Microtubule Organization During *in Vitro* Development of Rat Oocytes*

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Abstract Microtubule organization in developing rat oocytes were investigated through confocal microscopy. The inductive effects of some factors on microtubule modification were also studied. The results indicated that the meiotic cell cycle progression of rat oocyte is accompanied by extensive rearrangement of the microtubule network of the cell. Microtubule organization was modified by the treatment of oocytes with taxol, staurosporine, or okadaic acid. Rat oocytes released from the oviducts could undergo spontaneous parthenogenetic activation. They could also be induced to pseudo-cleave by cytochalasin B treatment. The microtubule configuration in rat oocytes undergoing spontaneous parthenogenetic activation or pseudo-cleavage was also observed.

Key words rat, oocyte, microtubule, meiosis

Mammalian oocytes are arrested at the diplotene stage of the first meiotic division. Fully-grown oocytes liberated from their follicles spontaneously reinitiate meiosis I *in vitro*, characterized by germinal vesicle breakdown (GVBD), and proceed to metaphase of the second meiotic division (M II), where they are arrested again. Only after fertilization or parthenogenetic activation will the eggs resume meiosis II, finish the meiotic division, and finally form pronuclei.

Though this developmental pattern of oocytes is conservative among mammalian species studied, some species-specific variations still exist. Most knowledge about mammalian oocyte development was obtained from the study of mouse, but the reports of our group and others indicated that the meiotic cell cycle of rat oocytes differ from mouse oocytes in several aspects. Released from the follicles, the rat oocytes developed to the M I stage earlier than the mouse oocytes, but MAP kinase activation in rat oocytes is even more delayed than in the mouse oocytes[1, 2]. Removed from oviducts and cultured *in vitro*, rat oocytes undergo spontaneous parthenogenetic activation, instead of being arrested at M II stage. The spontaneously activated oocytes extrude their second polar bodies (PB2), but they do not pass to interphase with the formation of pronuclei. Instead, they enter into a next metaphase-like arrest termed the third meiotic mataphase (M III)[3].

Due to these specialties, the studies on rat oocyte maturation, fertilization and activation were far fewer than those in mouse. Wassarman *et al.*[4] reported that mouse oocytes could be induced by cytochalasin B to undergo "pseudo-cleavage" *in vitro* into equally sized compartments. But whether the rat oocytes can response to this induction is unknown.

The cell cycle progression is accompanied by extensive rearrangement of the microtubule network of the cell. At interphase, long and relatively stable microtubules radiate throughout the cytoplasm. At metaphase, by contrast, microtubules are shorter and less stable and are restricted to the region of the spindle[5]. How the microtubule organization correlates to the meiotic maturation and activation of rat oocytes is a problem lacking systematic studies. This experiment aimed to elucidate the microtubule organization changes in rat oocytes during cell cycle progression. The inductive effects of some factors on microtubule organization were also studied. All these researches will be helpful to provide optimizing culture conditions for further studies on rat oocyte meiosis.

1 Materials and methods

1.1 Collection, culture and *in vitro* fertilization of oocytes

Wister strain rats 20~ 30 days old were primed with 20 U pregnant mare's serum gonadotropin (PMSG). Approximately 48 ~ 50 h later, fully grown GV-intact oocytes were collected from the ovaries in M2 medium containing 4 g/L bovine serum albumin (BSA) and cultured in this medium under paraffin oil at 37 ℃, 5% CO_2 in air. The cumulus cells surrounding the oocytes were removed by repeated pipetting before maturation culture.

Twenty-five-day old females and three-month old males were used for *in vitro* fertilization. Superovulation was induced by injecting PMSG (20 U per rat) subcutaneously 54 h before an intraperitoneal injection of hCG (20 U per rat). Animals were sacrificed by cervical dislocation 15 h after hCG injection. Cumulus-enclosed metaphase II (M II)

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oocytes were recovered from the oviducts. Cumulus cells were removed by brief exposure to 300 U/ml hyaluronidase (Sigma), and zona pellucida (ZP) was removed with acid Tyrode's solution (pH 2.5). On the day of collecting oocytes, male rats were sacrificed, and the cauda epididymides were dissected from animal, and minced slightly into 2 ml of Dulbecco's Modified Eagle Medium (DMEM) containing 20 g/L BSA, and then placed in a 5% CO_2 air incubator for 10 min at 37℃ to allow spermatozoa to swim up. We collected the top sperm suspension showing vigorously progressive motility and containing about 1.5×10^7 spermatozoa/ml. For capacitation, the sperm suspension in the concentrated form was kept in a 5% CO_2 air incubator for 3 h. Capacitated sperm suspension was diluted at 1:10 before insemination of M Ⅱ oocytes[6, 7].

1.2 Chemicals

The chemicals used in this experiment were purchased from Sigma Chemical Company except for those specially mentioned. Stock solutions of okadaic acid (OA, 0.2 mmol/L, Calbiochem), isobutylmathylxanthine (IBMX, 100 mmol/L), cytochalasin B (CB, 1 mmol/L), taxol (10 g/L), and staurosporine (3 mmol/L) were prepared with dimethyl sulfoxide (DMSO) and stored frozen at -20℃. They were diluted with M2 medium just prior to use.

1.3 Experimental design

Experiment 1: The kinetics of microtubule organization during the meiotic maturation and fertilization were studied. Oocytes at differential developmental stages were fixed for confocal microscopy examination (see below).

Experiment 2: To investigate the microtubule organization of rat oocytes after spontaneous pathenogenetic activation, M Ⅱ oocytes were collected from oviduct and cultured in M2 medium for 8 h before the staining of α-tubulin, and the rate of PB2 emission were recorded by observing through an inverted phase-contrast microscope.

Experiment 3: Microtubule assembly in M Ⅱ oocytes were induced by treatment of oocytes with 1 μmol/L microtubule disassembly inhibitor taxol for 10 min. In another approach, M Ⅱ oocytes were treated with 30 μmol/L protein kinase inhibitor staurosporine for 30 min to destruct the meiotic spindle. Some oocytes treated with staurosporine were further exposed to 1 μmol/L taxol for 10 min.

Experiment 4: To investigate the effects of protein phosphotase on micrutubule organization, some oocytes at G V or M Ⅱ stage were cultured in M2 medium containing 2 μmol/L OA for 4 or 6 h respectively. All these experiments were performed at 37℃. After each treatment, oocytes were collected for confocal microscopy.

Experiment 5: Induction of pseudo-cleavage was performed as reported in mouse[8]. Briefly, cumulus-free and G V intact oocytes were cultured in M2 medium containing 0.2 mmol/L IBMX (This drug is used to prevent spontaneous meiotic resumption) and 5 μmol/L CB overnight. To increase the rate of pseudo-cleaved oocytes, some ZP-free oocytes were cultured at the same condition for 3 h.

1.4 Confocal microscopy

Microtubule detection was based on the procedures reported by us previously[9, 10]. After removal of ZP in acidified Tyrode's solution (pH 2.5), oocytes were fixed in 3% formaldehyde, 2% sucrose in PBS for 30 min and then incubated in incubation buffer (0.5% Triton X-100 in 20 mmol/L Hepes, pH 7.4, 3 mmol/L $MgCl_2$, 50 mmol/L NaCl, 300 mmol/L sucrose, 0.02% NaN_3) for 30 min. After placed in methanol for 5 min at -20℃, the eggs were washed in PBS with 0.1% Tween 20 for three times and then incubated with 1:50 diluted FITC-anti-α tubulin (Sigma) for 1 h. The oocytes were rinsed three times before staining with 10 mg/L propidium iodide (PI) for 10 min. Finally, the eggs were mounted on glass slides and examined using a TCS-4D laser scanning confocal microscope (Leica Microsystems).

2 Results

2.1 Dynamics of microtubule organization during meiotic maturation and fertilization *in vitro*

In our culture system, 71.03% (76/107) oocytes underwent GVBD 3 h after releasing from follicles. These oocytes reach M Ⅰ stage 3 h after GVBD and extrude PB1 5~6 h after GVBD. Most oocytes (81.58%, 62/76) reached M Ⅱ stage 9~10 h after maturation culture. 78.31% (65/83) fertilized oocytes released their PB 2 within 2 h after insemination and 69.88% (58/83) of them formed pronuclei 6 h following fertilization.

Long and randomly-oriented microtubules were detected in G V oocytes, with a condensed distribution around the germinal vesicle (Figure 1a). Following GVBD, the long cytoplasmic microtubules were replaced by short microtubules radiating from the condensed chromosomes (Figure 1b). Two hours after GVBD, the condensed chromosomes separated from each other, but loosely connected by bundles of short microtubules (Figure 1c). Three hours after GVBD, the oocytes entered premetaphase, and the M Ⅰ spindle began to form. At this stage, the microtubules have already been organized into the shape of spindle, but there were still some chromosomes not being arranged to the middle of the spindle (Figure 1d and 1e). The dominant microtubule-containing structure in unfertilized rat oocytes arrested at mataphase of second meiosis is the meiotic spindle (Figrue 1f). But compared to the barrel-shaped

mouse oocyte spindle, the rat meiotic spindle was much more elongated and taper-shaped with distinct poles. Furthermore, the M II spindle is much smaller than the M I spindle. With the formation of pronuclei following fertilization, long microtubules reorganized in the cytoplasm (Figure 1g). At the same time of pronucleus movement and apposition, the cytoplasmic microtubules concentrated to the area between the male and female pronuclei (Figure 1h). At approximately 18 h after fertilization, nucleus envelop breakdown (NEBD) occurred. Then the microtubules concentrated to the condensed chromosomes with the much reduced length (Figure 1i).

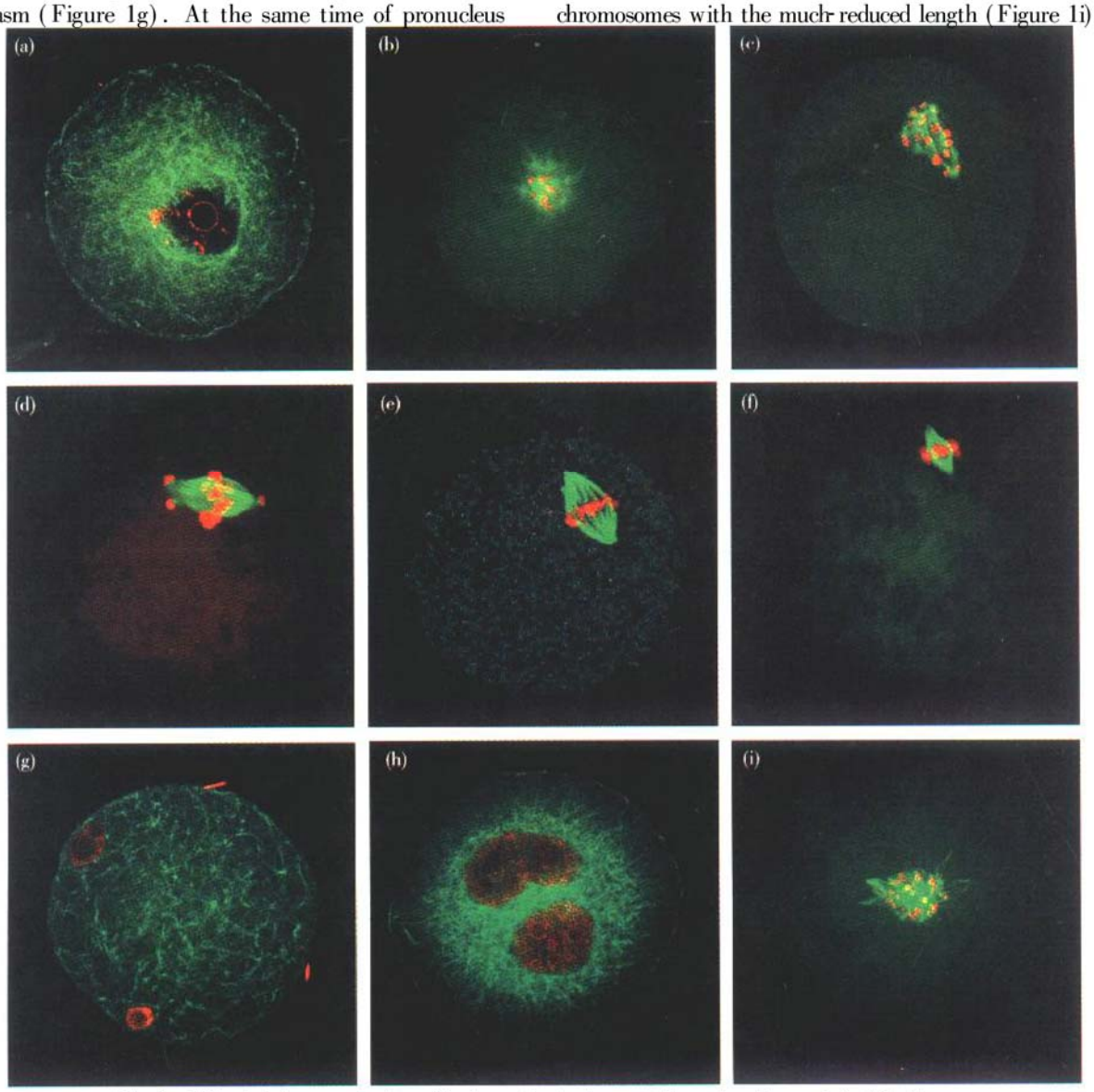

Fig. 1 Microtubule organization of rat oocytes during meiotic maturation and fertilization

(a) an oocyte at G V stage; (b) 1.5 h after GVBD; (c) 2 h after GVBD; (d) 3 h after GVBD; (e) an oocyte at M I stage; (f) an M II oocyte; (g) 6 h after insemination; (h) 16 h after fertilization; (i) a fertilized egg after pronucleus membrane breakdown. Green: α-tubulin; Red: DNA.

2.2 Spontaneous pathenogenetic activation of oocytes

Most M II oocytes (93.68%, 89/95) underwent spontaneous activation when cultured *in vitro* for 2 h, marked by PB2 emission. The results of confocal microscopy indicated that the chromosomes dispersed irregularly in these oocytes (Figure 2a). No pronucleus formation occurred 8 h after culture. Instead, oocytes entered a metaphase-like arrest (M III) and reformed a spindle-like structure. However, the organization of the M III spindle was not stable: the chromatids tend to

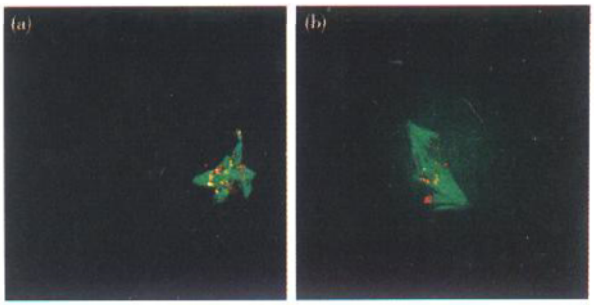

Fig. 2 Rat oocytes following spontaneous activation

(a) 2 h after activation; (b) 8 h after activation.

disperse and polymerization of new microtubules occurred (Figure 2b).

2.3 Induced assembly/disassembly of microtubules

As shown in Figure 3a, the spindle was significantly enlarged due to the excessive microtubule polymerization, and multiple microtubule asters formed in the cytoplasm after treating the oocytes with taxol. The spindle was destructed when the oocytes were treated with broad-spectrum protein kinase inhibitor staurosporine (Figure 3b). Furthermore, taxol cannot up-regulate the microtubule organizing ability of the spindle after the pretreatment with staurosporine. However, some short microtubule networks were detected in the cytoplasm after taxol treatment, though the cytoplasmic asters were not induced (Figure 3c).

The presence of OA during meiotic maturation significantly affected the cytoskeletal architecture of the oocytes. When the oocytes were cultured in 2 mmol/L OA for 2 h, comprising long microtubules arose from the area occupied by chromosomes (Figure 3d). When the M Ⅱ oocytes were treated with OA for 6 h, the spindle structure was significantly damaged (Figure 3e).

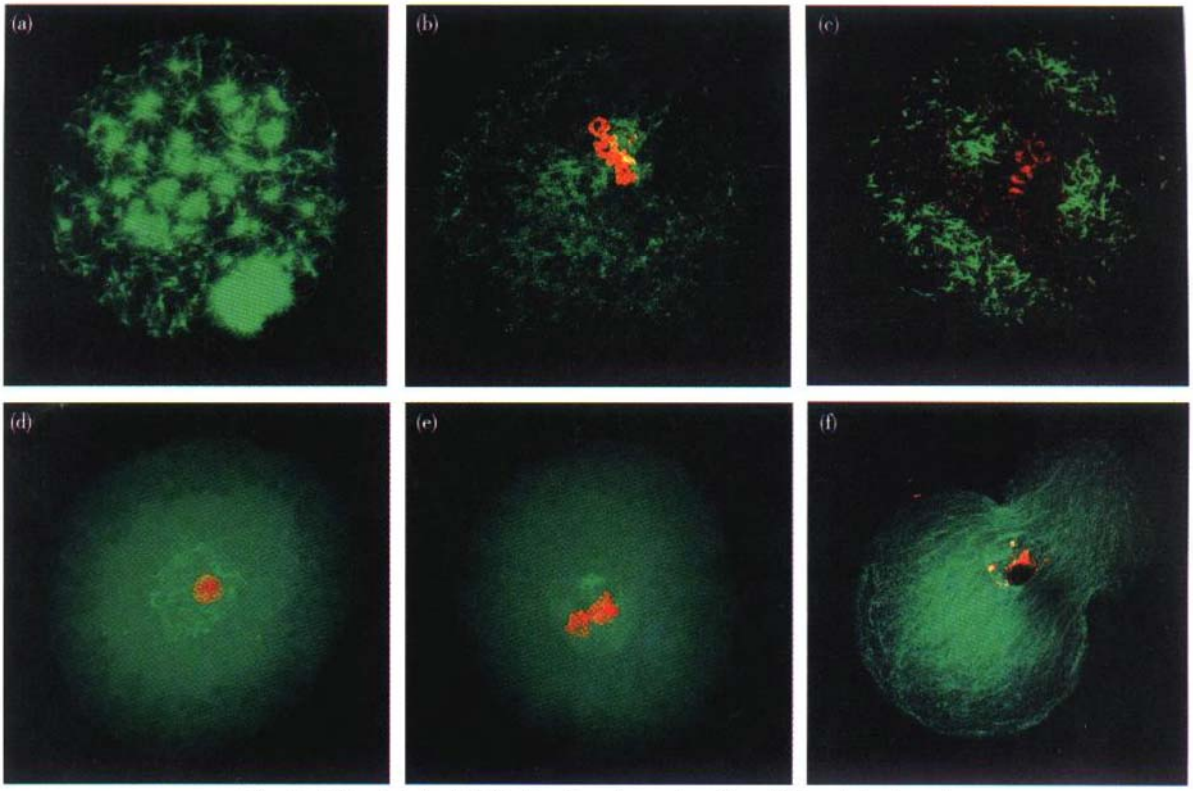

Fig. 3 Microtubule distribution in rat oocytes after different treatment

(a) 10 min after taxol treatment; (b) 30 min after staurosporine treatment; (c) treatment with taxol for 10 min after pretreatment with staurosporine; (d) a G Ⅴ oocyte cultured with 2 mmol/L OA for 4 h; (e) an M Ⅱ oocyte cultured with 2 mmol/L OA for 6 h; (f) a pseudo-cleaved oocyte induced by CB.

2.4 Induced pseudo-cleavage of G Ⅴ oocytes

48.24% (41/85) ZP-intact rat oocytes cultured overnight in the presence of both IBMX and CB slowly underwent pseudo-cleavage, which involved the division of the oocytes into 2 approximately equal-sized compartments, one of which contained an intact G Ⅴ and nucleolus. However, 79.59% (39/49) ZP-free oocytes underwent pseudo-cleavage within 3 h after culture. Confocal microscopy indicated that long microtubules existed in the pseudo-cleavaged oocytes, and most of them extend parallelly to the axis of pseudo-cleavage. No condensation of microtubules at the vicinity of division furrow was observed (Figure 3f).

3 Discussion

Our results showed that microtubule organization of rat oocytes exhibited several important differences as compared to the reports in mouse. In contrast to the barrel-shaped and anastral mouse M Ⅱ spindle, the rat M Ⅱ spindle is elongated, cone-shaped, and sharply ended with distinct poles. Furthermore, the GVBD in rat oocytes occurred later than in mouse oocytes (within 3 h compared to 2 h in mouse), but the rat M Ⅰ spindle formed earlier (3 h after GVBD) than that in the mouse (6~8 h after GVBD). This may be due to the delayed activation of MAP kinase as reported by others[11].

The mechanism of spontaneous activation is not fully known. It was argued that the cooling of the oocytes to room temperature or other changes in the oviduct after the animal's death can induce the resumption of meiosis, but further stimulation, such as the increase of cytoplasmic calcium, is necessary for the fully activation of oocytes characterized by pronucleus formation[12, 13]. In spontaneously activated eggs, bundles of short microtubules radiated from the chromosomes. Since no spindle poles are formed in this case, kinetochore of individual chromosomes may responsible for the microtubule assembly. We also proved that in rat oocytes arrested at M III, the activity of MAP kinase remain high, compared to the MAP kinase dephosphorylation observed after fertilization[11]. Since the activity of MAP kinase is an inhibitory factor of pronucleus formation, the M III arrest in rat oocytes may be maintained by this kinase.

Besides its function in inducing the spindle enlargement, taxol could also induce the formation of multiple cytoplasmic asters. This result was in agreement with other's argument that there were latent microtubule organizing centers existed in rat ooplasm. Although multiple potential MTOCs exist in the cytoplasm of rat eggs, the active microtubule assembly occurs only at spindle poles. The mechanism of this phenomenon is still not fully known until now. We found recently that some molecules important for microtubule nucleation, such as Polo-like kinase[9], MAPK, and gamma tubulin[14], localize exclusively on spinle poles in M II-arrested eggs. However, they concentrate to the cytoplasmic asters when the eggs were treated with taxol. So the MTOC functions may be regulated by relevant protein kinases and microtubule nucleating molecules. Staurosporine, a broad-spectrum protein kinase inhibitor, could induce the dessembly of spindle in mouse and pig oocytes[15]. Here we reported that it has the same effects in rat oocytes. Furthermore, taxol failed to induce the formation of cytoplasmic asters at the presence of staurosporine, suggesting that the microtubule assembly of cytoplasmic asters was also sensitive to this reagent.

In mouse[16], rat[11] and pig[17] oocytes, the activity of MAP kinase is regulated by an OA-sensitive protein phosphotase. In our experiment, OA leads to the distortion of microtubule configuration during multiple phases of meiosis. As indicated by our former reports, OA treatment resulted in super-activation of MAP kinase, which is the key regulator of meiotic spindle organization. We proved here that MAP kinase, and the OA-sensitive protein phosphotase, were responsible for the normal microtubule organization in maturing and M II-arrested rat oocytes. However, though OA could induce meiotic resumption by a MAPK-dependent but MPF-independent mechanism[18], spindle does not form in OA-treated oocytes, and an interphase network of microtubules develops with time. Thus, MAPK is unable to substitute fully for MPF in the control of microtubule dynamics when protein phosphatases are inhibited, its activity being insufficient to maintain the progression through meiotic maturation.

We proved that the GV-intact rat oocytes could be induced to undergo pseudo-cleavage by CB, and this response to the drug is influenced by the presence of an intact zona pellucida. The ZP-free oocytes response was more sensitive to the induction than the ZP-intact oocytes, which was a phenomenon also reported in mouse. The mechanism of pseudo-cleavage has not been fully known until now. Some researchers hypothesized that CB-induced pseudo-cleavage of mouse oocytes was a consequence of the interaction between the drug and the contractile components of the cell cortex[4]. In this experimental model the concentration of CB is crucial, since it is a drug inhibiting the actin polymerization. Only when the CB at the concentration that the microfilament assembly was partially, but not completely inhibited, will the cytoplasmic contractile ring be induced. Our results indicated that the pattern of microtubule organization in GV oocytes was not changed after pseudo-cleavage. Thus, the microtubules may not involve in the cytoplasmic division in this case.

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大鼠卵母细胞体外发育过程中的微管组装研究*

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摘要 大鼠卵母细胞的发育具有许多与其他哺乳动物不同的特点, 用激光共聚焦显微术研究了大鼠卵母细胞发育过程中微管的组装过程, 以及一些因素对微管组装过程的影响. 结果表明, 在大鼠卵母细胞减数分裂的细胞周期进程中, 细胞微管系统发生广泛而剧烈的重组, 紫杉醇、星形孢菌素和冈田酸等药物能显著改变卵母细胞内的微管组织状态. 大鼠卵母细胞可以在体外发生自发的孤雌活化, 也可以被细胞松弛素诱导发生胞质假分裂. 因此, 研究了在这些过程中微管结构的特殊构象, 以期更加深入认识这些特殊细胞事件的生化机理.

关键词 大鼠, 卵母细胞, 微管, 减数分裂
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