Ultrastructural study on mammalian oocytes during maturation process

สุวดี ชวนไชยะกูล, ปร.ด.
หัทยา เพชรพิบูลย์ไทย, วท.บ.

บทคัดย่อ การศึกษาโครงสร้างอย่างละเอียดของไข่ (cumulus oocyte complexes=COCs) ที่ดูดมาจาก antral follicle ของรังไข่หนู (rat) ด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน เพื่อเปรียบเทียบระหว่าง immature COCs และ mature COCs ที่ได้จากการเลี้ยง immature COCs ในน้ำเลี้ยงเชื้อนาน 8 ชั่วโมง พบว่า oocyte ของ immature COCs มี germinal vesicle (GV) และ cortical granules เป็นกลุ่มๆ ตรงริมเซลล์ oocyte ถูกล้อมรอบด้วย cumulus cells ที่มีลักษณะกลมหลายชั้น โดยเซลล์เหล่านี้จะส่ง processes ยื่นออกไปติดต่อกับทั้ง microvilli ของ oocyte และ cumulus cell ที่อยู่ข้างเคียง ส่วน mature COCs พบว่าการแบ่งตัวของ oocyte ดำเนินไปจนถึงระยะ metaphase II (M II) และพบ first polar body แยกตัวออกจาก oolemma แต่ไม่พบ germinal vesicle cytoplasm ของ oocyte มีลักษณะ homogeneous โดยมี cortical granules ไปเรียงตัวเป็นแถวอยู่ใต้ oolemma พร้อมที่จะปล่อยออกสู่ perivitelline space ทันทีที่เกิดมีการปฏิสนธิ ส่วน cumulus cells ที่พบมีลักษณะยาวขึ้นและอยู่กันอย่างหลวมๆ zona pellucida ของ mature COCs จะกว้างกว่าของ immature COCs และพบ microvilli น้อยกว่า แสดงให้เห็นว่าหลังจากเลี้ยง immature COCs ในน้ำเลี้ยงเชื้อเป็นเวลา 8 ชั่วโมง จะยังเกิดการเปลี่ยนแปลงได้ทั้งที่ nucleus และ cytoplasm ของ oocyte ซึ่งเราสามารถนำความรู้พื้นฐานนี้ไปประยุกต์ใช้ในการคัดเลือก mature oocyte ในการทำ *in vitro* fertilization ต่อไป

Abstract **Ultrastructural study on mammalian oocytes during maturation process**

Suwadee Chaunchaiyakul, Ph.D
Hattaya Petchpiboonthai, Ms.C

Transmission electron microscopy (TEM) was used to evaluate the fine structure of cumulus–oocyte complexes (COCs) of *in vitro* immature and *in vitro*

ภาควิชากายวิภาคศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
Department of Anatomy, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand

matured rat oocytes. COCs were aspirated from antral follicles (2–6 mm in diameter). Those with compact multilayered cumulus investment were cultured in drops of Tissue Culture Medium (TCM 199) for maturation. Intensive communications between spherical cumulus cells and immature oocyte and / or among cumulus cells can be confirmed by the presence of vesicle–filled of cell projections at the oocyte surface. The germinal vesicle (GV) is present. The oocyte contains cluster of cortical granules located in the periphery of ooplasm. Numerous microvilli projects from oocyte into the very narrow perivitelline space(PVS). TEM of mature oocyte revealed the elongated cumulus cells with loose connection. The cytoplasm of oocyte is homogeneous. The cortical granules can be observed lining up immediately beneath the oolemma and will be released into perivitelline space at the time of fertilization. The interruption of interaction between cumulus cells and oocytes may not stabilize the distribution of cortical granules. The perivitelline space of mature oocyte is wider than that of immature oocyte and shows only a few microvilli. The cell division continued and progressed to metaphase II (MII) and protruded the first polar body out of oocyte in final maturation. This study demonstrated that maturation of rat oocytes caused both nuclear and cytoplasmic changes after 8 hours of *in vitro* culture. The mature oocyte would be selected to use for in vitro fertilization.

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Introduction

The increasing application of biotechnologies for embryo production has emphasized the need of knowledge concerning the reproductive including biological fertilization and *in vitro* maturation (IVM), in vitro fertilization (IVF), and *in vitro* culture (IVC). There are only a few reports of a successful system for IVM, IVF and IVC in mammal. Therefore, *in vitro* techniques have been explored as an alternative route for embryo production. The process of oocyte maturation and fertilization appear to remain some of the major critical points when performed *in vitro*.

The biotechnology for increasing the number of the offspring by *in vitro* fertilization is interesting. Therefore, the morphology of the immature and mature rat oocytes at the electronmicroscopic level is needed to study. Ultrastructural investigations have revealed certain aspects of nuclear and cytoplasmic oocyte maturation in rat. However, only for human[1] and mouse[2] ultrastructural features of in vitro oocyte maturation have been compared with those of *in vivo* oocyte maturation, revealing only minor differences. The practical use of in vitro fertilization in rat relies on achieving proper mammal oocyte maturation *in vitro*. The objectives of this study were to provide an ultrastructural description of the nuclear and cytoplasmic changes taking place in the cumulus–oocyte complex during maturation *in vitro*.

Materials and Methods

Ten female rats were injected intraperitoneally with nembutal (sodium pentobarbiturate). Ovaries were removed immediately. The contents of follicles on the ovarian surface were aspirated from small vesicular follicles (2 mm in diameter) by using 5 ml disposable syringe with 18 gauge needle containing 1 ml of HEPES- buffered Tyrode's media (TALP-HEPES) and placed on glass petridishes. Follicular contents were viewed under a stereomicroscope, and the oocytes were collected by using fine drawn glass Pasteur pipette.

Oocyte were recovered within 2 h after slaughter. After recovery, oocytes were washed three times in TALP-HEPES supplemented with 10 % heat-treated fetal calf serum (HTFCS) and 50 g/ml gentamycin. After washing, oocytes were cultured in maturation medium (TCM-199 supplemented with 10% HTFCS, 15 g/ml FSH, 1 g/ml LH and 1 g/ml estradiol) in CO_2 incubator at 39 °c in 5 % CO_2, 95 % air with high humidity for 8 h in Falcon culture dishes.

Oocyte preparation for transmission electron microscopy

COCs were cultured for o h (before incubation) and 8 h in drops of TCM 199. After incubation, oocytes were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 4 °c and postfixed in 1% OsO_4 and 0.5% uranyl acetate, respectively. They were dehydrated in a graded series of ethanol and then transferred through propylene oxide and embedded in araldite. Oocytes were sectioned by using an ultramicrotome with glass knives. The semithin sections were stained with toluidine blue, and viewed by using light microscope while the ultrathin sections were stained with lead citrate and uranyl acetate observed under Hitachi H-300 transmission electron microscopy (TEM) at the accelerating voltage of 75 kV.

Results

Germinal vesicle (GV) stage (0 h before incubation)

Immature oocyte (before incubation) were surrounded by a compact multilayered cumulus investment. It showed eccentric and spherical nucleus or germinal vesicle (fig. A). Numerous dense mitochondria are located in the subplasmalemal area and in small cluster throughout the ooplasm. The cluster of cortical granules (CGs) were mainly located in the periphery of ooplasm (fig. C). The well develop golgi complexes and smooth endoplasmic reticulum were observed as numerous scattered aggregates. The cumulus cell process (CCP) projection penetrated through zona pellucida (ZP) to the perivitelline space (PVS). The cumulus cell process ending (CCPE) attached to the microvilli (MV) of the ooplasm and sometimes contacted directly to the oolemma by gap junction. Multivesicular body (MVB) was observed in the cumulus cell process ending.

The maturation stage (MII stage), (8 h of incubation)

At 8 h of maturation, the cumulus investment was completely expanded. TEM of mature oocyte revealed the loose connection of elongated cumulus cells. Only a few cumulus cell process endings were found in the perivitelline space and did not form gap junction, but they detached from oolemma. There were morphological changes of the maturation stage both nuclear and cytoplasmic maturation. The nuclear maturation showed the stage of metaphase II. Dense chromatin (metaphase II) was found in the eccentric area (fig.B). First polar

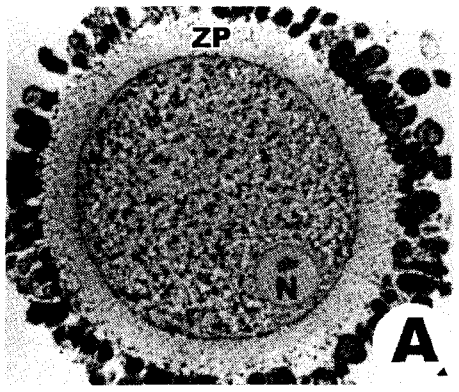

Figure A Micrograph of semithin section of a GV stage rat oocyte (o h before incubation). Note the healthy, compact multilayered cumulus investment surrounding the zona pellucida (ZP) and eccentric nucleus (N) or germinal vesicle (GV). The dense mitochondria are located in the subplasmalemmal area and in small clusters appear as dark sports throughout the ooplasm at this magnification. Lucent vesicles and semilucent lipid droplets are evenly distributed throughout the ooplasm. X400

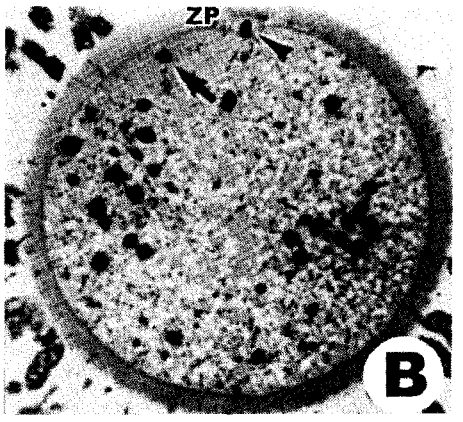

Figure B Micrograph of semithin section of a MII stage rat oocyte (8 h after incubation) showing the expanded cumulus investment. The chromosomes forming the second metaphase plate (arrow). The first polar body (arrowhead) is separated clearly from the oolemma. X400

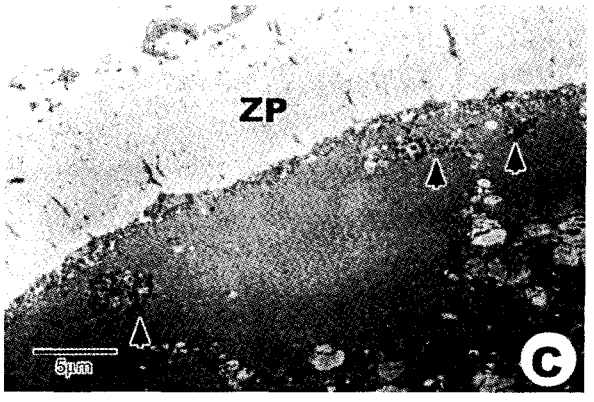

Figure C Electron micrograph of a part of immature oocyte showing the clusters of cortical granules (arrow) at the periphery. ZP = zona pellucida

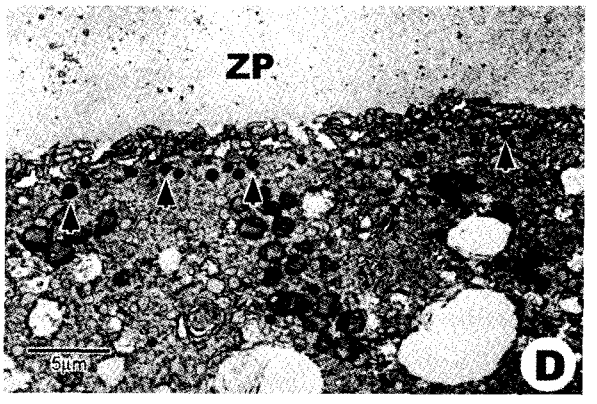

Figure D Electron micrograph of a subplasmalemmal detail of an MII stage rat oocyte. The cortical granules (arrow) can be observed immediately beneath the oolemma. ZP = zona pellucida

body containing condense chromatin configurations was at peripheral site and protruded out of the oocytes (fig.B). The cytoplasmic maturation showed the migration of individual cortical granules to solitary position along the oolemma (fig.D) and were released into the PVS at the time of fertilization.

Discussion

In immature oocytes at GV stage (0 h), there were the compact of cumulus investment. Cumulus cell process endings (CCPE) were observed in perivitelline space and contacted to the oolemma.

Multivesicular body (MVB) was found in the CCPE. Phillips and Dekel[3] suggested that there were communications in the cumulus oocyte complexes by the vesicle–filled in the cumulus cell projections at gap junctions which were often found between CCPE and oolemma. Gap junctions were always necessary for transferring amino acids, nucleosides and choline into the oocytes[4,7]

At the final oocyte maturation of metaphase II, the appearance of the first polar body indicated nuclear maturation. The cytoplasmic compartment was characterized by continued development of the lipid storage of the oocytes. Reduction in the golgi complex and alignment of the cortical granules form the structural background lead to block against polyspermy. These observations were similar in mature bovine oocytes[5,8,9,10]. The gradual increase in the lipid compartment of the oocytes throughout final maturation was probably of a great importance for the initial phase of the embryonic development. Thus, the lipid represents an energy pool comparable to the yolk in avian eggs, which was utilized up to the blastocyst stage [6,10].

In conclusion, both nuclear oocyte maturation and the sequence of structural events of cytoplasmic maturation of rat oocytes *in vitro* were described. These results will be used as the determinants for rat oocyte maturation. These morphological changes were the basic knowledge that lead to the successful *in vitro* maturation of oocytes. The higher percentage of oocyte maturation determines the higher fertilization rate and the higher yield of embryo transfer. The fertilization competency and the affected factors of oocyte maturation will be investigated in the further studies.

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