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Original Articles

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Abstract

Developmental capacity of domestic cat oocyte matured *in vitro* is poor when compared to that of *in vivo* matured oocyte. While cytoplasmic maturation of the oocytes is essentially required for embryo development, little is really known about distribution of cell components during the course of *in vitro* maturation (IVM). The aims of this study were to examine and to describe the changes in the distribution of microtubule and actin microfilaments in relation to stage of nuclear maturation during IVM.

Cumulus oocyte complexes were matured *in vitro* for 0, 6, 12, 18 and 24 h and then labeled with immunofluorescent dyes against microtubules, actin microfilaments and chromatin to enable examination of a series of changes in nuclear and cytoplasmic maturation. A total of 302 oocytes were examined with a confocal laser scanning microscope. Immature oocytes were arrested at germinal vesicle stage for approximately 6-12 h, where actin microfilaments were typically distributed throughout the ooplasm. Metaphase I plate (meiotic spindles) was then completely formed by 12-18 h. The meiotic spindle and actin microfilaments facilitated segregation of the homologous chromosomes resulting in extrusion of the first polar body. The second meiotic spindle was soon established, and the oocytes then finally reached MII stage. In conclusion, this study described a series of nuclear and cytoplasmic changes during *in vitro* maturation of cat oocytes. Nuclear maturation of cat oocytes asynchronously resumed and reached metaphase II, while examination of the oocytes every 6 h was sufficient to evaluate the redistribution of microtubules and actin microfilaments during the course of nuclear maturation.

Keywords : actin microfilament, cat, maturation, microtubule, oocyte,

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บทคัดย่อ

การเปลี่ยนแปลงของเซลล์โครงร่างในโอโอไซต์แมว ขณะเจริญพร้อมปฏิสนธิภายนอกร่างกาย

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ความสามารถในการเจริญถึงระยะตัวอ่อนของโอโอไซต์แมวที่เลี้ยงภายนอกร่างกายอยู่ในระดับต่ำเมื่อเปรียบเทียบกับโอโอไซต์ระยะพร้อมปฏิสนธิที่เก็บจากรังไข่ ถึงแม้ว่าความสมบูรณ์ของไซโตพลาสมของโอโอไซต์มีส่วนสำคัญในการสนับสนุนการเจริญของตัวอ่อน ข้อมูลที่เกี่ยวข้องกับการเปลี่ยนแปลงของไซโตพลาสมยังมีอยู่อย่างจำกัด การศึกษาในครั้งนี้มีวัตถุประสงค์เพื่อศึกษาการเปลี่ยนแปลงของโครงสร้างไมโครทูบูล แอคติน ไมโครฟิลาเมนต์ ในโอโอไซต์ขณะที่มีการเจริญพร้อมปฏิสนธิ โอโอไซต์แมวที่เลี้ยงนาน 0 6 12 18 และ 24 ชม. ถูกนำมาตรึงและย้อมสีเรืองแสงที่มีความจำเพาะต่อไมโครทูบูล แอคติน ไมโครฟิลาเมนต์และโครมาติน จากนั้นนำไปตรวจด้วยกล้องจุลทรรศน์คอนโฟคอลที่ใช้แสงเลเซอร์เป็นตัวกระตุ้นการเกิดสี พบว่าโอโอไซต์ที่เก็บได้จากรังไข่จะอยู่ในระยะไม่พร้อมปฏิสนธินานประมาณ 6-12 ชม. ซึ่งโอโอไซต์ระยะนี้จะมีการกระจายตัวของแอคติน ไมโครฟิลาเมนต์ ทั่วไปในโอโอไซต์ ส่วนไมโอติก สปินเดิล ที่เกิดจากการรวมตัวของไมโครทูบูลพัฒนาเสร็จสมบูรณ์เมื่อโอโอไซต์ถูกเลี้ยงประมาณ 12-18 ชม. ไมโอติก สปินเดิลนี้ทำหน้าที่ในการแยกชุดโครโมโซม จำนวน 1 ชุด โดยมีแอคตินไมโครฟิลาเมนต์ช่วย การศึกษาในครั้งนี้เป็นการศึกษาถึงการเปลี่ยนแปลงของโอโอไซต์ทั้งในระดับนิวเคลียสและไซโตพลาสม การพัฒนาพร้อมปฏิสนธิของโอโอไซต์เกิดขึ้นไม่พร้อมกัน ในขณะที่การตรวจโอโอไซต์ทุกๆ 6 ชม. ช่วยให้สามารถตรวจการเปลี่ยนแปลงของโครงสร้างไมโครทูบูล และ แอคติน ไมโครฟิลาเมนต์ ในขบวนการเจริญพร้อมปฏิสนธิได้

คำสำคัญ : แอคติน ไมโครฟิลาเมนต์ แมว การเจริญพร้อมปฏิสนธิ ไมโครทูบูล โอโอไซต์

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Introduction

Fundamental and application study of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture of embryo (IVC) in domestic cat has become increasingly important aspects for conservation of endangered felid species (reviewed by Swanson, 2006). In addition, the domestic cat has also been used as a model for biomedical research because cat genome is highly conserved and exhibits the most similarity to that of human (O'Brien et al., 1999). However, the efficiency of these techniques has met only limited success, especially when oocytes are matured *in vitro* (Swanson et al., 1994). During oocyte maturation, the oocyte must undergo a series of nuclear and cytoplasmic changes in order to complete its competence. Most *in vivo* matured oocytes acquire their developmental competence by bidirectional communication or interaction between the oocyte and companion somatic cells within the follicle (Matzuk et al., 2002). When these oocytes are fertilized and cultured *in vitro*, they demonstrate greater fertilization and blastocyst formation rates compared favorably to *in vitro* matured oocytes (Roth et al., 1994). More specifically, intrinsic factors within the ovary/follicle promote cytoplasmic competence that supports fertilization process and enhances embryo developmental capability. Currently, a number of strategies have been tested, aimed specifically to improve cytoplasmic competence of mammalian oocytes during oocyte maturation (see review: Gilchrist and Thompson, 2007). These strategies would reveal the mechanism underlying the process of oocyte maturation *in vitro*, and also to find potential markers that reflect their developmental competence. It is also worth to note that the synchronization between nuclear and cytoplasmic maturation is

critically important to determine oocyte's developmental competence, while most IVM studies have only focused on nuclear maturation and information regarding to the cytoplasmic maturation of the cat oocyte is very limited.

In vitro maturation of oocyte is a complex process that occurs spontaneously when an immature oocyte (germinal vesicle stage: GV) is removed from inhibitory factors within the follicle (Pincus and Enzmann, 1935). After condensation of chromatin, nuclear membrane then starts to disintegrate (germinal vesicle breakdown: GVBD) and microtubule-rich domain is formed and become the meiotic spindle. This meiotic spindle plays an essential role in segregating chromosomes during meiosis and also contributes to many aspects during fertilization (Kim et al., 1997). In addition, actin microfilaments support cell plasma membrane and also involve in several cell functions. These two cell cytoskeleton elements have been used to evaluate cytoplasmic changes during *in vitro* maturation and also cell damage after cryopreservation (Tremoleda et al., 2001; Comizzoli et al., 2004; Tharasanit et al., 2006).

To date, study of reorganization of cell cytoskeleton of cat oocytes has not yet been examined. The aims of this study were therefore to examine and to describe the distribution of oocyte's cytoskeleton in relation to nuclear maturation of cat oocytes during *in vitro* maturation.

Materials and Method

Collection and culture of cumulus oocyte complexes

Domestic cat ovaries from mixed breeds and various stages of estrous cycle were collected immediately after routine ovariohysterectomy (OVH) from the Veterinary Public Health, Division of the Bangkok Metropolitan Administration. The ovaries were then maintained and transported at room temperature in 0.9% (w/v) normal saline solution supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. Within 6 h after OVH, the ovaries were dissected free from connective tissue and washed twice in holding medium consisting of hepes buffered tissue culture medium 199 (M199), 1.0 mM pyruvate, 2.0 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 4 mg/ml bovine serum albumin (BSA). To obtain cumulus oocyte complexes (COCs), ovarian cortex was minced using a surgical blade. The COCs with darkened homogenous ooplasm and completely surrounded by at least 3-5 layers of cumulus cells were only used in this study.

For *in vitro* maturation, a group of 15-25 COCs were cultured at 38.5°C in humidified condition of 5% CO_2 in air. The maturation medium was consisted of $NaHCO_3$ buffered M199, 2.0 mM L-glutamine, 1.0 mM pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, 4 mg/ml BSA and 0.1 IU/ml recombinant human follicle stimulating hormone (FSH). After 6, 12, 18 and 24 h of culture, the cumulus cells were removed from oocytes by repeat pipetting in phosphate buffered saline (PBS) supplemented with 0.1% (w/v) BSA at 37°C. All chemicals were purchased from Sigma-Aldrich, USA, unless otherwise specified.

Immunofluorescent labeling of the meiotic spindle and chromatin

Denuded oocytes were incubated for 45 min at 37°C in a glycerol-based microtubule stabilizing solution containing 25% (v/v) glycerol, 50 mM KCL, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM 2-mercaptoethanol, 50 mM imidazol, and 4% Triton-x-100 (Simerly and Schatten, 1993). After a brief wash with PBS-BSA, the oocytes were fixed and stored in 4% (w/v) paraformaldehyde until analysis.

When required, oocytes destined to microtubule labeling were first incubated for 1 h at room temperature with a 1:100 solution of mouse monoclonal anti-α-tubulin antibody (clone B1-5-1-2: Sigma-Aldrich) in PBS-BSA. After a further wash with PBS-BSA, the oocytes were incubated for 1 h with a 1:100 solution of goat anti-mouse second antibody conjugated to tetramethylrhodamine isothiocyanate (TRITC) in PBS supplemented with 2%

(v/v) goat serum. To visualize actin microfilament, the oocytes were then incubated in a solution of 0.165 μM Alexa Fluor® 488 phalloidin (Molecular probes) in PBS-BSA for 30 min. The oocytes were finally stained with 0.1 μg/ml fluorescent DNA labeling (4'6' Diamidino-2-phenylindole dihydrochloride: DAPI) to visualize the chromatin configuration of the oocytes.

Confocal laser scanning microscopy (CLSM)

To assess cell cytoskeleton and chromatin configurations during *in vitro* maturation, fluorescent-labeled oocytes were mounted on glass microscope slides in a 2 μl droplet of antifade medium (Vectashield, Vector Labs, Burlingame, CA, USA) to retard photobeaching, and sealed under a coverslip using nail polish. Examination was performed using a confocal laser-scanning microscope (C1, Nikon, Japan). Three laser sources from Diote 408 nm, Argon 488 nm and HeNe 543 nm were used to simultaneously excite the fluorescent signals from DAPI, Alexa Fluor® 488 phalloidin (microfilaments) and TRITC (microtubules) respectively. The digital-micrographs produced using the sequential scanning mode for the 3 separate colors were merged into single panel using EZ-C1 software (Nikon, Japan). The resulting multi-color micrographs were subsequently examined using Adobe Photoshop CS (Adobe System Inc., Mountain View, CA, USA).

Experimental design

To establish normal pattern of cell cytoskeleton changes, a total of 302 oocytes were examined at the specific time point during the course of *in vitro* maturation (0, 6, 12, 18 and 24 h) to ensure that all important stages of nuclear and cytoplasmic changes of cat oocytes were analyzed. The fluorescent labeled oocytes were examined using a confocal laser scanning microscope. This microscope provides a significant improvement of image quality compared with conventional fluorescent microscopes and also allows spontaneous excitement of the three fluorescent colors (actin microfilament, microtubule and chromatin).

Result

Recovery rates for good quality COCs ranged from 2-5 COCs per ovary (data not shown). Although a large proportion of these COCs were arrested at GV stage (92.5%,), they asynchronously resumed meiosis through metaphase I (MI), anaphase I (AI), telephase I (TI) and finally metaphase II (MII) during 24 h of IVM (Table 1). The GV oocytes required approximately 12 h to reach MI stage while *in vitro* culture for 24 h was sufficient to complete nuclear maturation of cat oocytes (63% MII).

The fluorescently microtubule-, actin microfilament- and DNA-labeled oocytes were examined with a confocal laser scanning microscope. At 0-12 h of IVM, the chromatin of GV oocyte was unevenly distributed and confined within nuclear membrane (germinal vesicle, Fig. 1A). At this stage, actin microfilaments were typically distributed throughout the ooplasm with a moderate concentration beneath oolema, while the oocyte demonstrated a weak staining of microtubules (Fig. 1A). A microtubule-rich domain, however, developed rapidly following germinal vesicle breakdown without any obvious microtubule organizing center. Metaphase I plate (meiotic spindle) that is formed by polymerization of microtubules was completely developed at 12-18 h, and this was typified by the formation of a symmetrical barrel-shaped meiotic spindle with the two sets of homologous chromosomes aligned at its center (Fig. 1B). During Anaphase I and Telephase I, the sets of chromosomes redirected to each anastral poles of meiotic spindle, the actin microfilaments then formed a dense network between the segregated sets of chromosomes (Figs. 1C, 1D). Finally, the second meiotic spindle was soon formed and, with assistance of actin microfilaments, one set of chromosomes was extruded as the first polar body that enveloped by unorganized microtubules and actin microfilaments (Figs. 1E, 1F).

Table 1. Data demonstrate percentage of nuclear maturation of cat oocytes. The oocytes were matured in maturation medium for 0, 6, 12, 18 and 24 h.

Time (h) of IVM	No. of oocytes	GV (%)	MI (%)	Ana I/Tel I (%)	MII (%)	Degen/ Parthenote (%)
0	67	62 (92.5)	2 (3)	0	2 (3)	1 (1.5)
6	50	49 (98)	1 (2)	0	0	0
12	50	28 (56)	22 (44)	0	0	0
18	48	10 (20.8)	23 (47.9)	8 (16.7)	7 (14.6)	0
24	87	16 (18.4)	11 (12.6)	2 (2.3)	55 (63.2)	3 (3.4)

Discussion

This study described the redistribution of microtubule and actin microfilaments in relation to meiotic competence of cat oocytes. It is generally accepted that 50-60% of cat oocytes resume and reach MII stage during *in vitro* maturation (Luvoni and Oliva, 1993; Wood et al., 1995; Spindler and Wildt, 1999). However, developmental competence of these MII oocytes is poor compared to *in vivo* matured oocytes (Nagashima et al., 1996; Swanson et al., 1996). Thus, only nuclear maturation is not a good parameter to indicate development potential of the oocyte. As the two cytoskeleton elements play an important role during meiosis, fertilization and early embryo development, configuration and redistribution of these cell cytoskeletons have thus become one of the parameters used to access cytoplasmic maturation of the oocytes (Kim et al., 1998; Lee et al., 2000; Tremoleda et al., 2001). This was achieved by multiple labeling of cell cytoskeleton and examined with laser-assisted confocal microscopy that provides 2-fold benefits. The multiple fluorescent- labeled structures can be examined simultaneously and then merged into a single panel. In addition, reducing "out-of-focus" by a pin-hole markedly improves the quality of digital micrographs.

A large proportion (>90%) of cat oocytes, recovered soon after ovarian excision, remained at GV stage, similar to a report of Bogliolo et al. (2004). These GV oocytes displayed only weak-labeled microtubules, while actin microfilaments were distributed throughout the ooplasm and formed a tranzonal network (Fig. 1A), which was connected to moderate-intensified actin microfilament beneath the oolema. These tranzonal actin microfilaments exert intercommunication between cumulus-corona radiate cell complex and the oocyte (Hyttel, 1987; Schoevers et al., 2005), especially via gap junction to maintain the cAMP levels that control the initiation of meiosis resumption. Bogliolo et al. (2004) demonstrated that a large proportion of GV oocytes resumed and reached MI stage during 8-18 h of *in vitro* maturation which is comparable to our results. The resumption of meiosis was, however, not synchronized among cultured oocytes. Because M-phase promoting factor (MPF) and mitogen activated protein kinases (MAPK) increase coincidently with germinal vesicle breakdown and inactivation of these kinases releases the oocyte from MII arrest, these MPF and MAPK thus play an essential role to regulate the meiosis of oocytes in many species as well as in the domestic cat (Bogliolo et al., 2004; Gavin et al., 1994). It is however unknown whether treating cat oocytes with roscovitine, a specific cyclin dependent kinase inhibitor, would efficiently allow the synchronization of the nuclear maturation of cat oocytes and thereby improving their developmental competence.

During transition of GV to metaphase I stage, it was clear that the microtubule organizing center (MTOC) was absent in cat oocytes similar to a number of other mammalian species studied (pig: Kim et al., 1996; man: Kim et al., 1998; horse: Tremoleda et al., 2001). However, mouse oocyte displays cytoplasmic MTOC around the

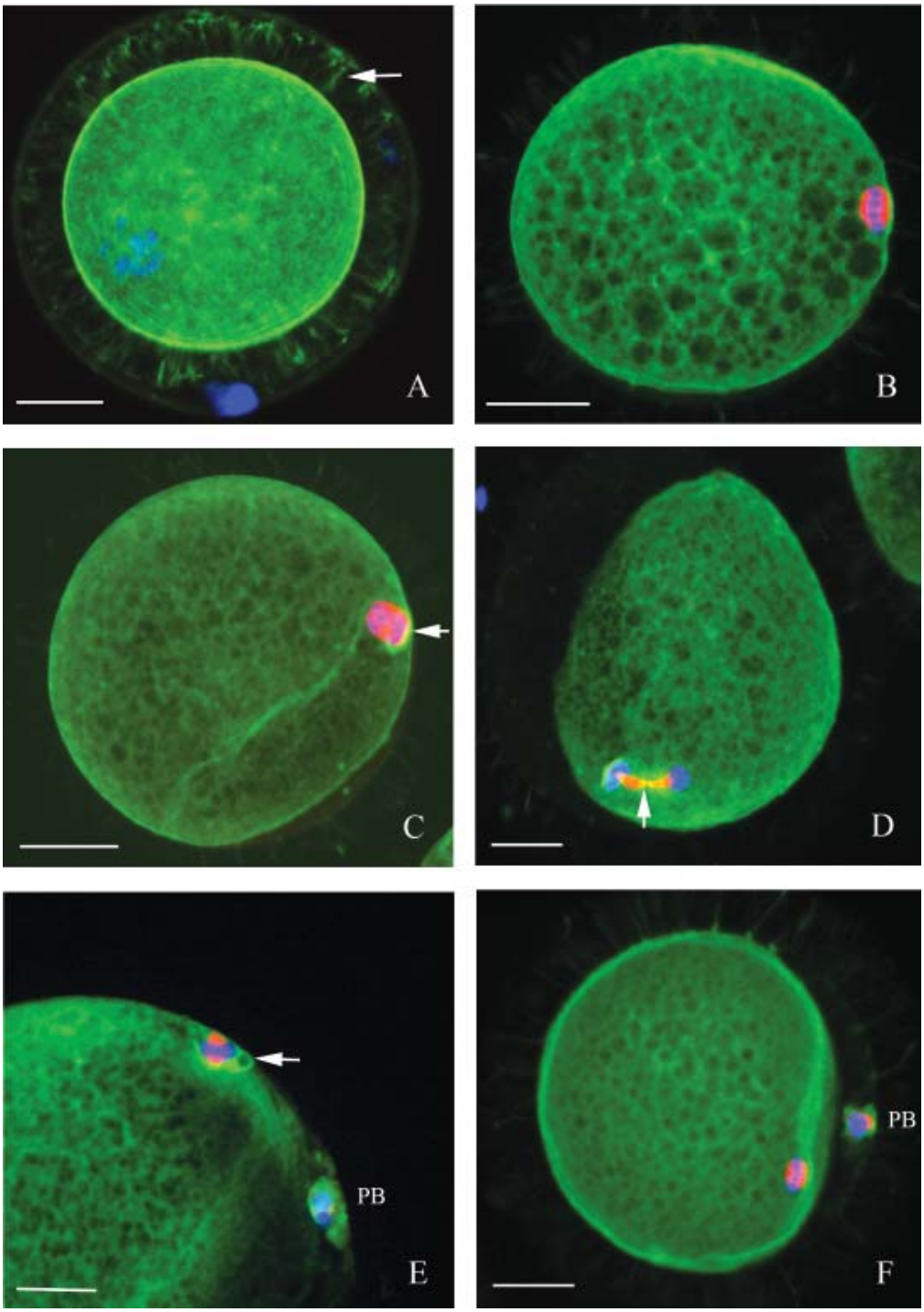

Figure 1 Confocal laser scanning photomicrographs of cat oocytes at different stages of *in vitro* maturation demonstrating the distribution of meiotic spindles (red), actin microfilaments (green) and chromatin (blue). (A) A germinal vesicle stage oocyte displays dense network of transzonal actin (arrow). (B) metaphase I: formation of a symmetrical barrel-shaped meiotic spindle with the two sets of homologous chromosomes aligned at its center. (C, D) anaphase I and telephase I: arrows indicate the role of actin microfilaments that form between the two sets of chromosomes, resulting in the extrusion of the first polar body. (E, F) metaphase II oocytes demonstrate metaphase II plate and the extruded 1st polar body (PB) surrounded by unorganized microtubules and actin microfilaments. Scale bars represent 25 μm.

time of GVBD that later facilitates the migration of female and male pronuclei after fertilization. In cat and other domestic animals, this syngamy of the pronuclei depends upon paternal (sperm) centrosomes (Sun et al., 2001). After metaphase I plate (meiotic spindle) was formed, it was clearly shown that actin microfilaments regulate the process of chromosome segregation by forming a "furrow" structure between the two sets of homologous chromosomes with a high concentration around the first polar body.

During *in vitro* maturation for 24 h, 63% of immature oocytes resumed and reached MII stage. However, it has been reported that nuclear maturation of cat oocytes occurs with 2-wave fashion. The first wave takes place by 26 h of IVM following with the second wave that occurs around 28-30 h (Katska-ksiazkiewicz et al., 2003). Of course, the maturation rate in this study would be increased if the oocytes were cultured longer than 24 h. It is generally acceptable to use 24 h IVM oocytes for *in vitro* fertilization, although it is relatively difficult to compare the results from different studies because the culture system used may affect both nuclear and cytoplasmic maturation of the oocytes. Prolonging *in vitro* maturation time, however, induces aging-like and also reduces viability and developmental capability in terms of fertilization and blastocyst formation rates (reviewed by Amstrong 2001). As meiotic spindle configuration has been used to evaluate the oocyte's quality (cat: Comizzoli et al., 2004; horse: Tharasanit et al., 2006), it is of great interest to examine whether good morphology of the meiotic spindle of cat oocytes would be correlated to its development competence, especially when "LH-free" rhFSH culture system was used. It is also important to compare the quality of oocyte's cytoskeleton between *in vivo* and *in vitro* matured oocytes whether or not rh FSH culture system would really affect on the quality of oocyte's cytoskeleton.

In conclusion, this study examined and described a series of nuclear and cytoplasmic changes during *in vitro* maturation of domestic cat oocytes by means of the redistribution of microtubule and actin microfilaments in relation to stages of nuclear maturation. Although the nuclear maturation occurred asynchronously, the examination of oocytes every 6 h provided useful information on cytoplasmic changes considering that proper redistribution and function of oocyte's cytoskeletons are essentially required during resumption of meiosis, fertilization and embryo development.

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