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Cortical Granule Distribution During *In Vitro* Maturation and Fertilization of Porcine Oocytes

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돼지난자의 체외성숙 및 수정시 일어나는 표층과립막세포의 분포변화에 관한 연구

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요 약

본 연구의 목적은 돼지난자의 체외성숙, 수정 및 단위발생시 일어나는 표층과립의 분포를 살펴보고, 그것들의 역할을 규명해 보고자 실시하였다. 난자의 표층과립은 형광염색을 실시한 후 laser scanning confocal microscope를 이용하여 관찰하거나 transmission electron microscope를 사용하여 관찰하였다. Germinal vesicle 단계의 돼지난자에서는 표층과립은 난자피질에 비교적 두꺼운 형태로 발견되었는데, germinal vesicle breakdown이 일어난 직후 피질 부근으로 표층과립의 움직임이 관찰되었다. Microfilaments의 중합화를 방해하는 cytochalasin B를 처리하였을 때 표층과립의 움직임은 관찰되지 않았다. 무처리군의 수정 및 단위발생을 유도한 난자에서는 표층과립 내용물들이 위란강내에 균질하게 관찰되었으나, cytochalasin B를 처리한 난자에서는 비정상적인 cortical granule reaction이 관찰되었다. 이러한 결과는 돼지난자의 성숙시 microfilaments가 cortical granule의 움직임을 관장하고 이러한 움직임이 수정시 다정자 침입을 막고 표층과립 반응에 영향을 미치는 것으로 사료된다.

I. INTRODUCTION

In the pig, several investigators have demonstrated the ability of porcine oocytes matured and fertilized *in vitro* to develop normally (Funahashi et al., 1994; Nagai et al., 1984) and the birth of piglets from embryos produced *in vitro* has been reported (Mattioli et al., 1989; Yoshida et al., 1993). However, the progress toward efficient *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) has been limited due to abnormally high incidence of polyspermy in the pig (Funahashi et al., 1994; Mattioli et al., 1988; Kim et al., 1996c,e; Nagai et al., 1984) and low incidence of early embryo development (Funahashi et al., 1994b; Nagai et al., 1990).

Recent results suggested that incomplete cortical granule exocytosis has been observed in porcine oocytes matured and fertilized *in vitro*, which may result in the abnormally high incidence of polyspermy in the pig (Cran and Cheng, 1986; Kim et al., 1996a). During oocyte maturation, centrifugal movement and an in-

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crease in number of cortical granules following ovulation appear to be a common feature in mammals (Cran et al., 1980; Kruip et al., 1983; Cran and Cheng, 1985, Yoshida et al., 1993). In sheep, treatment of matured oocytes with microfilament inhibitor, cytochalasin D caused profound internal structural changes to the cytoplasm of the cortical granules and, in some regions, induces a loss of association with the plasma membrane. Kim et al., (1996b) demonstrated abnormal patterns of microfilament organization of porcine oocytes following *in vitro* maturation as compared to those matured *in vivo*. The abnormalities of microfilament organization seem to be closely related to the culture system during *in vitro* maturation (Funahashi et al., 1996) and oocyte aging (Kim et al., 1996c). Therefore inadequate culture conditions during *in vitro* maturation may disturb the function of microfilaments and thus cause incomplete cortical reaction following sperm penetration.

However, information is largely lacking in regard to direct involvement of microfilament assembly on the cortical granule movement and exocytosis during oocyte maturation and fertilization. In this study we observed cortical granule distribution, and examined effects of microfilament inhibitor on the cortical granule distribution during oocyte maturation parthenogenetic activation and fertilization of porcine oocytes.

Ⅱ. MATERIALS AND METHODS

1. *In vitro* maturation

Porcine oocyte-cumulus complexes (OCC) with uniform ooplasm and a compact cumulus cell mass were prepared in HEPES buffered TALP medium containing 0.1% polyvinylalcohol (H-TL-PVA, Funahashi et al., 1994). Culture medium for *in vitro* maturation was BSA-free NCSU23 (NCSU23, Petters and Wall, 1993) supplemented with 10% (v/v) porcine follicular fluid (pFF), 10 IU/ml eCG and 10 IU/ml hCG. Fifty OCC were transferred to 500 μl of NCSU23, covered with paraffin oil in a four well culture plate, and then cultured for 44 h at 39℃ in an atmosphere of 5% CO_2 in air.

2. *In vitro* fertilization

Sperm-rich fraction (15 ml) was collected from a boar by the gloved-hand method, and kept at 20℃ for 16 h after adding antibiotic-antimycotic solution (GIBCO). The semen was washed 3 times with 0.9% (w/v) NaCl supplemented with 1 mg/ml BSA (Fraction V, Sigma) by centrifugation. At the end of washing, the pellets containing spermatozoa were resuspended at 2×10^8 cells/ml in modified Medium199 (Funahashi et al., 1994; mM199, Sigma) at pH 7.8. The sperm suspension was incubated for 90 min at 39℃ in an atmosphere of 5% CO_2 in air. Ten oocytes were washed three times with mM199 supplemented with 10 mM caffeine sodium benzoate and 4 mg/ml BSA at pH 7.4 and placed into a 50 μl droplet of the mM199 under paraffin oil. Fifty microliter of diluted preincubated sperm was added to 50 μl of the medium containing oocytes so that a final sperm concentration of 1×10^6 cells /ml was obtained. Oocytes were co-cultured with spermatozoa for 6 h at 39℃ in an atmosphere of 5% CO_2 in air. The oocytes were then transferred to 500 μl of fresh NCSU23 and cultured at 39℃ in an atmosphere of 5% CO_2 in air.

3. Oocyte activation

Electrical stimulation to induce activation was delivered via a BTX Electro Cell Manipulator to a chamber with two parallel platinum wire electrodes (200 mm OD) spaced 1 mm apart and overlaid with electroporation medium. Before electrical stimulation, ooytes matured *in vitro*

were denuded of cumulus cells, washed and preincubated 5 min in electroporation medium: 0.25 M mannitol supplemented with 0.01% polyvinyl alcohol, 0.5 mM HEPES, and 100 mM $CaCl_2 \cdot 2H_2O$ and 25 mM $MgCl_2 \cdot 6H_2O$ with pH 7.2. A single DC pulse of 1.2 kV/cm for 30 msec was used for electrical stimulation. After a 2 min recovery, the oocytes were transferred to 500 ㎕ of NCSU23 and cultured at 39℃ in an atmosphere of 5% CO_2 in air.

4. Cytochalasin B treatment

Effects of cytochalasin B, a microfilament inhibitor on the cortical granule movement and exocytosis were determined during maturation and following electrical stimulation. Stock solution of 5 mM of cytochalasin B (Sigma) in dimethyl sulfoxide was used. The stock solution was stored at −20℃ and diluted to and 20 ㎛M cytochalasin B in NCSU23 prior to treatment of oocytes.

5. Assessment of cortical granules distribution

At specific time points, cumulus cells were removed from oocytes by repeated pipetting. Denuded eggs were permeabilized in 25% glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 1 mM 2-mercaptoethanol, 50 mM imidazol, pH 6.7, with 4% Triton X-100 (Simerly & Schatten, 1993). The eggs were then fixed in methanol at −10℃. After several washes with PBS containing 0.5% Triton-X 100 and 0.5% BSA, eggs were incubated in a blocking solution (0.1 M glycine, 0.01% Triton X-100, 1% powdered milk, 0.5% BSA and 0.02% sodium azide) at 39℃ for 1 h. Blocking was followed by incubation in 20 ㎍/ml FITC labeled lectins (Arachis hypogaea, Sigma) at 39℃ for 1 h FITC labeled. DNA was fluorescently detected by exposure to 20 ㎍/ml propidium iodide (Sigma) for 1 h. The eggs were then mounted under a coverslip with antifade mounting medium (Vectashild, Vector Lab. Burlingame, CA, USA) to retard photobleaching. Slides were examined using laser-scanning confocal microscopy. Laser-scanning confocal microscopy was performed using a Bio-Rad MRC 1024 equipped with a Krypto-argon ion laser for the simultaneous excitation of fluorescein for cortical granules, rhodamine for microfilaments, and Cy5 for DNA. The images were recorded digitally and archived on an erasable magnetic optical disk.

6. Assessment of cortical granule distribution by Transmission electron microscopy

Several embryos were fixed in 2.5% glutaraldehyde in Phosphate buffer, pH 7.2, immersed on 1% osmium tetroxide for 10min, and washel. d again in the phosphate buffer. After osmication, the embryos were then dehydrated in a graded series of alcohols, embedded in Poly/Bed 812 resin Polysciences, Inc. Warrington, PA 18976), and left to polymerize at 60℃ in the oven. Thin sections were cut using an ultramicrotome and staind with uranyl acetate and lead citrate for 10 minutes. The sections were finally viewed under the transmission electron microscope (Hitachi 600).

7. Statistical Analysis

At least three replicates were conducted for each experiment. Statistical differences were determined by Fisher's protected least significant difference test. Probability of $P < 0.05$ was considered to be statistically significant.

Ⅲ. RESULTS

1. Ultrastructure of cortical granules

In immature pig oocytes with germinal vesicle, the cortical granules were located within

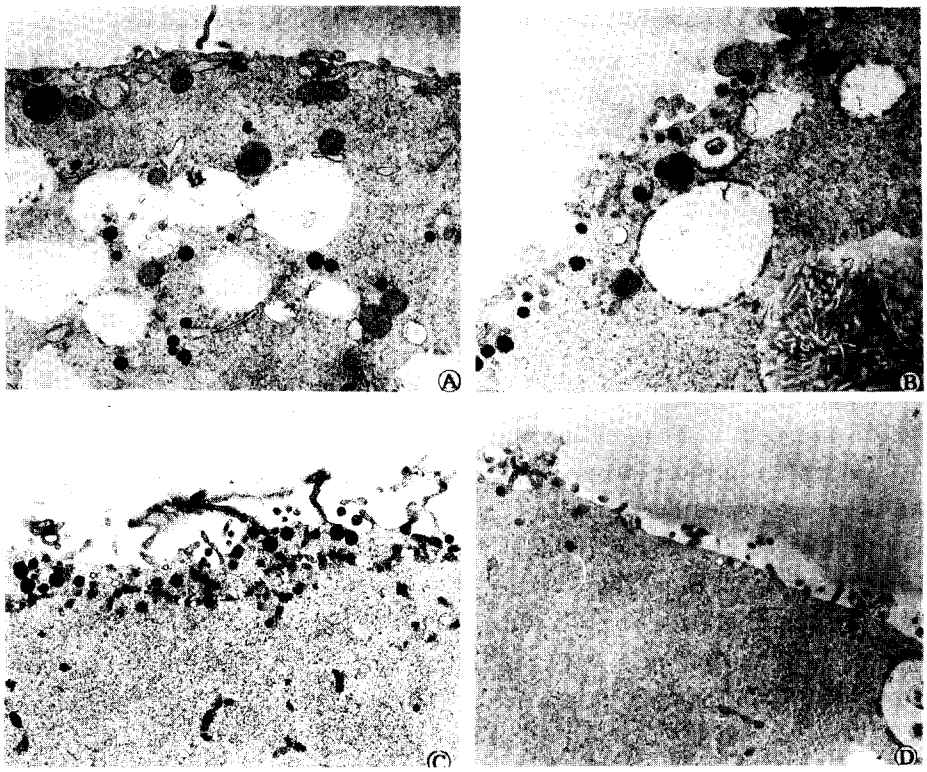

Fig. 1. Transmission electron micrograph of pig oocytes. A. At the germinal vesicle stage oocyte, most cortical granules are distributed in the thick area of cortex (× 8,000). B. Cortical granules are lying next to the plasma membrane and form a monolayers (× 10,000). C. Abnormal cortical granule reaction is observed in some oocytes matured *in vitro* (× 10, 000). D. Cortical granules are absent from ooplasm following sperm penetration(× 8,000).

the thick area of the cortical cytoplasm (Fig. 1A). Between 24 and 36 h of *in vitro* maturation, centrifugal migration took place in which most CGs came to lie next to the plasma membrane, where they formed a monolayer (Fig. 1B). At 6 h following in vitro insemination or at 1 h following parthenogenetic activation, cortical granule exocytosis occured(Fig. 1C).

2. Lectin binding structure of cortical granules

Table 1 shows kinetics of meiotic progression and cortical granule distribution in the presence and absence of cytochalasin B during *in vitro* maturation. In the presence of cytochalasin B, microfilament polymerization was inhibited, and movement of chromatin was not observed towards the cortex. In matured oocytes, cortical granules were distributed in the entire cortical cytoplasm (Fig. 2A), and the wide area with cortical granules was observed around first polar body (Fig. 2B). Cortical granule exudation following sperm penetration or parthenogenetic activation is evenly distributed throughout the entire perivitelline space (Fig. 2C). Abnormal cortical granule exudation is also observed in some *in vitro* matured oocytes following sperm penetration (Fig. 2D).

Table 1. Kinetics of meiotic progression and cortical granule distribution in the presence and absence of cytochalasin B (CB) during *in vitro* maturation of porcine oocytes

Time after culture	Culture with(+) or without(−) CB	No. of oocytes examined	No. of oocytes(%) developed				Cortical granule distribution	
			GV	GVBD	M I	M II	Cortex	Scattered
0	−	46	43(85)	3(7)	−	−		46(100)
11	+	43	32(74)	11(26)	−	−	−	32(100)
	−	39	31(79)	8(21)	−	−	3(8)	36(92)
22	+	42	19(45)	23(55)	−	−	3(7)	39(93)
	−	46	24(52)	20(43)	4(9)	−	13(28)	33(72)
33	+	49	11(22)	17(35)	21(43)	0(0)	5(10)	44(90)
	−	45	4(9)	5(11)	29(64)	7(16)	35(78)*	10(22)
44	+	39	5(13)	9(23)	25(64)	0(0)	8(21)	31(79)
	−	43	3(7)	1(2)	3(7)	36(85)*	39(91)*	4(9)

* $P<0.05$

GV: Germinal Vesicle, GVBD: Germinal Vesicle Breakdown, M I : Methaphase I , M II : Methaphase II , CB: Cytochalasin B.

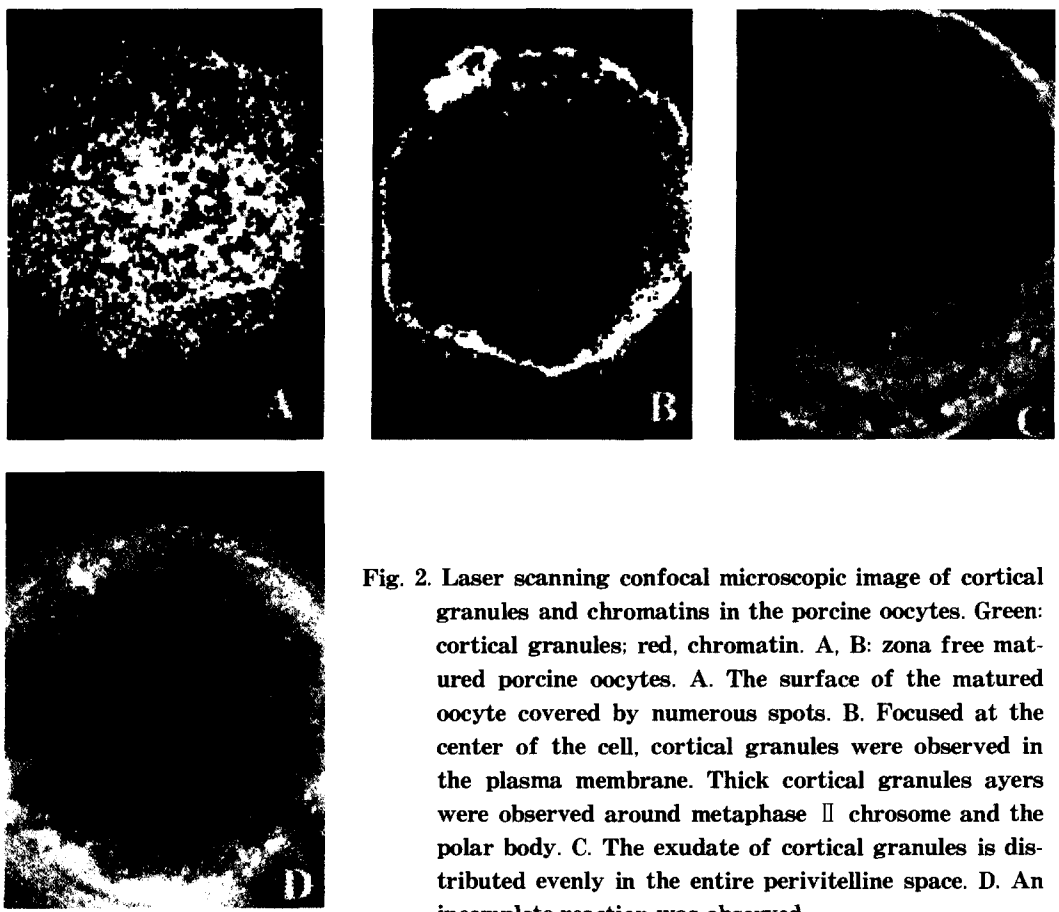

Fig. 2. Laser scanning confocal microscopic image of cortical granules and chromatins in the porcine oocytes. Green: cortical granules; red, chromatin. A, B: zona free matured porcine oocytes. A. The surface of the matured oocyte covered by numerous spots. B. Focused at the center of the cell, cortical granules were observed in the plasma membrane. Thick cortical granules ayers were observed around metaphase II chrosome and the polar body. C. The exudate of cortical granules is distributed evenly in the entire perivitelline space. D. An incomplete reaction was observed.

Ⅳ. DISCUSSION

During sperm penetration, cortical granules fuse with the overlying oolemma and release their contents into the perivitelline space. The cortical granule exudate appears to block polyspermy by changing the properties of the zona pellucida. It has been suggested that the cortical granule reaction in porcine ova followed current IVM and IVF procedures does not appear to be effective in the prevention of polyspermy (Sirard et al., 1993). Although electrical stimulation induced cortical reaction in porcine eggs matured *in vitro* (Sun et al., 1992), it did not block sperm penetration (Funahashi et al., 1993, 1995). Very recently Funahashi et al. (1996) demonstrated that the high concentration of NaCl in maturation medium has detrimental effects on microfilament organization. Therefore inadequate culture condition during maturation may disturb the function of microfilaments, and thus cause incomplete cortical reaction following sperm penetration.

The present study demonstrated centrifugal movement of cortical granules immediately following germinal vesicle breakdown. This result is consistent with previous observation by Yoshida et al. (1993) who reported that the migration takes place within 24 h of culture. The distribution of cortical granules during *in vivo* maturation of porcine oocytes that took place between 20 and 30 h post hCG injection corresponds to the time of the completion of germinal vesicle breakdown (Cran and Cheng, 1985). Therefore, in the pig, the timing of cortical granule migration appears to be similar for oocytes matured *in vitro* and *in vivo*. In contrast, a delay in the time of cortical granule movement in bovine oocytes matured *in vitro* as compared with those matured *in vivo* has been observed (Hyttel et al., 1989). Curiously, the centrifugal migration of cortical granule was not detected in the hamster and mouse during meiotic maturation (Ducibella et al., 1990). The centrifugal migration of cortical granules in the rodent has been observed during oocyte growth and thus has been suggested to be related to the relatively short time necessary for meiotic maturation (Yoshida et al., 1993).

In this study we observed that cytochalasin B prevented centrifugal movement of cortical granules during *in vitro* maturation. Cran and Cheng (1985) reported that microfilament-like structures became less distinct at a time when cortical granules were undergoing migration to the plasma membrane and increasing in number. In sheep unfertilized eggs, cortical granules were completely absent from the microfilament-free region, suggesting a direct relationship between the assembly of microfilaments and anchorage of cortical granules under the plasma membrane of the oocyte (Le Guen et al., 1989). In the present study we imaged a thick cortical granule area around the first polar body. It has been suggested that during polar body extrusion microfilaments play a role in the polar body extrusion and cell division in the mouse (Maro et al., 1985) and pig (Kim et al., 1996d,e). Collectively, our study and previous observations suggest that microfilaments may be directly linked with the cortical granule movement to the cortex during oocyte maturation.

Previously, Yoshida et al. (1993) observed that the cortical granule constituents aggregated and formed a reticulately banded structure following sperm penetration in the perivitelline space. However, in this study, we did not observed that type of structure, but instead evenly distributed cortical granule exudate in the entire perivitelline space following sperm penetration and parthenogenetic activation. This difference

may be due to different types of lectin used to stain cortical granules. In this study we used FITC labeled lectins, Arachis hypogaea, in contrast, Yoshida et al. (1993) treated oocyte with peanut agglutinin.

In summary, we have obsered distribution of cortical granules during maturation, fertilization and parthenogenesis. The centrifugal movement of cortical granules has been observed immediately following germinal vesicle breakdown. The inhibition of microfilament assembly prevented cortical granule movement during oocyte maturation, suggesting a role of microfilament on this event.

V. SUMMARY

The objectives of this study are to determine cortical granule distribution during *in vitro* maturation, parthenogenetic activation and *in vitro* fertilization of oocytes, and to investigate effects of microfilament inhibitor on the cortical granule distribution during *in vitro* maturation and fertilization of oocytes in the pig. The cortical granule distribution were imaged with fluorescent labeled lectin under laser scanning confocal microscope or detected by transmission electron microscope. At germinal vesicle stage, cortical granule organelles were located around the cell cortex and were present as a relatively thick area on the oolema. Microfilaments were also observed in a thick uniform area around the cell cortex. Following germinal vesicle breakdown, microfilaments concentrated to the condensed chromatin and cortical granules were observed in the cortex. Treatment with cytochalasin B inhibited microfilament polymerization and prevented movement of cortical granules to the cortex. Cortical granule exudate following sperm penetration was evenly distributed in the entire perivitelline space. Therefore, these results suggested that the microfilament assembly is involved in the distribution, movement and exocytosis of cortical granules during maturation and fertilization of porcine oocytes.

(Key words : cortical granule, porcine, maturation, fertilization)

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