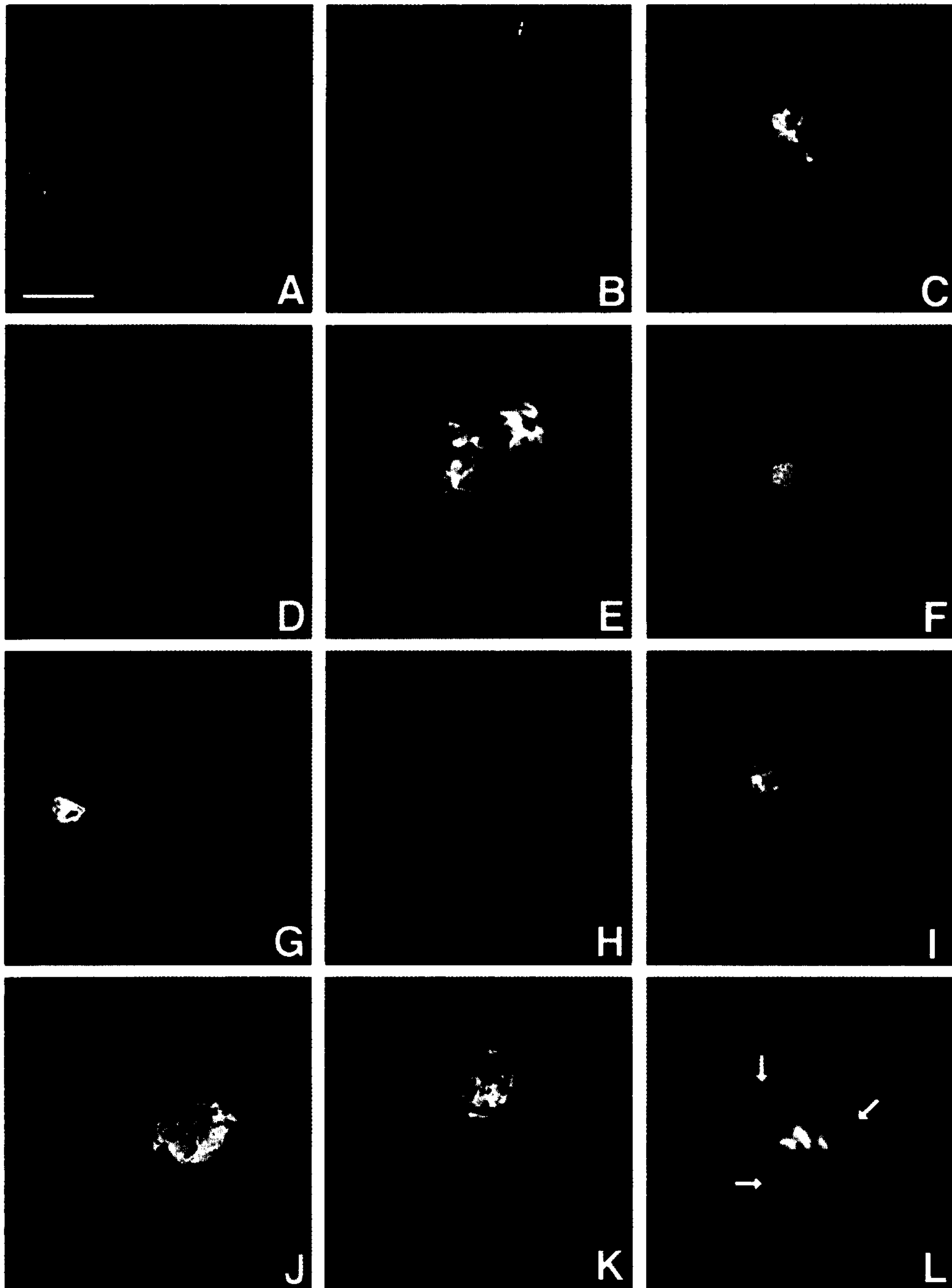
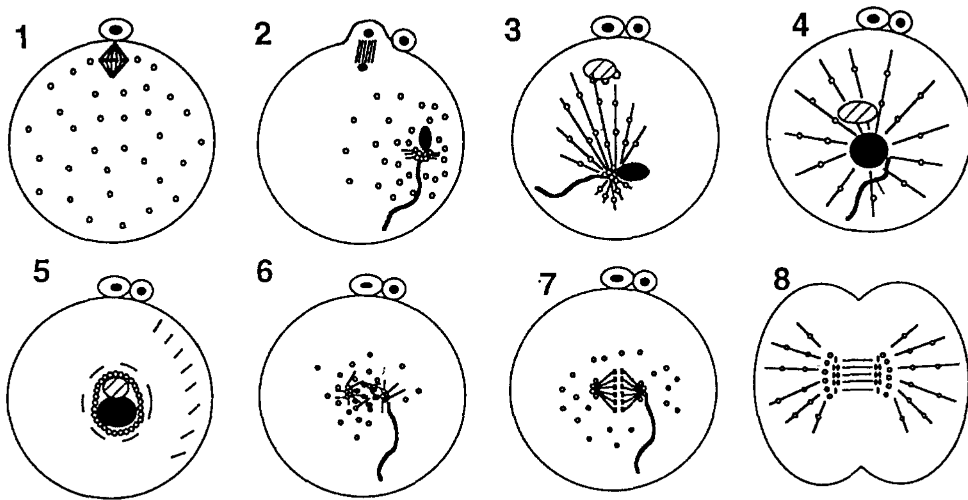


Fig. 4. Laser scanning confocal microscopic image of microtubules and chromatin in the porcine oocyte during parthenogenesis. Green, microtubules; red, chromatin, Bar = 20 μm. A. A dense microtubule network is observed in the parthenote at 5 h after activation. B. In the full grown pronuclear stage parthenote (18 h after stimulation), the microtubules are less

detectable. C. At prometaphase for mitosis, microtubules are found near the chromatin mass. D. The parthenote forms an anastral bipolar spindle at mitotic metaphase. E. The parthenotes complete mitosis and during anaphase, astral microtubules are present. F. Taxol treatment induce numerous microtuble foci which start to aggregate at 1.5 h after electrical activation. G, H. Same oocyte at different focal points. With focused at female nucleus (~ 40 μm from the surface), the microtubules are found around the female chromatin (G). A dence matrix of microtubules are found in the entire cytoplasm of taxol treated oocyte. J, K. During the pronuclear stage, taxol induce microtubules around the pronucleus. L. At mitotic pro-metaphase, a few cytoplasmic microtubules are induced by taxol treatment. Microtubules are organized at several points near the condensed chromatine (arrows).



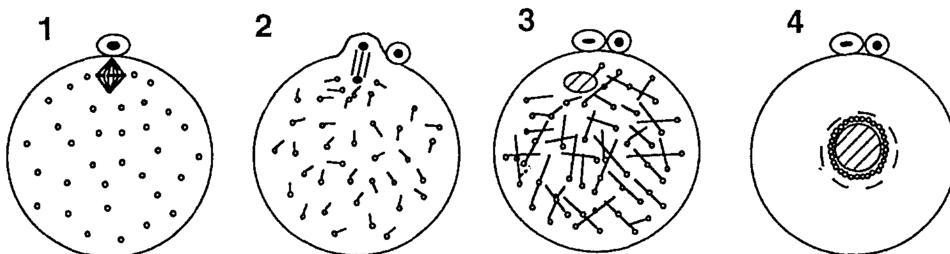


Fig. 5. Schematic of microtubule configuration in the porcine oocyte after fertlization and parthenogenetic activation. A. Fertilization. The mature, unfertilized oocyte has microtubules only in the meiotic spindle. Maternal centrosomal material seems to be in the cytoplasm, perhaps in or near the cortex(A-1). After sperm incorportion, the centrosomal material is

attracted to the sperm centrosome, and forms sperm aster(A-2). Sperm aster enlarges and reaches the female pronucleus (A-3). The female pronucleus moves toward the male pronucleus, and at the same time the mae pronucleus moves to the oocyte center (A-4). During pronuclear union, microtubule matrix is less detectable in the cytoplasm. The nuclear envelope seems to retain centrosomal material (A-5). During mitotic pro-metaphase, centrosomal material is dispersed in the cytoplasm. At that time, the condensed chromatin recruits nearby centrosomal materials, and organizes microtubules (A-6). The microtubule foci form eccentric mitotic metaphase spindle, which is anastral and fusiform (A-7). At anaphase and telophase microtubules extend into the cytoplasm from the each spindle pole (A-8). B. Parthenogenesis. Parthenogenetical stimulation activated maternal centrosomal material which forms numerous microtubule foci in the cytoplasm (B-1 & -2). During pronuclear formation, microtubule foci aggregated to each other and form disarrayed microtubule network (B-3). The maternal centrosomal material seems to be concentrated toward the pronucleus and becomes associated with the nuclear envelops (B-4).

ro et al., 1984; Zernicka-Goetz, 1993). Microfilaments are located mainly in the cell cortex overlying the meiotic spindle. In this microfilament-rich domain, the cleavage furrow forms for polar body extrusion following activation. When the oocyte enters interphase, this domain disappears and microfilaments concentrate around pronuclei. In aged mouse oocytes, the microfilament-rich domain overlying the meiotic spindle disappears (Webb et al., 1986). This is followed by migration of the spindle toward the center of the egg and spindle breakdown with the chromosomes no longer organized on a metaphase plate. These results suggest that the distribution of microfilaments is closely related to microtubule organization and is integrated and interacts with chromatin morphology. Webb et al. (1986) also demonstrated that the cytoskeletal organization of the egg during aging is related to the different types of parthenogenetic embryos obtained after activation.

Ⅴ. CONCLUSION

To summarize, microtubule organization during fertilization and parthenogenesis in porcine oocytes is diagrammed in Fig. 5. After sperm penetration, centrosomal material is attracted to the sperm neck area. During pronuclear formation, the sperm aster enlarges as the decondensing male and female chromatins move toward the center of the oocyte. In contrast, after electrical activation cytoplasmic centrosomal material is activated and forms a network of microtubules. After gamete union at fertilization and during pronuclear formation after activation, microtubules are less detectable in the cytoplasm. These data suggest that the nuclear envelope may retain the centrosomal material and, at that time, the ability to organize microtubules is lost. During mitotic metaphase, centrosomal material may disperse to the entire cytoplasm again where prometaphase chromatin attracts centrosomal material and forms a mitotic spindle.

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