STUDIES ON THE EFFECT OF INHIBITING FACTOR-HYPOXANTHINE ON THE *IN VITRO* MATURATION OF BOVINE OOCYTES USING LIGHT AND TRANSMISSION ELECTRON MICROSCOPY

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BUSABA PANYARACHUN : STUDIES ON THE EFFECT OF INHIBITING FACTOR-HYPOXANTHINE ON THE *IN VITRO* MATURATION OF BOVINE OOCYTES USING LIGHT AND TRANSMISSION ELECTRON MICROSCOPY. THESIS ADVISOR : KANOK PAVASUTHIPAISIT, M.D.,Ph.D., REON SOMANA, Ph.D.,M.D., AHNOND BOONYARATAVEJ, Ph.D., PRAPEE SRETARUGSA, Ph.D., SANGCHAI PRUTTIPUN, M.D., PRAKONG TANGPRAPRUTGUL, Ph.D.

The inhibitory effects of hypoxanthine (HX) on nuclear and cytoplasmic maturation of cumulus bovine oocyte complexes (COCs) were studied by light and transmission electron microscopy (LM and TEM). COCs were exposed to 4 mM HX in TCM 199 for 0,8,16 and 24 h. Nuclear stages during maturation of normal oocytes occur during various time were compared with those oocytes treated with HX in the same intervals. To study the reversibility of HX action, COCs were washed out of HX after 24 h treatment and re-cultured in the inhibitory-free culture medium (TCM 199) for 24 h. At 0,8,16 and 24 h of HX treatment, the percentages of oocytes in GV stage were 95, 93, 81 and 76, respectively whereas in the untreated group, all oocytes underwent GVBD by 8 h culture. GVBD occurred completely after 5 h incubation which was twice as fast as in the control medium (9 h). However, seventy-six percent of oocytes could reach metaphase II (M II) after 24 h incubation whereas 80% of control oocytes reached M II. However, the percentages of oocytes reaching M II in both groups at 24 h were not statistically significant. The morphological study at the beginning of treatment with HX (0 h) showed that GV was intact without chromosome condensation. But after 8,16 and 24 h treatments, oocytes were maintained in the GV stage with condensed chromosome and normal intact nuclear membrane. During the maturation period, the oocytes underwent a series of clearly defined nuclear meiotic events. Oocyte maturation was also characterized by cytoplasmic changes. The cortical granules (CG) were composed of heterogenous and homogenous particles demonstrated by both the different electron densities and the different sizes similar to those immature oocytes. In contrast, the oocytes after washing out of HX and recultured in control medium. There were more dense electron particles and similar size granules as observed in the normal mature oocytes. These results indicated that 4 mM HX could inhibit the nuclear membrane breakdown but it could not prevent chromosome condensation at 8,16 and 24 h after treatment. Furthermore, HX could also inhibit the cytoplasmic maturation of CG. The HX treated oocytes could be recultured to mature and normally reached M II after washing out of HX. The morphology of both nuclear and cytoplasmic matuation of HX treated oocytes and their reversibility was demonstrated by TEM. The distribution of CG and the stages of DNA during normal oocyte maturation were also revealed by the confocal laser scanning microscope.

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บุษบา ปันยารชุน : การศึกษาสารยับยั้งที่มีผลต่อการสุกของไข่วัวในจานแก้ว โดยกล้องจุลทรรศน์ธรรมดาและจุลทรรศน์อิเลคตรอนแบบส่องผ่าน (STUDIES ON THE EFFECT OF INHIBITING FACTOR-HYPOXANTHINE ON THE *IN VITRO* MATURATION OF BOVINE OOCYTES USING LIGHT AND TRANSMISSION ELECTRON MICROSCOPY) คณะกรรมการควบคุมวิทยานิพนธ์ : กนก ภาวสุทธิไพศิฐ, พ.บ.,Ph.D., เรือน สมณะ, Ph.D.,พ.บ., อานนท์ บุณยะรัตเวช, Ph.D., ประพีร์ เศรษฐรักษ์, Ph.D., แสงชัย พฤทธิพันธุ์, พ.บ., ประคอง ตังประพฤทธิ์กุล, Ph.D.

การศึกษาสารยับยั้ง, Hypoxanthine (HX) ที่มีผลต่อการเจริญของนิวเคลียสและไซโตพลาสซึมของไข่วัวด้วยกรรมวิธีทางจุลทรรศน์ธรรมดาและจุลทรรศน์อิเล็คตรอนแบบส่องผ่าน มีการประเมินผลไข่ที่ได้รับการเพาะเลี้ยงในน้ำยาที่มีสาร HX โดยดูระยะของนิวเคลียสทุกช่วงเวลา 0,8,16, และ 24 ชั่วโมง ในชั่วโมงที่ 8 ของการเพาะเลี้ยงในน้ำยาที่มีสาร HX พบว่า ระยะนิวเคลียสของไข่อยู่ในระยะ germinal vesicle (GV) 93% ในชั่วโมงที่ 16 และ 24 พบว่า HX ไม่สามารถยับยั้งระยะการเจริญของนิวเคลียสให้อยู่เฉพาะในระยะ GV เท่านั้น ไข่ 13% และ 17% สามารถเจริญไปอยู่ในระยะ germinal vesicle breakdown (GVBD) ได้ตามลำดับ ในการศึกษาการผันกลับฤทธิ์ของสารHX ไข่ที่ได้รับการเพาะเลี้ยงในน้ำยาที่มีสาร HX เป็นเวลา 24 ชั่วโมง นำมาล้างและทำการเพาะเลี้ยงต่อในน้ำยาปกติที่ไม่มีสาร HX เป็นเวลา 24 ชั่วโมงพบว่า เกิด GVBD อย่างรวดเร็วในชั่วโมงที่ 5 ของการเพาะเลี้ยง ในขณะที่ไข่ปกติจะเกิด GVBD ในชั่วโมงที่ 9 ซึ่งเร็วเกือบเป็น 2 เท่าของไข่ในกลุ่มควบคุม นอกจากนี้ไข่ในกลุ่มที่ล้าง HX ออกสามารถเจริญและสุกถึงระยะ metaphase II 76% ในขณะที่ไข่ในกลุ่มควบคุมสุก 80% ในชั่วโมงที่ 24 ในช่วงเวลา 8,16 และ 24 ชั่วโมงของการเพาะเลี้ยงในสาร HX พบว่า มีการเกิด chromosome condensation ขึ้นใน GV โดยที่เยื่อหุ้มนิวเคลียสยังปกติ ส่วนไข่ที่ถูกยับยั้งด้วยสาร HX เป็นเวลา 24 ชั่วโมง พบกลุ่มของคอร์ติคอลแกรนูลที่ประกอบด้วยสารเนื้อเดียวกันและสารเนื้อผสม และมีขนาดแตกต่างกัน ลักษณะนี้พบได้ในคอร์ติคอลแกรนูลในไข่ปกติที่ยังไม่สุก เมื่อนำไข่กลุ่มนี้มาล้างและเพาะเลี้ยงต่อในน้ำยาปกติ พบว่า ลักษณะของคอร์ติคอลแกรนูลจะเรียงตัวกันเป็นแนวเดียวและติดสีเข้ม ซึ่งลักษณะเช่นนี้พบได้ในคอร์ติคอลแกรนูลในไข่ปกติที่สุกแล้ว จากการวิจัยพบว่าสาร HX สามารถยับยั้งการสลายของเยื่อหุ้มนิวเคลียส แต่ไม่สามารถป้องกันการเกิด chromosome condensation และยับยั้งการเจริญของคอร์ติคอลแกรนูลในขณะที่การเปลี่ยนแปลงของออร์กาแนลอื่นๆในไซโตพลาสซึมไม่ปรากฏเด่นชัด ไข่ที่ถูกยับยั้งด้วยสาร HX เมื่อนำมาเพาะเลี้ยง ภายหลังจากล้าง HX ออก สามารถสุกถึงระยะM II ได้ตามปกติ การศึกษาการสุกของไข่ทั้งในนิวเคลียสและไซโตพลาสซึมที่เพาะเลี้ยงด้วยHX และภายหลังการล้างออกของ HX ได้ศึกษาด้วยกล้องจุลทรรศน์อิเลคตรอน นอกจากนี้ยังสามารถศึกษาระยะนิวเคลียสและคอร์ติคอลแกรนูลได้พร้อมกันในเวลาเดียวกันด้วยกล้อง confocal laser scanning microscope

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LIST OF ABBREVIATIONS

AC	adenylate cyclase
AL	alulate lamella
AMP	adenosine monophosphate
Ana I	anaphase I
cAMP	cyclic adenosine monophosphate
CC	cumulus cell
CCPE	cumulus cell process ending
CG	cortical granule
Chr	chromatin
CLSM	confocal laser scanning microscope
COC	cumulus oocyte complex
CON CHR	condense chromosome
°C	degree Celsius
6-DMAP	6-dimethylaminopurine
dcAMP	dibutyric cyclic adenosine monophasphate
DNA	deoxyribonucleic acid
DRB	Dichoro 1B-D-ribofuranosylbenzimidazole
E_2	estradiol
EM	electron microscope
FF	follicular fluid

LIST OF ABBREVIATIONS (continued)

FITC	fluorescien isothiocynante
FSH	follicular stimulating hormone
G	Golgi complex
GnRH	gonadotropin-releasing hormone
GV	germinal vesicle
GVBD	germinal vesicle breakdown
h	hour
HC	heterochromatin
HCG	human chorionic gonadotropin
HPRT	hypoxanthine phosphoribosyl transferase
HTFCS	heat-treated fetal calf serum
HX	hypoxanthine
IMP	inosine monophosphate
IVF	in vitro ferilization
IVM	in vitro maturation
LCA	Lens culinaris agglutinin
LH	luteinizing hormone
M	mitochondria
M I	metaphase I
M II	metaphase II
MAPK	mitogen activated protein kinase

LIST OF ABBREVIATIONS (continued)

mm	millimetre
mM	millimole
mg	milligram
ml	millilitre
MPF	maturation promoting factor
MV	micovilli
ng	nanogram
Nu	nucleolus
OMI	oocyte maturation inhibitor
OsO_4	osmium tetroxide
PB	polar body
PBS	phosphate buffer saline
PDE	phosphodiesterase
PKA	protein kinase A
PI	propidium iodide
PO	propylene oxide
PVS	perivitelline space
RNA	ribonucleic acid
RNAase	ribonuclease
SEM	scanning electron microscope
SER	smooth endoplasmic reticulum

LIST OF ABBREVIATIONS (continued)

TEM	transmission electron microscope
Telo I	telophase I
μ	micron
μg	microgram
μm	micromole
V	vesicle
ZP	zona pellucida

CHAPTER I

INTRODUCTION

Oocyte Maturation

Oocyte maturation is defined as the reinitiation and completion of the first meiotic division, subsequent progression to metaphase II and the completion of nuclear and cytoplasmic process essential for fertilization and early embryo development. Oocytes arrest in prophase I of meiosis during the fetal period. This nucleated stage persists until maturation begins. During this prolonged period, the oocyte enlarges and synthesizes RNA and protein as the follicle grows. The oocyte and follicle become sensitive to the actions of gonadotrophins. The follicle stimulating hormone (FSH) and luteinizing hormone (LH) surges in mid-cycle, or the external application of human chorionic gonadotrophin (HCG), initiate the onset of maturation in antral follicles and oocytes (1).

Completion of the first meiotic division takes place when oocytes have undergone an extensive growth in a cellular interaction with the granulosa and theca cells. The oocyte undergoes asymmetric cytokinesis and extrudes the first polar body containing a haploid chromosome complement. Immediately after the first meiotic division is completed, the second meiotic division inititates. The oocytes arrest in metaphase II until fertilization occurs and activates the mature oocytes. The follicular cells interact with gonadotrophin and steriod hormones as well as other substances such as growth factors, inhibitory factors (2) to affect the carefully integrated process

of oocyte maturation. Mechanisms of oocyte maturation are still under investigation. The in vitro models give insight into the importance of substances affecting oocyte maturation and its inhibition such as cAMP, calcium, cell cycle, proteins, growth factors, gonadotrophin-releasing hormone (GnRH), gonadotrophins, purines and steroids. Not all oocytes cultured after isolation from their follicles undergo maturation in vitro. The proportion of in vitro maturing oocytes appears to depend upon various factors such as culture medium, temperature, time, growth and inhibiting factors. In preparation of the oocytes for the in vitro fertilization (IVF), not only must meiotic oocur, but the cytoplasm of the oocyte must undergo critical changes in order to achieve competency of support sperm chromatin decondensation and subsequent male pronuclear formation (3). Some mature oocytes may lack the ability to decondense sperm chromatin and subsequently to form male pronuclei. Therefore, the preparation of the oocyte for in vitro maturation (IVM) is very critical to provide good quality oocytes for IVF.

The process designated as oocyte maturation is characterized by a series of morphological, and functional changes that take place within the nucleus, highlighted by the following events : (1) dissolution of the nuclear membranes manifested as germinal vesicle breakdown (GVBD) (2) chromatin condensation and the formation of distinct chromosomes, were clearly observed after GVBD (3) formation of the first meiotic spindle and translocation of the spindle to the peripheral region (Metaphase I stage = M I) (4) the appearance of chromosomal separation and movement to the opposite pole (Anaphase-Telophase I) (5) formation and extrusion of the first polar body (6) formation and positioning of the second meiotic division (Metaphase II stage

= M II) and rearrest at the second metaphase. Such development from prophase I to metaphase II is often referred to as the period of oocyte maturation. The second meiotic arrest at metaphase II is maintained until the mature egg is either fertilized or parthenogenetically activated, when meiosis is completed with extrusion of the second polar body. The events of oocyte maturation are controlled by maturation promoting factor (MPF) (4).

The mature oocyte is one of the largest cells in the body of mammals. It represents a highly specialized, which differentiates the cells. The diameter of the mammalian oocyte is between 60 and 150 μm;. They are composed of two compartments, the nucleus called germinal vesicle (GV) and the cytoplasm. The germinal vesicle is located in the center of oocyte in mammals. It migrates gradually to the periphery corresponding to the gradual growth of the oocytes. At first the GV is spherical but prior to LH surge the GV flattens against the plasma membrane. The rupture of the nuclear envelope (GVBD) occurs wherever the position of the GV. Descriptions of the ultrastructural morphology of in vivo and in vitro maturing bovine oocytes have previously been reported. Mitochondria of immature oocyte were located in a generally peripheral position whereas Golgi complexes, endoplasmic reticulum and numerous vesicles were evenly distributed in the cytoplasm. During maturation, the cluster of mitochondria were disappeared from the peripheral. They were dispersed in the ooplasm. At 22 to 24 h after incubation, the polar body was extruded and M II chromosome was presented. The Golgi complexes also decreased in size (5,6). The nuclear maturation is composed of

A) Meiotic competence

B) Meiotic resumption

A. Meiotic Competence

The growing oocyte becomes progressively capable of resuming meiosis called "meiotic competence," which appears only when the oocyte is about 80% of the size of the fully developed one. In vivo, studies have shown that specialized membrane contacts with follicular cells is necessary for oocyte growth. This growth is maintained when oocytes are cultured within the surrounding of follicular cells. The favorable effect of FSH or estradiol on the percentage of competent oocytes is probably related to the favorable effect of these hormones on the whole ovary particularly on the follicular cells rather to a specific action on the oocytes. However, the stage of competent bovine oocytes that bring about the high percentage of them reaching metaphase II is higher when culture medium was supplemented with heat-treated fetal calf serum (HTFCS), FSH, LH and estradiol. Growing mammalian oocytes (<90 μm in diameter) are unable to resume meiosis in vitro. A diameter of 120 μm cell represents fully grown oocyte size which is able to undergo further meiotic progression, the transition from the G2-to M-phase (7).

B. Meiotic Resumption

Meiotic resumption occurs when oocytes are isolated from their follicular environment and are placed in a simple maturation medium. The oocytes must be competent to resume meiosis. It has been shown that competent oocytes must reach a

minimum diameter before resuming meiosis. However, once the oocytes become competent, they need these factors to maintain meiotic arrest. It is generally recognised that follicular cells produce inhibitors necessary to maintain the oocytes in meiotic arrest. The removal of the oocyte from its follicular environment deprives the oocyte of inhibitory factors. Oocytes then resume meiosis (8). The period of meiotic progression is controlled by MPF (9). In horses, newly synthesized proteins are necessary for progression from M I to M II. These must include proteins essential for the completion of meiosis I as well as resumption of meiosis II. Formation of the M I plate is associated with high activity of MPF (histone H1 kinase) as reported in pigs (10). Histone H1 kinase activity increases during the M I to M II transition in pigs. The proteins needed for progression from M I may be related to activation of MPF, dephosphorylation of p34cdc2 or proteolysis of cyclin B or of cytostatic factor (CSF), which is a gamete-specific protein probably similar to the product of the gene, c-mos, stabilized the cyclin-B component of MPF. In mice, the c-mos product is required for resumption of meiosis II (11).

Oocyte Maturation Inhibitor (OMI)

Mammaian oocytes undergo spontaneous germinal vesicle breakdown when isolated from the ovarian follicle and cultured in vitro. Yet, the oocytes remain arrested in prophase I of meiosis in vivo unless stimulated by gonadotrophin to resume meiotic maturation. Thus, the follicular environment provides an inhibitory influence that maintains the oocyte in meiotic arrest (2).

During their growth phase mammalian oocytes are blocked in the diplotene/dictyate stage of the first meiotic prophase and present decondensed chromosomes distributed in the oocyte nucleus (12). Chromatin condensation oocurs in fully grown in oocytes in response to the preovulatory surge of gonadotropin hormones (13) and the subsequent activation of MPF in oocyte cytoplasm (14) Mammalian oocytes are arrested in late G2 of the first meiotic division. Resumption of meiosis depends on action protein synthesis. This suggests that proteins required for M phase transition have probably showed up in the prophase oocyte (15).

In 1955, Chang demonstrated that follicular fluid (FF) can block oocyte maturation in vitro (16). This finding, as well as subsequent observations, led to the concept that FF contains an oocyte maturation inhibitor (OMI) (17). The putative OMI was postulated to be a heat-stable peptide with a low molecular weight as cyclic nucleotide (18). Heating of the follicular fluid at 60 °C for 20 min, or of its low molecular weight fraction to 90 °C, did not destroy OMI activity but its activity was abolished by treatment of the follicular fluid by trypsin (19). An OMI was isolated from pig and mouse FF and identified as the purine, hypoxanthine (HX) (20). In vivo, the oocyte is exposed to the intrafollicular environment which maintains the oocyte arrested in the meiotic prophase. Meiosis is prevented in antral follicles by OMI produced inside. OMI inhibited the resumption of meiosis only in oocytes cultured within their intact cumulus, but failed to affect the maturation of denuded oocytes. The inhibitory action of OMI is apparently exerted through the mediation of cumulus cells and the signal conveyed through cumulus cell-oocyte gap junction. The cumulus cells and their junctional associations with the oocyte are essential for the

maintenance of meiotic arrest. It has been suggested that the follicular purine, HX which maintains meiotic arrest in mouse oocytes is a main inhibitory substance of oocyte maturation (21).

Although the issues on the existence and nature of these substances are still controversial, the importance of the physiological role on follicular environment in oocyte maturation has been increasingly gained in an effort to elucidate the active substance (s) responsible for the regulation of oocyte meiotic maturation.

It has been reported that HX inhibit meiotic resumption of oocytes in many mammalian species such as the mouse (22,23,24,25,26), cow (27) and monkey (28). Hypoxanthine or adenosine alone had little effect to prevent GVBD on mouse oocytes maturation. While there was a strong synergistic effect of hypoxathine and adenosine in meiotic arrest (22). Hypoxanthine and adenosine in combinations decreased the number of bovine oocytes undergoing GVBD at 6 h after incubation (27). Rhesus monkey oocytes were maintained in meiotic arrest for 40 hours by treatment with hypoxanthine and dbcAMP, and this effect was overcome by removal of inhibitors from the culture medium (28). The meiotic maturation of denuded oocytes was significantly inhibited by hypoxanthine and/or adenosine in a dose-dependent manner. GVBD was inhibited, when hypoxanthine was microinjected into the cytoplasm of denuded oocytes. On the contrary, a microinjection of adenosine was not effective in inhibiting GVBD. The inhibitory action of hypoxanthine in pig oocytes was dose dependent at the concentrations of 1-6 mM and was reversible following the subsequent culture in hypoxanthine-free medium (29).

Hypoxanthine and cyclic (cAMP) were to play a pivotal role in maintenance of mammalian oocyte meiotic arrest (30). In this respect, Downs et al., in 1989 postulated that the action of HX in meiotic arrest may be mediated by cAMP (2). A simplified representation of the cAMP regulation within the cell is shown in Figure 1. The protein kinase A (PKA) was dependent of cAMP. This kinase was part of a signal transduction pathway of cAMP including adenylate cyclase (AC) and phosphodiesterase (PDE). PKA was most effective in preventing GVBD in mouse oocytes. In hamster oocytes, PKA activity seems to be necessary to maintain meiotic arrest since the inhibition of PKA induce meiotic resumption (31). The key role played by cAMP in the control of meiotic is well established. The first study demonstrated the inhibitory effect of cAMP on meiotic resumption of mouse oocytes (32). High level of cAMP within the oocyte maintained meiotic arrest whereas a decrease of cAMP level within the mouse oocyte preceded meiotic resumption. It has also been suggested that a part of cAMP required could be synthesized by cumulus cells and transferred to the oocyte by the gap junction (33). It was also possible that the stimulation of cumulus cells induces the cAMP production at basal level able to maintian meiotic arrest (34). In the bovine, AC has been located in cumulus cells, specifically on the cumulus cell processes contacting the oocyte. Also, the oocyte membrane was provided with the very active PDE and its activity was regulated by PDE inhibitors. The expression and the specific regulation of PDE would be implicated in the control of cAMP level and in oocyte maturation (35). When these events was related to the role of PKA, it has been proposed that in bovine oocytes, the regulatory subunit of PKA could modulate transcription in cumulus cells. The

catalytic subunit could maintain meiotic arrest and phosphorylating regulatory key proteins. Biochemical events downstream of PKA activation by cAMP was not clearly elucidated (36).

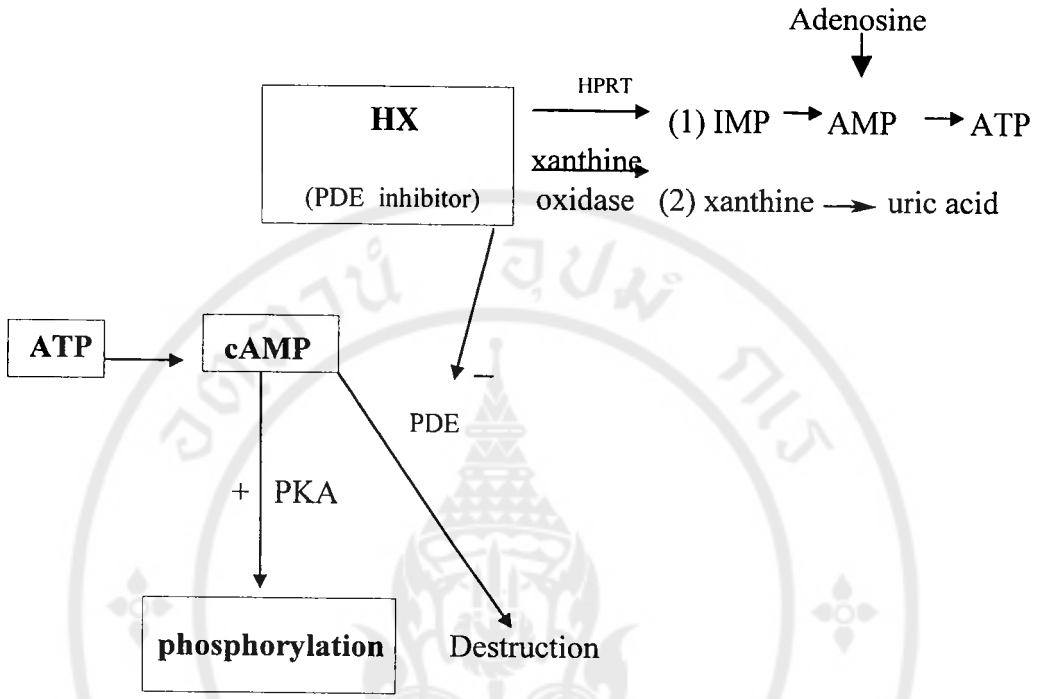


Figure 1. A simplified representation of action of HX and metabolism of HX in the oocyte. ATP = Adenosine triphosphate, cAMP = cyclic adenosine monophosphate, PKA = protein kinase A, PDE = phosphodiesterase, HPRT = hypoxanthine phosphoribosyltransferase (25,36).

Metabolism of HX by the oocyte or oocyte-cumulus cell complex may be important in the mechanism involved in the inhibitory action (Figure 1). Two possible routes of hypoxanthine metabolism exist : (1) salvage to inosine monophosphate (IMP) via the enzyme, hypoxanthine phosphoribosyltransferase (HPRT), (2) metabolism of HX of xanthine and uric acid by xanthine oxidase does not mediate the inhibitory action of this purine base on meiotic maturation (25). It is possible that IMP mediates the inhibitory action of this purine.

There were other inhibitors that were used to maintain meiotic arrest in mammalian oocytes maturation in vitro. Inhibitor of phosphorylation, 6-dimethylaminopurine (6-DMAP) affects on GVBD and maturation in mouse and bovine oocytes. Although the inhibitory effect of 6-DMAP was fully reversible, the morphology of M II chromosomes was abnormal in nearly all cases. The chromosomes were thinner and usually randomly arranged (37). Dichoro-1B-D-ribofuranosylbenzimidazole (DRB), a specific inhibitor or RNA polymerase II-mediated transcription has been used to arrest GVBD in bovine cumulus-oocyte complexes (COCs). There were limitations for DRB. It must be replaced frequently during the culture period (3 h intervals). This appears to be due to the degradation of the compound in the culture media, since the ability of DRB to inhibit meiosis was lost if the compound was incubated at 39 °C up to 8 h in maturation media (38). Other transcriptional inhibitor, α-amanitin was used to block GVBD (39,40). Alpha-amanitin was only partially reversed after its removal and was associated with an increased incidence of oocyte degeneration. Morphology of degenerative changes was identified in both murine and bovine embryos after exposure to even moderated

doses of α-amanitin (41). Vanadate is an inhibitor of protein tyrosine phosphateses that affects meiotic resumption in bovine oocytes. Vanadate completely blocked GVBD with most oocytes (84%) remaining at the GV stage after 24 h culture. The inhibitory effect of vanadate was in reversible, even at the lowest effective dose (42). Active protein synthesis is required for the moietic resumption in oocytes of pig, goat and cattle (15,43). Bovine oocytes did not undergo GVBD in media with protein synthesis inhibitors such as puromycin and cycloheximide. The inhibition of GVBD was fully reversible, since after washing the inhibitors, oocytes undergo GVBD (44,45). Moreover, after washing of cycloheximide oocytes matured in vitro and developed to blastocysts following in vitro fertilization (45). Recently roscovitine, a potent inhibitor of MPF kinase activity maintained cattle oocytes at the GV stage for a 24 h culture period. This inhibitory effect of roscovitine was fully reversible (46). It has also been reported that small dose of ethanol or dimethylsulphoxide (DMSO) could cause profound negative effects on bovine in vitro maturation and subsequent embryo development (47).

Comparing to other inhibitors, HX appears to be non-toxic to the mouse oocyte and could reversibly maintain cultured mouse oocytes in meiotic arrest (22). In addition, HX treated mouse oocytes were reversible to provide high rate of polar body (24). Inhibitory effect of HX has also been investigated in bovine and pig oocytes but their reversiblity and detail morphology has not been reported.

It is of interest to analyzed the effect of HX in the maintenance of bovine oocyte meiotic arrest and their reversibility to mature to metaphase II using light microscope (LM) and transmission electron microscope (TEM). The reports of

inhibitory effect of HX on meiotic arrest on bovine oocyte are limited comparing to other species. Moreover, the ultrastructure of bovine oocytes after HX treatment and its reversal during maturation has never been reported. Although the morphology of DNA and cortical granules (CG) during maturation in bovine oocytes could be demonstrated by TEM, this approach suffers from the major drawbacks that the experimental trunover is long and only relatively few oocytes could be examined for any particular treatment. DNA and CG were visualized in a confocal laser scanning microscopy (CLSM) by the use of fluorescently labelled propidium iodide and lectins (48,49). There are some advantages of using this technique when compare with that of routine TEM method for it allows rapid visualization and examination of DNA and CG distribution both in immature and mature oocytes Therefore, CLSM was also employed to verify the feasibility in determination of bovine oocytes maturation in the present experiment.

The hypothesis of the present study was whether hypoxanthine could be able to inhibit bovine oocyte maturation and its effect was reversible to allow the treated oocytes to reached M II normally after washing out of inhibitor.

CHAPTER II

OBJECTIVES

The aims of the present study were

1. To investigate the optimum concentration of hypoxanthine as bovine oocyte maturation inhibitor.
2. To determine the inhibitory effects of HX treatment and its reversibility for bovine oocyte maturation in vitro at various time interval.
3. To study the morphology of bovine oocyte maturation using LM and TEM in both normal and HX treatment.

CHAPTER III

MATERIALS AND METHODS

1. Oocyte collection and maturation in vitro (Figure 2,12)

Bovine ovaries showing follicular development were obtained from a slaughterhouse. Ovaries were removed within 30 min after slaughter and transported to the laboratory in a thermos (25 to 30 °C containing 20 ml of 0.9% NaCl with 100 iu/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone. The retrieved ovaries include the cows of both *Bos indicus and Bos taurus.* The small vesicular follicles (2 to 5 mm in diameter) visible at the ovarian surface were aspirated into a 20 ml disposable syringe (with an18 gauge needle) containing 1 ml of HEPES-buffered Tyrode's media (TALP-HEPES) and were placed in Petri-dishes. The oocytes were removed from the follicular content with a glass pipette under a stereomicroscope.

Oocytes recovered within 2 to 3 h after slaughterhouse were washed three times in TALP-HEPES supplemented with 10% heat-treated fetal calf serum (HTFCS) and 50 μg/ml gentamycin. They were then cultured in CO_2 incubator (Forma Scientific Co., USA) at 39 °C in 5% CO_2, 95% air with high humidity at 8, 16 and 24 h in 50 μl droplets of a maturation medium under 10 ml of paraffin oil in 60x15 mm Falcon culture dishes (Becton Dickinson Labware, Lincoln Park, NJ). Each droplet contained 5 to 10 cumulus-oocyte complexes (COCs). Maturation medium was TCM 199 supplemented with 10% HTFCS and 5 μg/ml follicle-

stimutating hormone (FSH), 1 μg/ml LH and 1 μg/ml estradiol diluted with ethanol. Although HEPES buffer in culture medium maintains proper pH level for a longer culture period outside a 5% CO_2 incubator, TCM 199 was used to maintain pH in CO_2 environment. The pH of the maturation medium with 2.20 mg/ml $NaHCO_3$ was about 7.2 to 7.3 after equilibration in CO_2 incubator and only slightly decreased at the end of maturation by 24 h by observing the color of the medium with phenol red as an indicator.

2. Hypoxanthine treatment (Figure 6,7)

In preliminary experiments, oocytes were cultured for 8 h in TCM 199 with 0.2, 2, 4 and 6 mM HX. High percentage of maintaining meiotic arrest at GV stage is the criteria for dose selection. For subsequent experiments a concentration of 4 mM HX was used. Treated oocytes were cultured in TCM 199 with 4 mM HX and supplement with 10% fetal calf serum, 5 μg/ml of FSH, 1μg/ml of LH and 1 μg/ml of estradiol. The nuclear status was observed at 0, 8, 16 and 24 h after HX treatment.

To test the reversible effect of HX. Oocytes were washed in inhibitor-free medium after being cultured for 8, 16 and 24 h in TCM 199 with 4 mM HX. The washed oocytes were cultured in inhibitor-free maturation medium for additional 24 h (Figure 7).

Statistical analysis

For statistical analyses the software program was used. The data were analyzed by Mann Whitney U test (nonparametric statistics). $P<0.05$ was chosen as the significant level.

3. Oocytes staining for fluorescent microscope (Figure 3)

Immature (0 h) and maturing oocytes (8, 16 and 24 h) were stripped of their cumulus cells by manual pipetting in 0.3 % hyaluronidase. Denuded oocytes were stainned with Hoechst 33342 (Sigma) for 10 min. The specimens were washed with phosphate buffer saline (PBS) and were examined with fluorescent microscopy.

4. Oocytes staining for confocal laser scanning microscope (CLSM) (Figure 4)

Immature (0 h) and maturing oocytes (8, 16 and 24 h) were stripped of their cumulus cells by manual pipetting in 0.3% hyaluronidase. Zona pellucida were removed by 0.1% pronase (Sigma). Then, these denuded oocytes were fixed in methanol at -10 °C. After several washes with PBS the oocytes were permeabilized in 0.1% triton x-100 and were incubated in 1 mg/ml RNAase (Sigma) to digest rRNA and ribosome in the cells. DNA was fluorescently detected by exposure to 20 μg/ml propidium iodide (Sigma) for 1 h. The specimens were washed with PBS and followed by incubation in 10 μg/ml fluorescien isothiocyanate (FITC) labelled lectin (Lens culinaris agglutinin = LCA; Sigma) for 1 h.

The specimens were washed with PBS and examined CLSM. This microscope was performed using a Bio-Rad MRC 1024 equipped with an argon ion laser for the simultaneous excitation of fluorescein, FITC labelled lectin for CG and propidium iodide for DNA. The images were digitally recorded and archived on an erasable magnetic optical disk.

5. Aceto-orcein staining for light microscope (Figure 5)

Immature (0 h) and incubated oocytes at 8, 16 and 24 h were stripped off their surrounding cumulus cells by manual pipetting in 0.3% hyaluronidase. Denuded oocyte were fixed in ethanol-acetic acid (3:1; v/v) overnight, stained with 1% orcein in 25% acetic acid and destained with ethanol-acetic acid. Oocytes were evaluated for stage of meiotic maturation by phase contrast microscopy.

Chromosomal configurations used for classification were categorized as follows :

- The germinal vesicle (GV) stage consisted of a round shaped nucleus containing a permanent nucleolus and fine filaments of chromatin. The presence of an intact nuclear membrane was observed.
- The geminal vesicle breakdown (GVBD) / prophase stage was chracterized by the appearance of condensing chromosome and the absence of nucleolus and nuclear membrane.
- Metaphase I was identified by the presence of single row of pair chromosome.
- Anaphase I was designated by the appearance of chromosomal separation and movement to opposite pole.

- Telophase I was identified by the appearance of two groups of chromosome.
- Metaphase II was classified when the oocytes extruded the first polar body into perivitelline space.

6. Preparation of oocytes for transmission electron microscopy (TEM)

(Figure 8)

The immature and maturing oocytes were primarily fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 for at least 1 h to overnight at 4 °C. They were washed in the same buffer and secondarily fixed in 1% OsO_4 in 0.1 M cacodylate buffer for 1 h at 4 °C. After washing for 3 times in 0.05 M sodium cacodylate buffer, oocytes were stained with 0.5% uranyl acetate and dehydrated in a graded series of ethanol. The ethanol was removed by propylene oxide. The oocytes were then infiltrated with propylene oxide. After treatment with the solution of 2:1 propylene oxide per epoxy resin for one hour, they were finally kept overnight in the solution of 1:2 propylene oxide per epoxy resin. On the following day, oocytes were carefully embedded in epoxy resin in a flatmole. This flatmole was kept in the oven at 45°C for two days and at 60 °C for 3 days to polymerize the resin. Oocytes were then sectioned with a microtome using the glass knives. The semithin sections were stained with toluidine blue, and ultrathin sections were observed under Hitachi H-300 TEM at the accelerating voltage of 75 kv.

7. Preparation of oocytes for scanning electron microscopy (SEM)

(Figure 9)

There were two groups of oocytes. The first group was immature and mature oocytes with surrounding cumulus primarily fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 for at least 1 h to overnight. In the second group, the cumulus cells were partially removed for SEM by manual pipetting in 0.3% hyaluronidase. Zona pellucida was exposed and not surrounded by cumulus cells. Then, these oocytes were fixed in the same procedure as the first group. Both group of oocytes were washed and secondarily fixed in 1% OsO_4 in 0.1 M cacodylate buffer for 1 h at 4 °C. After washing three times in 0.05 M sodium cacodylate buffer, oocytes were dehydrated in a series of increasing concentrations of ethanol and critical point dried. The specimens were then sputter coated with gold and observed with JEOL SEM at an accelerating voltage of 15 kv.

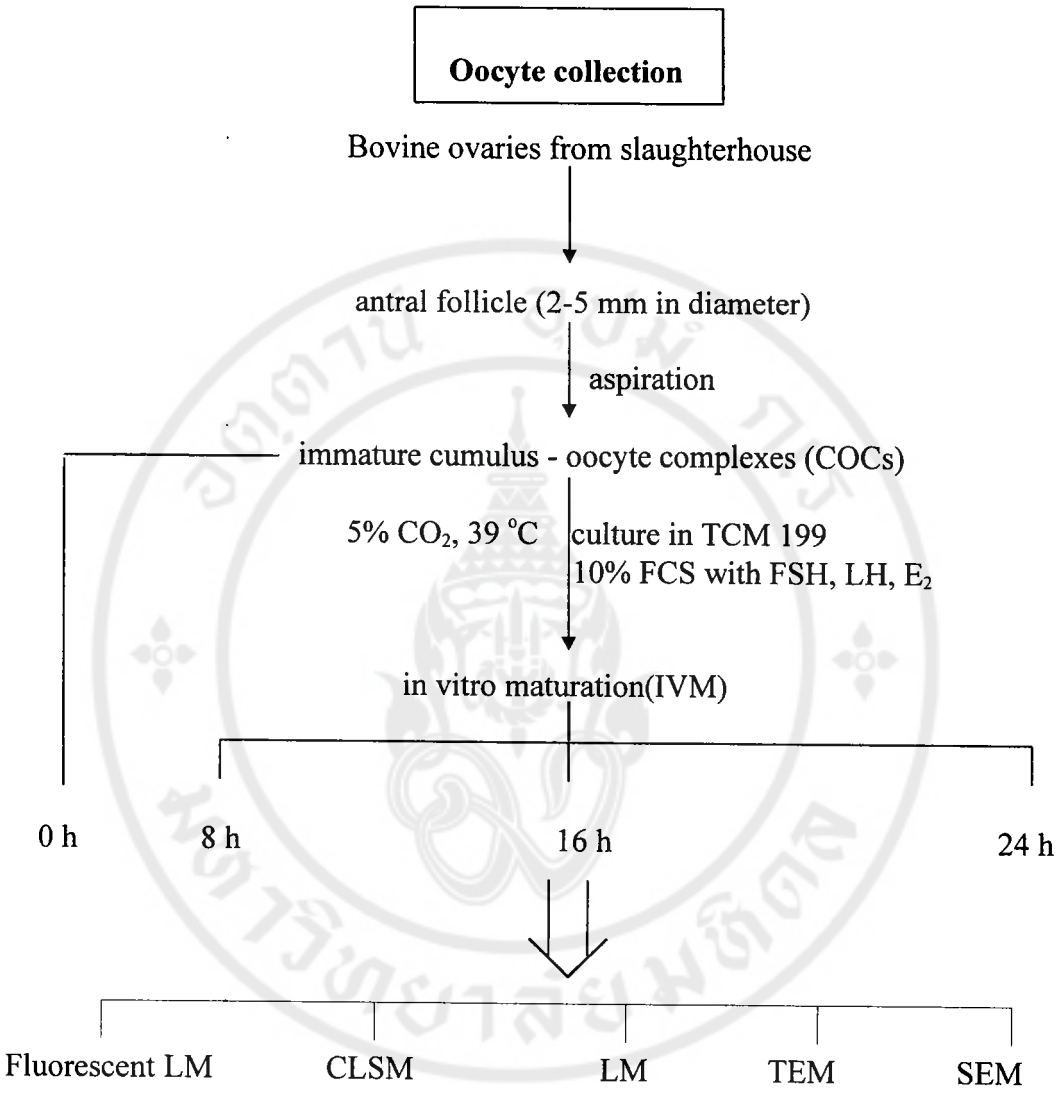


Figure 2. The diagram illustrating experimental plan. The bovine oocytes were recovery from ovaries. Immature (0 h) and maturing oocyte (8, 16 and 24 h) were stained for meiotic stage evaluation. Fluorescent LM = fluorescent light microscope, CLSM = confocal laser scanning microscope, LM = light microscope, TEM = transmission electron microscope, SEM = scanning electron microscope.

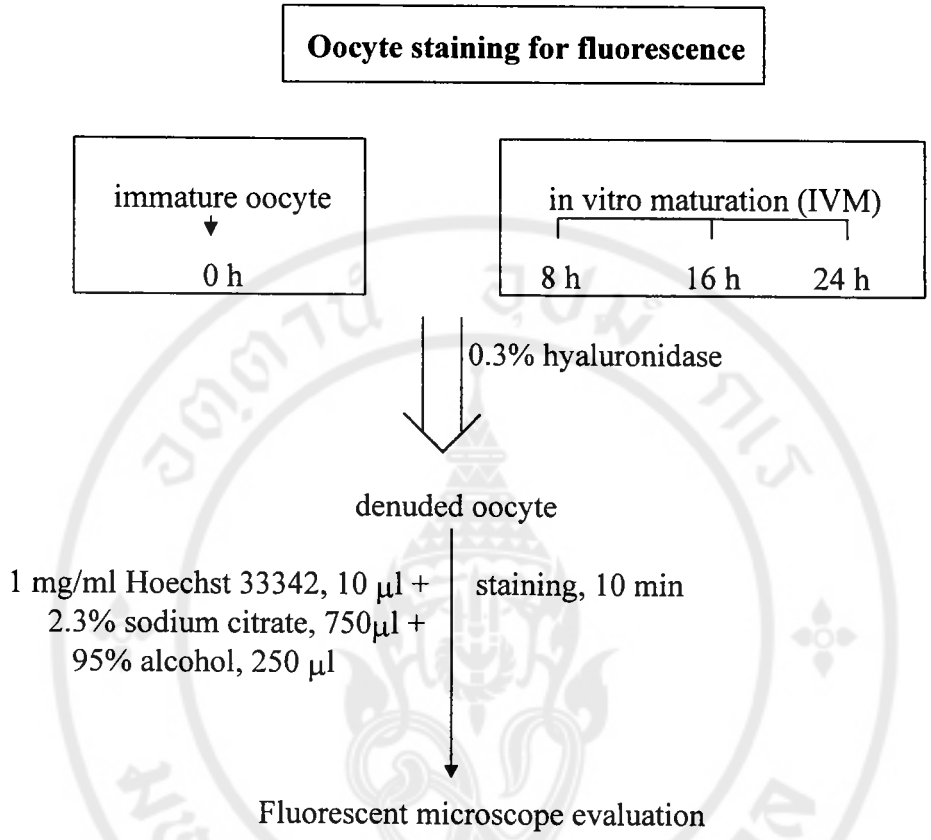


Figure 3. The diagram showing the oocyte preparation for fluorescent light microscope evaluation.

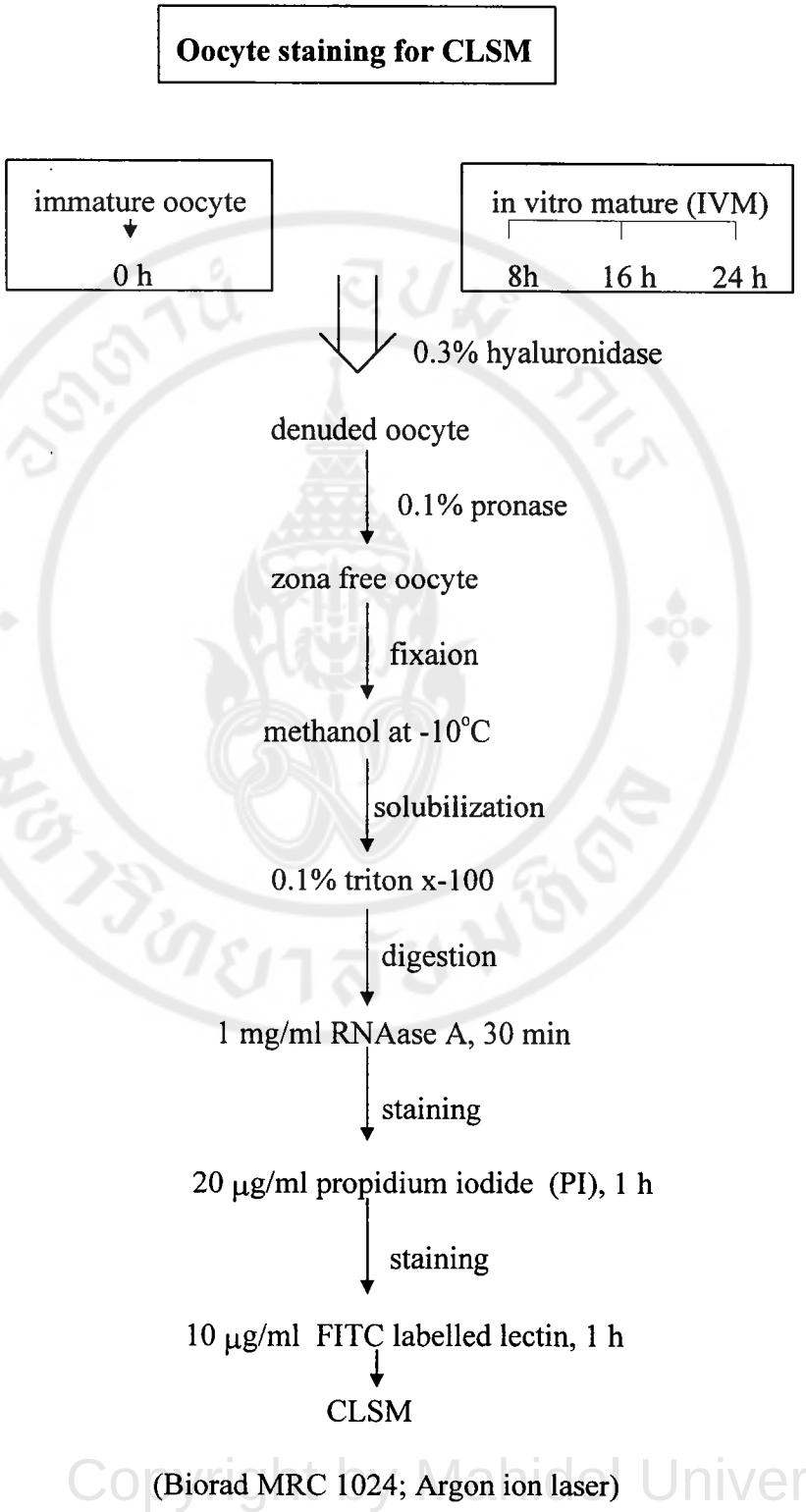


Figure 4. Diagram showing the oocytes staining PI, FITC for CLSM.

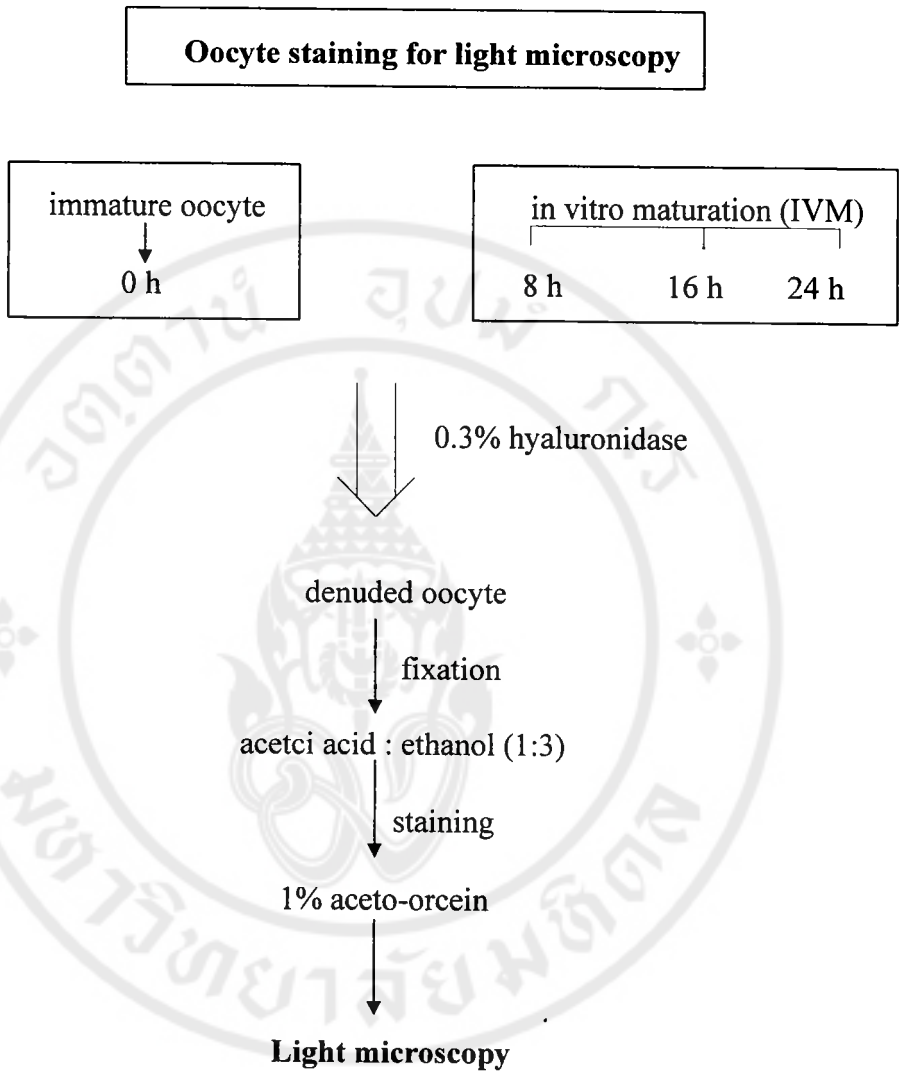


Figure 5. The diagram showing the oocyte staining using aceto-orcein for light microscopy.

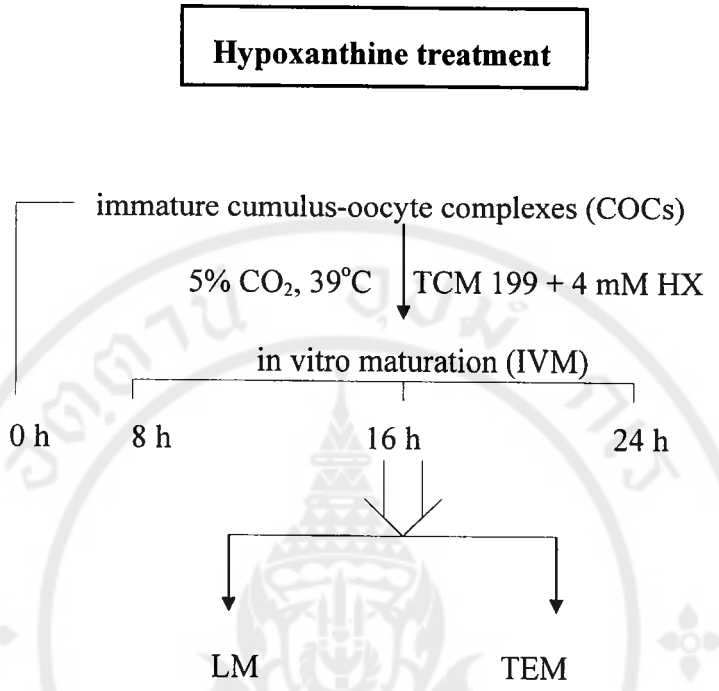


Figure 6. The diagram illustrating experimental plan of IVM oocyte treated with HX. COCs were incubated in 4 mM HX + TCM 199 for 0 h, 8 h, 16 h, and 24 h. Each group was evaluated by LM and TEM.

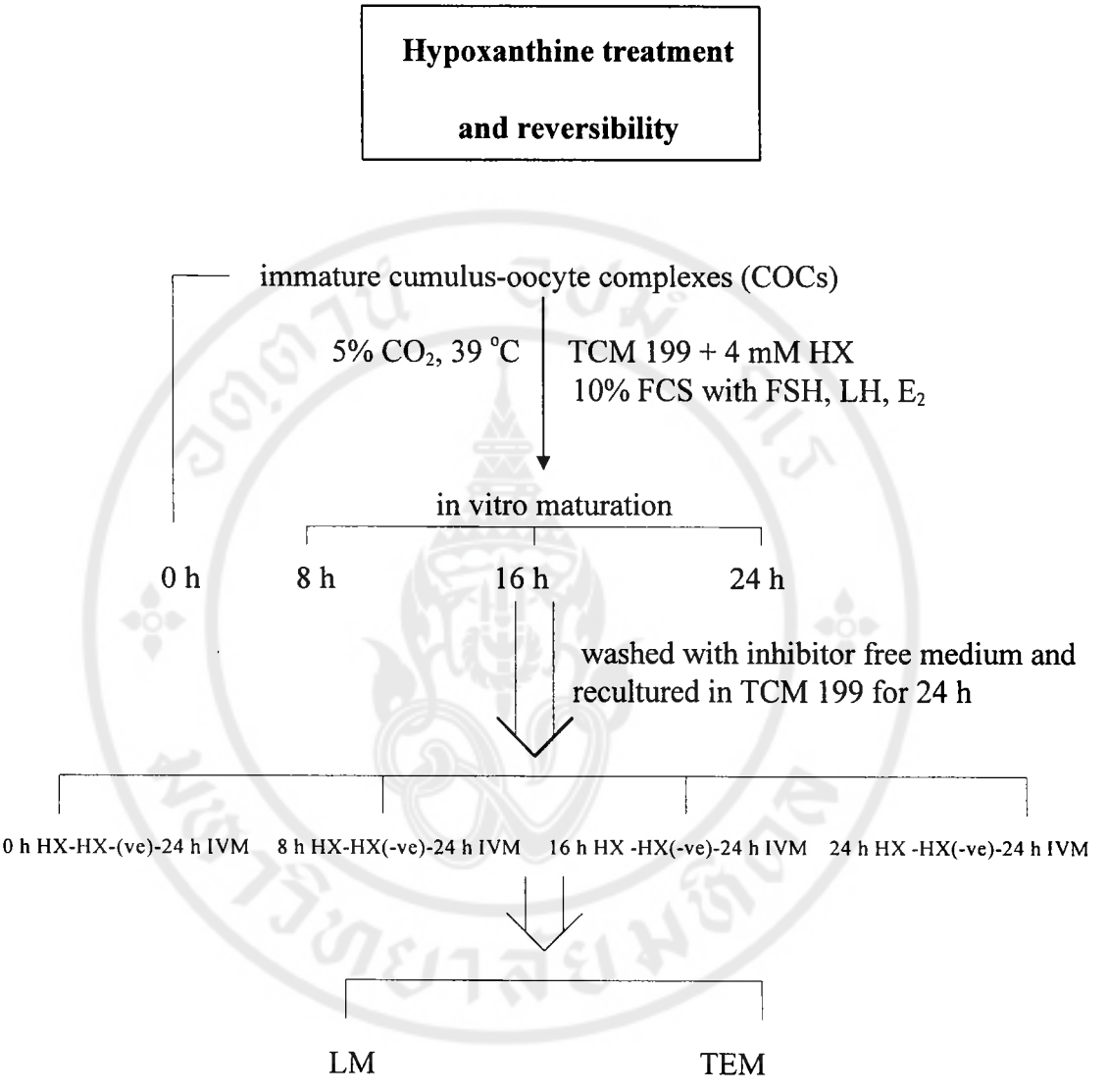
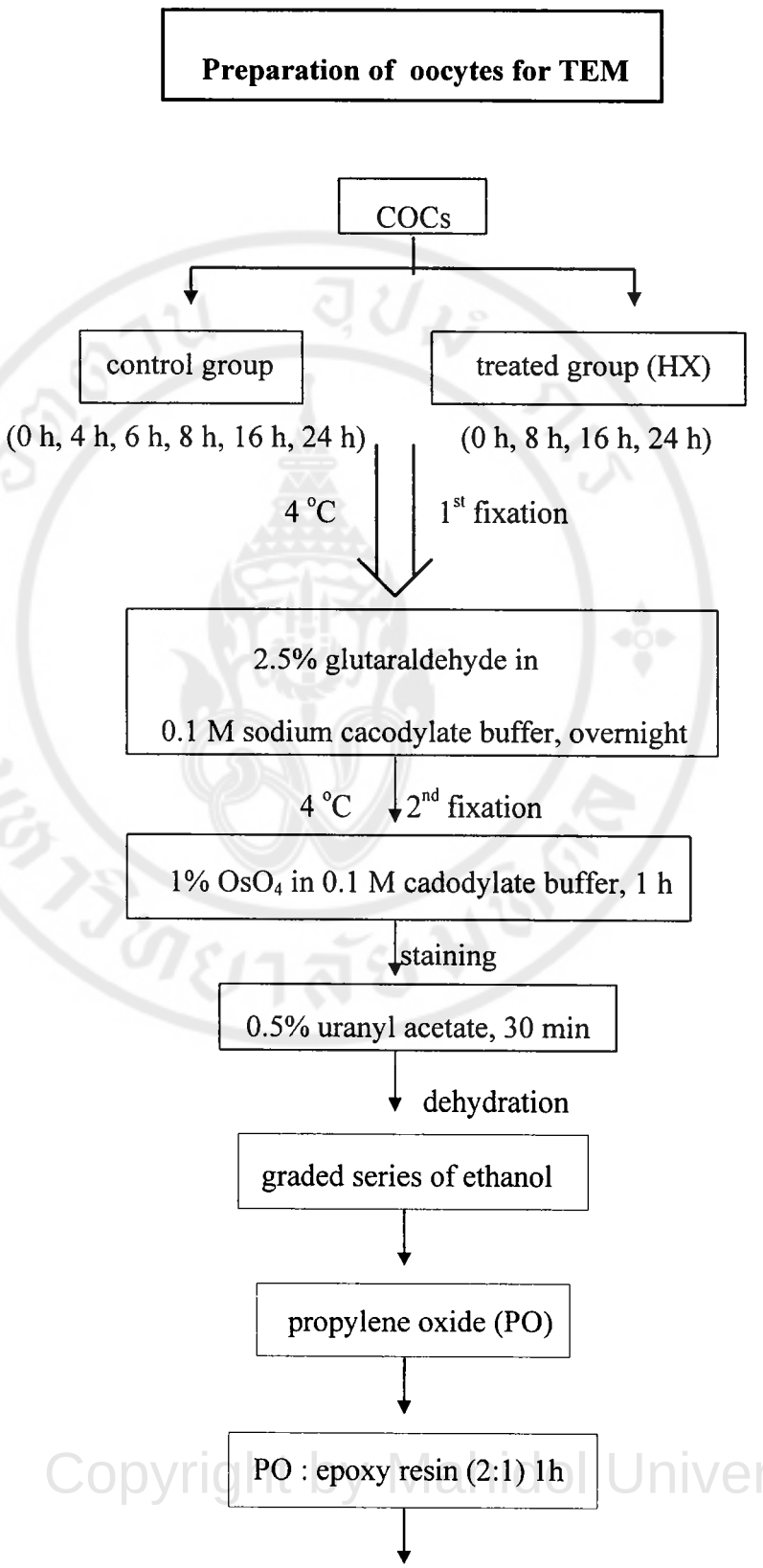


Figure 7. The diagram illustrating experimental plan of IVM oocytes treated with HX and reversibility. COCs cultured in TCM 199 containing HX for 0, 8, 16 and 24 h, washed and recultured in inhibitor free TCM 199 medium for 24 h. These oocytes were evaluated by LM and TEM.



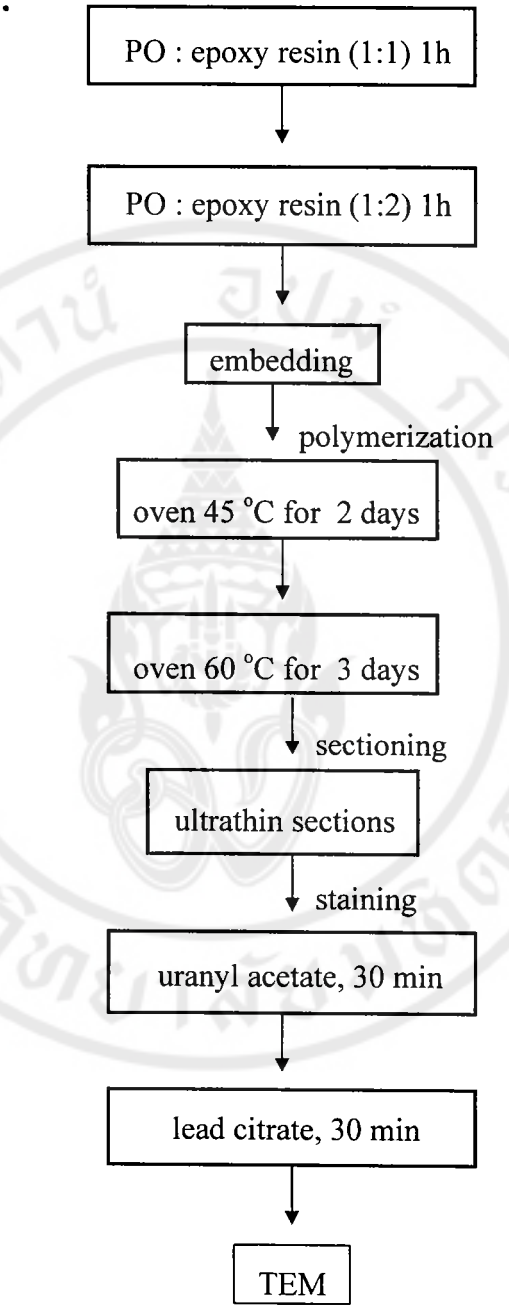


Figure 8. The diagram demonstrating the preparation of oocytes for TEM evaluation.

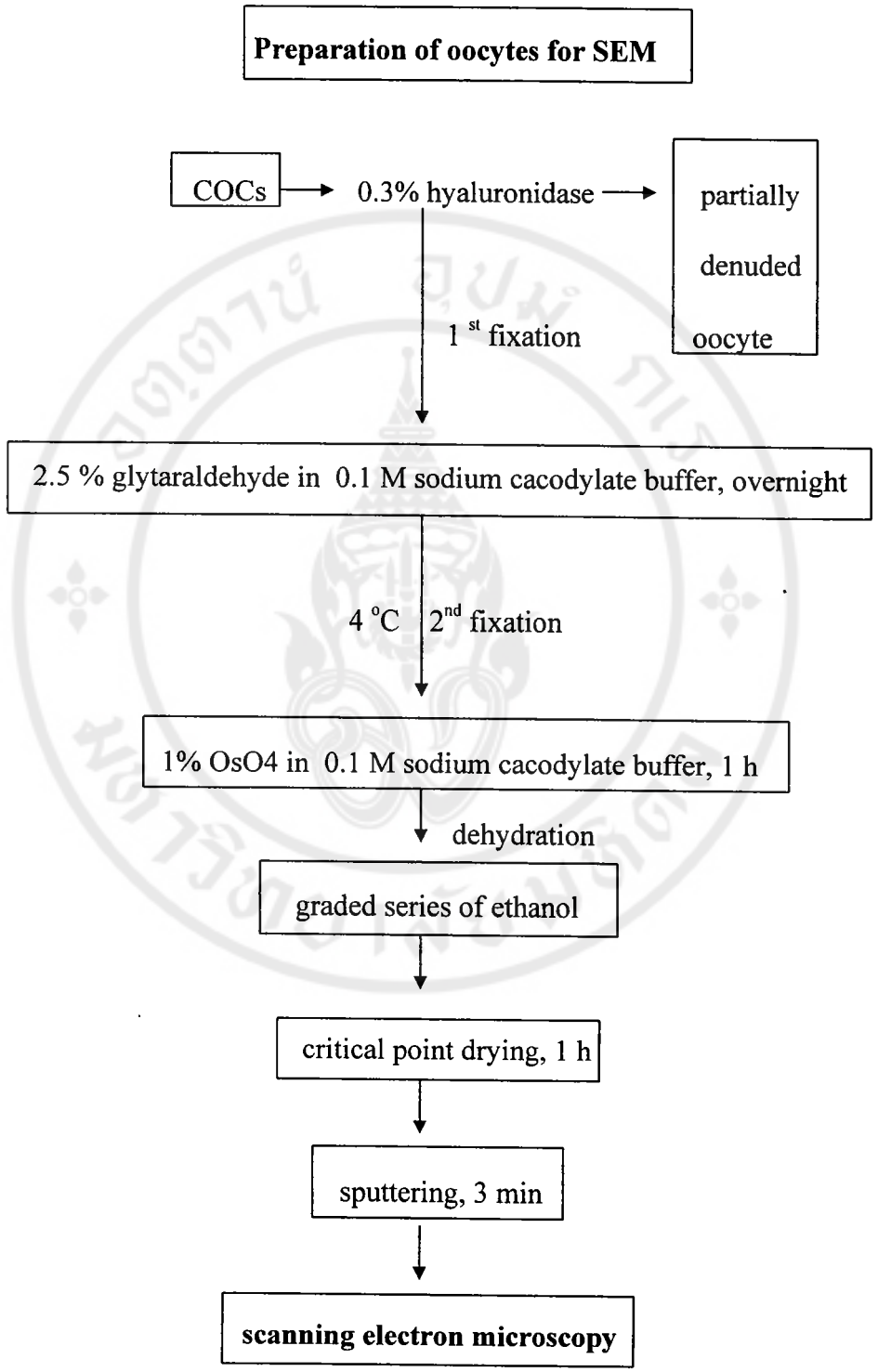


Figure 9. The diagram showing the preparation of oocytes evaluation for SEM.

CHAPTER IV
RESULTS

Dose effect of HX

Table 1 and Figure 11 showed a dose dependent effect of HX in inhibition of GVBD in bovine oocytes. After 8 h incubation, oocytes which underwent GVBD were scored. The inhibitory effect of HX was clearly dose dependent. At a concentration of 0.2 mM the percentage of GVBD was 94%(32/34) which was comparable to untreated group. HX had no effect on meiotic maturation. After increasing HX to 2, 4 and 6 mM the percentage of GVBD decreased to 56%(30/54), 19%(13/68) and 21%(13/61), respectively. The result of this study showed that the optimum dose effect of HX on blocking meiotic maturation was 4 mM HX. Therefore 4 mM of HX was chosen in the following experiment.

Nuclear status of normal maturation

Changes in nuclear status of oocytes throughout maturation were shown in Table 2. Ninety-five percent of immature oocytes were in GV stage. At 8 h after incubation, the percentage of oocytes underwent GVBD was 94%. At 16 h, the percentage of oocytes reaching metaphase I and anaphase-telophase I were 76%, and 18%, respectively and at 24 h oocytes reached metaphase II was 81%.

Effect of HX on nuclear stage of bovine oocytes

The effects of HX on nuclear stage were evaluated by treating oocytes for various time intervals during in vitro meiotic maturation process. All oocytes were exposed to 4 mM HX in TCM 199 for intervals of 0, 8, 16 and 24 h. Nuclear maturation stages of normal oocytes occur during various times were presented in Table 2 and composed of those oocytes treated with HX in the same intervals (Table 3).

At the beginning of incubation (0 h), ninety-five percent were at the GV stage. Ninety-three percent of oocytes were inhibited in GV stage and none of them progressed to GVBD by 8 h incubation. At 16 and 24 h, HX could not inhibit all oocytes at GV stage, thirteen and 17% of oocyte exposed to HX progreessed to GVBD stage.

Reversibility of HX treatment on GVBD, M II stage

To study the reversibility of HX action, COCs cultured in TCM 199 containing HX for 24 h, washed out of HX and re-cultured in the normal culture medium. After a long period of cultivation in the medium following removal of the inhibitor, GVBD was occurred completely for 5 h incubation. The time sequence of GVBD following removal of HX occurred twice as fast as in the control medium (9 h). However, 76% of oocytes could reach metaphase II after 24 h incubation. Eighty percent of control oocytes reach metaphase II (Table 6.)

Table 1. Dose-dependent effect of HX on GVBD. (3 replicates)

Concentration of HX (mM)	No. of oocytes	No. (%) of GVBD
0.2	34	32 (94)
2	54	30 (56)
4	68	13 (19)
6	61	13 (21)

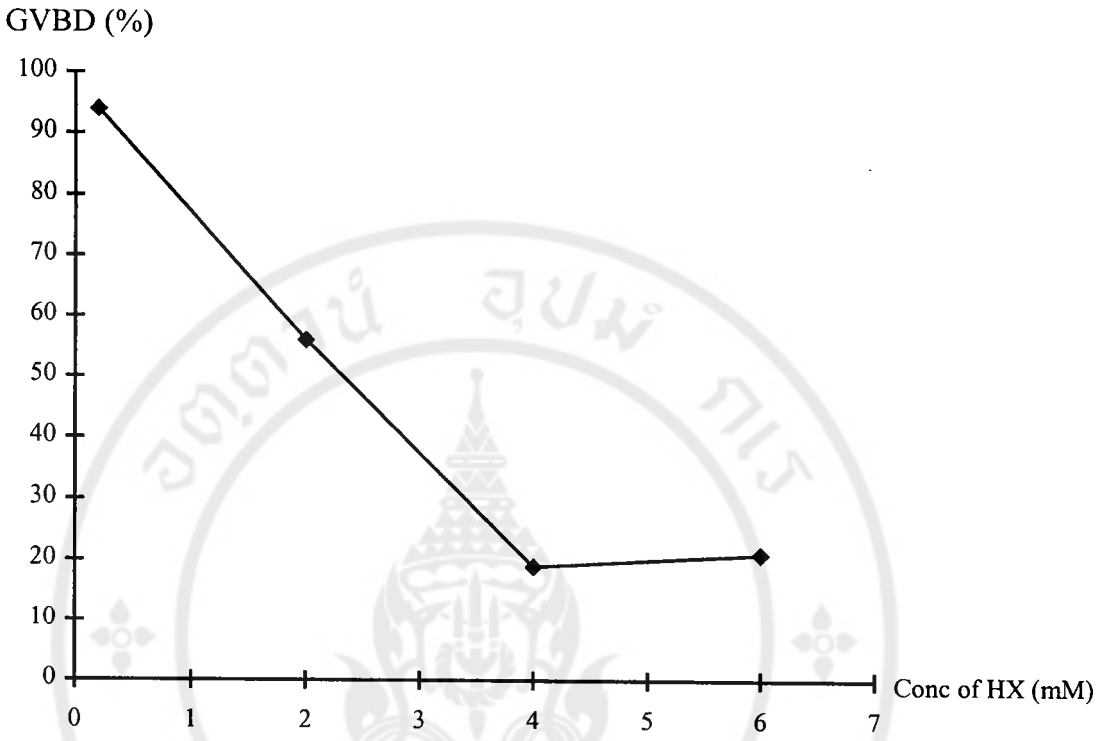


Figure 10. Dose-dependent effect of HX on GVBD in bovine oocytes. Oocytes were cultured in TCM 199 containing various concentration of HX for 8 h.

Table 2. Nuclear stages of normal bovine COCs on maturation in vitro in TCM 199 during interval time. (4 replicates)

Time (h) of culture	No. of oocytes	Stages of oocytes					
		GV N (%)	GVBD N (%)	M I N (%)	A-II N (%)	M II N (%)	Degenerate N (%)
0	115	108 (95±1.43)	-	-	-	-	7 (6±1.08)
8	132	-	124 (94±5.33)	-	-	-	8 (6±3.43)
16	202	-	-	154 (76±2.21)	36 (18±2.04)	-	12 (8±4.12)
24	215	-	-	28 (13±14.04)	7 (4±1.41)	174 (81+5.21)	6 (3±3.64)

Table 3. Nuclear maturation of hypoxanthine-treated oocyte in culture medium, TCM 199. (4 replicates)

Time (h) of HX treatment	No. of oocytes	Stages of oocytes					
		GV N (%)	GVBD N (%)	M I N (%)	A-I I N (%)	M II N (%)	Degenerate N (%)
0	116	110 (95±2.13)	-	-	-	-	6 (5±2.09)
8	133	124 (93±3.42)	-	-	-	-	9 (7±1.89)
16	211	172 (81±5.32)	28 (13±4.21)	-	-	-	11 (5±4.68)
24	224	173 (76±6.81)	39 (17±4.08)	-	-	-	12 (5±4.26)

Table 4. Time sequence of GVBD in normal oocyte maturation. (4 replicates)

Time (h)	No. of oocytes	No. (%) of GVBD
1 → 4	76	0 (0)
5	46	4 (9)
6	51	27 (53)
7	50	36 (72)
8	73	70 (96)
9	51	51 (100)

Table 5. Time sequence of GVBD in oocytes released from HX block, washed and subsequently cultured in TCM 199 medium. (4 replicates)

Time (h)	No. of oocytes	No. (%) of GVBD
1	29	0 (0)
2	37	3 (8)
3	68	28 (41)
4	69	58 (84)
5	56	56 (100)

Table 6. Reversibility of meiotic arrest in HX treated oocytes at 0, 8, 16 and 24 h, washing out and subsequently cultured in IVM medium free of HX for 24 h. (3 replicates)

oocyte group Time (h) HX ⟶ IVM (h)	No. of oocytes	No. (%) of M II
0 ⟶ IVM 24	68	55 (80±2.02)
8 ⟶ IVM 24	61	48 (78±2.28)
16 ⟶ IVM 24	81	62 (77±4.36)
24 ⟶ IVM 24	73	56 (77±4.88)

Morphological Study

DNA staining for fluorescent microscope

Immature oocyte (0 h before incubation) showed eccentric germinal vesicle (GV) in Figure 19 a. Germinal vesicle breakdown (GVBD) was appeared by 8 h incubation (Figure 19b). Metaphase I chromosome presented by 16 h incubation (Figure 19c). Mature oocyte showed metaphase II chromosome and polar body in the peripheral area (Figure 19d).

Cortical granules and DNA staining for confocal laser scanning microscopy (CLSM)

After labelling with FITC-labelled lectin, fluorescent spots was distributed in the entire cortical cytoplasm of immature oocyte (Figure 20a). GV is eccentric. The distribution of FITC-LCA labelling during in vitro maturation of bovine oocytes by 8 h incubation was shown in Figure 20b. In maturing oocyte (16 h incubation), fluorescent spots were located within the cortical cytoplasm of the oocytes. Metaphase I chromosome was shown in Figure 20c. The cortical granules (CG) have migrated towards the plasma membrane and form a monolayer. Metaphase II chromosome and first polar body indicated the mature oocyte (Figure 20d). DNA and the pattern of CG were detected in the same pictures. It takes advantage to the picture from fluorescent microscopy (Figure 19).

Light & Electron Microscope

Normal oocyte maturation

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

Immature oocyte, was surrounded by a compacted multilayered cumulus investment (Figure 15). The oocyte nucleus or GV presented a typically nonundulating nuclear membrane (Figure 22a). The GV typically displayed distinct nucleoli associated with disperse heterochromatin (Figure 26). In most cases, the nucleoli consisted of a dense spherical mass of fibrils, and a more loosely packed fibrils (Figure 27). The most predominant ooplasmic structures were mitochondria,

and vesicles. The mitochondria were found in small clusters throughout the ooplasm, but in greatest number in a narrow peripheral zone close to the oolemma (Figure 28,29). CG were in the cluster at the peripheral with composed of less and dense electron density, heterogenous and homogenous material (Figure 29). Numerous Golgi complexes were detected, both adjacent to the nucleus and in the periphery of the ooplasm (Figure 30,32,33). The surface of the oocytes was characterized by the presence of groups of cylindrical microvilli (Figure 31,36,39).

Flattened germinal vesicle (GV) stage

Flattened GV was characterized by undulated membrane of GV. The undulated membrane was adjacent to the oolemma. Flattened GV was observed at the beginning for 4 h after incubation (Figure 22b,38). The nuclear membrane was more wavy than the beginning by 6 h after incubation as shown in Figure 40.

Germinal vesicle break down (GVBD) stage (8 h after incubation)

GVBD was characterized by a gradually dense chromatin and more wavy nuclear membrane and disintegrated in the final. The cumulus investment displayed clear signs of expansion (Figure 22c). There were concomitantly enlargement expansion of the cumulus cell process and complete GVBD. The nuclear membrane displayed sharpedged undulation over the entire surface as well as deep indentations and areas in which the membrane folded back upon itself (Figure 41). Finally, nuclear membrane was disappeared and dense chromatin was observed (Figure 22c).

Metaphase I (M I) stage (16 h after incubation)

The cumulus investment was fully expanded, and none of the projections displayed contact with the oolemma. The metaphase I chromosomes were found at the periphery of the ooplasm (Figure 42). At the peripheral of some oocytes, the organelles were not found except the clusters of CG (Figure 43). Annulate lamella were observed in the oocytes, often in close association with aggregates of mitochondria. The annulate lamella appeared as stocks of cisternae with

constrictions at intervals along the membranes quite similar to those of the nuclear membrane (Figure 44). The mitochondria, vesicles, lipid droplets and smooth endoplasmic reticulum had migrated to a central ooplasm (Figure 45).

Metaphase II (M II) stage (24 h after incubation)

The mature oocyte were examined. The cumulus investment was completely expanded (Figure 46). The loose connection of elongated cumulus were observed (Figure 47). There were morphological changes of the maturation stage both nuclear and cytoplasmic maturation. The nuclear maturation showed metaphase II chromosome at the eccentric area. The polar body was observed in the Figure 48. The cytoplasmic maturation showed the migration of individual cortical granules to solitary position along the oolemma and were released to the perivitelline space at the time of fertilization. The mature cortical granules were composed of homogenous material in the similar size and were more dense electron density than those in the immature oocytes (Figure 49). Abundant vesicles mitochondria and other organelles were dispersed throughout the ooplasm.

HX treated oocytes

HX treated oocytes were cultured for 0, 8, 16 and 24 h. All interval time, GV were remained in the oocytes (Figure 23,24). At the beginning of treatment with HX (0 h), GV was intact without chromosome condensation (Figure 23a,50). But in the later by 8, 16 and 24 h after treatment, oocytes were rested in the GV stage with condensed chromosome (Figure 23b-d,52,54). In the group of HX 24 h, GV with highly condensed chromosome were observed in the oocyte (Figure 55). Highly condensed chromosome was still surrounded by nuclear membrane as shown in Figure 56. The shape of chromatin mass was irregular. The clusters of CG in 24 h HX were observed in the periphery (Figure 55). These cortical granules were composed of heterogenous and homogenous material, less and dense electron density in various size that similar to immature oocyte (Figure 58a). In contrast: the oocyte following washing and culture in control medium for 24 h were reversible to

metaphase II (M II) (Figure 57). The solitary CG line along the oolemma. They were more dense electron density in the similar size as the mature oocyte (Figure 58b).

Figure 11. Picture of the *Bos indicus* (arrowhead) and *Bos taurus* (arrows).

Figure 12. Pictures showing the oocytes recovery method

a. bovine oocytes aspiration from follicular of ovaries by using 18 gauge needle with 10 ml syringe.

b. the oocytes were recovered with fine drawn Pasteur pipette and cultured in microdrops of TCM 199 plus HX.

FIGURE 12

Figure 13. Micrographs by inverted microscope of

a. immature oocyte (0 h before incubation) showing compacted cumulus investment. (x400)

b. mature oocyte (24 h after incubation) showing expansion of multilayered cumulus investment. (x400)

FIGURE 13

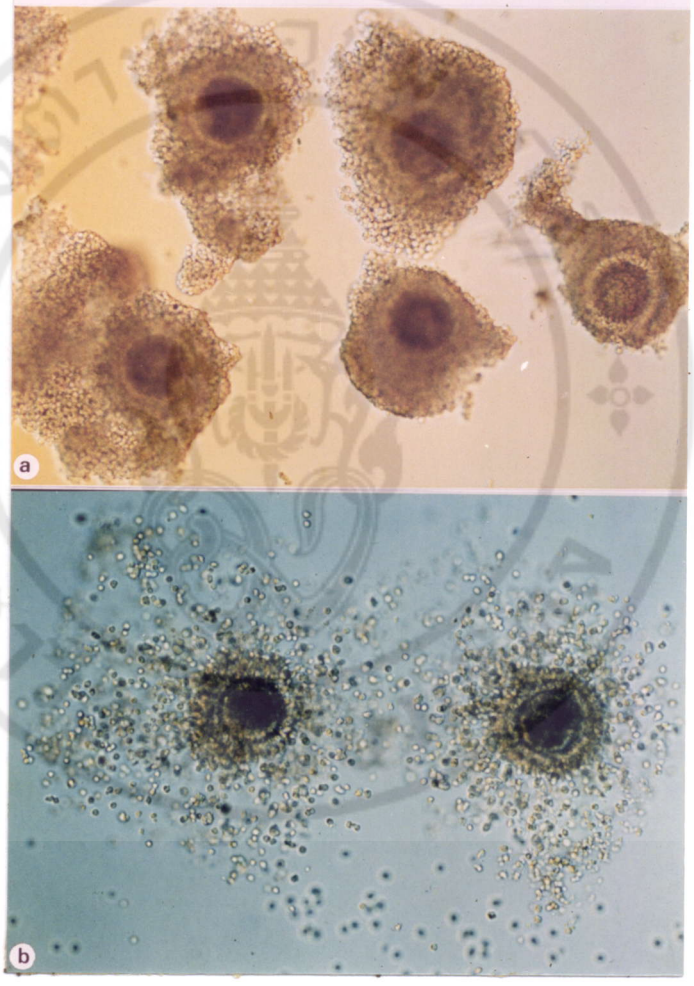


Figure 14. Micrographs by inverted microscope of

a. twenty-four h of HX treated oocytes (24 h after incubation in HX) showing unexpanded cumulus cells. (x200)

b. oocytes after HX treatment for 24 h, washed out HX and further cultured for 24 h in the inhibitory-free medium (24 h HX → HX (-ve) → 24 h IVM). Noted the expanded cumulus cells. (x200)

FIGURE 14

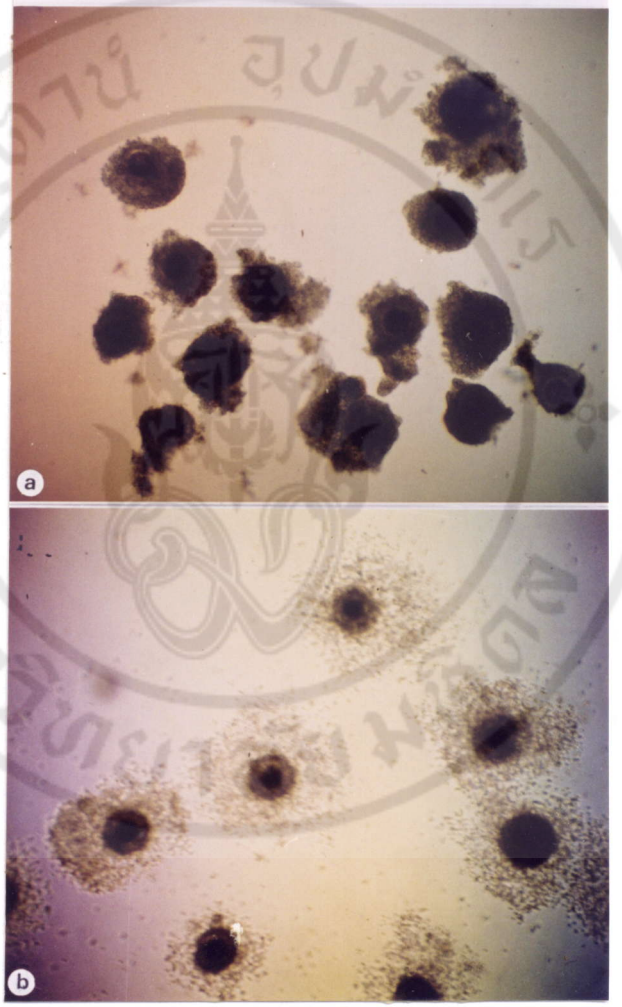


Figure 15. SEM micrograph showing the multilayers of cumulus cells (CC) covering the immature oocyte. Bar = 60 μm

Figure 16. SEM micrograph showing expanded cumulus cells (CC) covering mature oocyte. The intercellular spaces (arrows) among cumulus cells are observed. Zona pellucida (ZP) is surrounded by the CC. Bar = 60 μm

Figure 17. Higher magnification of SEM micrograph showing the cumulus cells (CC) attach zona pellucida (ZP) of immature oocyte. Bar = 10 μm

Figure 18. Higher magnification of SEM micrograph showing the cumulus cell processes (arrows). ZP = zona pellucida. Bar = 6 μm

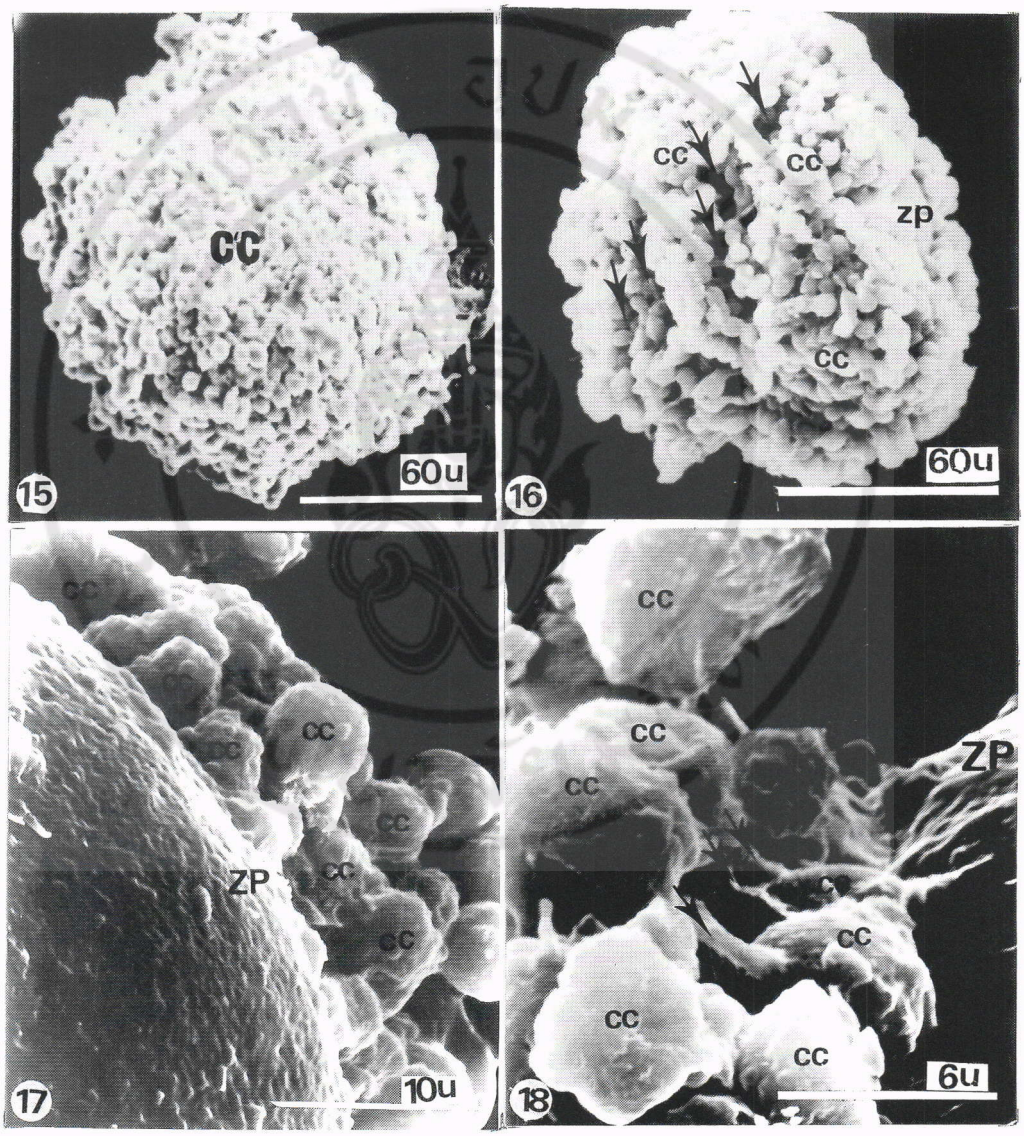


Figure 19. Fluorescent micrographs showing the stages of nuclear maturation. (x400)

a . Germinal vesicle (GV) stage in immature oocyte (0 h before incubation). Note the presence of nuclear membrane (arrowhead).

b . Germinal vesicle breakdown (GVBD) stage at 8 h after incubation.

c . Metaphase I (M I) stage at 16 h after incubation.

d . Metaphase II (M II) stage and first polar body (PB) at 24 h after incubation.

FIGURE 19

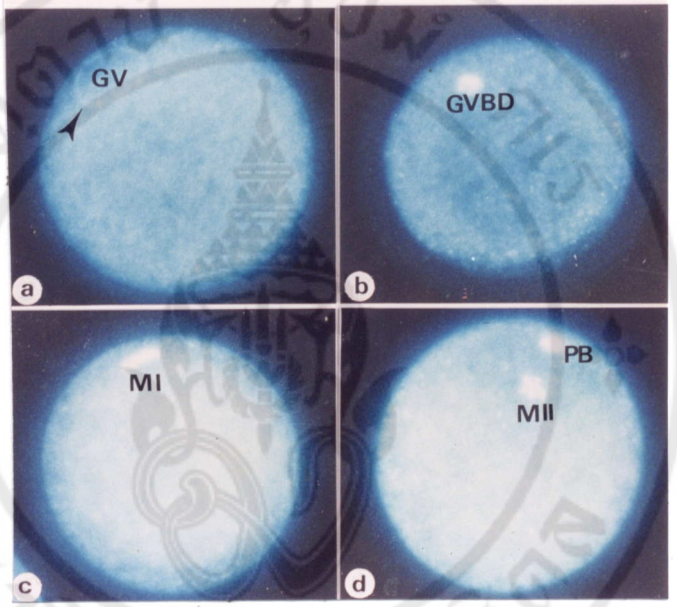


Figure 20. Confocal images of cortical granules (CG) and DNA in zona free oocyte labelled with FITC-LCA and PI. Green and red images show CG and DNA respectively. (x400).

a. Immature oocyte (0 h incubation) shows DNA in GV. Note the presence of nuclear membrane (arrow). Most distributes in green fluorescent spots in the cytoplasm. The depth of field in this optical section is about 28 μm from the zona surface..

b. Maturing oocyte (8 h after incubation) shows GVBD stage. CG are distributed in the cytoplasm. The depth of field in this optical section is about 45 μm from the zona surface.

c. Maturing oocyte (16 h after incubation) shows metaphase I stage. CG are mainly located nearer to the periphery than the central. The depth of field in this optical section is about 20 μm from zona surface.

d. Mature oocyte (24 h after incubation) shows metaphase II stage (arrowhead). First polar body is shown by arrow. Green fluorescence spots, CG have migrated towards the plasma membrane and form monolayer. The depth of field in this optical section is about 48 μm from zona surface.

FIGURE 20

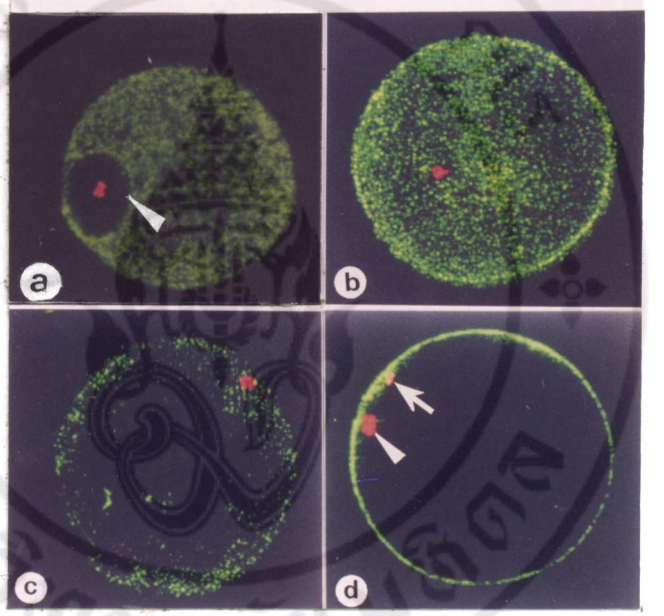


Figure 21. Light micrographs staining with aceto-orcein showing the different stages of oocyte maturation. (x400)

a. Germinal vesicle (GV) stage in immature oocyte (0 h before incubation). Note the presence of nuclear membrane (arrowhead).
b. Germinal vesicle breakdown (GVBD) stage at 8 h after incubation.
c. Metaphase I (M I) stage at 16 h after incubation.
d. Anaphase-Telophase I (A-TI) stage at 16 h after incubation.
e. Metaphase II (M II) stage and first polar body (PB) at 24 h after incubation.
f. Metaphase II (M II) stage and first polar body (PB) present at the periphery after 24 h incubation.

FIGURE 21

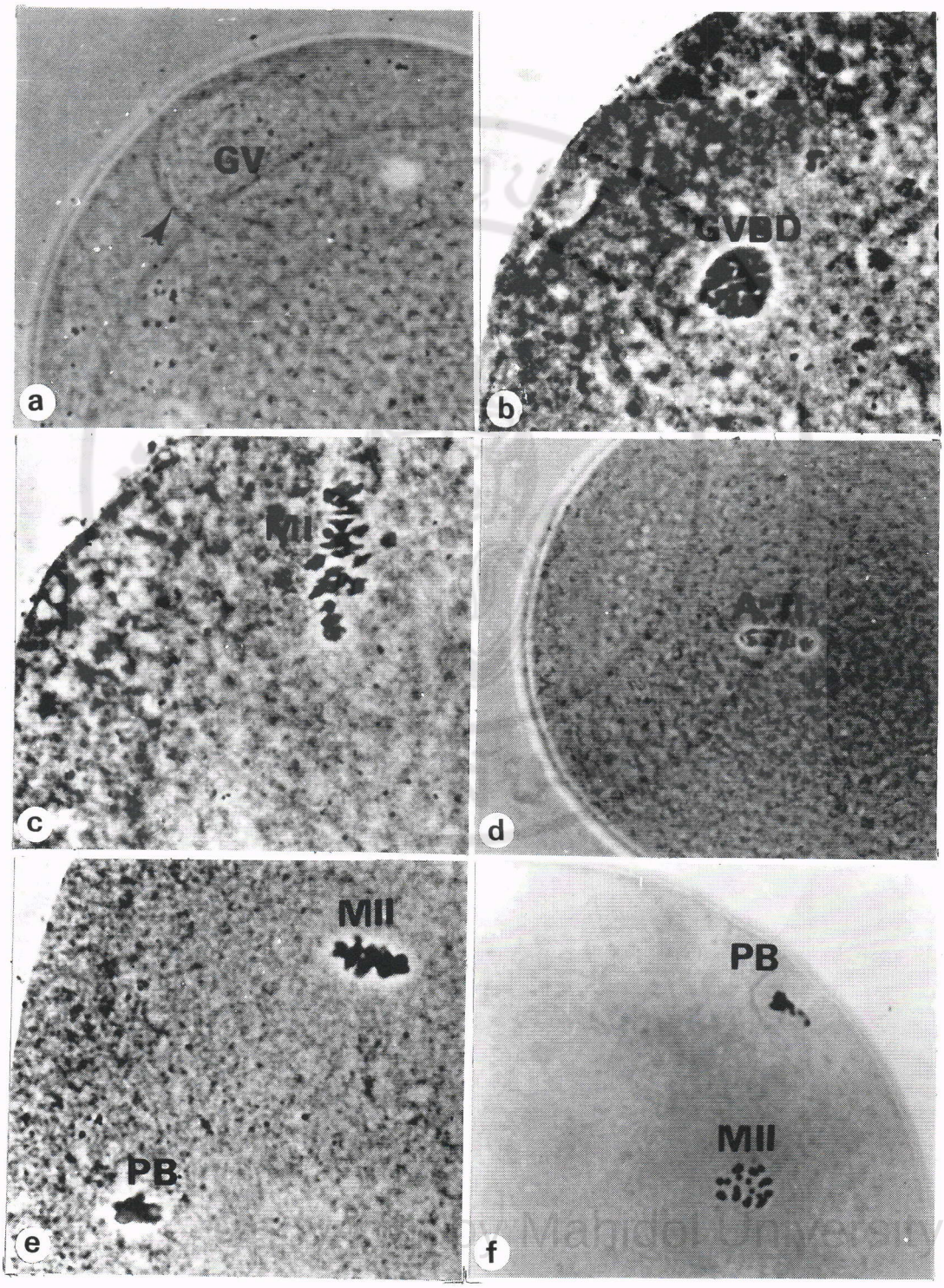


Figure 22. Light micrographs of semithin section oocytes showing time sequence of normal oocyte maturation in culture medium. (x400)

a. At 0 h; The multilayers of cumulus cells (CC) are surronding zona pellucida (ZP) and immature oocyte. The spherical nucleus or germinal vesicle (GV) is eccentric.

b. At 4 h; The flattened germinal vesicle (GV) is adjacent to the oolemma.

c. At 8 h; The germinal vesicle breakdown (GVBD) and the cumulus expansion occur concomitantly. The oocyte shows the condensed chromosomes (arrow) associated with dissolution of the nuclear membrane.

d. At 12 h; The organelles and vesicles disperse in the cytoplasm.

e. At 16 h; Most of the organelles and vesicles move to the central of oocyte.

f . At 24 h; The mature oocyte showing the expansion of the elongated cumulus cells. Polar body is observed (arrow).

FIGURE 22

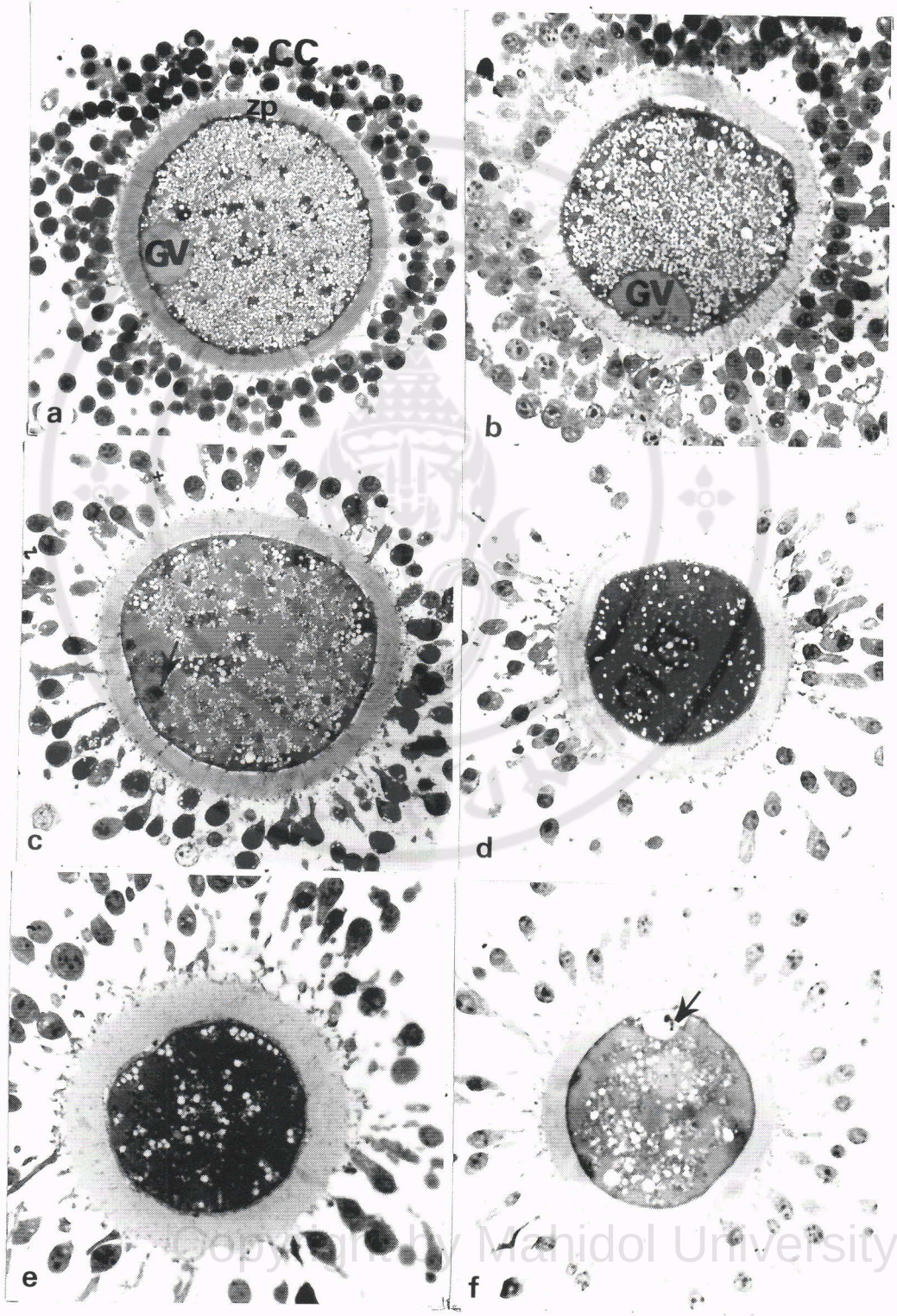


Figure 23. Light micrographs staining with aceto-orcein showing the nuclear stage of HX treated oocytes at different times after incubation. (x400 in Figure a, b, d; x200 in Figure c)

a. At 0 h, germinal vesicle (GV) stage showing intact nucleus, without chromosome condensation.

b, c, d. At 8, 16 and 24 h after incubation respectively, germinal vesicle (GV) stages showing chromosome condensation within nuclear membrane (arrows).

Figure 23. Light micrographs staining with aceto-orcein showing the nuclear stage of HX treated oocytes at different times after incubation. (x400 in Figure a, b, d; x200 in Figure c)

a. At 0 h, germinal vesicle (GV) stage showing intact nucleus, without chromosome condensation.

b, c, d. At 8, 16 and 24 h after incubation respectively, germinal vesicle (GV) stages showing chromosome condensation within nuclear membrane (arrows).

Figure 24. Micrographs of semithin section of HX treated oocytes by 0, 8, 16 and 24 h showing germinal vesicle (GV) stage in a, b, c and d respectively. GV stage and unexpanded cumulus cells were found in all time intervals. Most of vesicles were dispersed in the ooplasm similar as normal immature oocyte (0 h). (x400)

Figure 25. The oocyte after HX treatment-washing and further culturing for 24 h. (24h HX ⟶ HX(-ve)⟶24 h IVM) showing the characteristic of maturation by light microscopy. (x400)

a. Micrograph of aceto-orcein staining showing metaphase II chromosome (M II) and the polar body (PB).

b. Micrograph of semithin section showing metaphase II chromosome (arrow) and the protruded polar body (arrowhead).

Figure 26-33. Micrographs of immature oocytes (0 h before incubation)

Figure 26. Micrograph of semithin section showing compacted cumulus oocyte. Nucleolus (arrow) is clearly visible in spherical germinal vesicle (GV). (x400)

Figure 27. Higher magnification of TEM micrograph showing two parts of a nucleolus. It consists of a loosely packed fibrils (L) and a dense spherical mass of fibrils (D). Bar = 0.5 μm

Figure 28. TEM micrograph showing large cluster of mitochondria (M). They are mainly in the periphery. V = vesicle, ZP = zona pellucida. Bar = 5 μm

Figure 29. Higher magnification of TEM micrograph showing the cluster of immature cortical granules (CG). They are composed of heterogenous and homogenous material, less and dense electron density. Pleomorphic mitochondria are located mainly in the periphery. MV = microvilli, V = vesicle. Bar = 1 μm

Figure 30. TEM micrograph showing portion of a germinal vesicle (GV) in an immature oocyte. The outside surface of zona pellecuda (ZP) is more irregular than the inside. CC = cumulus cells. Bar = 5 μm.

Figure 31. TEM micrograph showing a detail of immature oocyte. Microvilli (MV) project to perivitelline space (PVS). The Golgi complexes (G), the smooth endoplasmic reticulum (SER), and the pleomorphic mitochondria (M) are observed. Double nuclear membrane is clearly visible by arrows. V = vesicle. Bar = 2.5 μm

Figure 32. TEM micrograph showing self duplicating mitochondria (arrow). Numerous pleomorphic mitochondria (M) are mainly in the peripheral of the oocyte. ZP = zona pellucida, G = Golgi complex, SER = smooth endoplasmic recticulum, V= vesicle. Bar = 2.5 μm

Figure 33. TEM micrograph showing well developed golgi complexes (G) in the ooplasm. SER = smooth endoplasmic reticulum, GV = germinal vesicle. Bar = 1.5 μm

Figure 34-35.TEM micrographs showing the elongated processes of the cumulus cells (CC) penetrate through zona pellucida (ZP). There are gap junctions between CC connection (arrows). Bar = 5 μm

Figure 36. TEM micrograph showing the close relationship of the cumulus cells process endings (CCPE) and the oolemma. There are illustrated in the micrographs. Cross sections of the CCPE are found surrounded by ooplasm just beneath the oolemma. Gap junctions (arrows) aid in maintaining the association between the oocyte and cumulus cells. Microvilli (MV) also help to maintain this association by extending over the process. Golgi complexes (G) are found aggregated just beneath the oolemma throughout the peripheral cytoplasm. The double nuclear membrane and nuclear pores long arrows are clearly seen. PVS = perivitelline space, ZP = zona pellucida, GV = germinal vesicle Bar = 1.5 μm

Figure 37. High magnification of illustrate showing cumulus cell process ending (CCPE). It is associated with microvilli (MV) of the oocyte. Multivesicular body (arrow) is observed in CCPE. Bar = 0.5 μm

Figure 38-41. TEM micrographs of the oocytes after 4, 6 and 8 h culture.

Figure 38. The GV flattens against the plasma membrane after 4 h culture. The nuclear membrane close to the plasma membrane forms folds, undulating membrane (arrows). Nucleolus (Nu) and heterochromatin (HC) are seen. Cumulus cell process endings (arrowheads) penetrate through the zona pellucida (ZP). Bar = 1.5 μm

Figure 39. TEM micrograph showing a detail of the organelles that are near the undulating membrane of flattened germinal vesicle (GV). Cumulus cells process ending (CCPE) contacted to microvilli (MV) by gap junction (arrows). Golgi complexes (G) and smooth endoplasmic reticulum (SER) are observed. ZP = zona pellucida, PVS = perivitelline space. Bar = 1.5 μm

Figure 40. TEM micrograph showing the oocyte after 6 h culture. Numerous golgi complexes (G), mitochondria (M) are located near the undulating membrane. ZP = zona pellucida, CCPE = cumulus cells process ending, GV = germinal vesicle. Bar = 1.5 μm

Figure 41. TEM micrograph showing germinal vesicle (GVBD) stage of oocyte after 8 h culture. More undulating of nuclear membrane (arrows) are shown. ZP = zona pellucida. Bar = 1.5 μm

FIGURE 23

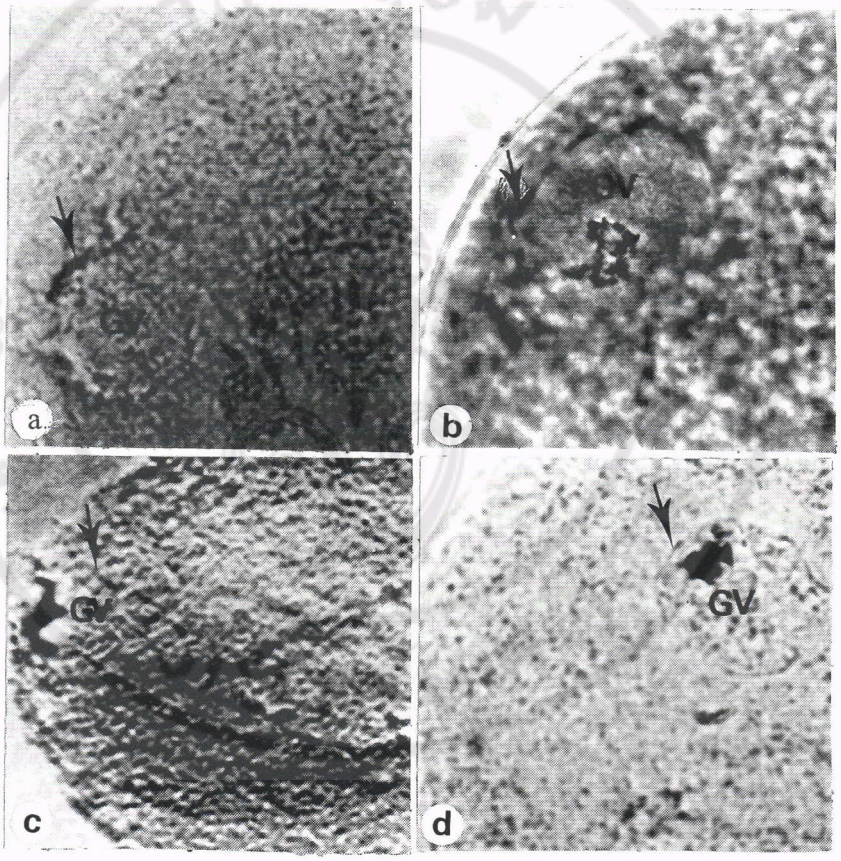


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FIGURE 24

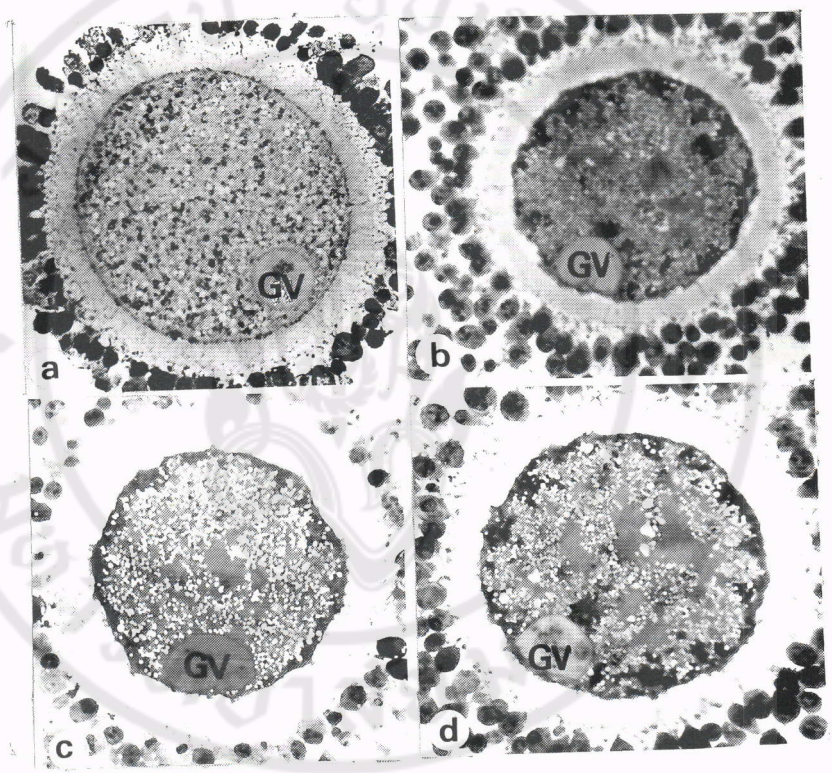
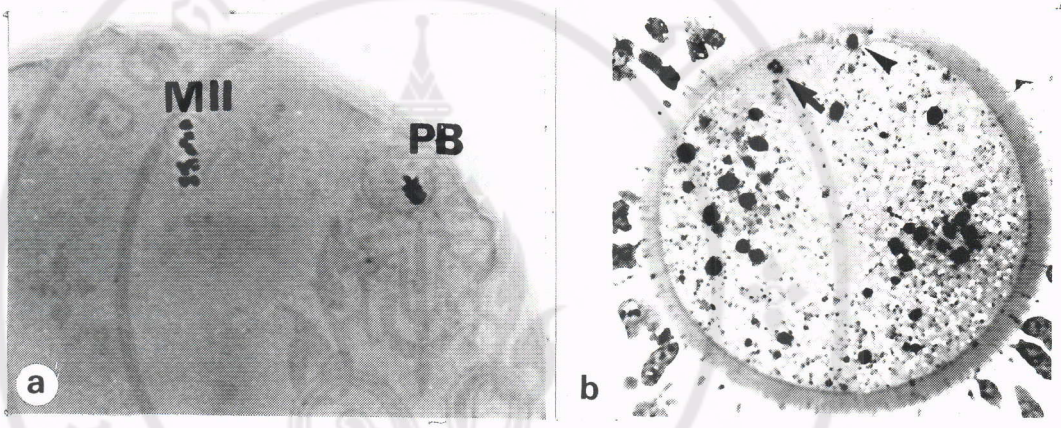


Figure 25. The oocyte after HX treatment-washing and further culturing for 24 h. (24h HX ⟶ HX(-ve)⟶24 h IVM) showing the characteristic of maturation by light microscopy. (x400)

a. Micrograph of aceto-orcein staining showing metaphase II chromosome (M II) and the polar body (PB).

b. Micrograph of semithin section showing metaphase II chromosome (arrow) and the protruded polar body (arrowhead).

FIGURE 25



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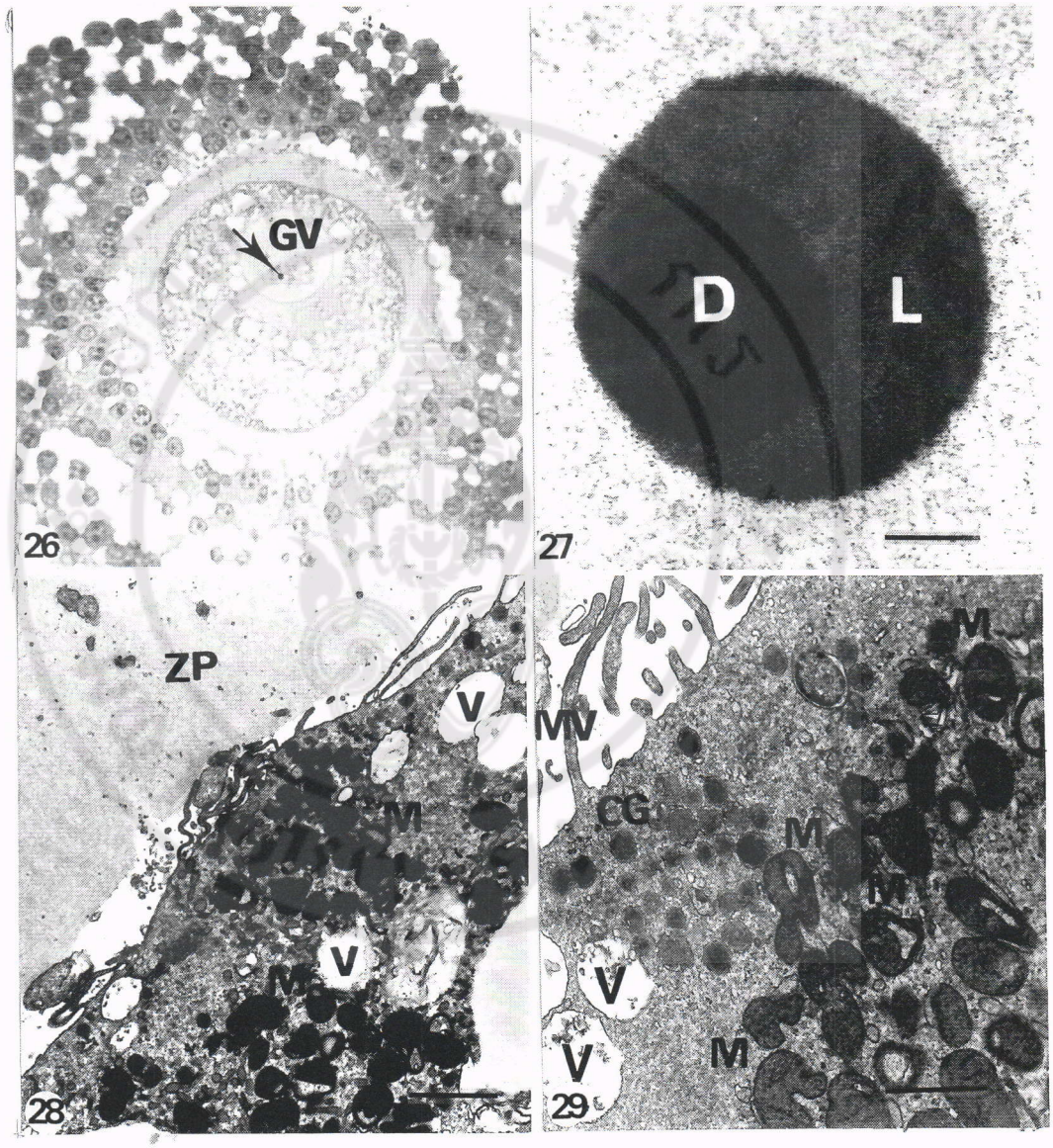

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Figure 33. TEM micrograph showing well developed golgi complexes (G) in the ooplasm. SER = smooth endoplasmic reticulum, GV = germinal vesicle. Bar = 1.5 μm

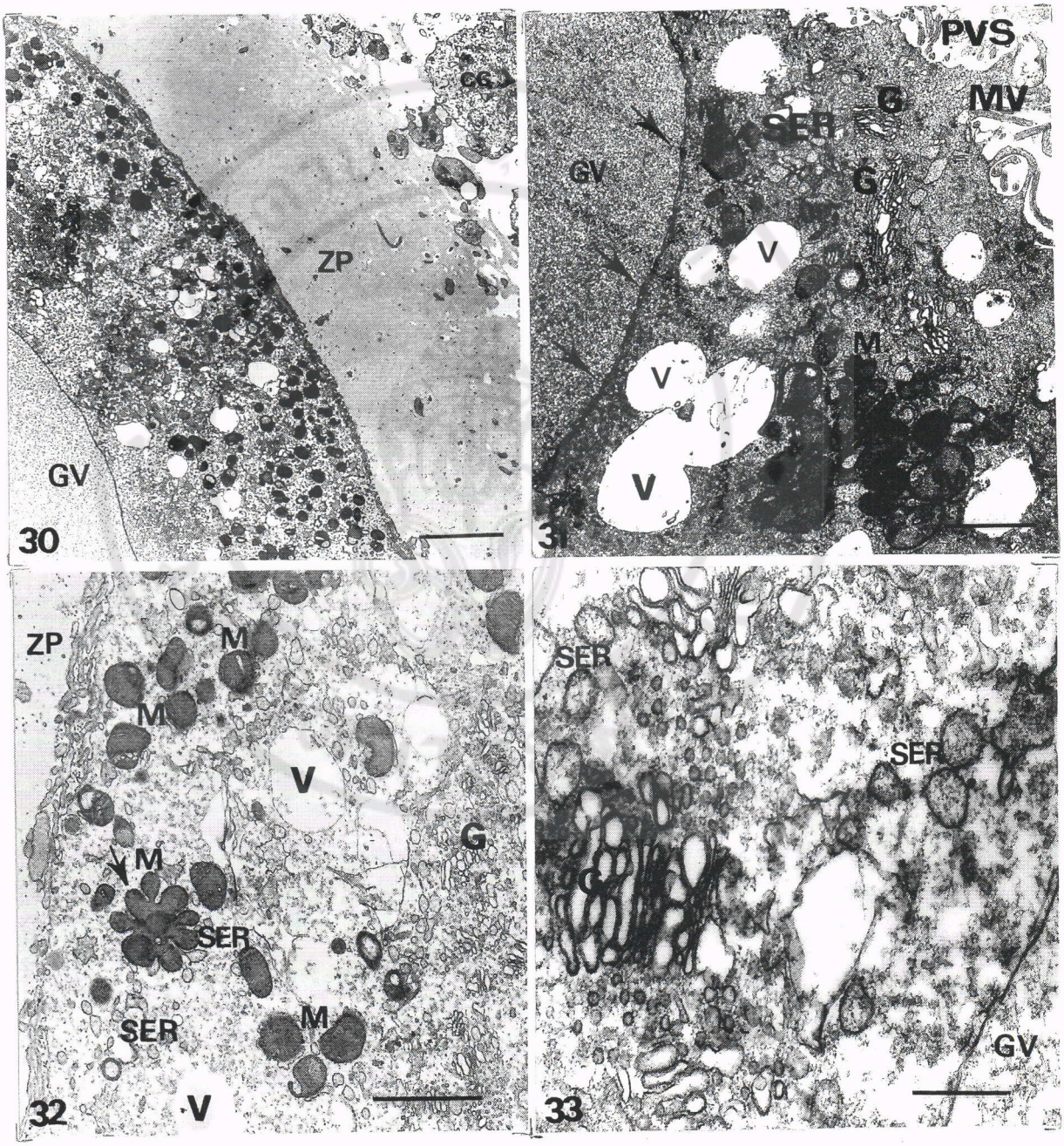

Figure 34-35. TEM micrographs showing the elongated processes of the cumulus cells (CC) penetrate through zona pellucida (ZP). There are gap junctions between CC connection (arrows). Bar = 5 μm

Figure 36. TEM micrograph showing the close relationship of the cumulus cells process endings (CCPE) and the oolemma. There are illustrated in the micrographs. Cross sections of the CCPE are found surrounded by ooplasm just beneath the oolemma. Gap junctions (arrows) aid in maintaining the association between the oocyte and cumulus cells. Microvilli (MV) also help to maintain this association by extending over the process. Golgi complexes (G) are found aggregated just beneath the oolemma throughout the peripheral cytoplasm. The double nuclear membrane and nuclear pores long arrows are clearly seen. PVS = perivitelline space, ZP = zona pellucida, GV = germinal vesicle Bar = 1.5 μm

Figure 37. High magnification of illustrate showing cumulus cell process ending (CCPE). It is associated with microvilli (MV) of the oocyte. Multivesicular body (arrow) is observed in CCPE. Bar = 0.5 μm

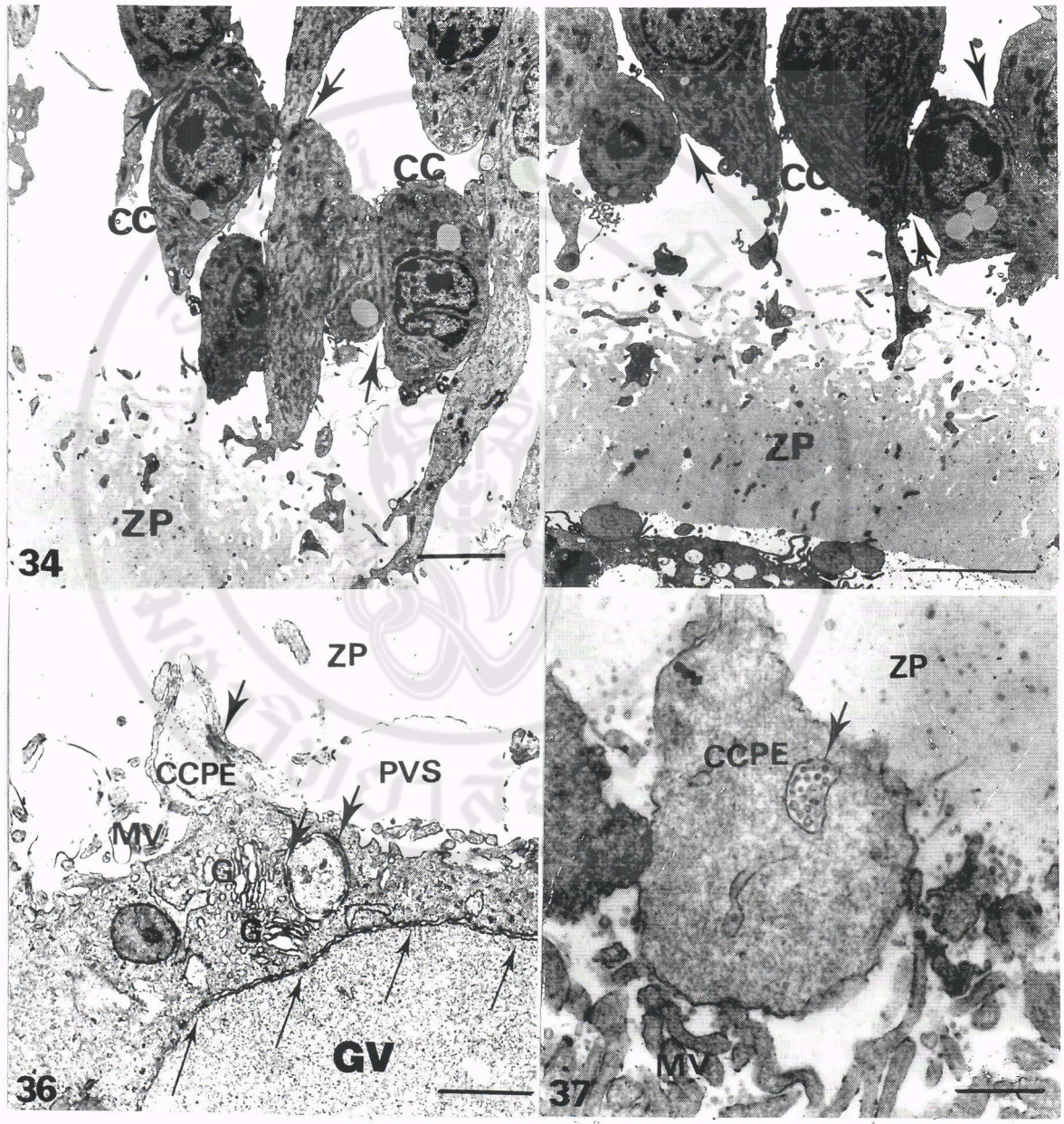

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Figure 38. The GV flattens against the plasma membrane after 4 h culture. The nuclear membrane close to the plasma membrane forms folds, undulating membrane (arrows). Nucleolus (Nu) and heterochromatin (HC) are seen. Cumulus cell process endings (arrowheads) penetrate through the zona pellucida (ZP). Bar = 1.5 μm

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Figure 40. TEM micrograph showing the oocyte after 6 h culture. Numerous golgi complexes (G), mitochondria (M) are located near the undulating membrane. ZP = zona pellucida, CCPE = cumulus cells process ending, GV = germinal vesicle. Bar = 1.5 μm

Figure 41. TEM micrograph showing germinal vesicle (GVBD) stage of oocyte after 8 h culture. More undulating of nuclear membrane (arrows) are shown. ZP = zona pellucida. Bar = 1.5 μm

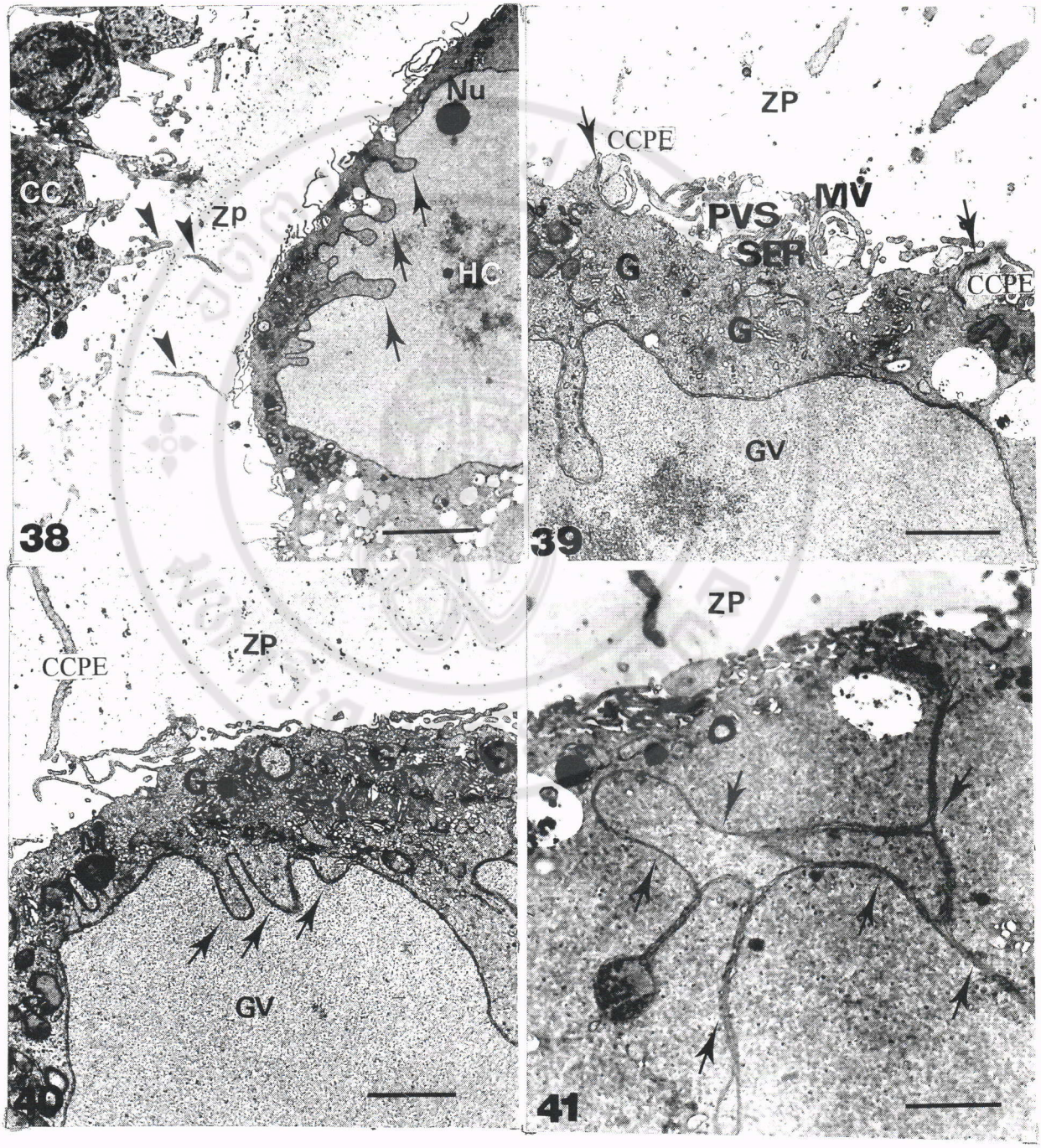


Figure 42-45. TEM micrographs showing the oocytes after 16 h culture.

Figure 42. TEM micrograph showing the metaphase I at the periphery. CC = cumulus cell, ZP = zona pellucida. Bar = 5 μm

Figure 43. TEM micrograph showing cumulus cell process ending (CCPE) of oocyte. The clusters of cortical granules (CG) are located in the peripheral area. Bar = 5 μm

Figure 44. TEM micrograph showing the retention of nuclear envelope fragments from dissolution at GVBD stage becomes to form the annulated lemella (AL). AL were dissolved into tubular structures (T) that were associated with mitochondria (M). Bar = 0.5 μm

Figure 45. TEM micrograph showing the pleomorphic mitochondria (M) in the ooplasm. SER = smooth endoplasmic reticulum. Bar = 0.5 μm

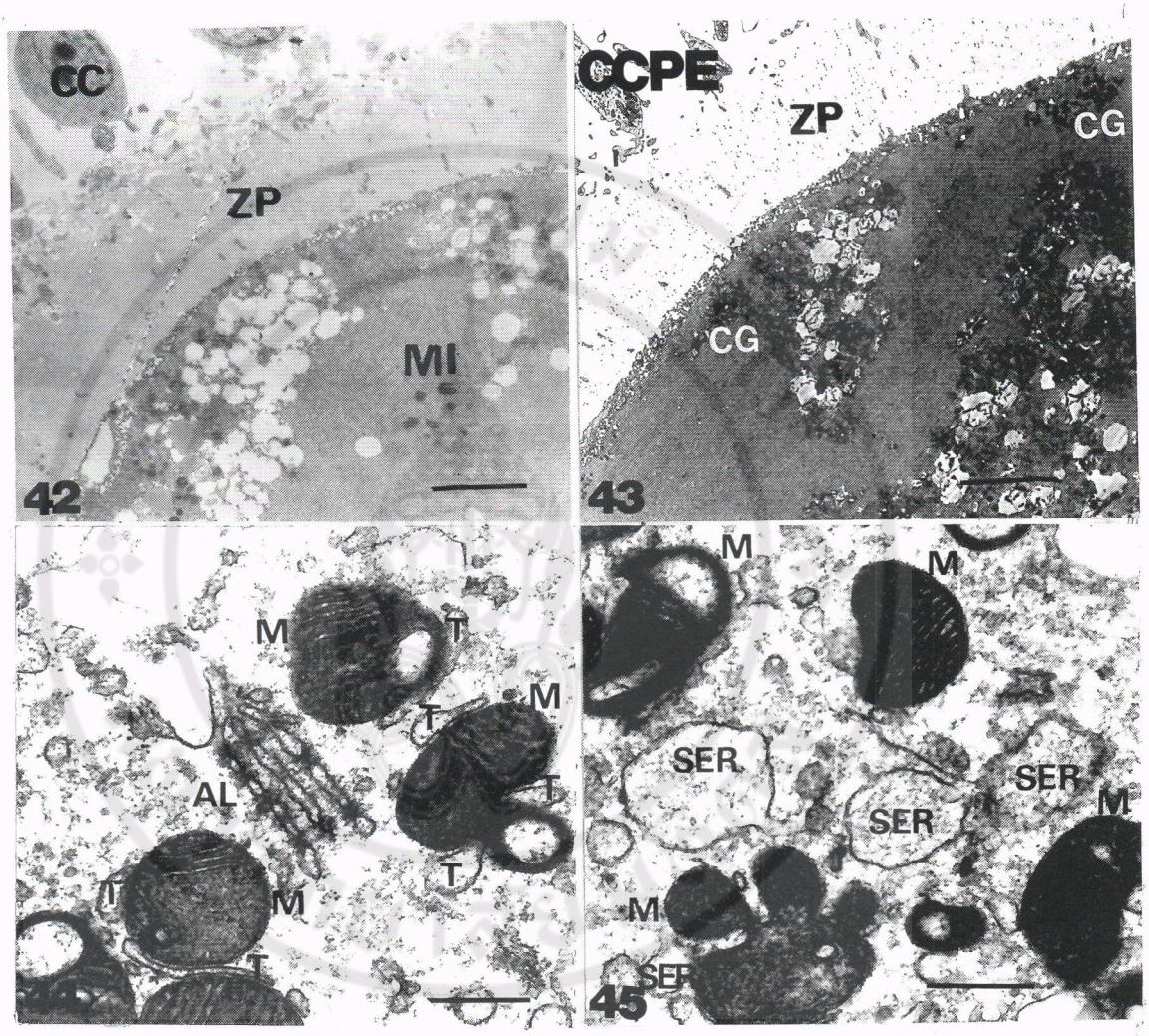


Figure 46-49. Semithin and TEM micrographs of mature oocyte after 24 h culture.

Figure 46. The cumulus investment is completely expanded. The first polar body is showed by arrow. (x400)

Figure 47. TEM micrograph showing expanded cumulus cells (CC). ZP = zona pellucida. Bar = 5 μm

Figure 48. TEM micrograph showing the mataphase II chromosome (M II) that is located near oolemma. The first polar body (PB) is located between oolemma and zona pellucida (ZP). Chromatin (Chr) in the PB is clearly observed. Arrows indicate border line of oolemma and the PB. Numerous smooth endoplasmic recticulum (SER) are presented in ooplasm. Bar = 1.5 μm

Figure 49. TEM micrograph showing solitary cortical granules (CG) lining along the oolemma. ZP = zona pellucida, M = mitochondria, SER = smooth endoplasmic recticulum, G = golgi complex, V = vesicle. Bar = 1.5 μm

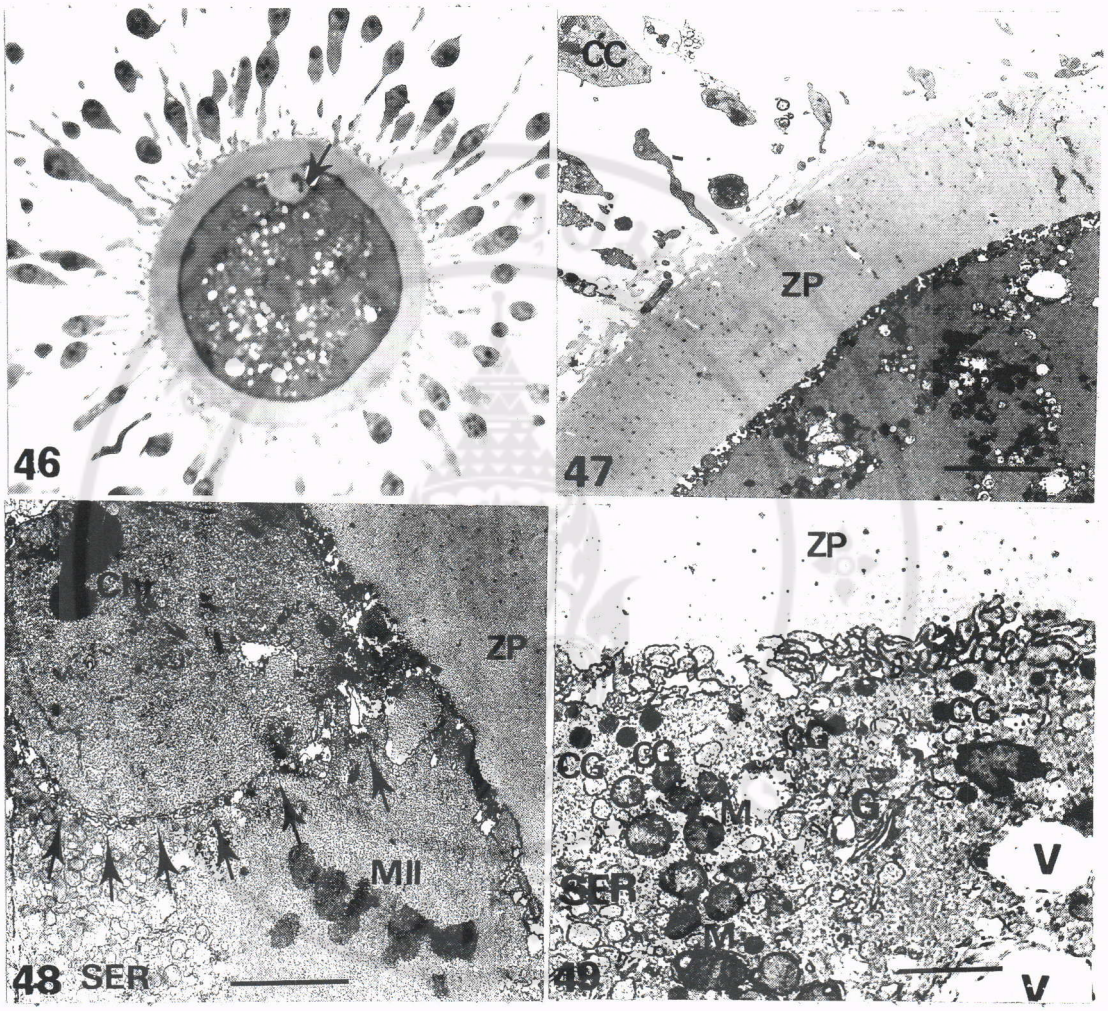


Figure 50-56. TEM micrographs of oocytes treated with HX for 0, 8, 16 and 24 h.

Figure 50. TEM micrograph of 0 h after HX treatment showing germinal vesicle (GV) stage. Mitochondria are observed at the periphery. ZP = zona pellucida,
Nu = nucleolus. Bar = 5 μm

Figure 51. TEM micrograph of 0 h after HX treatment showing the characteristic of cortical granules (CG) in cluster. They are composed of less electron and dense electron density granules. ZP = zona pellucida. Bar = 5 μm

Figure 52. TEM micrograph of 8 h after HX treatment showing germinal vesicle (GV) stage. Condense chromosome (COND CHR) is observed with nuclear membrane (arrows). ZP = zona pellucida. Bar = 5 μm

Figure 53. TEM micrograph of 8 h after HX treatment showing the pleomorphic mitochondria (M) in the ooplasm. V = vesicle, PVS = perivitelline space, MV = microvilli. Bar = 1 μm

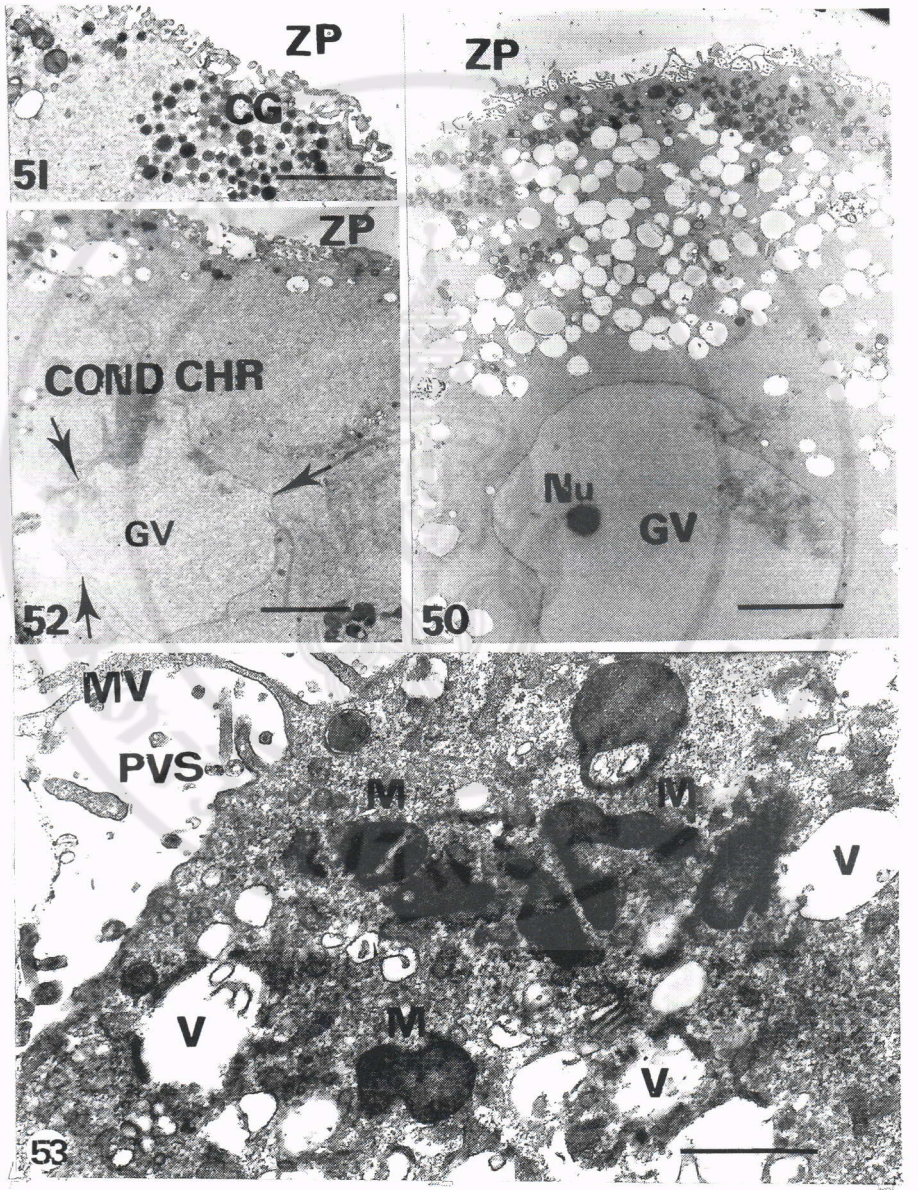


Figure 54. TEM micrograph of 16 h after HX treatment showing germinal vesicle (GV) stage. Condensed chromosome (COND CHR) are clearly vesicle with nuclear membrane (arrows). Bar = 2.5 μm

Figure 55. TEM micrograph showing highly condensed chromosome (COND CHR). The clusters of cortical granules (CG) are located at periphery. Bar = 5 μm

Figure 56. Higher magnification of condensed chromosome that is still surrounded by nuclear membrane (arrows). Bar = 0.5 μm

Figure 57. TEM micrograph of the oocyte after HX treatment, washing and further culturing for 24 h (24 h HX → HX(- ve) → 24 h IVM). It showed the characteristic of a mature oocyte. Metaphase II (M II) is observed and the solitary of cortical granules (CG) line along the oolemma. Bar = 2.5 μm

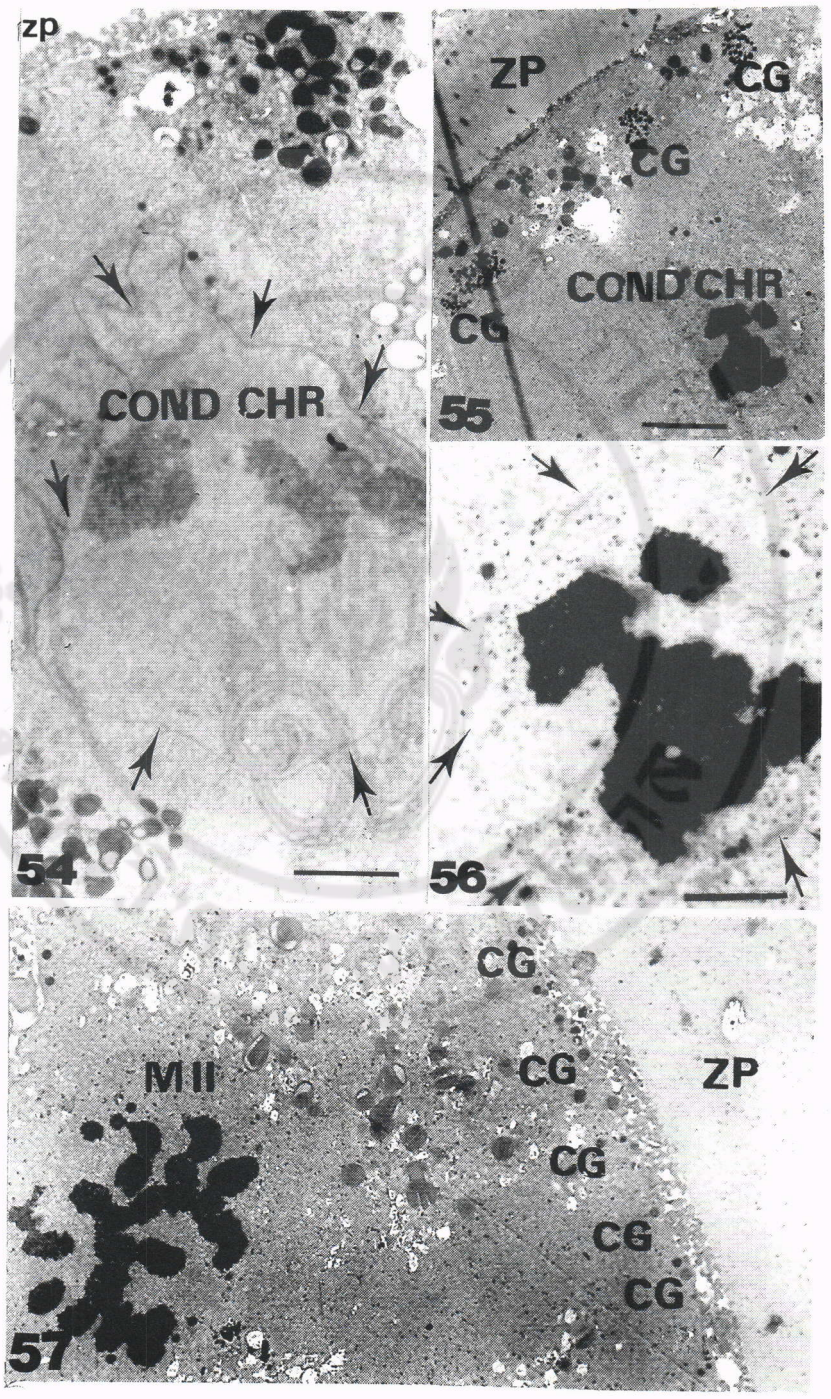


Figure 58. TEM micrographs showing the comparison of cortical granules (CG). Bar = 1 μm

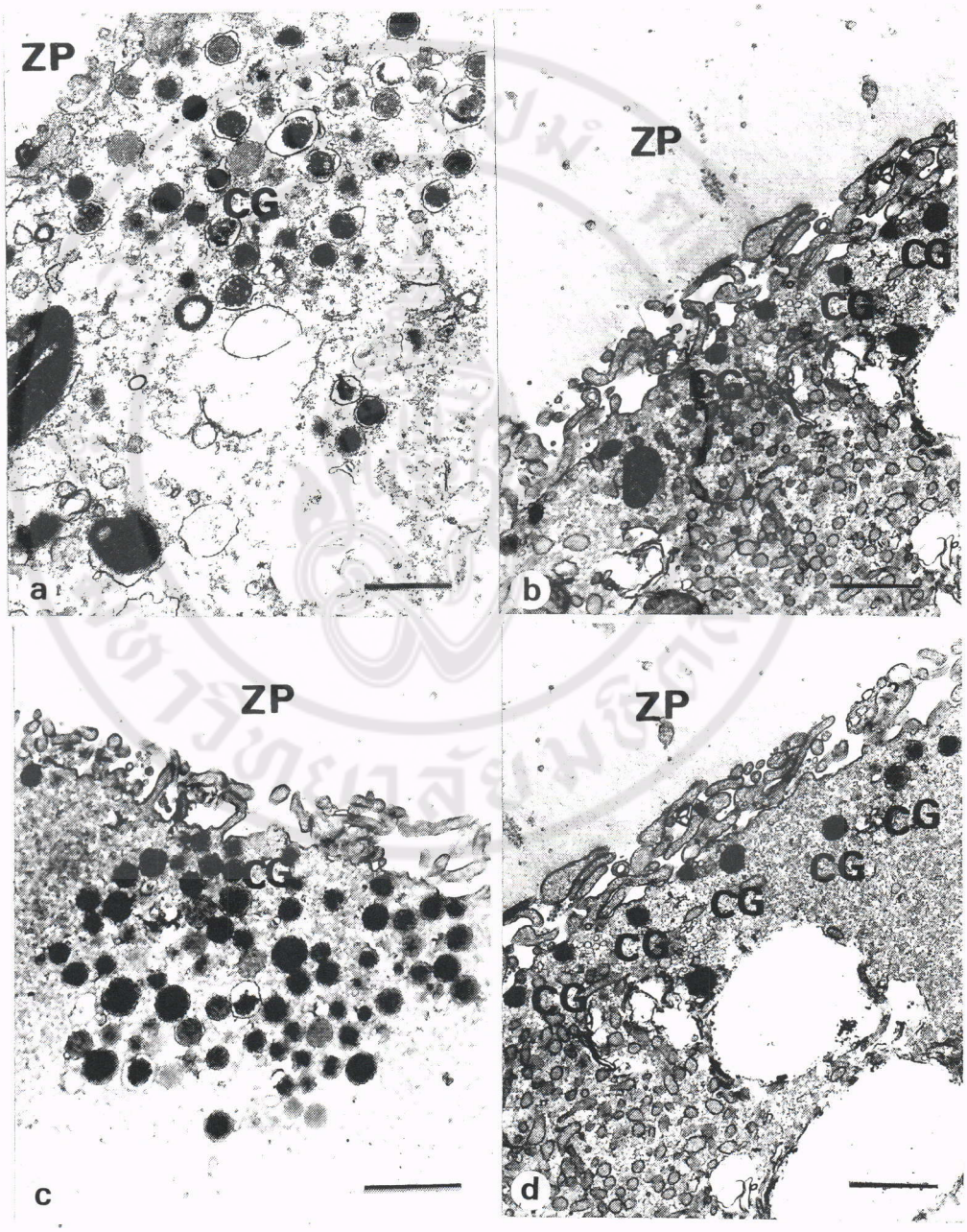
a : A cluster of cortical granules (CG) located near the periphery in an immature oocyte (0 h before incubation). They are composed of both heterogenous and homogenous, dense and less electron density in different size. ZP = zona pellucida.

b : Electron dense cortical granules (CG) line along the oolemma in an mature oocyte (24 h after incubation).

c : A cluster of CG in a 24 h HX oocyte (24 h after incubation), CG of different maturation accumulate into aggregates of different size as similar as CG in immature oocyte.

d : Oocytes after treatment with HX, washing and culture in control medium (24 h HX → HX(- ve) → 24 h IVM) solitary cortical granules (CG) line along the oolemma. It shows electron density and the uniformity in size. ZP = zona pellucida, MV = microvilli.

FIGURE 58



CHAPTER V

DISCUSSION

The present study has demonstrated that bovine cumulus oocyte complexes could be maintained in meiotic arrest *in vitro* up to 24 h by HX. Furthermore, after washing out of HX these oocytes were able to mature to the metaphase II stage following culture in HX-free TCM medium for 24 h. The concentration of HX in the present study (4 mM) was sufficient to maintain bovine oocytes in meiotic arrest.

In rodents, the percentages of mouse oocytes arrested at germinal vesicle (GV) stage in medium containing 2 mM of HX were 30 and 20% after 3 and 24 h incubation, respectively. In higher dose of HX (4 mM), 70 and 60% of mouse oocytes were arrested in GV stage by 3 and 24 h incubation (21). Shim et al., 1992 reported that the percentage of mouse oocytes arrested at the GV stage after 3 h culture in medium with 5 mM HX was 73% (24). Levesque and Sirard, 1995 reported that there were species difference between cattle and rats in the inhibitory action of HX on oocyte maturation. They showed that the inhibitory effect of HX was more sensitive in rodent than those in cattle. The percentage of bovine oocytes arrested at the GV stage after 6 h culture in medium with 2 mM HX was only 35%. At 21 h, the inhibitory effect was less effective, the percentage of oocyte arrested at GV stage decreased to 4%. In bovine oocytes the effect of cAMP-related compounds was reported to be only transient (50). In contrast, our study demonstrated that the effectiveness of inhibitory action of HX on bovine oocyte maturation was comparable

to that in mice. The discrepancy of our results and those reported by Sirard may be due to the difference of HX doses. HX (2 mM) employed by Sirard may be insufficient to maintain meiotic arrest in bovine oocytes. However, our result was agreeable with Miyano et al., 1985 who reported that all pig oocytes (100%) were maintained in GV stage by 4 mM HX in 24 h culture.

HX and adenosine, which present in mouse follicular fluid, interact in a synergistic manner to prevent spontaneous maturation in culture. HX (4 mM) and adenosine (0.75 mM) maintained more than 95% of mouse oocytes in GV stage for culture periods up to 24 h. The synergistic effect of these two purines may be resulted by both promoting cAMP synthesis (adenosine), and by preventing its hydrolysis (HX) (21). Shim et al., 1992 reported that 1 mM adenosine maintained 46% of mouse oocytes in GV stage after culture for 3 h whereas 5 mM HX inhibited meiosis in 63% of mouse oocytes. However, the percentage of mouse oocytes in GV stage was 93% when 1 mM adenosine and 5 mM HX were combined (24). HX and adenosine may be physiologically important as regulators of meiotic maturation in vivo. Adenosine has been reported to have toxic effects on cultured cells when it was used in high concentrations, presumably owing to the suppression of pyrimidine synthesis. This toxic effect was ameliorated by the addition of uridine to the culture medium (22). The evidence implicating cAMP in the maintenance of meiotic arrest, combined with the inhibitory action of purines on oocyte maturation, raises the possibility that purines act by modulating cAMP metabolism in the oocyte. Cyclic AMP mediated the action of HX in maintaining meiotic arrest (2). Warikoo and Bavister in 1989 studied the inhibitory effect of HX and cAMP combination on rhesus monkey oocyte

maturation. Eighty-two percent of rhesus monkey oocytes was arrested in the GV stage in the medium containing 100 μM cAMP and 0.2 mM HX whereas the percentage of oocytes in GV stage was only 28% in medium containing cAMP alone. The IBMX treatment that elevated the intracellular cAMP levels maintained oocytes in meiotic arrest (71% in GV). (51). In contrast, Bilodeau et al., in 1993 showed that bovine oocytes were difficult to be maintained in GV stage by cAMP-related compounds. They suggested that bovine oocytes might have an alternative pathway to induce maturation without any cAMP changes (33). Nevertheless, cAMP is accepted as a second messenger associated with the resumption of meiosis. There is a general consensus that a transient decrease in the cAMP level is an obligatory first step in the induction of oocyte maturation. This, in turn, decreases protein kinase A (PKA) activity, which leads to dephosphorylation of a putative maturation-inhibiting phosphorylated protein. Therefore, the fall in oocyte cAMP levels is important for GVBD (51). In fact, both inhibition and induction of oocyte maturation could be elicited by cAMP. When the oocyte is the target for cAMP, maturation is inhibited. At LH-stimulated cAMP event, it is the response of the cumulus cells to the nucleotide which results in breakdown of communication between oocytes and cumulus cells and leads to oocyte maturation (52). This observation is in agreement with the reported absence of LH-hCG receptors on the oocytes and their presence on cumulus cells (53).

In addition to HX and cAMP related compounds, other inhibitors were reported to inhibit maturation of bovine oocytes. Ninety-two percent of bovine oocytes was still in the GV stage after 9 h incubation in cycloheximide (a protein

synthesis inhibitor). The inhibitor of phosphorylation (6-DMAP) also maintained meiotic arrest in bovine oocytes. The percentage of oocytes in GV stage was 74% by incubation with 1 mM 6-DMAP.

In the present study, the inhibitory action of HX was reversed by its withdrawal. This finding of reversibility was consistent with the effects of HX in the oocytes of mice (22,24), rhesus monkeys (28) and pigs (29). The inhibitory effect of other inhibitors such as cycloheximide was also reversible in the cattle oocytes (44,45,54,55,), horses (56) and pigs (57). After washing out of cycloheximide, 80% of bovine oocytes reached metaphase II at 24 h culture comparing to 86% in the control group (58). In the present study, 76% of HX treated bovine oocytes reached metaphase II after culture in the HX free medium for 24 h comparing to 80% in the untreated oocytes incubation for the same duration. In contrast, goat oocytes relieved from cycloheximide block could not proceed beyond metaphase I stage under inhibitor-free conditions. It indicated that the requirement for meiotic progress beyond metaphase I of bovine oocytes as well as pigs and horses were different from goat. (15).

Downs et al., 1986, demonstrated that mouse oocytes were maintained in meiotic arrest for 12 h in medium containing maturation inhibitors (4 mM HX and 0.75 mM adenosine). After being washed free of inhibitor and cultured for 16 h in inhibitor-free medium to permit meiotic maturation, mature oocytes were inseminated at the end of this second culture period. Seventy percent of oocytes developed to 2- cell stage. Transfer of compacted morulae into pseudopregnant hosts produced live young at 19 days post insemination. Saeki et al., in 1997 reported that bovine oocytes

remained in the GV stage after culture for 24 h with cycloheximide or 6-DMAP. After washing out of the inhibitors cycloheximide-inhibited oocytes retained developmental competence whereas 6-DMAP-inhibited oocytes reduced their capacities for fertilization and further development. Two calves were obtained following the transfer of blastocysts derived from oocytes inhibited by cycloheximide to recipient heifers. In our studies, we attempted to demonstrate the inhibitory effect of HX on the ultrastructure of oocytes during maturation. The HX treated oocytes appeared normal in maturation after HX removal (59).

In the present study, bovine oocytes liberated from HX accomplished GVBD after 5 h culture in inhibitor-free medium, while untreated oocytes completed GVBD at 9 h incubation. The time sequence of GVBD following HX removal occurred twice as fast as in the control medium. An acceleration of the GVBD process after HX removal was previously described in pigs (29). A similar acceleration of GVBD was also reported in sheep, pig and bovine oocytes cultured in the medium containing cycloheximide (15,42,44,45,57,59). The time sequences of GVBD in sheep and pig oocytes were also two and three times faster respectively, then untreated oocytes (57). The acceleration of GVBD observed after the removal of the inhibitors may be explained by the incomplete inhibition of the inhibitors.

Resumption of meiosis normally occurs in the preovulatory follicle as a consequence of the LH peak or it can be initiated when oocytes are released from the inhibitory influences of the follicles and cultured in vitro (8). The two important indications of oocyte maturation in mammals are the breakdown of the nuclear envelope and chromosome condensation. The process of chromatin condensation is

differently controlled from membrane disassembly (9,60). Motlik et al., 1990 suggested that two groups of mammalian oocytes can be distinguished on the basis of their requirement for protein synthesis for GVBD. First, fully grown oocytes of rodents and rabbits possess all proteins essential for chromatin condensation and nuclear membrane breakdown. Second, the oocytes of pigs, sheep, and cattle show a slow sequence of GVBD and are equippes before resumption of meiosis for chromatin condensation only (7).

The entry of cells into the M phase induced by MPF was originally described by Masui and Markert in 1971 (61). MPF is reported to be responsible for the induction of nuclear membrane breakdown and chromatin condensation (62,63). Both events of nuclear membrane breakdown and chromatin condensation are commonly accepted to be controlled by MPF (64,65). MPF is known to be a heterodimer comprise of p34cdc2 and cyclin B (66). The catalytic subunit p34cdc2 displays a kinase activity, the regulation of which involved phosphorylation/dephosphorylation on specific residues (67,68,69) as well as its association with the regulatory subunit, (65,70). Treated bovine oocytes with HX in our study might allow a partial activation that is conpetent for chromatin condensation only. In the active form, MPF posses histone H1 kinase activity which is genenally used as biochemical assay for revealing MPF activity. The condensation of chromatin into chromosome during M-phase is accompanied by a specific phosphorylation of histones H1 and H3, suggesting that condensation is at least partially induced by these histone posttranslational modifications (65,66,71). Our finding in bovine oocytes confirmed the previous report of Miyano et al., 1995 in pig oocytes. They also observed the highly condensed

chromatin in the pig oocytes after culture in HX-supplemented medium. An increased H1 kinase activity in rabbit oocytes was significant and occurred concomitantly with the appearance of chromosome condensation (66). Coiling of chromosome is accompanied by the phosphorylation of all histone H1 molecules at five specific serine residues (51). Because of the role played by histone H1 in packing nucleosomes together, its phosphorylation seems likely to have a crucial role in this process of chromosome condensation. The increase in histone H1 kinase activity, accompanying chromatin condensation, did not affect the integrity of the nuclear membrane in oocytes (60,66)

Bovine oocytes that did not enter into M phase because of the presence of phosphodiesterase inhibitors such as HX or IBMX synthesized the cyclin protein of 46 kDa (72). Several evidences support an important role for the phosphorylation of protein substrates in the regulation of mitosis and meiosis (67,68,69,73). In fact, the control point of the cell cycle, the G2/M transition period of the meiotic and mitotic cells, is controlled by a complex cascade of protein phosphorylation and dephosphorylation events that leads to the activation of MPF (61,74)

The inhibitor of protein phosphorylation or protein kinase inhibitor (6-DMAP) specifically and reversibly blocked resumption of meiosis in oocytes from strarfish and mice presumable by inhibiting a protein kinase activity (75,76). Cattle oocytes were also sensitive to 6-DMAP and remained in the GV stage with highly condensed chromosome. The results imply that in cattle oocytes, enzymes responsible for chromatin condensation, mainly H1 kinase are more sensitive to 6-DMAP than the lamin kinases that are responsible for nuclear envelope disintegration (60,77).

Bovine oocytes that did not enter into M phase because of the presence of 6-DMAP did not synthesize proteins of average molecular sizes of 46 kDa. It is possible that the regulation of mammalian MPF depends on the synthesis of cyclin as demonstrated in the unicellular eucaryote and goldfish (78,79,80). Studies on 6-DMAP have indicated that it has no effect on protein synthesis (81), but it indirectly stimulates the tyrosine phosphorylation of P34 (82) consequently blocking MPF activity and the ability of cells to enter into M phase (83).

In the present study, HX could inhibit GVBD by maintaining nuclear membrane integrity but could not inhibit chromatin condensation. The results suggested that HX could not prevent all events involved in GVBD. These similar events were observed in cycloheximide treated oocyte of pig, sheep, horse and bovine (15,54,56,57). Both HX and cycloheximides experiments in inhibition of oocyte maturation in pig, cattle, and sheep imply that the activity inducing chromatin condensation is different from that inducing the breakdown of the nuclear envelope (60). The similar results of chromatin condensation were obtained after puromycin or cycloheximide treatment. The inhibition of protein synthesis by cycloheximide might lead to a partial MPF activation, that is also competent only for chromosome condensation (15).

Lamin is a protein of the nuclear envelope inner membrane that has a nucleoplasm (84,85). Recent evidence strongly implicates the pivotal regulator of mitosis, MPF, as playing an active and direct role in the phosphorylation of the lamins within the nuclear envelope (85). Thus, it appears that lamin disassembly at the onset of mitosis occurs owing to the phosphorylation of two specific sites on the nuclear

lamin protein by the MPF kinase (84,86,87,88). Nuclear membrane assembly at mitosis is regulated by a kinase/phosphatase system that is regulated by MPF kinase (51,63).

Inoue et al., 1998 reported the role of mitogen-activated protein kinase (MAPK) in the meiotic resumption of porcine oocytes according to the following evidences. (1) inactive MAPK was localized in the cytosol of immature GV oocytes (2) part of the activated MAPK translocated into the GV just before GVBD, translocation of MAPK into the GV was prerequisite for GVBD, and injection of active MAPK into GV induced GVBD and (3) exogenous MAPK maintained its activity level in the GV and induced GVBD. These evidences indicate that MAPK mediates the maturation-inducing signal from the cytoplasm into the nucleus to induce meiosis reinitiation. The activity of MAPK may be sustained in the nucleus, even on immature oocytes by the separation of MAPK from any cytoplasmic inhibitors (89). Although the direct target of MAPK in the GV is unclear, one of the candidates is nuclear lamins (85,88). MAPK was reported to phosphorylate nuclear lamins which lined the nuclear envelope (90). MAPK functioned as the inducer of GVBD in porcine oocytes. It had been postulated that the increase in phosphorylation before GVBD involved the elements necessary for nuclear dissolution. Precisely, the resulting inhibition of phosphatase activity by okadaic acid (OA) led to an increase in overall protein phosphorylation and allowed membrane dissolution (87). Moreover, continuous exposure to this substance resulted in an abortive M-phase without spindle formation as observed in mouse, pig, and cattle oocytes (91,92,93).

The successive culture in protein synthesis inhibitor, cycloheximide (20 h) and a protease inhibitor, p-aminobenzamidine (10 h) prevented GVBD in all pig oocytes. In contrast, when the oocytes washed after cycloheximide block (20 h) were cultured in p-aminobenzamidine enriched medium for 2-3 h and again for 6 h in cycloheximide medium, the nuclear membrane dissolved in more than 60% of oocytes. These data suggest that inhibition of protein synthesis in pig oocytes does not prevent the chromatin condensation in GV whereas nuclear membrane breakdown requires the successive protein synthesis and proteolysis (57). Newport et al., in 1987 described a stable cell-free mitotic extract derived from Xenopus egg containing the activities necessary for nuclear envelope breakdown and chromosome condensation. They demonstrated that nuclear envelope vesiculation, lamina solubilization and chromosome condensation were independent and separable biochemical processess. Lamin solubilization is enzymatically driven but chromosome condensation involves both binding proteins and enzymatic activities including topoisomerase II (60). Fleming et al., 1983 reported that cow oocytes are sensitive to inhibitory of protease. Proteolysis might play an important role in MPF activation in mammalian oocytes (94). Similar to starfish oocytes, proteolysis might also play an important role in MPF activation (95,96).

Taken together the inhibitory effect on oocytes maturation by HX and other inhibitory compounds mediate their actions through MPF as summarized in Figure 59. The activation of MPF through kinase and protein synthesis leads to chromosome condensation whereas the activation of proteolysis causes nuclear membrane breakdown.

Oocyte maturation appears to be characterized by changes in both nucleus and cytoplasm. Cytoplasmic maturation requires species-dependent protein synthesis at different steps of the meiosis process and leads to the activation of a universal complex, MPF, which is essential for the G2/M progression (97).

In the present study, the characteristics of cortical granules (CG) in HX treated bovine oocytes for 24 h were similar to that in immature normal oocytes. They consisted of both less and dense electron density particles in cluster. In contrast, CG in normal mature oocyte were composed of only dense electron density particles in solitary. These appearances indicated that the CG in treatment oocytes were not capable in progressing to mature CG. These CG could not function in the cortical reaction to prevent polyspermy. The numerous mitochondria were still located in the peripheral area and the characteristic of SER and Golgi complexes were similar to those in the untreated immature oocyte. However, the precise mechanism on changes of cytoplasmic maturation in HX treated oocytes was not reported in comparison to other inhibitors. Avery et al., 1998, demonstrated that CG in 6-DMAP treated bovine oocytes for 24 h were larger in size but less electron dense than those in normal mature oocyte. The ooplasmic vesicles progressed to maturation state than expected. Smaller and lesser vesicles with some fused together were demonstrated. The exact function of these vesicles were unknown (98). The 6-DMAP inhibited the activation of p34, one of the active MPF components, but it did not influence the synthesis of cyclin B or other proteins. Cyclin B protein was synthesized just before GVBD, and its level was critical for resumption of meiosis (99,100). The mechanism of inhibiting

cytoplasmic maturation in bovine oocytes by HX could be different from 6-DMAP treatment.

The distribution of CG and the stage of DNA during oocyte maturation were revealed by CLSM in the present study. It provides the clarity picture than that from fluorescent light microscope. Both DNA and CG during oocyte maturation were demonstrated at the same time with less time consuming in comparison to electron microscope (EM). The stages of normal oocyte maturation and distribution of CG were detected by this technique without any artifact section using EM. In addition to DNA and CG, CLSM could detect other organelles such as microtubule, endoplasmic reticulum, Golgi complex etc. Levels of Na^+, Ca^{++}, K^+ ion could also be detected by this technique (101).

The maintenance of meiotic arrest on bovine oocytes by HX and its reversibility is useful. Since the cattle is economically valuable animal in Thailand. Bovine oocytes maintained in meiotic arresting stage from superior breeder could be transported conveniently around the world. Good quality oocytes can be selected and maintained in the medium containing HX during transportation instead of oocyte cryopreservation in the cryoprotectants which are toxic to the oocytes.

The effect of HX on oocyte maturation inhibition is reversible and non toxic as revealed in our morphological studies. The HX treated oocytes will be fertilized and developed in vitro to the transferable stage embryos for transferring to the recipient cows in the further studies.

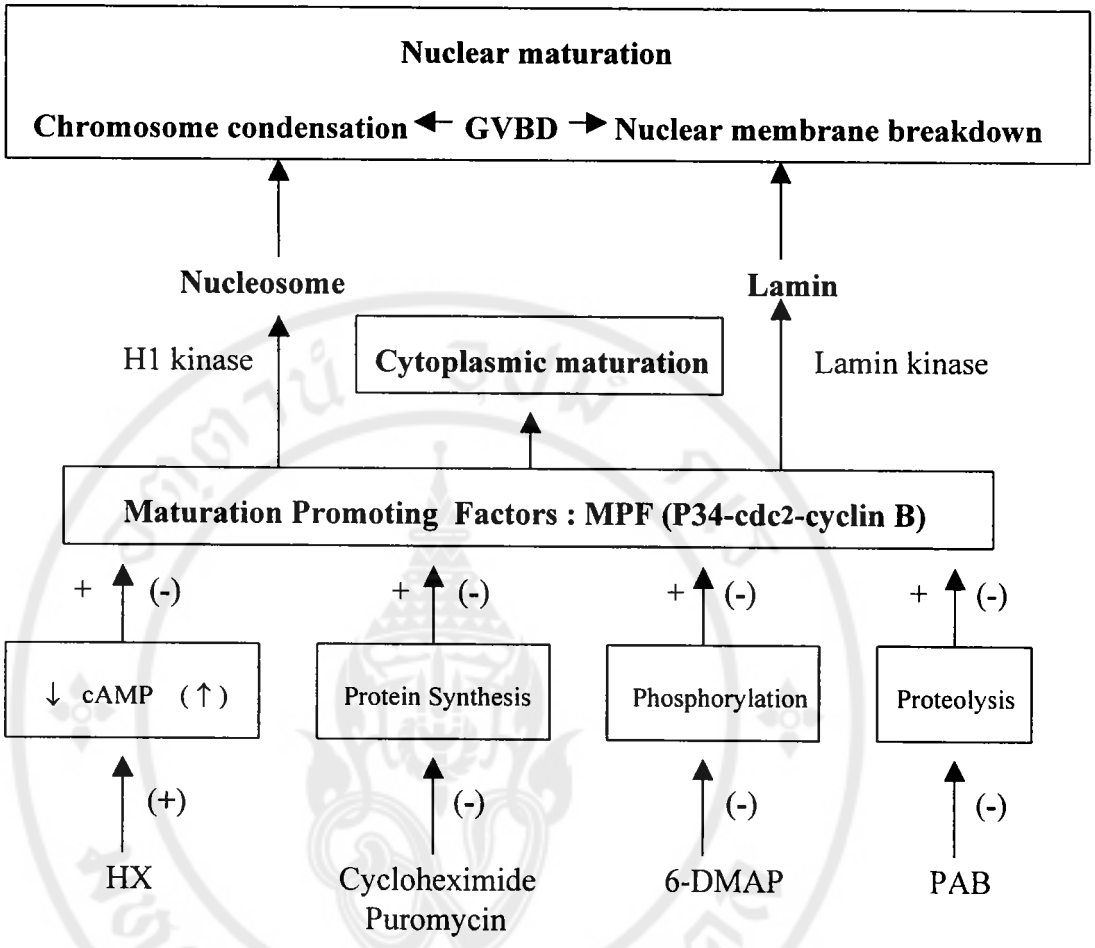


Figure 59. The diagram illustrating the hypothesis of HX and other inhibitors in affecting the nuclear and cytoplasmic maturation.

CHAPTER VI

CONCLUSIONS

1. HX effect is dose dependent and 4 mM is sufficient to inhibit both nuclear and cytoplasmic maturation on bovine oocytes.
2. HX can maintain meiotic arrest in GV stage at high percentages at 93, 81 and 76% by 8, 16 and 24 h incubation, respectively.
3. HX can not prevent all events involve in process of GVBD. HX inhibits nuclear membrane breakdown but it can not prevent chromosome condensation.
4. The inhibitory action of HX was reversed by its withdrawal and HX treated oocytes after HX washing reach M II similar to those untreated group.
5. Following the removal of HX and reculture in HX free culture medium, GVBD occures two times faster than the untreatment.

REFERENCES

1. Thibault C, Szollosi D, Gerard M. Mammalian oocyte maturation. Reprod Nutr Develop 1987;27(5):865-896.
2. Downs SM, Daniel SAJ, Bornslaeger EA, Hoppe PC and Eppig JJ. Maintenance of meiotic arrest in mouse oocytes by purines : Modulation of cAMP levels and cAMP phosphodiesterase activity. Gamete Research 1989;23:323-334.
3. Lonergan P, Monaghan P, Rizos D, Boland MP and Gordon I. Effect of follicle size on bovine oocyte quality and development competence following maturation, fertilization and culture in vitro. Mol Reprod Dev 1994;34:48-53.
4. Masui Y. Problems of oocyte maturation and the control of chromosome cycles. Dev Growth Differ 1985;27:295-309.
5. Hyttel P, Xu KP, Smith S, Greve T. Ultrastructure of in vitro oocyte maturation in cattle. J Reprod Fertil 1986a;78:615-625.
6. Hyttel P, Fair T, Callesen H and Greve T. Oocyte growth, capacitation and final maturation in cattle. Theriogenolgy 1977;47:23-32.
7. Motlik J and Kubelka M. Cell cycle aspects of growth and maturation of mammalian oocytes. Mol Reprod Dev 1990;27:366-375.

8. Pincus G, Enzman EV. The comparative behavior of mammalian eggs in vivo

and in vitro. The activation of ovarian eggs. J Exp Med 1935;62:665-675.

9. Christmann L, Jung T and Moor RM. MPF components and meiotic competence in growing pig oocytes. Mol Reprod Dev 1994;38:85-90.

10. Naito K and Toyoda Y. Fluctuation of histone H1 kinase activity during meiotic maturation in porcine oocytes. J Reprod Fert 1991;93:467-473.

11. Keefe SJ, Wolfes H, Kieesling AA and Cooper GM. Microinjection of c-mos oligonucleotides prevents meiosis II in the maturing mouse egg. Proceedings of the National Academy of Sciences USA 1989;86:7038-7042.

12. Szollosi MS, Debey P, Szollosi D, Rime H, Vautier D. Chromatin behaviour under influence of puromycin and 6-DMAP at different stages of mouse oocyte maturation. Chromosome 1991;100:339-354.

13. Eppig JJ. Maintenance of meiotic arrest and the induction of oocyte maturation in mouse oocyte-granulosa cell complexes developed in vitro from preantral follicles. Biol Reprod 1991;45:824-830.

14. Hashimoto N and Kishimoto T. Regulation of meiotic metaphase by a cytoplasmic maturation promoting factor during mouse oocyte maturation. Dev Biol 1988;126:242-252.

15. Le Gal F, Gall L, De Schmidt V. Changes in protein synthesis pattern during in vitro maturation of goat oocytes. Mol Reprod Dev 1992;32:1-8.

16. Chang MC. Fertilization and normal development of follicular oocytes in the rabbit. Science 1955;121:867-869.

17. Tsafriri A, Channing CP. An inhibitory influence of granulosa cells and

follicular fluid upon porcine oocytes meiosis in vitro. Endocrinology 1975;96:222.

18. Channing CP, Anderson LD, Hoover DJ, Kolena J, Osteen KG, Pomerantz SH, Tanabe K. The role of the nonsteroidol regulators in control of oocyte and follicular maturation. Rec Progr Horm Res 1982;38:331-408.
19. Tsafriri A, Dekel N and Ami S. The role of oocyte maturation inhibitor in follicular regulation of oocyte maturation. J Reprod Fert 1982;64:541-551.
20. Downs SM, Colemom DL, Ward Baily PF, Eppig JJ. Hypoxanthine is the principal inhibitor of murine oocyte maturation in a low molecular weight fraction of porcine follicular fluid. Proc Natl Acad Sci USA 1985;82:454-458.
21. Eppig JJ, Ward-Baily PF, Coleman DL. Hypoxanthine and adenosine in murine ovarian follicular fluid : concentrations and activity in maintaining oocyte meiotic arrest. Biol Reprod 1985;33:1041-1049.
22. Downs SM, Coleman DL, Eppig JJ. Maintenance of murine oocyte meiotic arrest : uptake of hypoxanthine and adenosine by cumulus cell-enclose and denuded oocytes. Dev Biol 1986;117:174-183.
23. Eppig JJ, Downs SM. The effect of hypoxanthine on mouse oocyte growth and development in vitro : Maintenance of meiotic arrest and gonadotropin-induced oocyte maturation. Dev Biol 1987;119:313-321.
24. Shim C, Lee DK, Lee CC, Cho WK and Kim KJ. Inhibitory effect of purine in meiotic maturation of denuded mouse oocytes. Mol Reprod Dev 1992;31:280-286.

25. Downs SM. Puring control of mouse oocyte maturation : evidence that nonmetabolized hypoxanthine maintains meiotic arrest. Mol Reprod Dev 1993;35:82-94.

26. Downs SM. Involvement of purine nucleotide synthetic pathways in gonadotropin-induced meiotic maturation in mouse cumulus cell-enclosed oocytes. Mol Reprod Dev 1997;46:155-167.

27. Sirard MA and First NL. In vitro inhibition of oocyte nulcear maturation in the bovine. Biol Reprod 1988;39:229-234.

28. Warikoo K, Bavister BD. Hypoxanthine and cyclic adenosine 5-monophosphate maintain meiotic arrest of rhesus monkey oocytes in vitro. Fertil Steril 1989;51:886-889.

29. Miyano T, Misa ebihara, Goto Y, Hirao Y, Nagai T and Kato S. Inhibitory action of hypoxanthine on meiotic resumption of denuded pig follicular oocytes in vitro. J Exp Zool 1995;273:70-75.

30. Sehultz RM, Montgomery RR, Belanaff J. Regulation of mouse oocyte maturation. Implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis. Develop Biol 1983;97:264-273.

31. Downs SM, Hunzicker-Dunn M. Differential regulation of oocyte maturation and cumulus expansion in the mouse oocyte-cumulus cell complex by site-selective analogues of cyclic adenosine monophosphate. Dev Biol 1995;172:72-85.

32. Cho W, Stern S, Biggers JD. Inhibitory effect of dibutyryl cAMP on mouse oocyte maturation in vitro. J Exp Zool 1974;187:183-186.

33. Bilodeau S, Fortier MA, Sirard MA. Effect of adenylate cyclase stimulation on meiotic resumption and cyclic AMP content of zona free and cumulus enclosed bovine oocytes in vitro. J Reprod Fertil 1993;97:5-11.

34. Bornslaeger EA, Mattei PM, Schultz RM. Involvement of cAMP-dependent protein kinase and protein phosphorylation in regulation of mouse oocyte maturation. Dev Biol 1986;114:453-462.

35. Rose-Hellekant TA, Bavister BD. Roles of protein kinase A and C in spontaneous maturation and foskolin or 3-isobutyl-1 methylxanthine maintained meiotic arrest of bovine oocytes. Mol Reprod Dev 1996;44:241-249.

36. Sirard MA, Richard F and Mayes M. Controlling meiotic resumption in bovine oocytes. Theriogenology 1998;49:483-497.

37. Fulka J, Leibfried-Rutledge ML, First NL. Effect of 6-dimethylaminopurine on germinal vesicle breakdown of bovine oocytes. Mol Repord Dev 1991;29:379-384.

38. Martus NS, Farin CE. Effectiveness of DRB in inhibiting GVBD in bovine oocyte maturation. Biol Reprod 1993; 48 (Suppl 1):102.

39. Osborn JC, Moor RM. Time dependent effect of α-amanitin on nuclear maturation and protein synthesis in mammalian oocytes. J Embryo Exp Morph 1983;73:317-338.

40. Meineke B, Meineke-Tillmamn S. Effects of α-amanitin on nuclear maturation of porcine oocytes in vitro. J Repro Fertil 1993;98:195-201.

41. Hunter AG, Moor RM. Stage-dependent effect of inhibiting ribonucleic acids and protein synthesis on meiotic maturation of bovine oocytes in vitro. J Dairy Sci 1987;70:1646-1652.

42. Lonergan P, Khatir H, Carolan C, Mermillod P. Bovine blastocyst production in vitro following inhibition of oocyte meiotic resumption for 24 h. J Reprod Fertil 1997;109:355-365.

43. Fulka J, Jr, Motlik J, Fulka J, Jilek F. Effect of cycloheximide on maturation of pig and mouse oocytes. J Reprod Fertil 1986a;77:281-285.

44. Motlik J, Lee B and Shioya Y. Two sensitvity levels of cattle oocytes to puromycin. Biol Reprod 1990;43:994-998.

45. Tatemoto H, Horiuchi T and Terda T. Effects of cycloheximide on chromatin condensations and germinal vesicle breakdown (GVBD) of cumulus-enclosed and denuded oocytes in cattle. Theriogenology 1994;42:1141-1148.

46. Mermilliod P, Tomanek M, Marchal R and Meijer L. High developmental competence of cattle oocytes maintained at the germinal vesicle stage for 24 hours in culture by specific inhibition of MPF kinase activity. Mol Reprod Dev 2000;55:89-95.

47. Avery B, Greve T. Effect of ethanol and dimethylsulphoxide on nuclear and cytoplasmic maturation of bovine cumulus-oocytes complexus. Mol Reprod Dev 2000;55:438-445.

48. Yoshida M, Cran DG and Pursel VG. Confocal and fluorescence microscopic study using lectins of the distribution of cortical granules during the

maturation and fertilization of pig oocytes. Mol Reprod Dev 1993;36:462-468.

49. Kim NH, Funnahashi H, Abeydeera LR, Moon SJ, Prather ES and Day BN. Effects of oviductal fluid on sperm penetration and cortical granule exocytosis during fertilization of pig oocytes in vitro. J Reprod Fert 1996;107:79-86.

50. Sirard MA and Bilodeau S. Granulosa cells inhibit the resumption of meiosis in bovine oocytes in vitro. Biol Reprod 1990;43:777-783.

51. Lveque JT and Sirard MA. Effects of different kinase and phosphatases on nuclear and cytoplasmic maturation of bovine oocytes. Mol Reprod Dev 1995;42:114-121.

52. Dekel N, Beers WH. Rat oocyte maturation in vitro : Relief of cAMP inhibition by gonadotropins. Proc Natl Acad Sci USA 1978;75:4369-4373.

53. Tsafriri A, Dekel N. Molecular mechanisms in ovulation. Molecular biology of the female reproductive system. New York : Acadamic Press 1994;207-220.

54. Lonergan P, Khatir H, Carolan C and Mermillod P. Bovine blastocyst produciton in vitro after inhibition of oocyte meiotic resumption for 24 h. J Reprod Fert 1997;109:355-365.

55. Saeki K, Nagao Y, Kirhi M and Nagai M. Development capacity of bovine oocytes following inhibition of meiotic arrest by cycloheximide or 6-dimethylaminopurine. Theriogenology 1997;48:1161-1172.

56. Alm H and Hinrichs K. Effect of cycloheximide on nuclear maturation of horse oocytes and its relation to initial cumulus morphology. J Reprod Fert 1996;107:215-220.

57. Kubelka M, Motlik J, Fulka J, Prochazka R, Rimkevicova Z and Fulka J. Time sequence of germinal vesicle breakdown in pig oocytes after cycloheximide and P-Aminobenzamidine Block. Gamete Research 1988;19:423-431.

58. Saeki K, Nagao Y, Kirhi M, Nagai M and Iritani A. Timing of completion of the first meiotic division in bovine oocytes after maintenance of meiotic arrest with cycloheximide and their subsequent development. J Vet Med Sci 1998;60(4)523-526.

59. Saeki K, Nagao Y, Kishi M, Nogai M and Iritani A. Yiming of completion of the first meiotic civistion in bovine oocytes after maintenance of meiotic arrest with cycloheximide and their subsequent development. J Vet Med Sci 1998;60(4):523-526.

60. Newport J, Spann T. Disasembly of the nucleus in mitotic extracts : Membrane vesicularization, lamina disassembly, and chromosome condensation are independent processes. Cell 1987;48:219-230.

61. Masui Y and Markert CL. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J Exp Zool 1971;177:129-146.

62. Masui Y. Problems of oocytes maturation and the control of chromosomes cycles. Dev Growth Diff 1985;27:295-311.

63. Pfaller R, Smythe C and Newport JW. Assembly/disassembly of the nuclear envelope membrane:cell cycle-dependent binding of nuclear membrane vesicles to chromatin in vitro. Cell 1991;65:209-217.

64. Hunt T. Maturation promoting factor, cyclin and the control of M-phase. Curr Opin Cell Biol 1989;1:268-274.

65. Murray AW and Kirschner MW. Dominoes and clock: The union of two views of the cell cycle. Science 1989;246:614-621.

66. Draetta G, Luca F, Westendorf J, Brizuela L, Ruderman J, Beach D. cdc2 protein kinase is complexed with both cyclin A and B : Evidence for proteolytic inactivation of MPF. Cell 1989;56:829-838.

67. Dunphy WG, Brizuela L, Beach D, Newport J. The *Xenopus* cdc2 protei kinase is a component of MPF, a cytoplasmic regulator of mitosis. Cell 1988;54:423-431.

68. Gautier J, Matsukawa T, Nurse P, Maller J. Dephosphorylation and activation of *Xenopus* p34cdc2 protein kinase during the cell cycle. Nature 1989;339:626-629.

69. Morla O, Draetta G, Beach D, Wang JY. Reversible tyrosine phophorylation of cdc2. Dephosphorylation accompanies activation during entry into meiosis. Cell 1989;58:193-203.

70. Gautier J, Minshull M, Lohka M, Glotzer M, Hunt T Maller JL. Cyclin is a component of MPF from *Xenopus*. Cell 1990;60:487-494.

71. Hirano T, Kobayashi R and Hirano M. Condensins, chromosome condensation protein complexes containing XCAP-C, XCAP-E and a *xenopus* homolog of the drosophila barren protein. Cell 1997;89:511-521.

72. Levesque JT and Sirard MA. Resumption of meiosis is initiated by the accumulation of cyclin B in bovine oocytes. Biol Reprod 1996;55:1427-1436.

73. Osmani SA, Pu RT, Morris NR. Mitotic induction and maintenance by over expression of a G2-specific gene that encodes a potential protein kinase. Cell 1988;53:237-244.

74. Kishimoto T, Kuriyama R, Kondo R, Kanatani H. Generality of the action of various maturation promoting factors. Exp Cell Res 1982;137:121-126.

75. Neant I, Guerrier P. 6-Dimethylaminopurine blocks starfish oocyte maturation by inhibiting a relevant protein kinase activity. Exp Cell Res 1988;176:68-79.

76. Rime H, Neant J, Guerrier P, Ozon R. 6-Dimethylaminopurine (6-DMAP) a reversible inhibitor of the cytoplasmic maturation-promoting factor (MPF) during the first maturation division of the mouse oocyte. Dev Biol 1989;133:169-179.

77. Murry AW, Kirschner MW. Dominoes and clocks : the union of two views of the cell cycle. Science 1989;246:612-621.

78. Yamashida M, Kaijiura H, Tanaka T, Onae S, Nagahama Y. Molecular mechanisms of the activation of MPF during goldfish oocyte maturation. Dev Biol 1995;168:62-75.

79. Tanaka T, Yamashita M. Pre-MPF is absent in immature oocytes of fished and amphibians except *Xenopus*. Dev Growth Differ 1995;37:387-393.

80. Jantzen H, Schulze I. Growth condition-induced precocious activation of p34cdc2 kinase inhibits the expression of developmental competence. Dev Biol 1994;166:311-322.

81. Neant I, Guerrier P. Meiosis reinitiation in the molusk patella vulagata. Regulation of MPF, CSF and chromosome condensation activity by intracellular pH, protein synthesis and phosphorylation. Development 1988;102:505-516.

82. Jessus C, Rime H, Haccard O, Vanlint J, Goris J, Merlevede W, Ozon R. Tyrosine phosphorylation of p34cdc2 and p42 during meiotic maturation of Senopus oocyte antogonistic action of okadaic acid and 6-DMAP. Development 1991;111:813-820.

83. Fulka J, Leibefried-rutledge ML, First NL. Effect of oocytes. Mol Reprod Dev 1991;29:379-384.

84. Collas P. Nuclear envelope disassembly in mitotic extract requires functional nuclear pores and a nuclear lamina. J of Cell Sci 1998;111:1293-1303.

85. Spann TP, Moir RD, Goldman AE, Stick R and Goldman RD. Dirsruption of nuclear lamin organization alters the distribution of replication factors and inhibits DNA synthesis. J Cell Biol 1997;136-:1201-1212.

86. Jelinkova L, Kubelka M, Motlik J and Gurerrier P. Chromatin condensation and histone H1 kinase activity during growth and maturation of rabbit oocytes. Mol Reprod Dev 1994;37:210-215.

87. Fulka J, Jr, Motlik J, Fulka J, Crozet N. Activity of matuation pormoting factor in mammalian oocytes after its dilution by single and multiple fusion. Dev Biol 1986b;118:176-181.

88. Yang L, Farin CE. Identification of cDNAs encoding bovine cyclin B and cdk 1/cdc2. Gene 1994;141:283-286.

89. Inoue M, Naito K, Nakayama T and Sato E. Mitogen-activated protein kinase translocated into the germinal vesicle and induces germinal vesicle breakdown in porcine oocytes. Biol Reprod 1998;58:130-136.

90. Peter M, Sanghera JS, Pelech SL, Nigg EA. Mitogen-activated protein kinase phosphorylate nuclear lamins and display sequence specificity overlapping that of mitotic protein kinase p34cdc2. Eur J Biochem 1992;205:287-294.

91. Rime H, Ozon R. Protein phosphatases are involved in the in vivo activation of histone H1 kinase in mouse oocyte. Dev Biol 1990;141:115-122.

92. Alexandre H, Van Cauwenberge A, Tsukitani Y, Mulnard J. Pleiotropic effect of okadaic acid on maturing mouse oocytes. Development 1991;112:971-980.

93. Kalous J, Kubelka M, Rimkevicova Z, Guerrier P, Motlik J. Okadaic acid accelerates germinal vesicle breakdown (GVBD) and overcomes cycloheximide and 6-dimethylaminopurine block in cattle and pig oocytes. Dev Biol 1993;157:448-454.

94. Fleming AD, Khalil W, Armstrong DT. Porcine follicular fluid does not inhibit maturation of rat oocytes in vitro. J Reprod Fertil 1983;69:665-670.

95. Kishimoto T, Clark TG, Kondo HK, Shirai H, Kanatani H. Inhibition of starfish oocyte maturation by some inhibitors of proteolytic enzymes. Gamete Res 1982;5:1-18.

96. Clark TG, Kanatani H. Effect of some inhibitors of proteolytic enzymes on maturation of starfish oocytes. Biol Bull 1975;149:423.

97. Masui Y. Towards understanding the control of the division cycle in animal cells. Biochem Cell Biol 1992;70:920-945.

98. Avery B, Hay-schmidt A, Hyttel P, Greve T. Embryo development, oocyte morphology and kinetical of meiotic maturation in bovine oocytes exposed to 6-dimethylaminopurine prior to in vitro maturation. Mol Reprod Dev 1998;50:334-344.

99. Chesnel F and Eppig JJ. Snthesis and accumulation of p34cdc2 and cyclin B in mouse oocytes during acquisition of competence to resume meiosis. Mol Reprod Dev 1995;40:503-508.

100. Wu B, Lgnotz G, Bruce Currie W and Yang X. Dynamics of maturation promoting factors and its constituent proteins during in vitro maturation of bovine ocoytes. Biol Reprod 1997;56:253-259.

101. Lefevre B, Pesty A and Testart J. Cytoplasmic and Nucleie calcium oscillations in immature mouse oocytes : Evidence of wave polarization by confocal imaging. Exp Cell Res 1995;218:166-173.

APPENDIX

Solutions and Embedding Media for TEM

Fixatives

1. 2.5% Glutaraldehyde in 0.1 M cacodylate buffer, pH 7.8.

5% glutaraldehyde	5 ml
0.2 M sodium cacodylate	4 ml
0.2 M calcium acetate	1 ml

2. 1% Osmium tetroxide in 0.1 M cacodylate buffer, pH 7.8.

2% Osmium tetroxide	5 ml
0.2 M cacodylate buffer	5 ml

Buffer Solution

1. 0.2 M Sodium cacodylate buffer, pH 7.8.

sodium cacodylate	4.28 g
single distill water	100 ml

2. 0.2 M calcium acetate

calcium acetate	3.16 g
single distilled water	100 ml

3. 0.1 M cacodylate buffer, pH 7.8.

0.2 M sodium cacodylate buffer	24 ml
0.2 M calcium acetate	1 ml
single distilled water	25 ml

Embedding Media

Vinylcyclophene dioxide (ERL 4206)	10	gm
Diglycidyl ether of polypropylene glycol (DER 736)	6	gm
Nonenyl succinic anhydride (NSA)	26	gm
Dimethylaminoethanol (DMAE)	0.4	gm

Staining Solutions

5% Uranyl acetate

Uranyl acetate	5	gm
Methanol, 70%	100	ml

Lead citrate

Lead nitrate	0.166	gm
Distilled water	3.5	ml

Mixed lead nitrate and distilled water together, shaked until completely dissslove, then add

Sodium citrate	0.220	gm

Gently shake until homogeneous, then titrate with

Sodium hydroxide	1	ml

Add distilled water for final volume is 6.5 ml

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