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THE ROLE OF NITRIC OXIDE IN SWAMP BUFFALO OOCYTE MATURATION

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การศึกษาครั้งนี้มีวัตถุประสงค์ เพื่อศึกษาบทบาทและกลไกการออกฤทธิ์ของไนตริกออกไซด์ (NO) ในระหว่างการเจริญของโอโอไซต์ของกระบือปลักที่นำมาเลี้ยงภายนอกร่างกาย โดยนำโอโอไซต์ของกระบือปลักแบบที่มีคูมูลัสเซลล้อมรอบ (COCs) กับแบบที่เอาคูมูลัสเซลที่ล้อมรอบโอโอไซต์ออกหมด (DOs) มาเลี้ยงในน้ำยาที่มี S-nitroso-L-acetyl penicillamine (SNAP) ซึ่งเป็นสารที่ให้ไนตริกออกไซด์ ที่ความเข้มข้นต่าง ๆ กัน ผลการทดลองพบว่าทั้ง COCs และ DOs ที่นำมาเลี้ยงในน้ำยาที่มี SNAP เป็นเวลา 8 ชั่วโมง จะมีเปอร์เซ็นต์ของ COCs และ DOs ที่มีการสลายของเยื่อหุ้มนิวเคลียสลดลงเมื่อเปรียบเทียบกับกลุ่มควบคุม ในทำนองเดียวกันเมื่อนำ COCs และ DOs มาเลี้ยงในน้ำยาที่มี SNAP เป็นเวลา 24 ชั่วโมง พบว่าเปอร์เซ็นต์ของ COCs และ DOs ที่เจริญจนถึงระยะเมตาเฟส II มีจำนวนลดลงเช่นเดียวกัน โดยผลที่เกิดขึ้นจะแปรตามปริมาณของ SNAP ที่ใช้ และพบว่า SNAP จะมีผลยับยั้งการเจริญของนิวเคลียสใน DOs มากกว่า COCs การศึกษากลไกการออกฤทธิ์ของ NO ใน COCs และ DOs โดยใช้ 1H-[1,2,4] oxadiazolo-[4,3,-α]quinoxalin-1-one (ODQ) ซึ่งเป็นสารยับยั้งการทำงานของเอนไซม์ soluble guanylyl cyclase (sGC) ที่ความเข้มข้น 10 ไมโครโมล่าร์ ในกลุ่ม COCs พบว่า SNAP ทำให้เปอร์เซ็นต์การสลายของเยื่อหุ้มนิวเคลียสลดลง ODQ ลดผลที่เกิดจาก SNAP โดยทำให้เปอร์เซ็นต์ของโอโอไซต์ที่มีการสลายของเยื่อหุ้มนิวเคลียสเพิ่มขึ้นเมื่อเปรียบเทียบกับ COCs ที่เลี้ยงในน้ำยาที่มี SNAP เพียงอย่างเดียว และ ODQ ทำให้เปอร์เซ็นต์การสลายของเยื่อหุ้มนิวเคลียสเพิ่มขึ้นเมื่อเทียบกับกลุ่มควบคุม ซึ่งผลการทดลองในกลุ่ม DOs จะแตกต่างจากกลุ่ม COCs โดยที่ ODQ ไม่สามารถจะไปลดผลที่เกิดจาก SNAP ใน DOs เมื่อวัดปริมาณ cyclic guanosine monophosphate (cGMP) ภายในโอโอไซต์ของ COCs และ DOs พบว่า SNAP ทำให้ปริมาณ cGMP ภายในโอโอไซต์ของ COCs มีปริมาณเพิ่มมากขึ้น ODQ จะทำให้ปริมาณ cGMP ภายในโอโอไซต์ของ COCs ที่เพิ่มขึ้นเนื่องมาจากผลของ SNAP นั้นมีปริมาณลดลง ในขณะที่ SNAP และ ODQ ไม่มีผลต่อระดับ cGMP ภายในโอโอไซต์ของ DOs นอกจากนี้ระดับ cyclic adenosine monophosphate (cAMP) ภายในโอโอไซต์ของ COCs และ DOs ทั้งในกลุ่มที่เลี้ยงในน้ำยาที่มี SNAP และในกลุ่มควบคุมไม่แตกต่างกัน โดยระดับ cAMP ภายในโอโอไซต์ของทุกกลุ่มจะลดลงหลังจากนำมาเลี้ยง 2 ชั่วโมง จากผลการทดลองที่ได้แสดงให้เห็นว่า NO มีบทบาทในการยับยั้งการเจริญของนิวเคลียสของโอโอไซต์กระบือปลัก โดยมีกลไกการออกฤทธิ์ได้ทั้งผ่าน cGMP และไม่ผ่าน cGMP และ NOไม่มีผลต่อปริมาณ cAMP ภายในโอโอไซต์ นอกจากนี้ NO ยังมีผลต่อการเจริญของไซโตพลาสซึมของโอโอไซต์ของกระบือปลักโดยทำให้ระดับ Maturation Promoting Factor (MPF) และ Mitogen-Activated Protein Kinase (MAPK) เพิ่มขึ้นจนถึงระดับที่จะทำให้เกิดการสลายของเยื่อหุ้มนิวเคลียสช้าลง และระดับ MAPK จะต่ำกว่ากลุ่มควบคุมตลอด 24 ชั่วโมงของการศึกษา จากการศึกษาโครงสร้างภายในโอโอไซต์ด้วยกรรมวิธีทางจุลทรรศน์อิเล็คตรอนแบบส่องผ่านพบว่า NO มีผลยับยั้งการเจริญของไมโตคอนเดรียและคอร์ติคอล แกรนูล

สาขาวิชา สรีรวิทยา (สหสาขาวิชา) ลายมือชื่อนิสิต.....
ปีการศึกษา 2547 ลายมือชื่ออาจารย์ที่ปรึกษา.....
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SIRIMA THONGRUAY: THE ROLE OF NITRIC OXIDE IN SWAMP BUFFALO OOCYTE MATURATION. THESIS ADVISOR: ASSOC. PROF. PRAKONG TANGPRAPRUTGUL, Ph.D., THESIS CO-ADVISOR: PROF. MONGKOL TECHAKUMPHU, Ph.D. AND SUNPETCH SOPHON, Ph.D., 129 pp. ISBN 974-53-1674-1.

The purposes of this study were to investigate the role of nitric oxide (NO) and its signaling pathway in nuclear and cytoplasmic maturation of swamp buffalo oocytes cultured *in vitro*. Cumulus-oocyte complexes (COCs) and denuded oocytes (DOs) were cultured in the basal medium M199 supplemented with S-nitroso-L-acetyl penicillamine (SNAP, a NO donor) at 10^{-7} M, 10^{-6} M, 10^{-5} M, and 10^{-4} M. SNAP inhibited the spontaneous nuclear maturation, significantly delayed germinal vesicle breakdown (GVBD) during the first 8 h and further delayed metaphase II (M II) stage at the 24 h of culture in a dose-dependent manner. Cumulus-oocyte complexes and DOs were cultured in the basal medium alone or supplemented with 10^{-4} M SNAP or 10 μM 1H-[1,2,4] oxadiazolo-[4,3,-α] quinoxalin-1-one (ODQ), a soluble guanylyl cyclase (sGC) inhibitor, or in combination with SNAP and ODQ. The results showed that SNAP significantly decreased the percentages of germinal vesicle breakdown (GVBD) in both COCs and DOs whereas ODQ could reverse SNAP-inhibited spontaneous oocyte maturation in only COCs. The treatment with SNAP determined an increase intraoocyte cyclic guanosine monophosphate (cGMP) concentration, which was attenuated by the combined treatment with ODQ only in COCs. Intraoocyte cyclic adenosine monophosphate (cAMP) concentrations were not correlated with cGMP concentrations, cAMP declined to basal levels after 2 h of culture in both SNAP and control groups. These results suggested that NO had an inhibitory effect on nuclear maturation and NO exerted its effect partly by cGMP which was not mediated by cAMP in COCs. In the contrary, NO might inhibit nuclear maturation of DOs through another signaling pathway. The effect of NO on cytoplasmic maturation was also characterized by the activities of maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK). MPF and MAPK were activated around the time of GVBD. Nitric oxide decreased MPF activity only at the time of GVBD, while MAPK activity was suppressed throughout the period of culture. Transmission electron microscopic study showed that immature forms of mitochondria and cortical granules were more abundant in NO treated group which implied the delay of cytoplasmic maturation. It is therefore suggested that NO plays a role in delaying both nuclear and in swamp buffalo oocytes. The inhibitory effect of NO may act through NO/cGMP dependent and independent signaling pathways but is not mediated by cAMP.

Field of study: Physiology (Inter-Department) Student's signature…….…….

Academic year: 2004 Advisor's signature…….

Co-advisor's signature……

Co-advisor's signature……

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LIST OF ABBREVIATIONS

AGT	=	aminoglutethimide
ANA I	=	anaphase I
cAMP	=	cyclic adenosine monophosphate
Cdk	=	cyclin dependent kinase
CG	=	cortical granule
cGMP	=	cyclic guanosine monophosphate
CL	=	corpus luteum
COC	=	cumulus-oocyte complex
Cx	=	connexin
dbcAMP	=	dibutyril cyclic AMP
DMSO	=	dimethyl sulfoxide
DO	=	denuded oocyte
DW	=	distilled water
eCG	=	equine chorionic gonadotrophin
EDTA	=	ethylenediaminetetraacetic acid
eNOS	=	endothelial nitric oxide synthase
FF-MAS	=	follicular fluid-meiosis activating sterol
FSH	=	follicle stimulating hormone
GMP	=	guanosine monophosphate
GnRH	=	gonadotrophin releasing hormone
GH	=	growth hormone
GV	=	germinal vesicle
GVBD	=	germinal vesicle Breakdown
h	=	hour(s)
hCG	=	human chorionic gonadotrophin
HMG-CoA	=	hydroxy-methyl-glutaryl-coenzyme A
IBMX	=	3-isobutyl-1-methylxanthine
IGF	=	insulin-like growth factor
iNOS	=	inducible nitric oxide synthase
IVM/ IVF/IVC	=	in vitro maturation/in vitro fertilization / in vitro culture
LH	=	luteinizing hormone

L-NAME	=	N-omega-nitro-L-arginine methyl ester
M I, M II	=	metaphase I, metaphase II
MAPK	=	mitogen-activated protein kinase
MAS	=	meiosis activating sterols
min	=	minute(s)
ml, µl, l	=	milliliter, microliter, liter
µM, M	=	micromolar, Molar
mg, µg, ng, g	=	milligram, microgram, nanogram, gram
µm	=	micrometer
MPF	=	maturation promoting factor
mRNA	=	messenger ribonucleic acid
MV	=	microvilli
NaF	=	sodium fluoride
NO	=	nitric oxide
nNOS	=	neuronal nitric oxide synthase
ODQ	=	1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one
$p34^{cdc2}$	=	catalytic subunit of MPF of 34 kDa
PBS	=	phosphate buffered saline
PDE	=	phosphodiesterase
PDE3A	=	type 3A phosphodiesterase /
PG/PGE2/PGF2α	=	prostaglandin /prostaglandin E2/ prostaglandin F2α
pH	=	hydrogen potential
PKA/PKG	=	protein kinase A/ protein kinase G
PMSG	=	pregnant mare serum gonadotrophin
PR	=	progesterone receptor
PVS	=	perivitelline space
RNA	=	ribonucleic acid
rRNA	=	ribosomal RNA
SEM	=	standard error of mean
sec	=	second
SDS-PAGE	=	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SNAP	=	S-nitroso-L-acetyl penicillamine
SNP	=	sodium nitroprusside

TCM-199	=	tissue culture medium-199
TEL I	=	telophase I
TEM	=	transmission electron microscope
v:v	=	volume:volume
w/v	=	weight/volume
5' –AMP	=	5'–adenosine monophosphate
8-Br-cAMP	=	8-bromo-3',5'-cAMP
°/ °C	=	degree/ degree celsius
%	=	percentage

CHAPTER 1

INTRODUCTION

The buffalo has been identified as a domestic animal of great economic importance for Asia and Mediterranean. During the past 24 years, swamp buffalo population in Thailand has decreased by 70% as reported by OAE (2000), from 6 million heads in 1979 to 1.7 million heads in the year 2000. Current economic demand for buffalo milk and meat has dictated breeding strategies which necessitate the extension of modern technologies such as *in vitro* maturation (IVM), *in vitro* fertilization (IVF), embryo transfer (ET) and cloning to this species. Most embryos, produced *in vitro*, originated from oocytes collected from ovaries obtained from the slaughterhouse. These ovaries collected post-mortem provide an inexpensive source of a large number of immature oocytes that allow a large-scale production of embryos *in vitro*. However, limited success has been achieved with low developmental competence (ability of oocytes to develop to the blastocyst stage). A possible cause of developmental failure is the incompetence of oocytes from smaller follicles to complete maturation. If these oocytes are to be used in the technologies, then it becomes essential to learn how to control their maturation. A better understanding of the meiotic control mechanism will be required to improve the *in vitro* developmental competence in buffalo immature oocytes.

When a cumulus oocyte complex (COC) containing a meiotic competent oocyte is released from its follicular environment, the oocyte spontaneously resumes meiosis up to the M II stage. This process is defined as *in vitro* oocyte maturation. Spontaneous maturation fundamentally involves removal of the inhibitory influence imposed by the follicular environment.

A properly matured oocyte is essential for a successful fertilization and subsequent embryo development. The majority of knowledge on oocyte maturation of mammalian species is based on *in vitro* maturation of immature oocytes from small and medium sized follicles. Those oocytes are collected for the *in vitro* production of embryos of domestic species in particular in the bovine, sheep, goat and pig. In order to improve the efficiency of the *in vitro* embryo production, in the past decades many

studies are conducted on the effect of particular hormones and growth factors on the *in vitro* maturation of oocytes and the subsequent *in vitro* embryo development following fertilization. There are various factors involved in oocyte maturation such as gonadotrophins, steroids, meiosis activating sterols (MAS) and growth hormone (GH).

Developmental competence of the oocyte increases with follicular size (Blondin and Sirard, 1994). Several studies supported that oocyte might require time to acquire developmental competence during meiotic arrest. The oocytes collected from large follicles have been shown to posse better developmental competence than those of oocytes collected from small follicles (Pavlok et al.,1992; Lonergan et al., 1994; Ceozt et al., 1995). Oocytes cultured in intact follicles for 48 h appeared to acquire a greater developmental competence than those matured directly following aspiration (Fouladi et al., 1998). For this reason, the developmental competence of immature oocytes *in vitro* can be improved by arresting them at the germinal vesicle (GV) stage (meiotic arrest).

One of the important factors that maintain the oocytes in GV stage is cyclic adenosine 3',5'-monophosphate (cAMP). Studies have shown that follicular fluid (Leibfried-Rutledge and First, 1980; Sirard and First, 1988), granulosa or theca cells (Kotsuji et al., 1994; Richard and Sirard, 1996), and follicle wall (De Loose et al., 1994) maintained meiotic arrest in bovine oocytes.

Nitric oxide (NO) is produced from enzymes known as nitric oxide synthases (NOS). The isoforms of NOS are neuronal NOS (or nNOS), endothelial NOS (or eNOS) and inducible NOS (or iNOS). Van Voorhis et al. (1995) demonstrated that iNOS and eNOS but not nNOS mRNA are expressed in the theca and granulosa cells. Specifically, roles of NO in ovulation (Ellman et al., 1993; Shukovski and Tsafriri, 1994; Bonello et al., 1996; Zackrisson et al., 1996; Jablonka-Shariff and Olson 1998), estradiol synthesis (Van Voorhis et al., 1994; Olson et al., 1996), and follicular survival (Chun et al., 1995) have been clarified. However, the potential role of NO in the regulation of oocyte maturation has been investigated with controversial results. Several studies have demonstrated that NO stimulates meiotic maturation to metaphase II stages in mice (Jablonka-Shariff and Olson, 1998; Zhang et al., 2001)

rats (Jablonka-Shariff et al., 2002) and bovine (Matta et al., 2002). However, it has also been reported that NO has no effect on the oocyte maturation in rabbits (Yamauchi et al., 1997). On the other hand, NO has been reported to have an inhibitory effect on oocyte maturation in the preovulatory follicles and denuded rat oocytes (Nakamura et al., 2002). Therefore, the role of NO in oocyte nuclear maturation is still unclear. To know the role of NO, which is one of the important follicular factors in mediating ovarian function, will be beneficial to oocyte maturation in *in vitro* system.

It is well established that several physiological actions of NO are mediated by the activation of soluble guanylyl cyclase (sGC) and the consequent increase in the concentration of cyclic guanosine monophosphate (cGMP) in target cells (Murad, 1994). Nitric oxide increased cGMP concentration in preovulatory follicles, and maintained intrafollicular cGMP level to inhibit oocyte meiotic resumption (Nakamura et al., 2002). Early studies on resumption of meiosis have shown that cGMP or 8-Br-cGMP blocked oocyte maturation in starfish (Meijer et al., 1989), hamsters (Hubbard and Terranova, 1982) and rats (Tornell et al.,1990). An increase in cGMP could evoke a concomitant increase in cAMP by inhibiting its phosphodiesterase-3 (PDE3)-catalyzed hydrolysis to AMP in human platelets, vascular smooth muscle cells (Maurice and Haslam, 1990; Trovati et al., 1999) and sperms (Revelli et al., 2002) (Figure 1-1). In addition, the inhibitor of PDE3 can prevent the oocyte meiotic resumption of mice (Shitsukawa et al., 2001), rats (Richard et al., 2001), bovine (Mayes and Sirard, 2002; Thomas et al., 2002), and macaques (Jensen et al., 2002). Thus, it is postulated that NO may inhibit oocyte maturation via NO/cGMP signaling pathway.

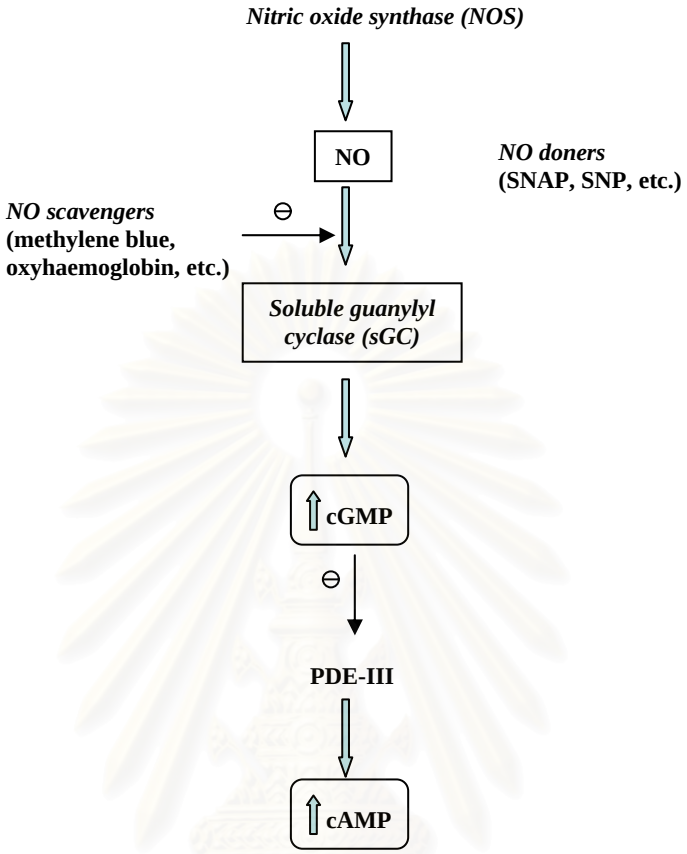


Figure 1-1 Schematic representation of the NO/cGMP pathway (Revelli et al., 2002)

Nitric oxide might be one of the follicular factors that maintained meiotic arrest or delayed oocyte nuclear maturation by increasing intracellular cAMP level. The oocytes maintained in meiotic arrest or delayed nuclear maturation had beneficial effect in promoting cytoplasmic maturation of the oocytes. Previous study showed that the intracellular cAMP concentration during the interval between oocyte isolation from the follicle and the beginning of *in vitro* maturation is critical for requiring optimal development competence (Guixue et al., 2001). The oocytes which were aspirated from follicles, their ability to synthesize protein were not affected, but they lost the capacity to make RNA in less than 2 h unless meiotic arrest was maintained artificially (Fouladi et al., 1998). The acquisition of development potential involved the accumulation of important factors in the form of protein or stable mRNA. Furthermore, the maintenance of meiotic arrest from superior breeder could be transported conveniently around the world. Good quality oocyte can be maintained in

the meiotic arrested medium during transportation instead of preservation in the cryoprotectants which are toxic to the oocytes.

However, there was still unclear experimental data about the role of NO in oocyte maturation and there were only a few reports in domestic species. Therefore, this study was performed to investigate the role of NO in oocyte maturation, and its signaling pathway in the swamp buffalo oocyte.

The aims of the present study were

1. To study the effect of nitric oxide on nuclear maturation rate in swamp buffalo oocyte .
2. To determine the signaling pathway of nitric oxide.
3. To evaluate the effect of nitric oxide on the kinetics of nuclear maturation and cytoplasmic maturation.

CHAPTER 2

LITERATURE REVIEW

The ovary

In mammal, the ovary is the female gonad responsible for differentiation and release of mature oocytes for fertilization and successful propagation of the species. The ovary is an endocrine organ that produces steroid to allow the development of female secondary sexual characteristics and support pregnancy.

The swamp buffalo ovary is elongated and considerably smaller than that of cattle. The average dimensions of the swamp buffalo left and right ovaries were 2.12 x 1.38 x 0.97 cm^3 and 2.19 x 1.43 x 1.10 cm^3; the average left and right ovaries weight were 2.24 and 2.28 g (Lohachit et al., 1981). The corpus luteum (CL) of the swamp buffalo showed predominantly brown colour. The colour changes from redbrown to yellowishgrey in different stages of estrous cycle (Lohachit, 1987). The dimension of CL in left and right ovaries at ovulation point were 1.05 x 1.36 x 0.72 cm^3 and 0.91 x 1.14 x 0.34 cm^3; the average weight of CL in left and right ovaries were 1.1 and 0.84 g (Lohachit et al., 1981).

The follicle

The ovarian follicle is comprised of several layers of somatic cells surrounding an antrum in which the oocyte surrounded by follicular cells is bathed. The fluid in the antrum is known as follicular fluid. The oocyte and the follicular unit maintain close association throughout development from primordial to preovulatory stages. The walls of mature preovulatory follicles consist of granulosa, theca interna and theca externa.

Granulosa cells are cells of epithelial origin consisting of the corona radiata, cumulus cells, mural and antral granulosa cells. The cumulus cells are the subpopulation of ovarian granulose cells that surrounds the oocyte. The cumulus cells in close contact with the oocyte are known as corona radiata. They are in close contact with the oocyte through cytoplasmic extension across the zona pellucida. The corona radiata communicates the oocyte with other cumulus cells through gap junctions. The granulosa

cells closet to the basement membrane are known as mural granulosa cells, while those closed to the follicular antrum are known as antral granulosa cells. The subpopulation of granulosa cells differ in their distribution of receptors and steroidogenesis.

The basement membrane or lamina propia is an acellular layer present between theca and granulosa cells. The theca interna and theca externa are stroma or fibroblastic cells that constitute the outermost coat of the preovulatory follicle. The theca interna are the major source of androgens. Follicles can be classified as primordial, preantral (primary and secondary) antral and preovulatory follicles.

Follicular development

Danell (1987) reported that buffalo ovaries have a similar number of primordial follicles in left and right (49.3% and 50.7%). The population of primordial follicles in buffalo was estimated to be 12,636 primordial follicles in cyclic buffalo heifers, and more atresia in buffalo follicles (66.66%) than in bovine follicles (50%). In cattle, the estimated mean number of primordial follicles decreased from 133,000 at birth to less than 3000 at 20 years of age (Erickson, 1966). Although the number of primordial follicles appears to be considerably lower in buffaloes than in cattle. In addition, buffalo ovaries contain only about 20% of the number of antral follicles found in cattle ovaries (48 versus 233; Le Van Ty et al., 1989).

The average number of follicles visible on surface of each ovary was 5.2 ± 1.0 (Kumar et al., 1997). In Surti buffaloes, the average proportion of antral follicles that appeared to be atretic, histologically, was 71% (Danell, 1987), compared to 82% (71.4% during follicular phase and 94.4% during luteal phase) in swamp buffaloes (Ocampo et al., 1994).

Follicular development in cattle was shown as schematic drawing in Figure 2-1 and 2-2 (Hyttel et al., 1997).

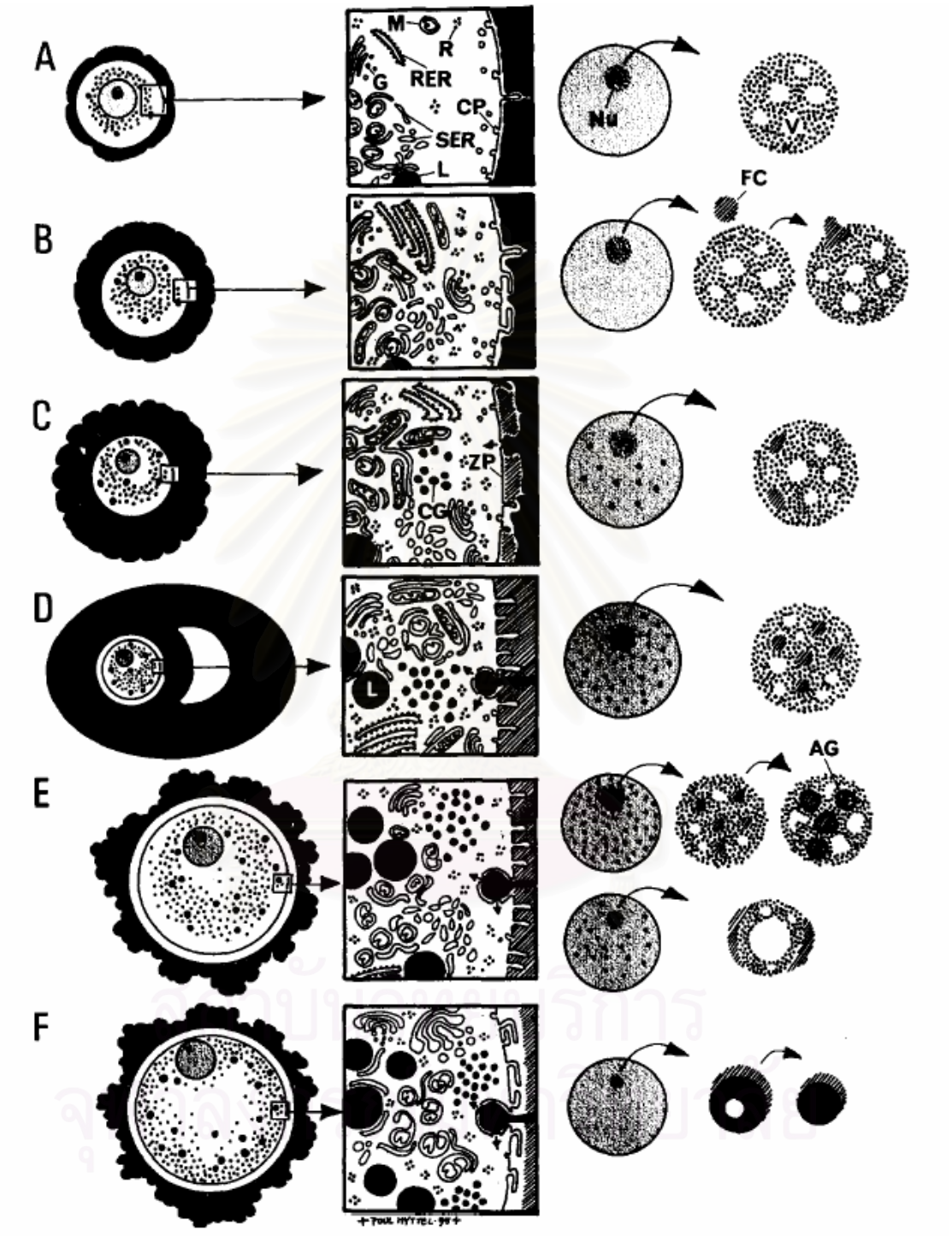


Figure 2-1 Schematic drawing of aspects of bovine oocyte growth with respect to general ultrastructure, autoradiographic labelling of the nucleus on semithin (2μm) sections following 30 min incubation with 3H-uridine, and nucleolar ultrastructure (Hyttel et al., 1997).

(A) Primordial follicle (34.6 ± 3.7 μm). The oocyte (27.9 ± 3.3 μm) is surrounded by a single layer of flattened granulosa cells. The central oocyte nucleus is surrounded by round mitochondria (M), smooth (SER) and rough (RER) endoplasmic reticulum and small Golgi complexes (G). The oocyte cortex presents numerous coated pits (CP) and vesicles. The oocyte nucleus lacks autoradiographic labelling and the nucleolus (Nu) only presents the granular component interspersed with vacuoles (V).

(B) Primary follicle (46.l ± 6.1 μm). The oocyte (31.6 ± 4.3 μm) is surrounded by a single layer of cuboidal granulosa cells, and it presents some microvilli and some elongated mitochondria. The eccentrical oocyte nucleus lacks autoradiographic labelling. Close to the granular oocyte nucleolus frbrillar centers (FC) appear and gradually start to invade the nucleolus.

(C) Secondary follicle (101.7 ± 41.8 μm). The oocyte (45.6 ± 14.0 μm) is surrounded by more than one layer of cuboidal granulosa cells. Small patches of zona pellucida (ZP) material have appeared, gap junctions (opposed arrows) have developed between the oocyte and the granulosa cells, and the oocyte microvilli have become more erect, The number of coated pits and vesicles at the oocyte cortex have decreased. In the oocyte, the first small clusters of cortical granules (CG) have developed and some membrane bounded vesicles have appeared (not shown). The oocyte nucleus including the nucleolus displays moderate autoradiographic labelling as indicated by the black dots. The fibrillar centres have become completely incorporated into the periphery of the nucleolus forming a tibrillo-granular nucleolus.

(D) Early tertiary follicle up to about l mm. The antrum folliculi has developed and the oocyte (up to about 80 urn) is located in the cumulus oophorus surrounded by cumulus cells and the innermost layers of corona radiata cells, which possess projections that penetrate the zona pellucida, invaginate the oolemma and make gap junctional (opposed arrows) contact to it. In the oocyte, the organelles have attained a more even distribution throughout the ooplasm, elongated mitochondria have become more numerous, lipid droplets have become common, and the number and size of the cortical granule clusters have increased as has the number of vesicles (not shown).

The erect microvilli have become embedded within the zona pellucida. The oocyte nucleus including the nucleolus displays distinct autoradiographic labelling. The nucleolus is fibrillo-granular and presents numerous evenly distributed fibrillar centers.

(E) Tertiary follicle up to about 3 mm as represented by oocytes at 80 to 110 μm in diameter. The number of lipid droplets in the oocyte has increased. In oocytes at less than 100 pm, the nucleus including the nucleolus displays distinct autoradiographic labelling and the fibrillo-granular nucleolus gradually develops aggregations of electron-dense granules (AG) around the fibrillar centers. In oocytes 100 to 110 μm, the nucleus displays moderate autoradiographic labelling excluding the nucleolus, and the fibrillo-granular nucleolus develops a large central vacuole and marginalized fibrillar centers.

(F) Late tertiary follicles as represented by oocytes at more than 110 μm in diameter. In the oocyte, the organelles have been dislocated to the periphery, the number of lipid droplets and vesicles have increased as have the size of the Golgi complexes. The microvilli have been released from the zona pellucida and pile up in stacks in the perivitelline space. The peripheral oocyte nucleus lacks autoradiographic labelling. The nucleolus consists of a sphere of densely packed fibrilles with a fibrillar center attached as a halo. In oocytes at 110 to 120 μm, the fibrillar sphere may still be vacuolated.

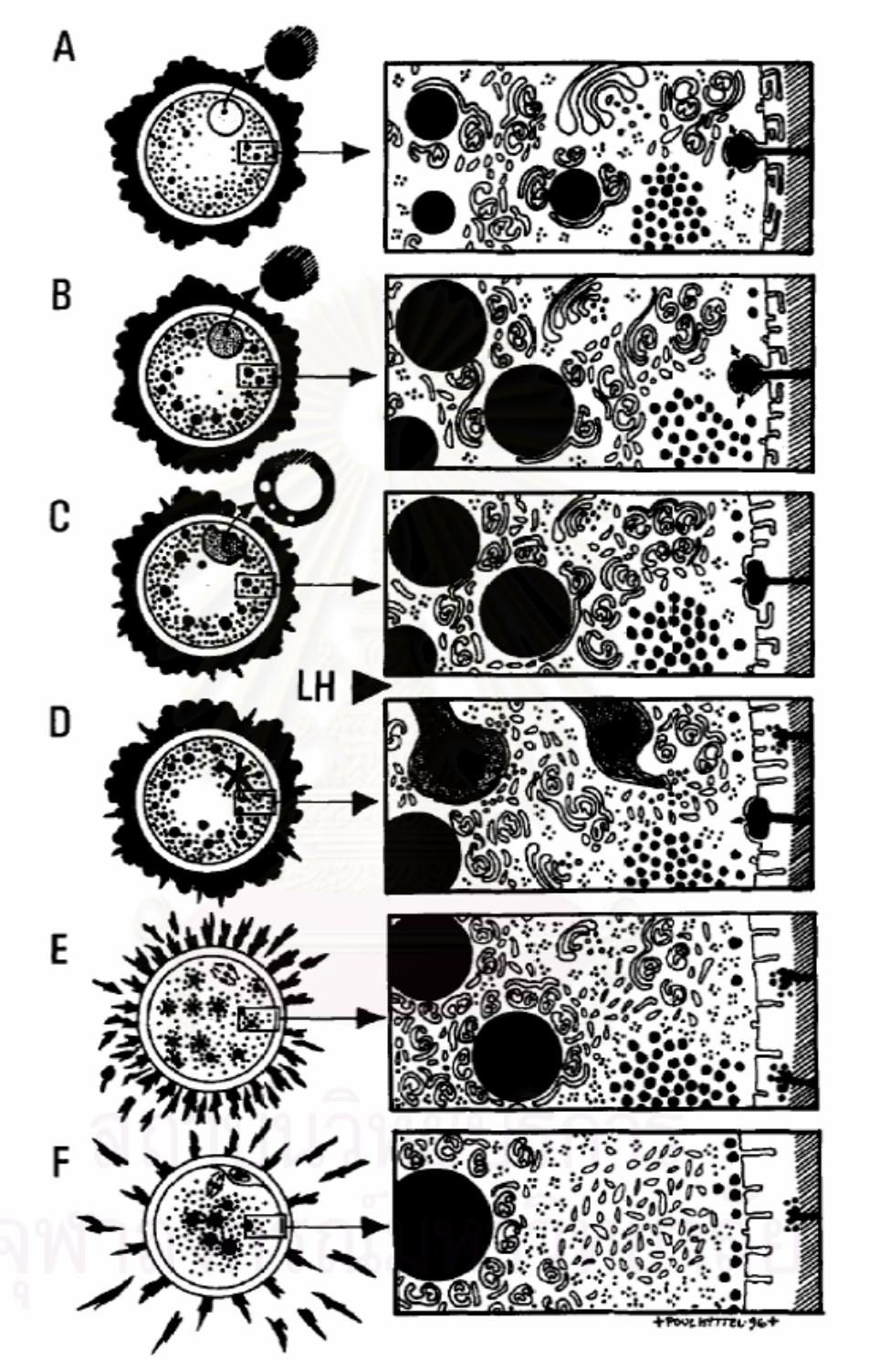


Figure 2-2 Schematic drawing of ultrastructural aspects of oocyte capacitation in the dominant follicle up to the LH peak and final oocyte maturation after the peak. See Figure 2-1 for legends (Hyttel et al., 1997).

(A) Oocyte from a dominant follicle on Day 3 post ovulation. The general ultrastructure is identical with that obtained at the end of oocyte growth.

(B) Oocyte from a dominant follicle on Day 7 post ovulation. The number of microvilli stacks have decreased as have the size of the Golgi complexes, the amount of lipid droplets has increased, and the cortical granule clusters have dislocated to a more superficial location.

(C) Oocyte from a dominant follicle on Day 9 post ovulation and prostaglandin treatment on Day 7. Some individual corona cells display elongation and the corona cell projections have been retracted to a more superficial location, the perivitelline space has enlarged, the microvilli have become more erect, and the size of the Golgi complexes has been further reduced. Moreover, the envelope of the oocyte nucleus has become undulating and the nucleolus has transformed into a ring-like structure including the fibrillar centre with a central and several secondary vacuoles.

(D) Oocyte at “oocyte nucleus breakdown” from an ovulatory follicle at 9-12 h after the LH peak. The perivitelline space develops further and in the oocyte the mitochondria tend to arrange around the lipid droplets and the nuclear envelope is dissolved into tubules of smooth endoplasmic reticulum and microtubules appear adjacent to the condensing chromosomes.

(E) Oocyte at M I from an ovulatory follicle at about 15 h after the LH peak. The number and size of the lipid doplets has increased and mitochondria have assembled around the droplets and these conglomerates have attained a more even distribution throughout the ooplasm. Numerous ribosomes have appeared especially around the chromosomes and the size of the Golgi complexes has decreased further.

(F) Oocyte at M II from an ovulatory follicle at about 24 h after the LH peak. The bulk of the cortical granules are distributed at solitary positions along the oolemma. The lipid droplets and mitochondria have attained a more central location in the ooplasm leaving a rather organelle free peripheral zone in which the most prominent

features are large clusters of smooth endoplasmic reticulum. Golgi complexes are practically absent.

It has been proposed that buffalo have the same pattern of follicular dynamics as was observed in cattle. Baruselli et al. (1997) found that ovarian follicles in buffalo develop in waves similar to those observed in cattle. The most frequently observed cycle in buffalo was the 2-wave cycle (63.3%).

The first wave starts on the 3rd day of the estrous cycle and ends on the 13th day. The second wave begins on the 9th day and supplies the ovulatory follicle. Le Van Ty et al. (1989) recorded the number of antral follicles in swamp buffalo; they found only 20% of those observed in cattle under similar conditions (47.5 $\pm$ 23.8 vs 233.0 $\pm$ 95.8 ; $P < 0.002$). The number of nonatretic follicles (> 1.7 mm) was between 1 and 5 (average 2.9) for buffalo, and 17 and 32 (average 22.1) for cattle.

The maximum diameter of the first dominant follicle was significantly larger than that of the second dominant follicle (1.33 $\pm$ 0.18 vs 1.ll $\pm$ 0.21 cm); the second dominant follicle was found around the maximum progesterone levels. The influence of progesterone during the luteal phase result in decreasing the diameter of the second dominant follicle. Ovarian follicular dynamics in buffalo are similar to those in cattle.

Although the 2-wave cycle was the most common in buffalo, both 3-wave and l-wave cycles were also observed. The number of waves in a cycle is associated with the luteal phase and with estrous cycle length (Baruselli et al., 1997). Unlike that in cattle, there were no 4-wave cycles in buffalo (Savio et al., 1988; Sirois and Fortune, 1988).

For cattle, it is not yet clear if the 3-wave cycle (Savio et al., 1988; Sirois and Fortune, 1988) or the 2-wave cycle (Ginther et al., 1989; Knopf et al., 1989) occurs more frequently. However, the number of follicular waves per cycle is correlated with cycle length.

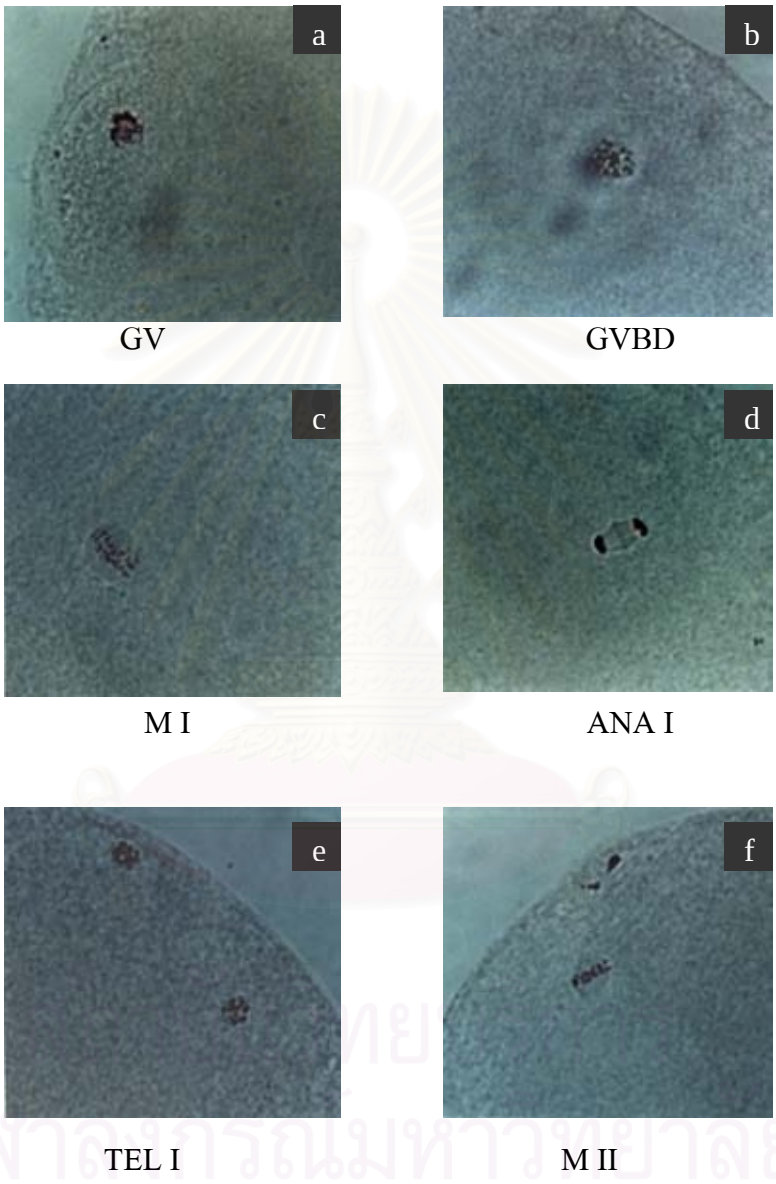
Oocyte maturation

Oocyte maturation is defined as the reinitiating and completion of the first meiotic division, subsequent progression to metaphase II, and the nuclear and cytoplasmic processes, which become essential for fertilization and early embryo development. Oocyte maturation is a complex phenomenon during which the oocyte progresses from the diplotene to the metaphase II stage (nuclear maturation). The oocyte resumes meiosis in response to the ovulatory LH surge (Channing et al., 1978; Callesen et al., 1986) or removal from the follicle (Pincus and Enzmann, 1935). The first morphological sign of meiotic resumption is the breakdown of the nuclear membrane, a process termed germinal vesicle breakdown (GVBD): the disappearance of nuclear envelope, the chromosome condense, meiosis progresses to the metaphase I (M I), Anaphase I (ANA I), Telophase I (TEL I), and the metaphase II (M II). The oocyte remains arrested at the M II stage until fertilization takes place and the oocyte completes meiosis and forms the pronucleus. Oocyte maturation also involves transformations at the cytoplasmic level, preparing the cell to support fertilization and early embryonic development (cytoplasmic maturation). The completion of nuclear maturation alone does not guarantee subsequent embryo development (Yang et al., 1998; Sirard, 2001).

Nuclear maturation

Nuclear maturation refers to the progression of the oocyte nucleus from the GV to the M II stage. Nuclear maturation involves GVBD, condensation of chromosomes, metaphase I spindle formation, separation of the homologous chromosomes with extrusion of the first polar body and arrest at M II. In buffalo, the oocytes had undergone GVBD at 6 h (Raminder and Majumdar, 1992) and M II at 24-28 h of culture (Chuangsoongneon and Kamonpatana, 1991). It appears that nuclear maturation follows the same pattern *in vivo* and *in vitro*. The ability of the oocyte to complete meiosis is known as meiotic competence. Meiotic competence is acquired gradually during follicular growth. Oocytes first acquire the capacity to undergo GVBD and chromosome condensation, then further follicular development is required to acquire the ability to progress to the M I (Tsafriri and Channing, 1975) and finally they acquire the ability to reach M II. Meiotic competence is closely correlated with oocyte size, which in turn is correlated with follicle size (Hendriksen

et al., 2000; Marchal et al., 2002). The size of the antral follicle at which the oocyte acquires meiotic competence is species specific. Buffalo oocytes acquire the ability to culture by the time at antral follicle reaches 3 mm in diameter (Naik et al., 2002).



GV =Germinal Vesicle ; GVBD = Germinal Vesicle Breakdown; M I= Metaphase I ; ANA I = Anaphase I ; TEL I = Telophase I ; M II =Metaphase II

Figure 2-3 The stage of oocyte nuclear maturation (Sophon, 1996)

Structural changes during oocyte maturation

Germinal vesicle breakdown

The nucleus of an oocyte is the germinal vesicle (GV) (Figure 2-3a). The most striking event of the reinitiation of meiosis is the disappearance or breakdown of the GV (GVBD). The acquisition of competence to undergo GVBD is a multistep or atretic degeneration of follicles, but when they are removed from their antral follicles or after removal of the entire oocyte-cumulus cell complex, spontaneous gonadotropin-independent maturation may occur in culture media as well. Within a few hours of culture *in vitro* fully-grown oocytes undergo complete GVBD (Figure 2-3b). GVBD begins with undulations of the nuclear envelope. These undulations may be correlated to the onset of chromosome condensation.

Meiotic maturation and chromosome formation

Chromosome condensation and spindle formation are the following steps in the scheduled program of the maturing oocyte to complete meiosis. Directly subsequent to GVBD - when the nuclear envelope and its inner lining, the fibrillar network of laminae, start to dissolve - chromosomes have moved from the center of the nucleus towards the undulating membranes, where condensation takes place. Chiasmata move to the ends of the chromosomes and chromatin becomes heterochromatic. After completion of condensation the chromosomal bivalents appear V-shaped and telocentric. They are often attached to fragments of the nuclear envelope. Being highly condensed, chromosomes become arranged in the center of the oocyte, waiting to line up on the metaphase spindle. During GVBD and chromosome condensation, kinetochores and the microtubule system appear to organize the spindle formation. The spindle does not display centrioles as is typical for mitotic cells; rather, it derives from so-called pericentriolar material which forms the spindle poles during prometaphase. The spindle apparatus increases in size and moves to the periphery of the oocyte. The barrel-shaped spindle is surrounded by mitochondria, vacuoles, and granules. Metaphase I (Figure 2-3c) lasts for a few hours and leads to ANA I (Figure 2-3d), when chromosomal bivalents move towards the opposite ends of the spindle and the whole spindle rotates 90^{o}. During TEL I (Figure 2-3e) the extrusion of the first polar body is prepared. Homologous chromosomes become separated, and one half is extruded with cytoplasmic material

such as mitochondria, ribosomes, and cortical granules into the perivitelline space. This takes place in late telophase. The oocyte has reached M II (Figure 2-3f). Progressive maturation beyond M II marks the beginning of fertilization or indicates parthenogenetic activation of the oocyte.

The timing of meiotic events in buffalo oocytes was investigated by Datta and Goswami (1999) to determine the chromosomal status from 0 to 24 h of culture. They demonstrated that GVBD, occurred between 6 and 10 h of culture. After 12 h culture the M I stage was dominant, whereas at 16 h M I, ANA I and TEL I were equally prevalent. The M II stage was first observed at 8 h but was only observed in a large number of oocytes after 16 h of culture and reached a peak value after 24 h.

Cytoplasmic maturation

Cytoplasmic maturation describes both the acquisition of developmental competence of the oocyte and the ultrastructural changes that take place in the oocyte from the GV to the M II stage (Hyttel et al., 1986; Ducibella et al., 1990).

Cytoplasmic maturation may be regulated by kinases involved in the initiation and progression of meiosis. The activation of MPF and MAPK, which share numerous substrates including proteins involved in nuclear membrane formation, chromatin condensation, and spindle assembly, results in resumption, progression, and arrest of meiosis at M II. Lower levels or inappropriate activation of MPF and MAPK may negatively influence the developmental capacity of oocytes (Salomone et al., 2001)

In mammalian oocyte, migration of cortical granules (CG) has been used as an important criterion to evaluate cytoplasmic maturation. CG are distributed throughout the cytoplasm of oocytes at the germinal vesicle stage (immature), as maturation proceeds, a progressive centripetal migration of CG to the oocyte cortex in mouse (Ducibella et al., 1990; Connors et al., 1998), and bovine (Wang et al., 1997).

Mitochondrial maturation and redistribution are crucial processes for activation, fertilization, and further successful development (Van Blerkom and Runner, 1984). Mitochondria translocate to the perinuclear region during formation of

the first metaphase spindle and subsequently disperse during abstriction of the first polar body. After maturation, mitochondria appeared as larger clumps that were not any more located only in the periphery of the bovine oocytes (Stojkovic et al., 2001).

Oocyte developmental competence

Developmental competence is usually expressed as the percentage of oocytes that can develop to the blastocyst stage. Multiple studies have been carried out to examine factors affecting the developmental competence of the oocyte. The size and the quality of the follicle of origin (Blondin and Sirard, 1994) influence the developmental capacity of bovine oocytes. The fate of individual oocyte according to the specific follicle has been corroborated that developmental competence of the oocyte increases with follicular size (Blondin and Sirard, 1995). It appears that the oocyte requires an additional "prematuration" to express this competence (Hendriksen et al., 2000; Sirard, 2001). *In vivo*, this prematuration occurs during preovulatory growth before the LH surge. The potential of the oocytes to become fertilized and successfully developed not only depends on meiotic/nuclear maturation event, but it is also critically influenced by the quality and maturity of the cytoplasm. Usually, the cytoplasm maturation prepares for fertilization and subsequent embryonic development in parallel with nuclear maturation.

The role of cyclic adenosine monophosphate (cAMP) in oocyte maturation

Cyclic AMP, which is present in the oocyte, appears to be a candidate involved in maintenance of meiotic arrest, since the mode of cAMP action is known. Cyclic AMP activates a cAMP-dependent protein kinase A (PKA). An inhibitory basal level of cAMP within the oocyte activates PKA. The active form of the heterotetramer PKA is the catalytic subunits after binding of cAMP to the inhibitory subunits of the PKA complex. PKA phosphorylates oocyte proteins which are necessary for GVBD. Continuous phosphorylation of proteins maintains the meiotic arrest. Resumption of meiosis, on the other hand, is triggered by a decrease in the inhibitory level of oocyte cAMP mediated through the action of cAMP phosphodiesterase (PDE). Cyclic AMP phosphodiesterase promotes the reassociation of the active catalytic subunits of PKA with its regulatory subunits, which prevents PKA from carrying on with phosphorylation. Oocyte proteins become dephosphorylated and

meiotic maturation will be initiated. Some experiments indicate the involvement of different substances in regulating the oocyte cAMP levels. Spontaneous maturation of mouse oocytes does not occur *in vitro* in the presence of cAMP analogues such as dbcAMP or phosphodiesterase inhibitors such as isobutyl methylxanthine (IBMX) and hypoxanthine. Forskolin maintains oocytes in meiotic arrest via direct stimulation of adenylate cyclase to increase the cAMP level. Phosphodiesterase activity which can increase or decrease cAMP levels in the oocyte is responsible for the onset or arrest of maturation. The origin of active cAMP is still unclear. Cumulus cell-free oocytes produce cAMP on stimulation by forskolin, but forskolin treatment produces only a delay of GVBD. Thus, higher amounts of cAMP are needed to maintain meiotic arrest. It is still unclear if sufficient cAMP is created by the oocyte itself or originates in granulosa cells. As mentioned above, oocytes are coupled to granulosa and cumulus cells by gap junctions. Gap junctions allow diffusion of small molecules from one cell to another, so cAMP may possibly diffuse from granulosa cells to the oocyte. Another theory is that stimulated granulosa cells promote the oocyte to produce cAMP itself.

Furthermore, it is suggested that other types of protein kinases (PK) may be involved in the regulation of oocyte maturation. Stimulation of the PKC system with phorbol esters and diacylglycerol also results in a transient inhibition of GVBD.

Although cAMP seems to play a major role in oocyte maturation and meiotic arrest, other substances present in the follicle are also likely to participate in meiotic arrest. Hypoxanthine and adenosine were detected in mouse oocytes and follicular fluid. Hypoxanthine inhibits GVBD when applied to denuded mouse oocytes. Moreover, the inhibitory effect of purines is increased in cumulus cell-enclosed oocytes. This observation suggests that the intercellular gap junction pathway to the oocyte provides regulation of uptake and metabolism of those putative granulosa cells generated substances. Adenosine displays a transiently inhibitory effect on GVBD similar to that of forskolin in culture, but when augmented by hypoxanthine the inhibitory effect persists. Direct injection of adenosine into the oocyte does not show any effect. The theory is that hypoxanthine inhibits cAMP phosphodiesterase and therefore prevents hydrolysis of oocyte cAMP. Adenosine promotes the cAMP

formation, acting at the oocyte surface by stimulating the adenylate cyclase. Purines may also be involved, to regulate the suppressive effects of guanyl compounds on oocyte maturation. Guanosine monophosphate (GMP), plays an essential role in the maintenance of meiotic arrest. GMP is itself converted to guanosine triphosphate (GTP), which interact subsequently with G-proteins present on the oolemma and membranes of the cumulus cells, GTP is able to suppress GVBD as well, as binding to G-proteins mediates an increase of cAMP levels within the oocyte.

The role of maturation-promoting factor (MPF) in oocyte maturation

MPF has been shown to be active during oocyte maturation of all studied species. Active MPF induces GVBD in mouse oocytes (Choi et al., 1991), porcine oocytes (Naito and Toyoda, 1991), and bovine oocytes (Tatemo and Terada, 1996). MPF is a protein kinase whose activity triggers a series of reactions ultimately leading to nuclear membrane breakdown, chromosome condensation and spindle formation and therefore entry into meiosis and second meiotic arrest. MPF is a protein complex composed of two subunits: cyclin B and $p34^{cdc2}$. Cyclin B is the regulatory component and $p34^{cdc2}$ is the catalytic component of MPF. In bovine oocytes, cyclin B is the limiting factor for meiotic resumption. The activation of MPF requires first the formation of the $p34^{cdc2}$-cyclin B complex and second, phosphorylation and dephosphorylation of $p34^{cdc2}$ (Figure 2-4). Cyclin B is synthesized and accumulated during interphase, while $p34^{cdc2}$ is always present in the cytoplasm (Solomon et al., 1990). The cyclic nature of MPF is explained by the phases of cyclin B synthesis and degradation. The degradation of cyclin B initiates the metaphase-anaphase transition and the decline in $p34^{cdc2}$ activity (Hashimoto and Kishimoto, 1988). Thus, the levels of MPF in the GV oocyte are low, but rise at GVBD and are at a maximum as M I is reached; there is then a rapid loss in activity at anaphase-telophase followed by a further rise as the oocyte enters M II (Fulka et al., 1992; Naito and Toyoda, 1991). This second level is maintained until completion of M II at fertilization (Fulka et al., 1992; Hashimoto and Kishimoto, 1988). The peak levels of MPF activity in bovine oocytes correspond with the two metaphases: 9-12 h and after 18 h of culture.

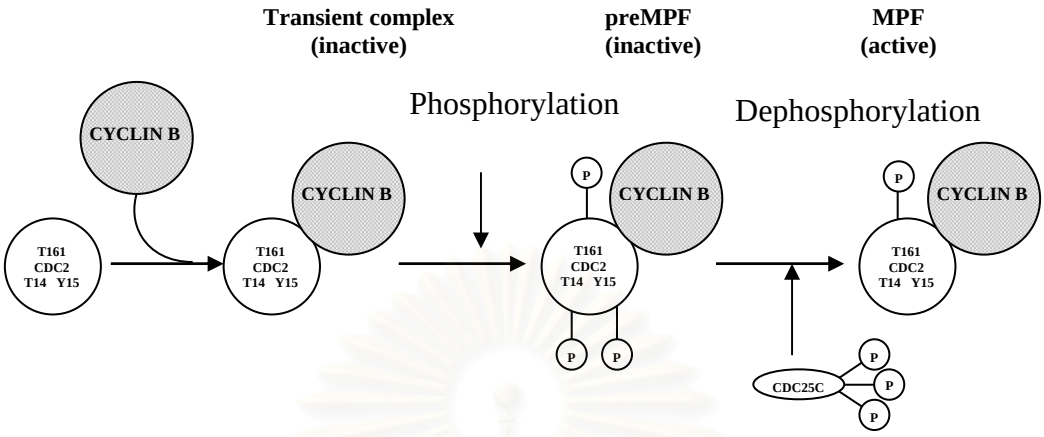


Figure 2-4 Regulation of MPF by phosphorylation and dephosphorylation (Basi and Draetta,1995)

The role of mitogen-activated protein kinase (MAPK) in oocyte maturation

MAPK is a serine-threonine kinase, which is phosphorylated and activated by MAPK kinase (MEK). MAPK was also found to be necessary for the onset of GVBD and metaphase progression during oocyte maturation (Gotoh et al., 1995; Choi et al., 1996a). MAPK is activated by a kinase cascade that includes several upstream activators; one of them is the product of the proto-oncogene mos. In bovine oocytes, injection of Mos RNA elicited a rapid maximal activation of MAPK that resulted in accelerated resumption of meiosis and GVBD. MAPK- activating cascade that can be initiated by the Mos protein (Fissore et al., 1996). In bovine oocytes, MPF and MAPK were activated at approximately the same time of GVBD.

The mechanism of meiotic resumption

In vivo, LH is essential for oocyte meiotic maturation. During the normal ovulatory cycle, the physiological stimulus for meiotic resumption is the LH surge. Pharmacological blockade of the LH surge with Nembutal, GnRH antagonists, or hypophysectomy prevents oocyte maturation. Similarly, inactivation of the LH receptor is associated with a failure of the oocyte to undergo meiotic resumption and ovulation. Because gonadotropin receptors are not detectable in the oocyte, it has been concluded that LH stimulates granulosa cells and that this stimulation removes

the inhibitory constraint on meiotic resumption, perhaps by blocking the production of an inhibitory substance (Figure 2-5).

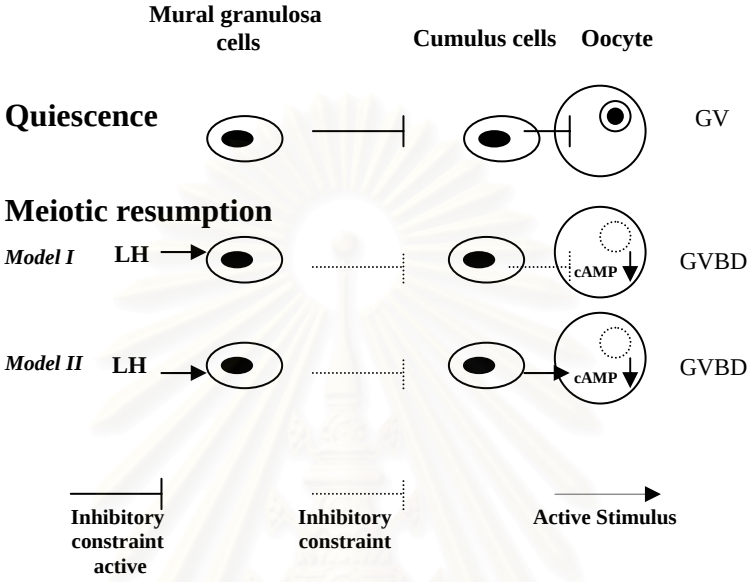


Figure 2-5 Possible mechanisms involve in resumption of meiosis in mammals (Conti et al., 2002)

Some of the data thus far published are also compatible with a more complex model II whereby cumulus cells send a positive signal that activates the oocyte, overcoming the inhibitory cAMP levels. This scenario is suggested by findings with an *in vitro* mouse model that is often used to study oocyte maturation. In this experimental paradigm, cumulus oocyte complexes (COC) are maintained in culture in the presence of hypoxanthine or dbcAMP, and meiotic resumption is induced by FSH, thus demonstrating that cumulus cells can send a positive stimulus to the oocyte that overrides the cAMP inhibitory effects. However, it is not clear whether this positive stimulus is effective in the presence of high cAMP levels or whether a decrease in cAMP is obligatory for the resumption of meiosis. Measurement of PDE present in the oocyte at different times during spontaneous maturation showed a transient increase in activity prior to GVBD (Richard et al., 2001). This increase was blocked by PDE3 inhibitors, but not PDE4, indicating that the oocyte PDE3 is involved in this regulation. Measurement of the PDE activity in oocyte derived from

intact follicles incubated *in vitro* for 2 h with hCG also showed an increase in PDE3 activity. Thus, an increase in oocyte PDE activity precedes both spontaneous and hCG-induced oocyte maturation. These findings support the concept that LH send an active signal is responsible for oocyte maturation and that an increase in PDE activity may be a component of the signaling cascade involved in oocyte maturation.

Hormonal regulation of oocyte maturation

A properly matured oocyte is essential for a successful fertilisation and subsequent embryo development. The majority of knowledge on oocyte maturation of mammalian species is based on *in vitro* maturation of immature oocytes from small and medium sized follicles. Those oocytes are collected for the *in vitro* production of embryos of domestic species in particular in the bovine, sheep, goat and pig. In order to improve the efficiency of the *in vitro* embryo production, in the past decades many studies are conducted on the effect of particular hormones and growth factors on the *in vitro* maturation of oocytes and the subsequent *in vitro* embryo development following fertilization.

Gonadotrophins

Currently most *in vitro* maturation (IVM) protocols use LH or FSH or a combination of them. However, the effect of gonadotrophins and their relative importance on maturation and subsequent fertilizing and early development is still controversial. Supplementation of maturation media with LH and/or FSH is based on the presumptive involvement of these hormones in the *in vivo* maturation process.

FSH and LH use the cAMP system as the intracellular second messenger, one can pose the question whether the regulating action of FSH on IVM of oocytes from small and medium size follicles is essentially different from the action of LH on oocytes from preovulatory follicle. The action of LH on its target cells is mediated through binding to specific receptors on the cell membrane. Because there are no LHRs on the surfaces of oocytes, the involvement of cumulus cells may explain this effect of LH.

In vivo LH surge stimulate preovulatory follicles in which LHRs had been expressed in cumulus cells, leading to meiotic resumption of mammalian oocytes. On

the other hand, oocytes for IVM are usually collected from early antral follicles. When COCs from the antral follicles were cultured with LH, the level of cAMP in the cumulus cells surrounding oocytes was not increased, whereas FSH raised cAMP level in cumulus cells of mouse COCs (Andersen et al., 2001). Thus, LH may not involve in the process of *in vitro* oocyte maturation. However, Shimada et al. (2003) examined whether LHRs were expressed in cumulus cells during *in vitro* maturation. The addition of FSH to the maturation medium induced a significant increase in the binding level of hCG to COCs. The increase of hCG binding level in COCs was not observed when COCs were cultured without FSH. These reports indicated that the addition of FSH to the medium produced LH receptors mRNA expression, which induced an increase in the level of LHRs on cumulus cells surrounding oocytes.

Meiosis activating sterol (MAS)

The molecular mechanism down-stream to receptor activation by which the gonadotrophins induce oocytes to resume meiosis is, however, partly understood. It has been shown that FSH induced a synthesis of a signal in the cumulus cells, meiosis activating sterol (MAS) (Figure 2-6), which induced oocyte maturation *in vitro* even in oocytes depleted of cumulus cells (Byskov et al., 1997). Gonadotrophins dependent activity of the rate-limiting enzyme P450-Demethylase (P450-DM, the enzyme which converts lanosterol to MAS) in COCs provided an indications for a possible role of MAS in oocyte maturation. In immature and female rats, PMSG induced a 2-fold increase of P450-DM in the ovary (Yoshida et al., 1996). Whereas the expression of P450-DM was increased 2.5 times after hCG stimulation (Vaknin et al., 2001).

Since MAS may represent the physiological signal that originates in the somatic compartment of the follicle and instructs the oocyte to reinitiate meiosis. Many studies, using follicle-derived or synthetic follicular fluid meiosis activating sterol: FF-MAS, have been carried out to observe the efficacy of FF-MAS in inducing maturation. FF-MAS have been shown to stimulate germinal vesicle breakdown (GVBD) in meiotically arrested rodent oocytes (Hegele-Hartung et al., 1999; 2001; Downs et al., 2001). These studies have shown that FF-MAS, in a dose-dependent manner, induced resumption of meiosis in culture in the presence of different meiosis-inhibiting factors. Moreover, Ketoconazole (an inhibitor of MAS

production) 10^{-7}-10^{-3} M inhibited the effect of FSH on resumption of meiosis (Lu et al., 2000), and this is consistent with the physiological role of FF-MAS in oocyte maturation.

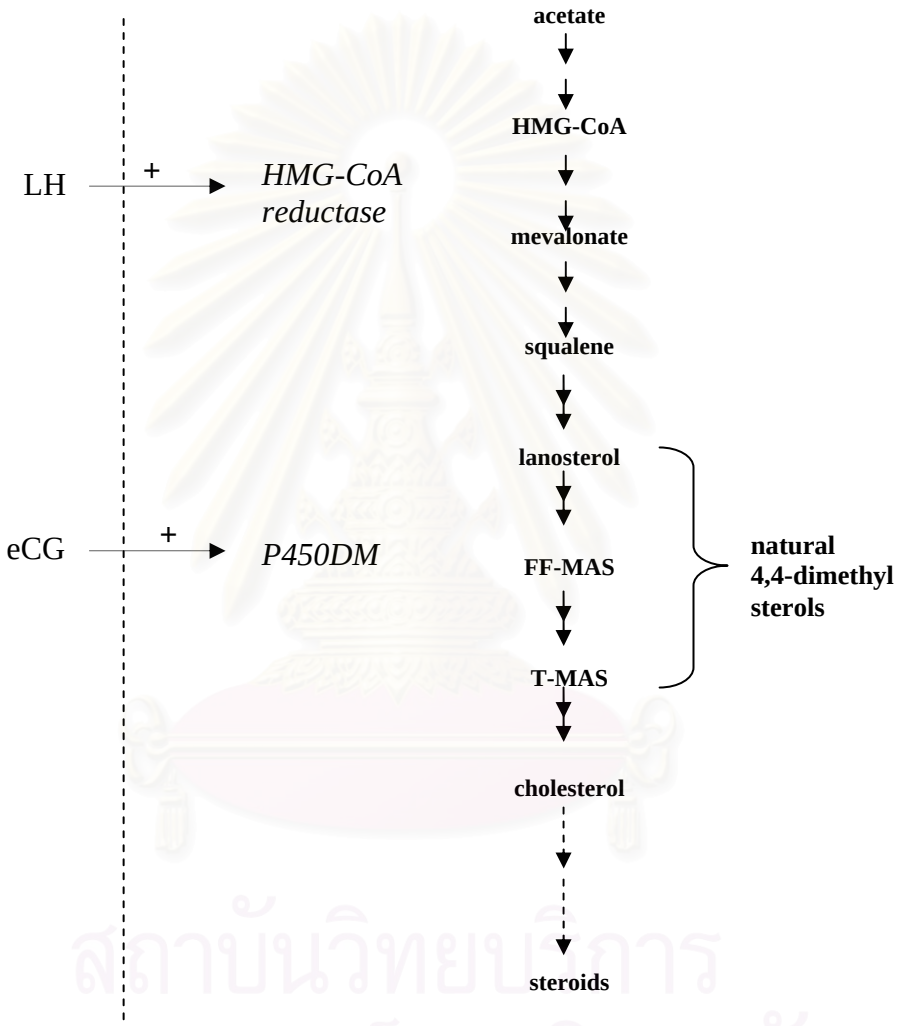


Figure 2-6 Simplified outline of the postsqualene sterol biosynthesis pathway in the endoplasmic reticulum and its regulation by gonadotrophins. Sterols with reported meiotic activity are in bold. Names of enzymes are in italics. Single arrows denote a single enzymatic conversion, whereas double arrows denote a multienzymatic step. Broken lines between cholesterol and the steroids indicate that cholesterol is transported to the mitochondria, where steroidogenesis takes place (Byskov et al., 1997).

The fact that MAS can induce maturation of cumulus-cell deprived oocytes indicated that receptors for MAS may exist in the oocyte. Using tritiated FF-MAS and high-resolution autoradiography, it was recently demonstrated that a specific binding of FF-MAS preferentially took place to the oolemma of oocytes from marmoset, cow and mouse (Faerge et al., 2001). In denuded oocytes the entire oolemma was labeled whereas in cumulus oocyte complexes (COC), FF-MAS only bound to areas which were not covered by cumulus cells. Labeling of COCs with thick cumulus investment showed a gradual decreased from its periphery towards to oocyte. It was suggested that the cumulus cells formed a barrier to exogenous MAS, which partly explained why COC appeared to be less responsive (Grøndahl et al., 1998) or unresponsive (Downs et al., 2001) to *in vitro* maturation with MAS. This suggestion was experimentally supported by Downs et al. (2001) who found that only very little radioactive lanosterol was taken up by cultured oocytes enclosed in cumulus cells during a 6 h culture period, whereas the cumulus cells as well as denuded oocytes contained somewhat more radioactivity.

It is, however, possible that MAS-mediated oocyte maturation was dependent on a G-protein coupled receptor mechanism. Addition of cholera toxin, which resulted in activation of adenylyl cyclase through G-proteins and increases cAMP, significantly inhibited MAS-mediated mouse oocyte maturation, but not spontaneous oocyte maturation (Grøndahl et al., 2000). These results also supported the notion that the mechanisms involved in induced and spontaneous meiosis are different.

Recently, the effect of FF-MAS on chromosome segregation was studied in oocytes matured in FF-MAS, it was noted that FF-MAS induced full nuclear maturation to M II, and chromosomes segregate with high fidelity. FF-MAS appeared to protect mammalian oocytes from precocious chromatid segregation (Cukurcam et al., 2003).

Steroids

In nonmammalian species resumption of meiosis is triggered by progesterone or a progesterone derivative. In mammals the role of steroids in oocyte maturation is still controversial. It has been reported that cumulus cells of bovine COCs matured *in vitro* are able to secrete estradiol and progesterone when matures in medium supplemented with gonadotrophins and BSA, and that this steroidogenesis can be modulated by steroids. This study reinforced the suggestion that it may not be necessary to add estradiol to the maturation medium (Mingoti et al., 2002).

The role of estradiol in oocyte maturation

Estradiol has been traditionally added to the medium for *in vitro* maturation (IVM) of mammalian oocytes. The specific role of estrogens in follicular and oocyte maturation, ovulation and embryo development seems to be species dependent and is currently unknown in various species. In cattle, Beker et al. (2002) reported that estradiol negatively affected bovine oocyte nuclear maturation as reflected by the decrease of proportion of M II oocytes. FSH appeared to suppress the negative effect of estradiol on nuclear maturation, when oocytes were cultured in the presence of estradiol and FSH, resulting in the same percentage of M II oocytes at the end of culture, a similar proportion of blastocysts formed was observed, as compared to oocytes cultured only in the presence of FSH. These findings indicated that estradiol did not affect cytoplasmic maturation in terms of blastocyst formation and they strongly suggested omission of estradiol in routine maturation protocols of bovine oocytes. Furthermore, the treatment of denuded oocyte with estradiol resulted in the strongest effect in terms of nuclear aberrations, indicated that estradiol exerted its direct effect on the oocyte, and not via the cumulus cells. Since the effect was less in COCs and even weaker in COCs cultured in the presence of FSH, it can be concluded that the cumulus cells counteracted to reduce the action of estradiol on the oocyte. This may be due to uptake of estradiol by the cumulus which was enhanced in the presence of FSH, resulting in a lower concentration of estradiol, or a signal generated by cumulus cells which was amplified in the presence of FSH, that counteracted the effect of estradiol.

In summary, estradiol added to a serum free maturation medium negatively affects bovine oocyte nuclear maturation and subsequent embryo development. This is reflected by a lower percentage of oocytes in M II stage, a higher percentage of nuclear aberrations, and a lower percentage of blastocysts. Although in the presence of FSH the effect is attenuated.

Dode and Graves (2002) reported that addition various concentrations (0–3000 ng/ml) of estradiol to IVM medium had no significant variation in the proportion of oocytes completing nuclear or cytoplasmic maturation.

In 2003, Dode and Graves demonstrated that estradiol was not involved in maturation of pig oocytes by blocking estradiol action with estadiol antagonist (1-p-dimethylaminoethoxyphenyl-1,2-diphenyl-1-butene: tamoxifen) or aromatase inhibitor (4-hydroxy-4-androstene-3-17-dione) in culture TCM199 supplemented with LH and FSH. The results showed that percentage of tamoxifen-treated oocytes that underwent nuclear maturation was similar to the control group. These results indicated that no steroids needed to be added to the medium during maturation of pig oocytes. Yet these results did not provide evidence for a specific role for steroids during maturation. If there is some beneficial effect of these hormones, it is possible that the amount of steroids produced by the COCs is sufficient to cause the biological response.

The role of progesterone in oocyte maturation

Evidence for examing the role of progesterone in meiotic resumption was studied. The addition of LH and FSH induced progesterone receptors (PRs) expression in cumulus cells, concomitant with increased progesterone production. When, an inhibitor of progesterone production, aminoglutethimide (AGT), was administered to the medium with LH and FSH, progesterone production was significantly suppressed in a dose-dependent fashion. When COC were cultured with LH, FSH and 0.5×10^{-3} mol/l AGT, almost complete inhibition of progesterone production and of GVBD was seen. However, this inhibitory effect on GVBD was overcome by additional progesterone. Moreover, 0.5×10^{-3} mol/l AGT also suppressed the reduction in connexin43, a gap junctional protein, in cumulus cells after 28 h cultivation (Shimada and Terada, 2002).

Previous studies in pigs demonstared that a significantly positive correlation between the proportion of oocytes undergoing GVBD and that of COC exhibiting a loss of the gap junctional communication between the cumulus cells of outer layers (Isobe et al., 1998; Isobe and Terada, 2001). In addition, a disruption of gap junctional communication in the outer layers of cumulus cells resulted in the depletion of connexin43 expression in those layers (Shimada et al., 2001). Furthermore, connexin43 expression in the rat endometrium is regulated by progesterone during early pregnancy (Grummer et al., 1994). It has also been shown that progesterone down-regulated the expression of the connexin43 gene in human myometrial cells (Zhao et al., 1996). These studies supported that the binding of progesterone, which was secreted by LH- and FSH-stimulated cumulus cells, to its newly synthesized receptors induced GVBD in porcine oocytes, possibly through a reduction of connexin43 in cumulus cells.

Yamashita et al. (2003) demonstrated that a high level of progesterone produced by cumulus cells was responsible for an acceleration of GVBD in porcine oocytes. Progesterone secreted by COCs played a positive role in GVBD induction in porcine oocytes. The addition of ketoconazole, which suppressed the sterol biosynthetic pathway, decreased the rate of GVBD, as well as progesterone production in COCs cultured. However, the suppression of GVBD by ketoconazole was overtaken by the addition of progesterone.

Taken together, it seems that different mechanisms of progesterone-induced meiotic resumption exist between amphibian and mammalian oocytes; in amphibian oocytes progesterone acts at the oocyte surface, and in mammalian oocytes (or at least in porcine oocytes), progesterone stimulates meiotic resumption through the action of the progesterone receptor in cumulus cells.

Growth Hormone (GH)

In vitro maturation of bovine oocytes by culture of COCs from small to medium sized follicles for 24 h in tissue culture medium M199 supplemented with 100 ng/ml of bovine GH accelerated the process of GVBD (Izadyar et al., 1996). Similarly the percentage of M II oocytes was increased in oocytes matured in the presence of GH.

However, the final amount of oocytes that reached to the M II stage after 24 h of culture was not affected by GH. These observations indicated that GH affected the kinetics of meiosis rather than increased the proportion of oocytes that reached the M II stage after *in vitro* maturation. Presence of GH during *in vitro* maturation also induced expansion of the cumulus cells leading to an average diameter increase of about 25%, although the effect was small as compared to the effect of FSH which caused a diameter increase of about 75% in the COCs (Izadyar et al., 1996).

The expression of connexin (Cx) 43 was examined in COCs matured in the presence or absence of GH. The percentage of gap junctions was significantly reduced by GH treatment, with Cx43 being mainly localized to the cells of the corona radiata. This localization pattern was an indication that the communication between the oocyte and the cumulus cells was maintained in the presence of cumulus cell expansion. Because it has already been demonstrated that GH accelerated nuclear maturation in oocytes, the reduction of gap junctions could result in alterations in the transport of signal transducers and metabolites in the cumulus cells. Thus, resumption of meiosis and nuclear maturation of the oocyte might be stimulated. This is supported by the fact that Cx43-mediated communication between cumulus cells played a crucial role in maturation of bovine oocytes (Vozzi et al., 2001) and that expression of Cx43 in oocytes was developmentally regulated (Granot et al., 2002). This could be related to modifications in intercellular communication resulting in an acceleration of maturation in the oocyte.

Recent evidence indicated that, percentage of proliferating cells was significantly ($P<0.05$) higher in COCs matured in the presence of GH (12.6%) compared with the COCs matured in the control media TCM 199 and TCM 199/ECS (9.9% and 9.0%, respectively). The supplementation of the maturation medium with a combination of GH and IGF-I or IGF-I alone did not significantly alter the proliferation rate compared with the control groups. When the maturation medium was supplemented with GH and IGF-I, the average proportion of proliferating cells was similar (8.9%) to supplementation with IGF-I alone (8.7%). In the negative controls (testing the specificity of the used antibodies), no signal was visible (Kölle et al., 2003). These results imply that the effects of GH on bovine cumulus cell proliferation are not

mediated by IGF-I. Thus, GH was able to directly modulate cell proliferation. This was supported by the fact that cumulus expansion induced by GH treatment was not inhibited by an IGF-I antibody. Cumulus cells played an essential role in transportation processes and nutrition of the oocyte. Therefore, the higher number of cumulus cells in the presence of GH was likely to stimulate the feeding of the oocyte, resulting in a higher viability of the oocyte. This could also contribute to the fact that fertilizability and developmental capacity of oocytes were increased by GH treatment.

The difference in response of COCs and denuded oocytes on treatment with GH points to the existence of the GH receptors (GHRs) in cumulus cells as well as in the oocyte. Although the presence of mRNA was indicative but not a proof for a functional receptor, together with the observed responses of GH on oocyte maturation it can be assumed that bovine cumulus cells and the bovine oocyte possess a functional GHRs. GHRs transcript and immunoreactivity in bovine cumulus cells and in the oocyte has also recently been reported (Kölle et al., 1998).

Most of the actions of GH are believed to be mediated by IGF-I that is secreted by various tissues in response to GH. In the rat the stimulatory effect of GH on *in vitro* oocyte maturation is mediated by IGF-I probably produced by the cumulus cells. However in the cow the effect of GH on oocyte maturation is a direct action of the hormone itself and not a process mediated by IGF-I since addition of an antibody direct against IGF-I did not affect the stimulatory effect on the nuclear maturation and the degree of cumulus expansion while the antibody completely inhibited the effect of IGF-I on nuclear maturation and cumulus expansion (Izadyar et al., 1997).

It is well documented that cAMP plays a key role in the control of oocyte maturation in mammals. In the mouse, as well as in other mammalian species, a high level of cAMP inside the oocyte is associated with transient maintenance of the meiotic arrest *in vitro*. This may be achieved by using different products, like invasive adenylate cyclase and dibutyryl cyclic AMP which act similar to cAMP, or by using protein kinase A or phosphodiesterase inhibitors which act downstream cAMP (Sirard, 2001). Addition of 100 μM 2',3'- dideoxyadenosine (DDA), a specific inhibitor of adenylate cyclase, at the onset of maturation of bovine oocytes

completely inhibited the stimulatory effect of GH on nuclear progression and suppressed the cumulus expansion exerted by the hormone. DDA also suppressed cumulus expansion by FSH, which is mediated by the cAMP transduction pathway.

These findings are strong evidence for cyclic AMP signaling in GH induced maturation and are consistent with the observation that the stimulatory effect of GH on oocyte maturation and cumulus expansion is exerted via GH receptor located in the cumulus cells.

Nitric oxide (NO)

Nitric oxide recognized as the "Molecule of the Year" by Science magazine in 1992, acts as an important signalling molecule in many tissues to regulate a diverse range of physiological processes (Dixit and Parvizi, 2001). Typical beneficial properties of NO include the regulation of vascular tone, the protection of cells against apoptosis, the modulation of immune responses, and the killing of microbial pathogens.

Nitric oxide is a fairly short-lived molecule (with a half-life of a few seconds) produced from enzymes known as nitric oxide synthases (NOS). These enzymes convert arginine into citrulline, producing NO in the process. The general mechanism of NO production from NOS is illustrated in Figure 2-7.

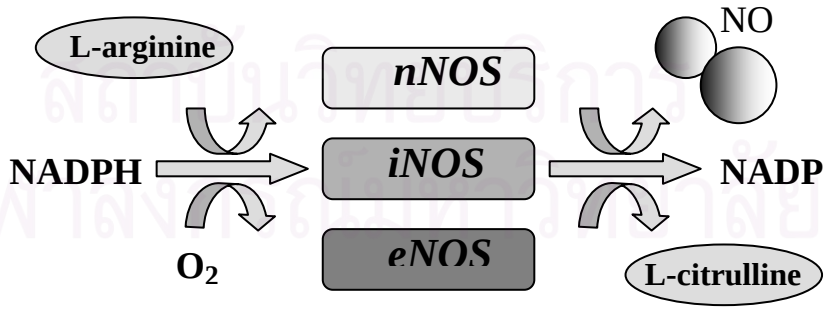


Figure 2-7 Mechanism of NO synthesis

Nitric oxide synthase (NOS)

Nitric Oxide Synthase (NOS) is the enzyme responsible for NO synthesis. NOS has three isoforms, named according to their activity or the tissue type in which they were first discovered. The isoforms of NOS are neuronal NOS (or nNOS), endothelial NOS (or eNOS) and inducible NOS (or iNOS). These enzymes are also sometimes referred to by number, so that nNOS is known as NOS1, iNOS is known as NOS2 and eNOS is NOS3. Despite the names of these enzymes, all three isoforms can be found in a variety of tissues and cell types. Two of the enzymes (nNOS and eNOS) are constitutively expressed in mammalian cells and synthesis NO in response to increases in intracellular calcium levels. iNOS activity is independent of the level of calcium in the cell, however its activity - like all of the NOS isoforms - is dependent on the binding of calmodulin. Increases in cellular calcium leads to increases in levels of calmodulin and the increased binding of calmodulin to eNOS and nNOS leads to a transient increase in NO production by these enzymes. By contrast iNOS is able to bind tightly to calmodulin even at very low cellular concentration of calcium. Consequently iNOS activity is not able to respond to changes in calcium levels in the cell. As a result the production of NO by iNOS lasts much longer than from the other isoforms of NOS, and tends to produce much higher concentrations of NO in the cell.

The production of NO by iNOS was controlled through transcription. In most cell types iNOS protein levels are either very low or undetectable. However, stimulation of these cells with, for example, cytokines or growth factors, can lead to increased transcription of the iNOS gene, with subsequent production of high concentration of NO.

Intraovarian is present of eNOS and iNOS in different compartments of ovary (Zackrisson et al., 1996), but nNOS has not been found (Jablonka-Shariff and Olson, 1997; Van Voorhis et al., 1995).

In the rat, immunofluorescent staining localizes eNOS specifically within the granulosa cells, thecal layer, ovarian stroma and the surface of oocytes enclosed in developing follicles (Zackrisson et al., 1996; Jablonka-Shariff and Olson, 1997;

Jablonka-Shariff et al., 1999; Yamagata et al., 2002) while iNOS was localized only in somatic cells of follicle and luteal cells (Jablonka-Shariff and Olson, 1997; Yamagata et al., 2002).

In the mouse, both eNOS and iNOS were localized in M II oocytes cytoplasm, but the secretion was quite weak (Nishikimi et al., 2001). Mitchell et al. (2004) also found that both eNOS and iNOS were distributed in oocytes and theca of mouse follicles as well as in granulosa cells and that iNOS mRNA occurred predominantly in granulosa cells and oocytes.

In the porcine, the immunoreactivity of eNOS was increased from small, medium to large follicle-enclosed oocytes. Cumulus cells from large follicles showed weak eNOS immunoreactivity but those from small or medium follicles not. In corpus luteum, eNOS-positive staining was shown in granulosa lutein cells. In corpus albican, it was in some parenchymal cells. In contrast, no immunoreactivity for iNOS was found in primordial, early antral follicle or the COCs aspirated from small and medium follicles. The large follicle-enclosed oocyte showed weak immunoreactivity. In corpus luteum, some granulosa lutein cells showed iNOS-positive cytoplasm. Such immunostaining was not found in corpus albican (Tao et al., 2004).

The expression of both iNOS and eNOS is regulated by gonadotrophins (Jablonka-Shariff and Olson, 1997) since both PMSG and hCG have been shown to influence eNOS and iNOS concentrations, thus confirming that both isoforms participate in the ovarian functions. Additionally, eNOS appears to be involved in mediating the number of oocytes recovered following superovulation (Jablonka-Shariff and Olson, 1998).

Nitric oxide and its mechanisms

NO is a small molecule which is able to diffuse rapidly across cell membranes and, depending on the conditions, is able to diffuse distances of more than several hundred microns. The biological effects of NO are mediated through the reaction of NO with a number of targets such as heme groups, sulfhydryl groups and iron and zinc clusters. Such a diverse range of potential targets for NO explains the

large number of systems that utilize it as a regulatory molecule. As a consequence of this abnormal regulation or control of NO synthesis is capable of affecting a number of important biological processes.

Many of the biological effects of NO may result from the alteration of multicomponent signal transduction pathways and are exerted via different mechanisms; three of which seem to be the most important.

The first one, NO is capable of donating electrons, which allows reactions with transitions metals such as iron, copper and zinc. The formation of metal-nitrosyl complexes might lead to the activation or inhibition of the function of proteins that contain prosthetic groups or metal-sulfide clusters. Well-studied examples are the activation of soluble guanylyl cyclase, where NO disrupts the bond between the ferrous iron of the heme and histidine 105 of the enzyme, thereby increasing the synthesis of cyclic GMP (Murad, 1994).

Second, Nitric oxide also can induce the S-nitrosylation of thiol groups of free amino acids, peptides or proteins. The formation of S-nitrosothiols is suitable to alter the tertiary structure of complex proteins and therefore forms a valid mechanism for the signaling effects of NO (Kelly et al., 1996).

Third, NO can react with other radicals. The reaction with $\cdot O_2$ results in the formation of peroxynitrite (ONOO–), which is a strong oxidant capable of oxidizing thiol residues to sulfenic and sulfonic acids and nitrating peptides and proteins at the phenyl side chain of tyrosine residues. Another rapid radical–radical interaction of NO has been described for biologically relevant tyrosyl ($Tyr^{\cdot}$) and tryptophanyl ($Trp^{\cdot}$) radicals in free amino acids, peptides and proteins.

Taken together, the available information suggests that the effects of NO are strictly dependent on its concentration, as well as on the presence of metals, proteins, and low-molecular-weight thiols in a given cell (Davies et al., 1995).

Nitric oxide and its control of ovarian function

Among the multiple functions controlled by NO within the female reproductive system, one of the most intriguing is represented by NO involvement in ovarian physiology, which has been supported by multiple findings. Cyclic changes in expression of NOS subtypes in ovary lends support to the role of NO in directly affecting the ovarian function. NO is hypothesised to play a role in folliculogenesis; steroidogenesis; ovulation and oocyte maturation.

The role of NO in folliculogenesis

It is well known that both folliculogenesis are regulated by a variety of factors, such as cytokines, growth factors, and locally produced substances, among which NO seems to play an important role.

Matsumi et al. (1998) observed iNOS is predominantly localised in granulosa cells in most healthy immature follicles and a decrease in iNOS mRNA levels induced by PMSG in granulosa cells from immature rat follicles. On this basis, they suggested that NO might have antiapoptotic actions in granulosa cells by autocrine/paracrine mechanisms, thus preventing the premature atresia of developing follicles. This hypothesis has been reinforced by results from a more recent study in the rat (Matsumi et al., 2000), which also show a GnRH induced reduction in iNOS mRNA levels. Moreover, NO has been shown to act as an antiproliferative agent (Kuzin et al., 1996) and to inhibit mitosis (Takagi et al., 1994) in other mouse cell types.

A mechanism through which NO may be involved in the control of follicular development is its effects on apoptosis, the programmed cell death by which the majority of ovarian follicles are lost during postnatal life (Kiess and Gallaher, 1998; Li et al., 1998). High NO levels have been shown to reduce apoptosis in both swine (Ponderato et al., 2000) and bovine (Basini et al., 1998) granulosa cells, whereas an opposite effect has been induced by low NO levels in more differentiated granulosa cells (from large follicles).

The effects of NO on folliculogenesis suggest that locally produced NO contributes to modulate follicle development and possibly prevents apoptosis, at least at low concentrations, whereas high levels may promote cell death.

The role of nitric oxide in steroidogenesis

Nitric oxide has been shown to exert negative effects on steroidogenesis, possibly through a direct action on steroid-secreting cells rather than via an effect on local ovarian blood flow (Dave et al., 1997).

Jablonka-Shariff and Olson (1998) reported a 5-fold higher concentration of estradiol in eNOS knockout mice compared to wild type (WT) mice, while no change in progesterone concentration was noticed. Administration of NO donor, spermine NONOate increased progesterone production. Furthermore in cultured rat ovaries, challenged with PMSG, NO donor causes a dose-dependent increase in progesterone synthesis with a concomitant decrease in estradiol secretion (Dong et al., 1999).

The impairment of steroid production by NO has been demonstrated in different species and in different conditions (rat, Dave et al., 1997; human, Van Voorhis et al., 1994; Rosselli et al., 1998; porcine, Masuda et al., 1997; Matsumi et al., 2000; Ponderato et al., 2000; bovine, Basini et al., 1998; Basini and Tamanini, 2000). The negative effect of NO on steroid production has been demonstrated by treating cultured granulosa-luteal cells with SNAP, an NO donor, or with L-NAME, an NOS inhibitor, which markedly decrease or stimulate, respectively, both estradiol and progesterone release.

As for the mechanism(s) of action, several lines of evidence suggest that most effects of NO are exerted by binding iron-containing enzymes: on this basis, its regulatory role on steroidogenesis can be exerted through a direct inhibition of the cytochrome P450 steroidogenic enzyme aromatase (Van Voohris et al., 1994)

The role of nitric oxide in ovulation

Studies conducted on eNOS and iNOS knockout mouse models provide a further support to the role of NO in ovulation. In eNOS deficiency mouse has been shown to reduced ovulatory potential after a superovulatory treatment (Hefler et al., 2002). Mature eNOS knockout female mice have a smaller litter size and have a higher stillborn rate as compared to wild type females (Jablonka-Shariff and Olson, 1998). Moreover, eNOS knockout females show a significant 63% reduction in ovulation induced by hCG when compared to WT females.

Local administration of iNOS inhibitors has been reported to suppress the ovulatory process in the rat, an effect reversed by sodium nitroprusside (Shukovski and Tsafriri, 1994). Similar results have been reported in hCG-treated rabbits (Hesla et al., 1997) and the systemic administration of NO blockers inhibited ovulation and suppressed the positive effect of IL-1 on LH-induced ovulation rate (Bonello et al., 1996). The role of eNOS in ovulation seems more important than that of iNOS (Mitsube et al., 1999), eventhough the results are still conflicting (Faletti et al., 1999). In fact, both rat thecal and stromal compartments present high eNOS levels around ovulation (Zackrisson et al., 1996).

Furthermore, a possible mechanism by which NO stimulates the ovulatory process involves the production of prostaglandins (which contribute to enhancing the inflammatory process in the periovulatory period). Effectors of NO production stimulate the synthesis of PGs by direct activation of cyclooxygenases (Salvemini, 1997). Blocking the intraovarian NO production by NOS inhibitors diminishes the production of PGE_2 and $PGF_{2\alpha}$ in response to hCG injections, while intrabursal injection of NO donor stimulates prostaglandin synthesis (Faletti et al., 2003). A cross talk between the NO and PG biosynthetic pathways, as well as a stimulatory effect of NO on $PGF_{2\alpha}$ production by large bovine follicles, has been recently reported (Basini and Tamanini, 2001). It has been suggested that NO might contribute to follicle rupture by also increasing the intrafollicular pressure (Matousek et al., 2001), either by increasing the vascular flow and the transudation of fluid to the follicular antrum or by stimulating the contractile elements of the ovarian follicle.

The role of nitric oxide in oocyte maturation

Jablonka-Shariff and Olson (1998) demonstrated that ovulated oocytes from eNOS- knockout mice, remained in metaphase I or atypical (degenerate) morphology. According to a study on *in vitro* maturation, COCs from eNOS-knockout mice or wildtype mice treated with NOS inhibitor (N-omega-nitro-L-arginine methyl ester: L-NAME) resulted in a lower percentage of oocyte at M II stage and a higher percentage of oocyte at M I or atypical stages (Jablonka-Shariff and Olson, 2000). In another study, rats treated with NOS inhibitor 3 h before and 3 h after hCG injection had significantly greater percentage of oocytes displayed atypical morphology (Jablonka-Shariff et al., 2002). Zhang et al. (2001) reported that in mice treated with L-NAME, most of the oocytes were at the metaphase I stage. These results indicated that ovarian nitric oxide is required for normal oocyte meiotic maturation during ovulation. Using an *in vitro* culture system added with sodium nitroprusside (SNP), NO donor, and NOS inhibitor (L-NAME), the results showed that low concentrations of SNP (10^{-7} M) significantly stimulated meiotic maturation to metaphase II stages in cumulus enclosed oocytes. While 10^{-3} and 10^{-5} M L-NAME demonstrated a significant suppression in resumption of meiosis (Sengoku et al., 2001).

On the other hand, Nakamura et al. (2002) showed that culture of COCs from preovulatory follicles or denuded oocytes(DOs) in NO donor (S-nitroso-L-acetyl penicillamine : SNAP 5 x 10^{-4} M) resulted in GVBD delay. They suggested that NO may be one of the regulators of oocyte maturation.

CHAPTER 3

MATERIALS AND METHODS

1. Oocyte collection

Buffalo ovaries were obtained from a local slaughterhouse, and were transported to the laboratory in 0.9 % NaCl solution in a thermocontainer at 30-35°C. Within 2 h after slaughter, the oocytes from 3-6 mm diameter follicles were harvested by aspiration, using a 18 gauge needle connected with a 10 ml disposable syringe.

Oocytes covered with multilayers of cumulus cells (≥ three cumulus layers) were selected and separated into two groups, one in which the oocytes remained cumulus enclosed (COCs) and the other in which the oocytes were denuded.

Denuded oocytes (DOs) were obtained by removing the surrounding cumulus cells from COCs. Selected COCs were mechanically pipetted in the presence of 0.1% hyaluronidase, and those with a homogeneous cytoplasm were allocated to their respective treatments.

DOs and COCs were washed five times in the medium and cultured in 100 µl droplets of experimental medium (10 oocytes per droplet) covered with mineral oil at 38.5°C in 5% CO_2 and humidified air.

2. Culture medium

The basal medium (M199) for the manipulation of the oocytes was medium 199 with Earle's salts (Gibco BRL, Life Technologies Inc., USA), supplemented with 0.5 mM sodium pyruvate (Sigma, USA), 25 mM sodium bicarbonate (Sigma, USA), 100,000 U/L penicillin G (Sigma, USA), and 100 mg/L streptomycin (Sigma, USA). The pH of M199B after equilibration with 5%CO_2 in air is 7.4. All media were filtered through 0.2 µm acrodisc filters (Pall Cooperation, USA).

In nitric oxide treatment groups, the culture medium was the basal medium supplemented with NO donor; S-nitroso-L-acetyl penicillamine (SNAP).

3. Fixation of oocytes

At the end of each treatment, the cumulus cells were removed. Denuded oocytes were recovered under a stereomicroscope and transferred onto a glass slide in a small drop of medium. A vaseline:paraffin wax mixture were used to maintain the coverslip in contact with the oocytes. The slides were immersed in a fixative solution (ethanol:acetic acid, 3:1) for a minimum of 24 h. Oocytes were then stained with 1% aceto-orcein and examined for nuclear morphology with a phase contrast microscope at 400 x magnification.

4. Assessment of stages of nuclear maturation

Stages of nuclear maturation were defined as described by Hunter and Polge (1966) as followed:

4.1 Germinal vesicle (GV)

Germinal vesicle is the dictyate stage in which the form of nucleus is unchanged from that seen in primary oocytes recovered at earlier stages of the oestrus cycle. The large nucleus is located eccentrically or peripherally and contains fine granular material. The nuclear membrane is intact, and the nuclear area is quite distinct. The chromatin strands are arranged in the form of a ring or housed hoe around a single large nucleous, again eccentrically placed.

4.2 Prometaphase (PM)

This term has used to cover the stage between late prophase and early metaphase. The appearance of the nucleus has changed considerably. The nuclear membrane can only be detected as a very fine strand, or is distinct, and the nuclear is less conspicuous due to a loss of granular material. The chromatin is undergoing condensation into either lump or into smaller discrete fragments, and the nucleolus is no longer appearance.

4.3 Metaphase I (M I)

There is no trace of the nuclear membrane. The chromosomes are formed and arrange in an orderly sequence on the equator of a bipolar spindle. The long axis of the spindle is orientated radially to the surface of the egg.

4.4 Anaphase I (ANA I)

The chromosomes are seen to be migrating towards the poles of an elongated spindle.

4.5 Telophase I (TEL I)

The chromosome have reached the polar regions of the spindle, and the mid-body of the spindle is now distinct. The first polar body is being formed, but is still clearly attached to the spindle.

4.6 Metaphase II (M II)

The first polar body has been abstracted from the egg (secondary oocyte), and the chromosomes of the latter are once more arranged on the equator of the spindle.

5. Measurement of cGMP

5.1 Test Principle

This assay is based on the principle of radioimmunoassays for cyclic nucleotides. High specificity and sensitivity (5 fmol/100 µl) of the test allows the direct determination of cGMP in plasma and urine. The assay is also suitable for the determination of cGMP in the guanylate cyclase assay in the presence of high GTP concentrations.

In the cGMP radioimmunoassay the samples (or standards) are incubated over-night with 125I-labeled cGMP and the pre-precipitated antiserum. There is competition between the radioactive and the nonradioactive antigen for a fixed number of antibody binding sites. The amount of 125I-labeled antigen bound to the antibody is inversely proportional to the analyze concentration of the sample. When the system is in equilibrium, the reaction is stopped by the addition of a co-precipitating solution and a centrifugation step. The precipitate is counted in a gamma counter.

Quantification of unknowns is achieved by comparing their activity with a response curve prepared using known standards.

5.2 Materials

5.2.1 Tracer

cGMP 125I-Tracer, lyophilized

Activity: < 750 kBq (20.3 µCi). Contains 0.1 % NaN_3

5.2.2 Antiserum : cGMP Antiserum

Contains cGMP Antiserum (rabbit), anti-rabbit antiserum (goat), 0.1 % NaN_3

5.2.3 Standard

Contains cGMP at 0.07; 0.2; 0.6; 1.8; 5.4; 15.5 pmol/ml , 0.1 % NaN_3.

5.2.4 Control, lyophilized

Contains 0.1 % NaN_3

5.2.5 Separation Reagent Concentrate (10x)

Contains 0.9 % NaN_3

5.2.6 Assay Buffer, Concentrate (5x)

5.2.7 NSB Solution

5.3 Sample Preparation

In each sample, 20 COCs were denuded and 20 DOs were kept at -70^{o}C. Before cGMP measurement, the oocytes were transferred to 0.5 ml of 95% ethanol, the oocytes were frozen and thawed in liquid nitrogen and sonicated for a few seconds. The samples were incubated for 30 min at 4^{o}C and then removed the precipitate by centrifugation. The supernatant were dried in a vacuum at 50^{o}C, and dissolved in 100 µl assay buffer. The intraoocyte cGMP were analysed by radioimmunoassay kit, purchasing from IBL Immuno-Biological Laboratories, Cat No. RE 29071.

5.4 Test Procedure

5.4.1 First Day

5.4.1.1 Pipette 100 µl of each Standard into the respective tubes

5.4.1.2 Pipette 100 µl of each extracted oocytes sample and control into the respective tubes

5.4.1.3 Pipette 100 µl of Assay Buffer into the NSB tubes and B0 tubes and into the tubes of the plasma. Do not pipette Assay Buffer into the tubes of the standards

5.4.1.4 Mix NSB Solution before use. Pipette 200 µl of NSB Solution into the NSB tubes

5.4.1.5 Pipette 100 µl of prepared 125I-Tracer into each tube. Include two tubes for Total Activity (T)

5.4.1.6 Mix Antiserum before use. Pipette 200 µl of Antiserum into each tube. (Except T, except NSB)

5.4.1.7 Vortex all tubes. Incubate 20-24 h at 2-8°C

5.4.2 Second Day

5.4.2.1 Pipette 1 ml of prepared cool Separation Reagent into each tube (Except T)

5.4.2.2 Centrifuge all tubes for 15 min at 2000-3000 x g. The use of a refrigerated centrifuge is recommended

5.4.2.3 Decant the tubes carefully. (Except T) Drain upside down with blotting paper. Remove any liquid carefully

5.4.2.4 Count the tubes in a Gamma counter for 1 min

5.4.2.5 Calculate the B/B0% for each standard, control and sample as follows:

$$B/B_0\% = \frac{CPM\ (calibrator/sample) - CPM\ (NSB)}{CPM(B_o) - CPM\ (NSB)} \times 100$$

Calculate B_0/T : $$B_0/T\% = \frac{CPM\ (Bo) - CPM(NSB)}{CPM(TotalCount\ (T))} \times 100$$

Calculate NSB/T: $$NSB/T = \frac{CPM\ (NSB)}{CPM(TotalCount(T))} \times 100$$

The standard concentrations are given in pmol/100µl.

The concentration of the samples can be read directly from the standard curve.

For the final concentration of the oocyte extract has to be considered:

Oocyte extract (pmol/20 oocyte) = Conc. (Standard pmol/100µl))

$$\text{Oocyte extract (fmol/oocyte)} = \frac{\text{Conc. (Standard (pmol/100µl)) x 1000}}{20}$$

The assay can be declared valid if the following criteria are met: Bmax/T > 20 %; NSB/T < 5 %.

6. Measurement of cAMP

6.1 Test Principle

This assay is based on the principle of radioimmunoassays for cyclic nucleotides described by Steiner et al. (1972), modified by Harper and Brooker (1975). The kit includes two standard ranges. The less sensitive method (1.5 - 450 pmol/ml) is applicable for urine and other samples with high cAMP levels. The highly sensitive method (0.05 - 15 pmol/ml) includes a simple acetylation step and is suitable for plasma and tissue extracts.

In the cAMP radioimmunoassay the samples (or standards) are incubated over-night with 125I-labeled cAMP and the pre-precipitated antiserum. There is competition between the radioactive and the nonradioactive antigen for a fixed number of antibody binding sites. The amount of 125I-labeled antigen bound to the antibody is inversely proportional to the analysed concentration of the sample. When the system is in equilibrium, the reaction is stopped by the addition of a co-precipitating solution and a centrifugation step.

The precipitate is counted in a gamma counter. Quantification of unknowns is achieved by comparing their activity with a response curve prepared using known standards. Because of its high specificity and sensitivity the assay is also suitable for the determination of cAMP in the adenylate cyclase assay in the presence of high concentrations of ATP.

6.2 Materials

6.2.1 Tracer

cAMP 125I-Tracer, lyophilized

Activity: ≤ 150 kBq (4.1 µCi). Contains 0.02 % Thimerosal

6.2.2 Antiserum

cAMP Antiserum pre-precipitated complex of cAMP antiserum (rabbit), antirabbit antiserum (goat), 0.02 % Thimerosal

6.2.3 Standard

0.05; 0.15; 0.5; 1.5; 5; 15; 50; 150; 450 pmol/ml

Contains cAMP, acetate buffer, BSA, 0.02 % Thimerosal

6.2.4 Plasma Control, lyophilized

Contains 0.1 % NaN_3

6.2.5 Separation Reagent Concentrate (10x)

Contains 0.9 % NaN_3

6.2.6 Assay Buffer, Concentrate (5x)

6.2.7 NSB Solution

6.2.8 Triethylamine

6.2.9 Acetic Acid Anhydride

6.3 Sample Preparation

In each sample, 50 COCs were denuded and the 50 DOs were kept at -70^{o}C. Before cAMP measurement the oocytes were transferred to 95% ethanol and were frozen and thawed in liquid nitrogen and sonicated for a few seconds. The samples were incubated for 30 min at 4^{o}C and then removed the precipitate by centrifugation. The supernatant were dried in a vacuum at 50^{o}C, and dissolved in 100 assay buffer. The intraoocyte cAMP were analysed by radioimmunoassay kit which purchased from IBL Immuno-Biological Laboratories, Cat No. RE 11071

6.3.1 Dilution of Samples

Extracted oocytes generally diltuted in assay buffer 1 : 5 prior to acetylation step.

6.3.2 Preparation of Acetylation Reagent

Mix Acetic Acid Anhydride and Triethylamine in the relation 1:2 in glass tubes. This reagent is very unstable and has to be prepared just prior to use. Work in a well ventilated area

6.3.3 Acetylation of Standards and Samples

For the determination of cAMP in oocyte extract samples, the test was performed with acetylation and only Standard 0.05-15 pmol/ml were

used. Acetylation had to be performed at room temperature. Prepared oocyte extract samples have to be assayed the same day.

6.3.3.1 Pipette 100 µl of oocyte extract samples and 400 µl of diluted Assay Buffer into the polypropylene tubes

6.3.3.2 Pipette 20 µl Control Plasma and 480 µl of diluted Assay Buffer into the polypropylene tubes

6.3.3.4 Pipette 500 µl of diluted Assay Buffer (serves as zero standard and nonspecific binding) and 500 µl of the Standards 0.05 – 15 pmol/ml into tubes of the same quality as for samples. Total volume of each tube is now 500 µl

6.3.3.5 Add 25 µl of freshly prepared Acetylation Reagent to each tube. Place each single tube on a vortex mixer ("press to mix") and add the reagent just above the surface of the solution. Mix immediately for at least 5 seconds after addition.

6.4 Test Procedure

6.4.1 First day

6.4.1.1 Pipette 100 µl of acetylated Standard 0-15 pmol/ml, acetylated Control Plasma and acetylated oocyte extract samples into the respective polystyrene tubes

6.4.1.2 Pipette 100 µl of acetylated Standard 0-15 pmol/ml, acetylated Control Plasma and acetylated oocyte extract samples into the respective polystyrene tubes

6.4.2.3 Pipette 100 µl of acetylated Assay Buffer into the NSB tubes and Bo tubes

6.4.1.4 Mix NSB Solution before use. Pipette 200 µl of NSB Solution into the NSB tubes

6.4.1.5 Pipette 100 µl of prepared 125I-Tracer into each tube. Include two tubes for Total Activity (T)

6.4.1.6 Mix Antiserum before use. Pipette 200 µl of Antiserum into each tube. (Except T, except NSB.)

6.4.1.7 Vortex all tubes. Incubate 20-24 h at 2-8°C

6.4.2 Second Day

6.4.2.1 Pipette 1 mL of prepared cool Separation Reagent into each tube (Except T)

6.4.2.2 Centrifuge all tubes for 15 min at 2000-3000 x g by a refrigerated centrifuge

6.4.2.3 Decant the tubes carefully. (Except T) Drain upside down with blotting paper. Remove any liquid carefully

6.4.2.4 Count the tubes in a Gamma counter for 1 min

6.5 Calculation of results

Calculate the B/B0% for each standard, control and sample as follows:

$$B/B_0\% = \frac{CPM\ (calibrator/sample) - CPM\ (NSB)}{CPM(B_o) - CPM\ (NSB)} \times 100$$

Calculate B_0/T : $B_0/T\% = \frac{CPM\ (Bo) - CPM(NSB)}{CPM(TotalCount\ (T))} \times 100$

Calculate NSB/T: $NSB/T = \frac{CPM\ (NSB)}{CPM(TotalCount(T))} \times 100$

The obtained $\%B/B_0$ of the standards (y-axis, linear) are plotted against their concentration (x-axis,logarithmic) either on semi-logarithmic graph paper or using an automated method. A good fit is provided with cubic spline, 4 Parameter Logisitcs or Logit-Log.

For the calculation of the standard curve, apply each signal of the standards (one obvious outlier of duplicates might be omitted and the more plausible single value might be used).

The concentration of the samples can be read directly from the standard curve. For the final concentration of the samples the dilution factor (1: 5 for oocyte extract) has to be considered:

oocyte extract (pmol/50 oocyte) = Conc. (Standard Curve (pmol/100 µl)) x 5

oocyte extract (fmol/oocyte) = Conc.(Standard Curve(pmol/100 µl)) x 5 x 1000

The assay can be declared valid if the following criteria are met: Bmax/T > 20 %; NSB/T < 5 %.

7. MPF and MAPK Assay

7.1 Sample preparation

Eight oocytes from each time point were denuded and transferred to 8 µl cold (4°C) collection buffer (see Appendix) in Eppendorf tubes, and kept at -70°C.

7.2 Kinase Asaay

The samples were placed on ice, and 10 µl of homogeneous buffer (see Appendix) were added to each sample. The samples were then incubated at 37°C for 15 min. After incubation, 8 µl of kinase buffer (see Appendix) were added. Samples were incubated for an additional 30 min at 37°C, and the reaction were terminated by the addition of 18 µl double-strength SDS sample buffer.

The samples were heated at 95°C for 5 min and then 5 µl of samples were separated by 10 % linear gradient SDS-PAGE. Gels (Bio-rad, Mini-PROTEAN II system).

7.3 Separation of Protein (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis : SDS-PAGE)

The most widely used method for qualitative analysis of a protein mixture is SDS-PAGE. With this method, it is possible to determine both the purity and the relative molecular weight of an unknown isolated protein or proteins. In the process, proteins migrate in response to an electrical field through pores in the gel matrix and separate based on molecular size.

The polyacrylamide gel was prepared using N,N-methylene-bis acrylamide as cross-linker in the amount corresponding to the weight of acrylamide. The resolving gel contained 10 % acrylamide (see Appendix). The polymerize was allowed to incubate at room temperature for at 45 min. After the separating gel is solid (polymerize) then the 3 % stacking acrylamide (see Appendix) gel would be added. Then leaving the gel for 15 min at room temperature prior to electrophoresis was performed.

7.4 Electrophoresis

After polymerization was complete, the samples were load up into polyacrylamide gel. The electrophoresis apparatus was attached to an electric power supply. The power supply was used at 150 V. The gel was run until the dye front reaches the bottom of the gel, approximately 45 min. After that the gel from the glass plate was removed into a staining dish**.**

7.5 Staining and Destaining

Polypeptides separated by SDS-PAGE can be simultaneously fixed with methanol : glacial acid and stained with Coomassie Brilliant Blue (R-250, Sigma) (see Appendix). The gel was covered with Coomassie staining solution and placed on the shaker and allows the gel to stain for at 5 min. Decant off the dye and add destaining solution to cover the gel was performed. Excess background stain was removed by soaking the gel in several changes of destaining solution (see Appendix) until the background color was clear.

7.6 Drying

When destaining was complete, the gel was washed briefly in deionized water, transferred onto a cellophan sheet, and leave the gel at room temperature for overnight. After that the gel were processed for autoradiography.

7.7 Autoradiography

The gel was placed on X-ray film cassette. The lights were turned off and a sheet of autoradiography film 18 x 24 cm (Hyperfilm TM-ECL, Amersham Pharmacia Biotech, UK) carefully placed on the top of the gel, the cassette was closed and exposed for 20-30 min. The hyperfilm then were developed and the autoradiographs then were subjected to image scanner (Amersham, Pharmacia Biotech, UK). The activities measured were expressed as percentage of the maximum level of histone H1 or MBP kinase activity, respectively.

8. Ultrastructural study

For transmission electron microscopic study, 20-30 oocytes of each treatment were fixed in 3%glutaraldehyde in 0.1 M phosphate buffer saline at pH 7.4 for overnight at 4^{o}C. Then they were rinsed in buffer and post-fix in 2% osmium tetroxide for 1 h. Next, the specimen were dehydrated in graded series of alcohol, transferred to propylene oxide and embedded in Epon 812. Ultrathin sections were cut with an ultramicrotome. The ultrathin sections were stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope.

Experimental protocol

Experiment 1: The effect of NO-donor (SNAP) concentrations on the percentage of GVBD and M II in COCs and DOs

This experiment was designed to investigate the different concentrations of SNAP on the maturation rate of swamp buffalo oocyte cultured *in vitro*. COCs and DOs (30 oocytes in each treatment) were cultured in the basal medium alone or supplemented with various concentrations of S-nitroso-L-acetyl penicillamine; SNAP (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} M) (Sengoku et al., 2001; Nakamura et al., 2002), and the percentages of GVBD and M II were observed at 8 and 24 h, respectively. After culture for 8 or 24 h, the COCs were denuded as described above. The oocytes were fixed, stained with aceto-orcein and then evaluated with an inverted phase-contrast microscope.

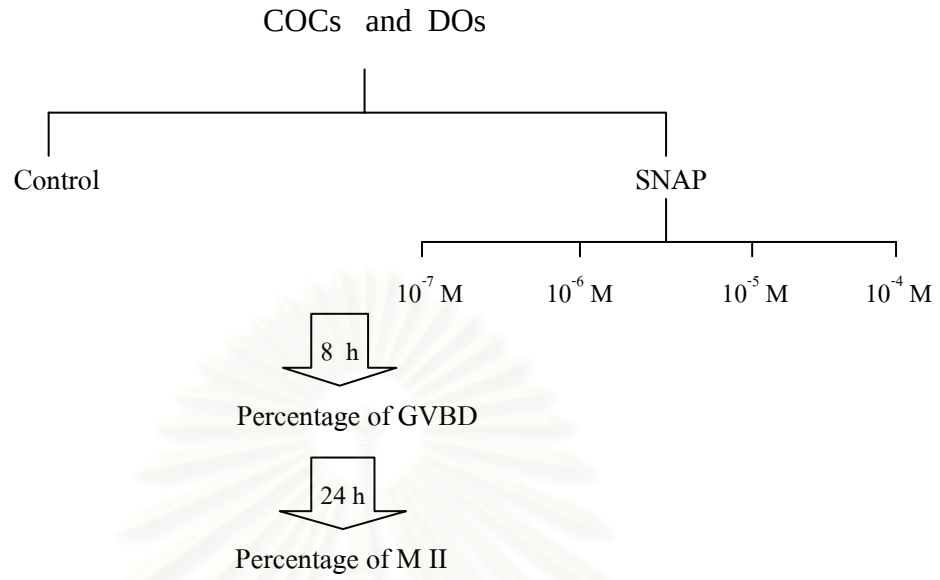


Figure 3-1 Experimental protocol to study the effect of NO-donor (SNAP) concentrations on the percentages of GVBD and M II stage in COCs and DOs

Experiment 2 : The signaling pathway of NO

Experiment 2.1 The effects of SNAP, SNAP with soluble guanylyl cyclase inhibitor; 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) and ODQ on the percentages of GVBD in COCs and DOs

This experiment was designed to determine whether the inhibitory effect of SNAP on spontaneous meiotic maturation was reversible by ODQ. COCs and DOs were cultured in the basal medium alone or supplemented with SNAP (at the most effective concentration from Experiment 1), SNAP+ODQ (10 μM) or ODQ(10 μM). After culture for 8 h, COCs were stripped free of cumulus cells. All of oocytes were evaluated for the percentages of GVBD.

Experiment 2.2 The effects of SNAP, SNAP with ODQ and ODQ on intraoocyte cGMP concentrations in COCs and DOs

This experiment was designed to determine whether the inhibitory effect of SNAP on spontaneous meiotic maturation was mediated by cGMP. COCs and DOs were cultured in the basal medium alone or supplemented with SNAP (at the most effective concentration from Experiment1), SNAP+ODQ(10 μM) or ODQ(10 μM).

After culture for 8 h, COCs were stripped free of cumulus cells. Samples of oocytes were stored at -70^{o}C until measurement of intraoocyte cGMP concentrations.

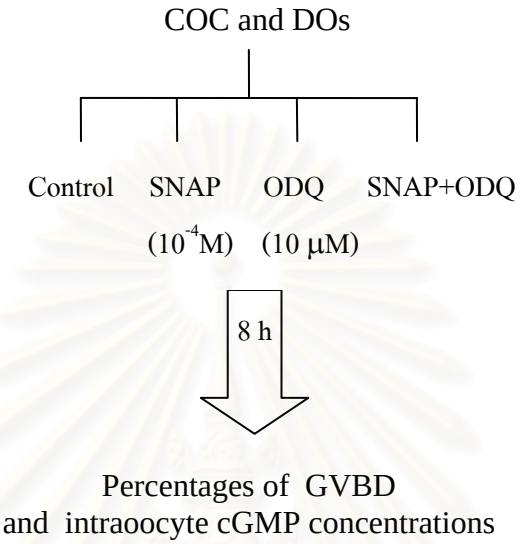


Figure 3-2 Experimental protocol to study the effects of SNAP, SNAP with ODQ and ODQ on the percentages of GVBD and intraoocyte cGMP concentrations in COCs and DOs

Experiment 2.3 The effects of SNAP on intraoocyte cAMP concentrations in COCs and DOs

This experiment was designed to determine whether the inhibitory effect of SNAP on spontaneous meiotic maturation was mediated by cAMP. COCs and DOs were cultured in the basal medium alone or in the presence of SNAP for 0, 2, 6, 12 h. At the end of culture, COCs were stripped free of cumulus cells. Samples of oocytes were stored at -70^{o}C until measurement of intraoocyte cAMP concentrations.

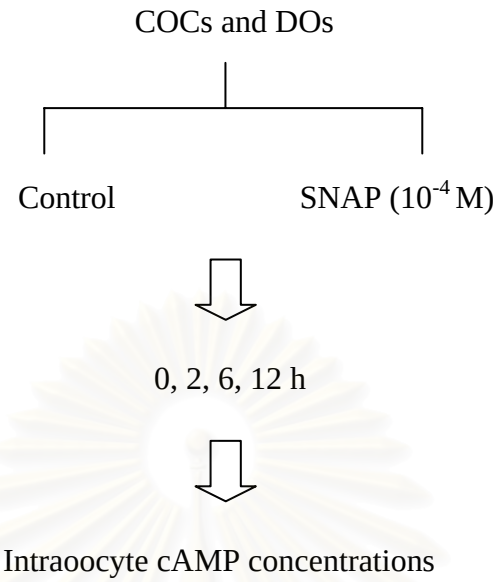


Figure 3-3 Experimental protocol to study the effect of NO-donor (SNAP) on intraoocyte cAMP concentrations in COCs and DOs

Experiment 3 : The effect of SNAP on the kinetics of nuclear maturation and cytoplasmic maturation

Experiment 3.1 : The effect of SNAP on the kinetics of nuclear maturation in COCs from 2-24 h

This experiment was designed to determine the effect of SNAP the kinetics of nuclear maturation. COCs (30 oocytes at each time point) were cultured in the basal medium alone or in the presence of 10^{-4} M SNAP. COCs were continuously observed for maturation stage at 2, 6, 8, 10, 12, 14, 16, 18, 20 and 24 h. The oocyte were fixed, stained with aceto-orcein and then evaluated for the stages of nuclear maturation (GV, GVBD, M I, ANA I, TEL I, and MII) with an inverted phase-contrast microscope.

Experiment 3.2 : The effect of SNAP on MPF and MAPK activities

This experiment was designed to determine the effect of SNAP the cytoplasmic maturation. COCs (30 oocytes at each time point) were cultured in the basal medium or alone or in the presence of 10^{-4} M SNAP. COCs were cultured for 2, 6, 8, 10, 12, 14, 16, 18, 20 and 24 h. Following culture, cumulus cells were stripped free from COCs. All of oocytes were analysed for MPF and MAPK activities at each time point.

Experiment 3.3 : The effect of SNAP on mitochondrial and cortical granule changes

COCs (20-30 oocytes in each treatment) were cultured in the basal medium alone or in the presence of 10^{-4} M SNAP for 24 h. Following culture, oocytes were assessed morphological changes of mitochondria and cortical granules by using transmission electron microscopy.

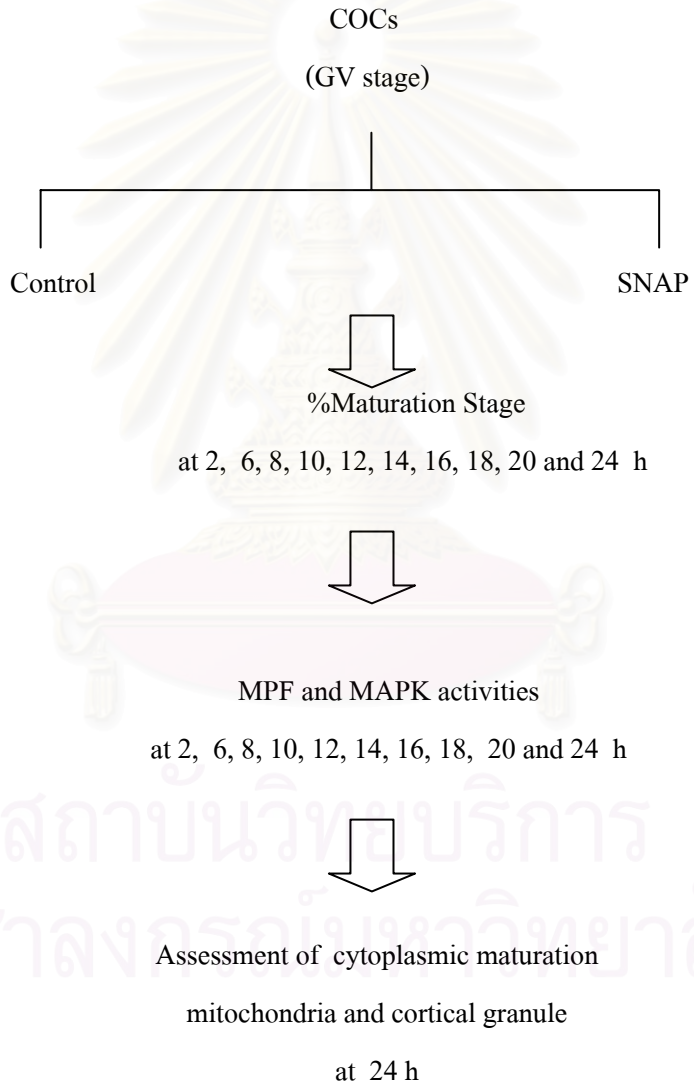


Figure 3-4 Experimental protocol to study the effect of NO-donor(SNAP) on the kinetics of nuclear maturation, MPF and MAPK activities and cytoplasmic maturation

Data analysis

Data were expressed as mean ± standard error (SEM). The comparison of the difference between the means of treatments were analysed by the Analysis of Variance (ANOVA) techniques using the procedures of Statistical Analysis System Institute (SAS). The difference between means were compared by Duncan's multiple range test.

The 2-way analysis of variance (Snedecor and Cochran, 1967) were used to compare the differences between the effects of NO treatment in COCs and DOs, which were divided into the main effect and their interactions. According to the procedures of SAS, the single degree of freedom orthogonal comparisons were : 1) COCs versus DOs and 2) control versus NO. The statistical significance were declared when probability (p) is below 0.05.

CHAPTER 4

RESULTS

The results are divided into 3 experiments as follows:

Experiment 1 : The effect of SNAP concentrations on nuclear maturation

The results in this experiment are divided as follows:

- The effect of various concentrations of SNAP on the percentages of GVBD stage in COCs and DOs
- The effect of various concentrations of SNAP on the percentages of M II stage in COCs and DOs

Experiment 2 : The signaling pathway of NO

The results in this experiment are divided as follows:

- The effect of SNAP, SNAP with ODQ, and ODQ on the percentages of GVBD in COCs and DOs
- The effect of SNAP, SNAP with ODQ and ODQ on intraoocyte cGMP concentrations in COCs and DOs
- The effect of SNAP on intraoocyte cAMP concentrations in COCs and DOs at 0-12 h

Experiment 3 : The effect of SNAP on the kinetics of nuclear maturation and cytoplasmic maturation

The results in this experiment are divided as follows:

- The effect of SNAP on the kinetics of nuclear maturation of COCs from 2-24 h
- The effect of SNAP on MPF and MAPK activities
- The effect of SNAP on mitochondrial and cortical granule changes

Experiment 1 : The effect of SNAP concentrations on nuclear maturation

The effect of various concentrations of SNAP on the percentages of GVBD stage in COCs and DOs

Evaluation of the status of nuclear maturation showed that COCs and DOs incubated in the basal medium alone had resumed meiosis spontaneously after 8 h of culture, the majority of the oocytes underwent GVBD. After culturing in the basal medium alone or supplemented with either 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M SNAP for 8 h the percentages of COCs underwent GVBD stage were 76.7 $\pm$ 5.7, 53.3 $\pm$ 5.8, 46.7 $\pm$ 5.8, 37.8 $\pm$ 3.8 and 24.1 $\pm$ 5.3, respectively (Table 4-1). The percentages of DOs underwent GVBD stage were 63.3 $\pm$ 5.8, 48.1 $\pm$ 3.2, 39.3 $\pm$ 5.6, 34.4 $\pm$ 5.1 and 27.8 $\pm$ 6.9, respectively (Table 4-2). The supplementation of SNAP from 10^{-7} to 10^{-4} M in the basal medium significantly decreased the percentages of GVBD in both COCs and DOs. The highest concentration of SNAP resulted in the lowest GVBD rates compared with the other groups.

Table 4-1 The percentages of COCs at GVBD stage after cultured in either basal medium alone or supplemented with various concentrations of SNAP for 8 h

Doses of SNAP	Percentages of COCs at GVBD stage
0 M	76.7 $\pm$ 5.7 ^{a}
10^{-7} M	53.3 $\pm$ 5.8 ^{b}
10^{-6} M	46.7 $\pm$ 5.8 ^{b}
10^{-5} M	37.8 $\pm$ 3.8 ^{c}
10^{-4} M	24.1 $\pm$ 5.3 ^{d}

The statistical differences are indicated at $P<0.05$

Different letters show the significant differences among groups

The mean number of COCs used in each treatment was 30

Data are expressed as means ± S.E.M. of three replicates

Table 4-2 The percentages of DOs at GVBD stage after cultured in either basal medium alone or supplemented with various concentrations of SNAP for 8 h

Doses of SNAP	Percentages of DOs at GVBD stage
0 M	63.3 ± 5.8^{a}
10^{-7} M	48.1 ± 3.2^{b}
10^{-6} M	39.3 ± 5.6^{bc}
10^{-5} M	34.4 ± 5.1^{c}
10^{-4} M	27.8 ± 6.9^{d}

The statistical differences are indicated at $P<0.05$

Different letters show the significant differences among groups

The mean number of COCs used in each treatment was 29

Data are expressed as means ± S.E.M. of three replicates

Comparisons of the percentages of GVBD between COCs and DOs were shown in Figure 4-1. In control group, COCs had the percentages of GVBD significantly higher than DOs, while there were no significant difference in the percentages of GVBD between COCs and DOs treated with SNAP.

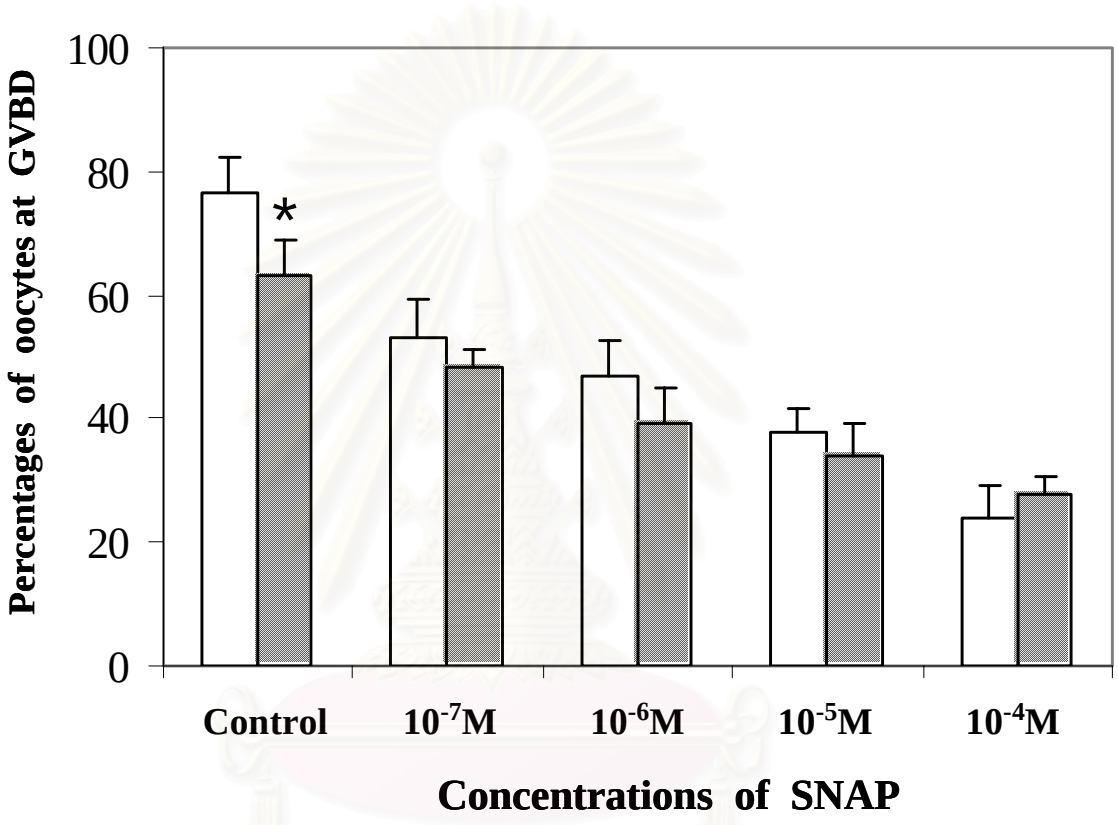


Figure 4-1 Comparisons of the effect of SNAP on the percentages of GVBD stage between COCs and DOs. COCs and DOs were cultured in either basal medium alone or supplemented with 10^{-7}, 10^{-6},10^{-5} or 10^{-4} M SNAP for 8 h. (Data from Table 4-1 and 4-2)

* significant difference compared with COCs at $p<0.05$

The effect of various concentrations of SNAP on the percentages of M II stage in COCs and DOs

When the culture period was extended to 24 h, the percentages of M II stage have been evaluated, the percentage of COCs reaching M II stage were 34.3 ± 3.1, 33.3 ± 5.8, 25.0 ± 5.0, 25.5 ± 7.6 and 19.2 ± 5.4, respectively (Table 4-3). Supplementation of SNAP from 10^{-6} to 10^{-4} M significantly decreased the percentages of COCs at M II stage but SNAP at 10^{-7} M had no significant effect on the percentages of COCs reaching M II stage after 24 h of culture. While in DOs, the percentages of DOs reaching MII stage in the culture medium alone or supplemented with either 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M SNAP were 30.8 ± 6.3, 23.1 ± 2.7, 17.8 ± 1.9, 10.4 ± 0.6 and 14.1 ± 5.1, respectively (Table 4-4). SNAP at all concentrations significantly decreased the percentages of DOs reaching M II stage compared with the control group.

Table 4-3 The percentages of COCs reaching M II stage after cultured in either basal medium alone or supplemented with various concentrations of SNAP for 24 h

Doses of SNAP	Percentages of COCs at M II stage
0 M	34.3 ± 3.1 [a]
10^{-7} M	33.3 ± 5.8 [a]
10^{-6} M	25.0 ± 5.0 [b]
10^{-5} M	25.5 ± 7.6 [b]
10^{-4} M	19.2 ± 5.4 [b]

The statistical differences are indicated at $P<0.05$

Different letters show the significant differences among groups

The mean number of COCs used in each treatment was 32

Data are expressed as means ± S.E.M. of three replicates

Table 4-4 The percentages of DOs reaching M II stage after cultured in either basal medium alone or supplemented with various concentrations of SNAP for 24 h

Doses of SNAP	Percentages of DOs at M II stage
0 M	30.8 ± 6.3^{a}
10^{-7} M	23.1 ± 2.7^{b}
10^{-6} M	17.8 ± 1.9^{bc}
10^{-5} M	10.4 ± 0.6^{c}
10^{-4} M	14.1 ± 5.1^{c}

The statistical differences are indicated at $P<0.05$

Different letters show the significant differences among groups

The mean number of COCs used in each treatment was 32

Data are expressed as means ± S.E.M. of three replicates

Comparisons of the percentages of M II stage between COCs and DOs were shown in Figure 4-2. In the control group and the SNAP treatment groups, there were no significant differences in the percentages of M II stage in COCs and DOs. Only SNAP at 10^{-5} M, COCs had percentages of M II significantly higher than DOs.

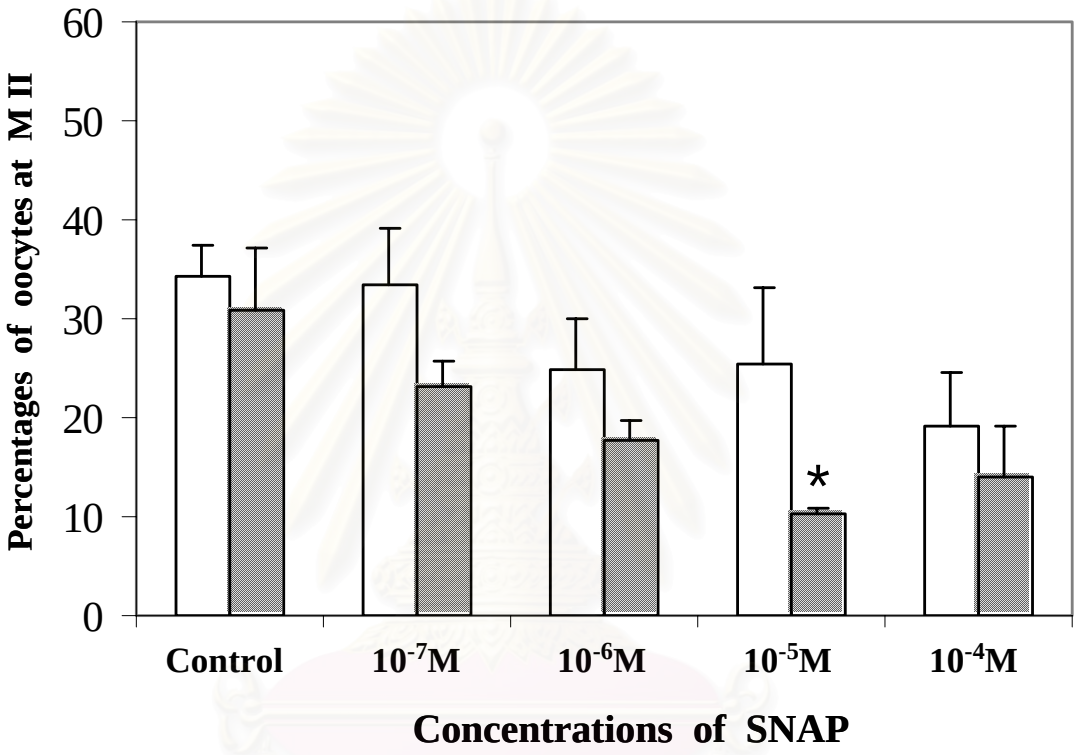


Figure 4-2 Comparisons of the effect of SNAP on the percentages of M II stage between COCs and DOs. COCs and DOs were cultured in either basal medium alone or supplemented with 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M SNAP for 24 h (Data from Table 4-3 and 4-4)

* significant difference compared with COCs at $p<0.05$

Experiment 2: The signaling pathway of NO

The effect of SNAP, SNAP with ODQ and ODQ on the percentages of GVBD stage in COCs and DOs

As shown in Experiment 1, SNAP had inhibitory effect on percentages of GVBD stage in both COCs and DOs. To determine the effect of SNAP, SNAP with ODQ, and ODQ on the percentages of GVBD stage in COCs, COCs were cultured in the basal medium alone or supplemented with either 10^{-4} M SNAP, combination of 10^{-4} M SNAP and 10 μM ODQ or 10 μM ODQ alone for 8 h. The results in COCs showed that the percentages of GVBD stage in SNAP, combination of SNAP and ODQ significantly decreased compared with control and ODQ alone (25.5 $\pm$ 3.2 and 54.9 $\pm$ 8.4 vs 74.6 $\pm$ 5.1 and 91.5 $\pm$ 7.6, respectively (Table 4-5).

Table 4-5 The percentages of COCs at GVBD stage after cultured in either basal medium alone or supplemented with 10^{-4} M SNAP, 10^{-4} M SNAP with 10 μM ODQ or 10 μM ODQ alone for 8 h

Treatments	Percentages of COCs at GVBD stage
Control	74.6 $\pm$ 5.1 [a]
10^{-4} M SNAP	25.5 $\pm$ 3.2 [b]
10^{-4} M SNAP + 10 μM ODQ	54.9 $\pm$ 8.4 [c]
10 μM ODQ	91.5 $\pm$ 7.6 [d]

The statistical differences are indicated at $P<0.05$

Different letters show the significant differences among groups

The mean number of COCs used in each treatment was 57

Data are expressed as means ± S.E.M. of three replicates

While DOs were cultured in basal medium alone or supplemented with either 10^{-4} M SNAP, combination of 10^{-4} M SNAP and 10 μM ODQ or 10 μM ODQ alone, the percentages of DOs at GVBD stage were 61.1 ± 10.7, 21.1 ± 2.7, 25.9 ± 8.2 and 66.9 ± 5.8, respectively. The results in DOs showed that the percentages of GVBD stage in groups of SNAP, combination of SNAP and ODQ significantly decreased compared with control and ODQ alone (21.1 ± 2.7, 25.9 ± 8.2 vs 61.1 ± 10.7 and 66.9 ± 5.8, respectively) (Table 4-6).

Table 4-6 The percentages of DOs at GVBD stage after cultured in either basal medium alone or supplemented with 10^{-4} M SNAP, 10^{-4} M SNAP with 10 μM ODQ or 10 μM ODQ alone for 8 h

Treatment	Percentages of DOs at GVBD stage
Control	61.1 ± 10.7 [a]
10^{-4} M SNAP	21.1 ± 2.7 [b]
10^{-4} M SNAP + 10 μM ODQ	25.9 ± 8.2 [b]
10 μM ODQ	66.9 ± 5.8 [a]

The statistical differences are indicated at $P<0.05$
Different letters show the significant differences among groups
The mean number of COCs used in each treatment was 52
Data are expressed as means ± S.E.M. of three replicates

Comparisons of the effect of SNAP, SNAP with ODQ or ODQ on the percentages of GVBD stage between COCs and DOs. The percentages of GVBD stage had no significant differences between COCs and DOs in control and SNAP group. In contrast, the combination of SNAP with ODQ or ODQ alone had the percentages of GVBD in COCs significantly higher than in DOs (Figure 4-3).

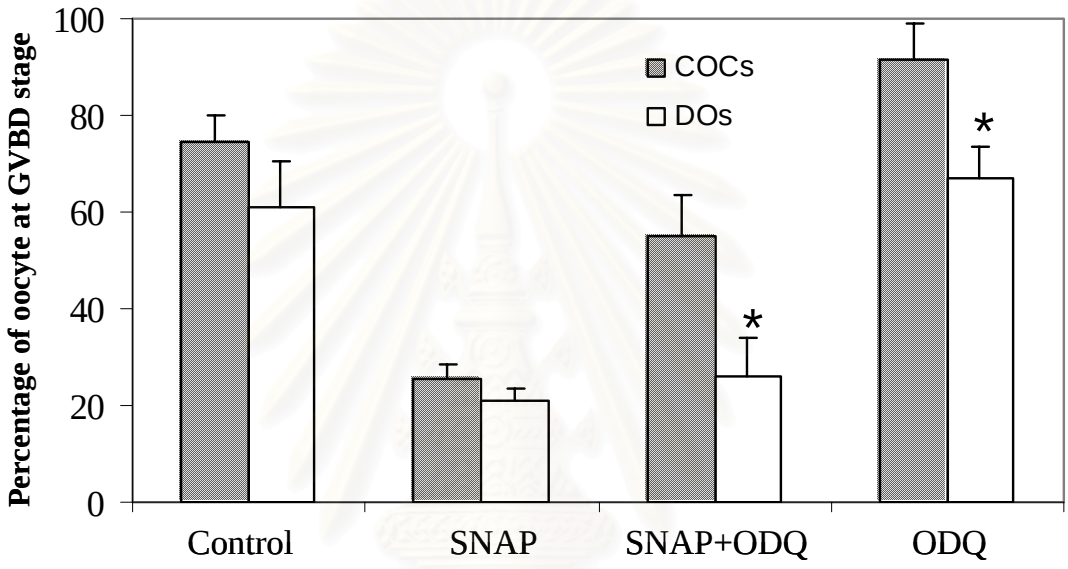


Figure 4-3 Comparisons of the effect of SNAP, SNAP with ODQ or ODQ alone on the percentages of GVBD stage between COCs and DOs. COCs and DOs were cultured in either basal medium alone or supplemented with 10^{-4} M SNAP, 10^{-4} M SNAP with 10 μM ODQ or 10 μM ODQ alone for 8 h (Data from Table 4-5 and 4-6)

* significant difference compared with COCs at $p<0.05$

The effect of SNAP, SNAP with ODQ and ODQ on intraoocyte cGMP concentrations in COCs and DOs

Intraoocyte cGMP concentrations were measured from COCs after cultured in either basal medium alone or supplemented with 10^{-4} M SNAP, 10^{-4} M SNAP with 10 μM ODQ or 10 μM ODQ alone for 8 h. The results showed significantly increased intraoocyte cGMP concentrations in SNAP, SNAP with ODQ compared with the control at $p<0.05$ (6.95 ± 1.34 and 4.58 ± 1.33 vs 2.15 ± 0.50 fmol/oocyte, respectively. There were no significant difference between ODQ and control (2.77 ± 0.98 vs 2.15 ± 0.50 fmol/oocyte, respectively (Table 4-7).

Table 4-7 Intraoocyte cGMP concentrations in COCs after cultured in either basal medium alone or supplemented with 10^{-4} M SNAP, 10^{-4} M SNAP with 10 μM ODQ or 10 μM ODQ alone for 8 h

Treatment	Intraoocyte cGMP concentrations (fmol/oocyte)
Control	2.15 ± 0.50^{a}
10^{-4} M SNAP	6.95 ± 1.34^{b}
10^{-4} M SNAP + 10 μM ODQ	4.58 ± 1.33^{c}
10 μM ODQ	2.77 ± 0.98^{a}

The statistical differences are indicated at $P<0.05$

Different letters show the significant differences among groups

The mean number of COCs used in each treatment was 60

Data are expressed as means ± S.E.M. of three replicates

Intraoocyte cGMP concentrations measured from DOs after cultured in either basal medium alone or supplemented with 10^{-4} M SNAP, 10^{-4} M SNAP with 10 μM ODQ or 10 μM ODQ alone for 8 h were 1.33 ± 0.59, 2.07 ± 0.56, 1.68 ± 0.41, and 1.90 ± 0.64 fmol/oocyte, respectively (Table 4-8). There were no significantly different in all treatment compared with the control.

Table 4-8 Intraoocyte cGMP concentrations in DOs after cultured in either basal medium alone or supplemented with 10^{-4} M SNAP, 10^{-4} M SNAP with 10 μM ODQ or 10 μM ODQ alone for 8 h

Treatment	Intraoocyte cGMP concentrations (fmol/oocyte)
Control	1.33 ± 0.59
10^{-4} M SNAP	2.07 ± 0.56
10^{-4} M SNAP + 10 μM ODQ	1.68 ± 0.41
10 μM ODQ	1.90 ± 0.64

There was no significantly different among groups at $P>0.05$
The mean number of DOs used in each treatment was 60
Data are expressed as means ± S.E.M. of three replicates

Comparisons of the effect of SNAP, SNAP with ODQ or ODQ on the intraoocyte cGMP concentrations between COCs and DOs. COCs and DOs were cultured in either basal medium alone or supplemented with 10^{-4} M SNAP, 10^{-4} M SNAP with 10 μM ODQ or 10 μM ODQ alone for 8 h. The COCs had intraoocyte cGMP concentrations significantly higher in SNAP and SNAP with ODQ (Figure 4-4).

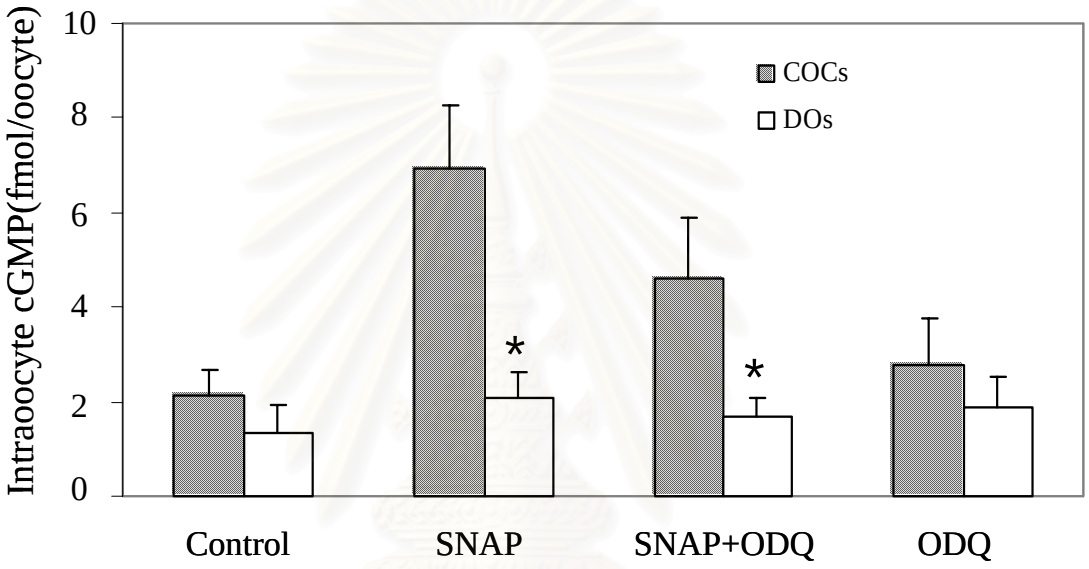


Figure 4-4 Comparisons of the effect of SNAP, SNAP with ODQ or ODQ on intraoocyte cGMP concentrations between COCs and DOs. COCs and DOs were cultured in either basal medium alone or supplemented with 10^{-4} M SNAP, 10^{-4} M SNAP with 10 μM ODQ or 10 μM ODQ alone for 8 h (Data from Table 4-7 and 4-8)

* significant difference compared with COCs at $p<0.05$

The effect of SNAP on intraoocyte cAMP concentrations in COCs and DOs at 0-12 h

During the course if *in vitro* maturation, intraoocyte cAMP concentrations measured from COCs after cultured in basal medium at 0, 2, 6 and 12 h of culture were 1.63 ± 0.24, 0.36 ± 0.04, 0.29 ± 0.16, and 0.44 ± 0.08 fmol/oocyte, respectively (Table 4-9). Intraoocyte cAMP concentrations significantly dropped during the first 2 h of culture and maintained at low level until 12 h of culture.

Table 4-9 Intraoocyte cAMP concentrations in COCs after cultured in basal medium for 0 – 12 h

Culture Time (h)	Intraoocyte cAMP concentrations (fmol/oocyte)
0	1.63 ± 0.24 [a]
2	0.36 ± 0.04 [b]
6	0.29 ± 0.16 [b]
12	0.44 ± 0.08 [b]

The statistical differences are indicated at $P<0.05$

Different letters show the significant differences among groups

The mean number of COCs used in each treatment was 150

Data are expressed as means ± S.E.M. of three replicates

The intraoocyte cAMP concentrations in DOs after cultured in basal medium at 0, 2, 6 and 12 h of culture were 1.13 $\pm$ 0.42, 0.49 $\pm$ 0.18, 0.33 $\pm$ 0.12, and 0.37 $\pm$ 0.05 fmol/oocyte, respectively (Table 4-10). Intraoocyte cAMP concentrations significantly dropped during the first 2 h of culture and maintained at low level until 12 h of culture.

Table 4-10 Intraoocyte cAMP concentrations in DOs after cultured in basal medium for 0 – 12 h

Culture Time (h)	Intraoocyte cAMP concentrations (fmol/oocyte)
0	1.13 ± 0.42^{a}
2	0.49 ± 0.18^{b}
6	0.33 ± 0.12^{b}
12	0.37 ± 0.05^{b}

The statistical differences are indicated at $P<0.05$

Different letters show the significant differences among groups

The mean number of DOs used in each treatment was 150

Data are expressed as means ± S.E.M. of three replicates

Intraoocyte cAMP concentrations measured from COCs after culture in basal medium supplemented with 10^{-4}M SNAP for 0, 2, 6 and 12 h of culture were 1.66 $\pm$ 0.35, 0.42 $\pm$ 0.06, 0.48 $\pm$ 0.22 and 0.39 $\pm$ 0.15 fmol/oocyte, respectively (Table 4-11). Intraoocyte cAMP concentrations significantly dropped during the first 2 h of culture and maintained at low level until 12 h of culture.

Table 4- 11 Intraoocyte cAMP concentrations in COCs after cultured in basal medium supplemented with 10^{-4}M SNAP for 0 – 12 h

Culture Time (h)	Intraoocyte cAMP concentrations (fmol/oocyte)
0	1.66 $\pm$ 0.35 [a]
2	0.42 $\pm$ 0.06 [b]
6	0.48 $\pm$ 0.22 [b]
12	0.39 $\pm$ 0.15 [b]

The statistical differences are indicated at $P<0.05$

Different letters show the significant differences among groups

The mean number of COCs used in each treatment was 150

Data are expressed as means ± S.E.M. of three replicates

Intraoocyte cAMP concentrations measured from DOs after culture in basal medium supplemented with 10^{-4}M SNAP for 0, 2, 6 and 12 h of culture were 1.87 $\pm$ 0.36, 0.48 $\pm$ 0.24, 0.35 $\pm$ 0.16, and 0.31 $\pm$ 0.11 fmol/oocyte, respectively (Table 4-12). Intraoocyte cAMP concentrations significantly dropped during the first 2 h of culture and maintained at low level until 12 h of culture.

Table 4-12 Intraoocyte cAMP concentrations in DOs after cultured in basal medium supplemented with 10^{-4}M SNAP for 0 – 12 h

Culture Time (h)	Intraoocyte cAMP concentrations (fmol/oocyte)
0	1.87 $\pm$ 0.36 [a]
2	0.48 $\pm$ 0.24 [b]
6	0.35 $\pm$ 0.16 [b]
12	0.31 $\pm$ 0.11 [b]

The statistical differences are indicated at $P<0.05$

Different letters show the significant differences among groups

The mean number of DOs used in each treatment was 150

Data are expressed as means ± S.E.M. of three replicates

Experiment 3: The effect of SNAP on the kinetics of nuclear maturation and cytoplasmic maturation

The effect of SNAP on the kinetics of nuclear maturation of COCs from 2-24 h

COCs were cultured in basal medium progressed to different stage of nuclear maturation. The times required for maximum percentages of GVBD, M I, ANA I, TEL I and M II stage were 8, 10-12, 18, 18-20 and 20-24 h, respectively (Table 4-13).

Table 4-13 The percentages of COCs progression in each nuclear maturation stage after cultured in basal medium for 24 h

Culture time	N	Percentages of COCs at each nuclear maturation stage					
		GV	GVBD	M I	ANA I	TEL I	M II
2	28	78.6	21.4	0.0	0.0	0.0	0.0
6	24	66.7	29.2	4.2	0.0	0.0	0.0
8	25	12.0	84.0	4.0	0.0	0.0	0.0
10	29	13.8	24.1	62.1	0.0	0.0	0.0
12	30	6.7	13.3	66.7	10.0	3.3	0.0
14	27	3.7	14.8	48.1	14.8	11.1	7.4
16	28	3.6	10.7	57.1	17.9	7.1	3.6
18	29	0.0	3.4	17.2	34.5	41.4	3.4
20	21	0.0	0.0	14.3	9.5	42.9	33.3
24	21	4.8	0.0	19.0	14.3	23.8	38.1

While COCs cultured in basal medium supplemented with 10^{-4}M SNAP were also progressed to different stages of nuclear maturation. The times required for maximum percentages of GVBD, M I, ANA I, TEL I and M II stage were 10, 12, 18, 24 and 24 h, respectively (Table 4-14). In addition, the percentages of COCs treated with SNAP at each stage were lower than the control.

Table 4-14 The percentages of COCs progression in each nuclear maturation stage after cultured in the basal medium supplemented with 10^{-4}M SNAP for 24 h

Culture time	N	Percentages of COCs at each nuclear maturation stage					
		GV	GVBD	M I	ANA I	TEL I	M II
2	29	86.2	13.8	0.0	0.0	0.0	0.0
6	29	86.2	10.3	3.4	0.0	0.0	0.0
8	28	69.0	27.6	3.4	0.0	0.0	0.0
10	29	10.3	41.4	41.4	3.4	3.4	0.0
12	27	18.5	22.2	48.1	11.1	0.0	0.0
14	24	20.8	16.7	37.5	16.7	8.3	0.0
16	28	7.1	14.3	46.4	25.0	7.1	0.0
18	28	7.1	7.1	14.3	35.7	35.7	0.0
20	27	18.5	29.6	11.1	7.4	29.6	3.7
24	27	11.1	3.7	14.8	18.5	37.0	14.8

The effect of SNAP on MPF activities

COCs were cultured in basal medium from 2 to 24 h, MPF activity of COCs started to significantly increase at 8 h of culture and reached maximum activity at about 8 to 10 h and then declined between 12 to 16 h and increased activity again at 18 h of culture and maintained high activity at 18 to 24 h (Figure 4- 5).

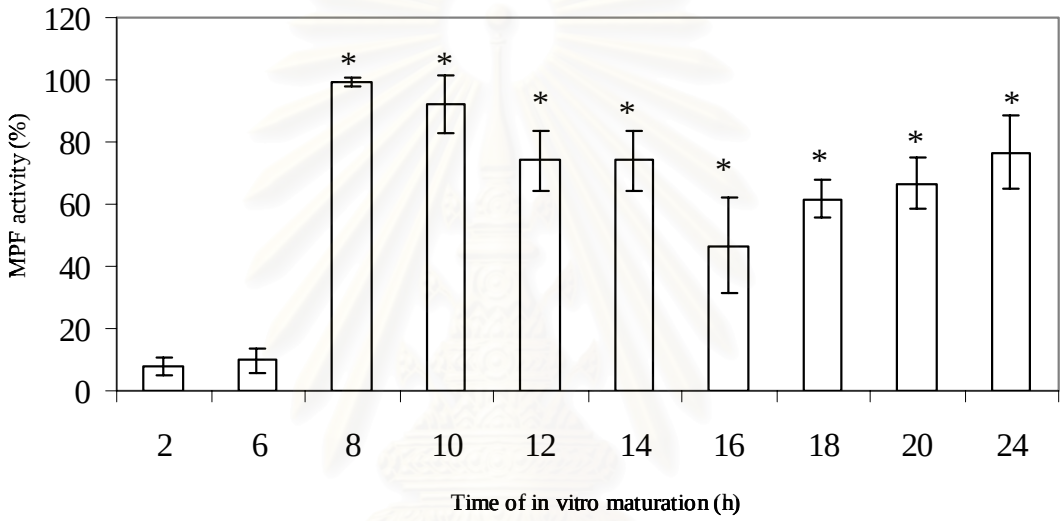


Figure 4-5 The MPF activity in COCs after cultured in basal medium for 24 h. COCs were collected at each time point and assayed for MPF activity after removal of cumulus cells. Eight COCs were used in each time point. Data are expressed as means ± S.E.M. of three replicates.

* significant difference compared with 2 h at $p<0.05$

In basal medium supplemented with 10^{-4}M SNAP, similar patterns of MPF activity was shown, MPF activity started to significantly increase at 8 h of culture and reached maximum activity at about 10 to 12 h and then declined between 14 to 18 h and increased activity again at 20 h of culture (Figure 4-6).

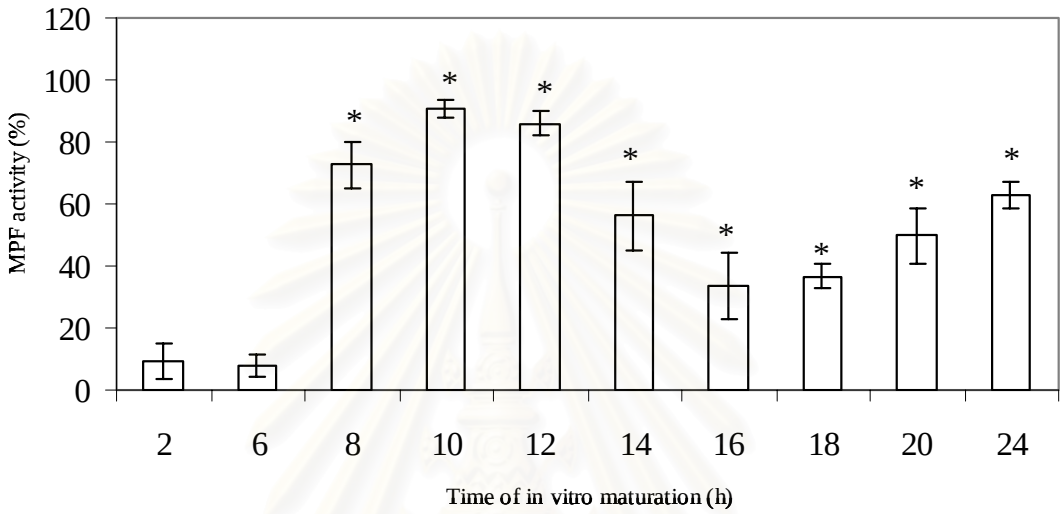


Figure 4-6 The MPF activity in COCs after cultured in basal medium supplemented with 10^{-4} M SNAP for 24 h. COCs were collected at each time point and assayed for MPF activity after removal of cumulus cells. Eight COCs were used in each time point. Data are expressed as means ± S.E.M. of three replicates.

* significant difference compared with 2 h at $p<0.05$

Comparisons of the effect of SNAP on MPF activity in COCs. The MPF activity significant higher in the control group at 8 and 18 h. However, the MPF activities in the control group were higher than the SNAP treatment at 14 to 24 h of culture (Figure 4-7).

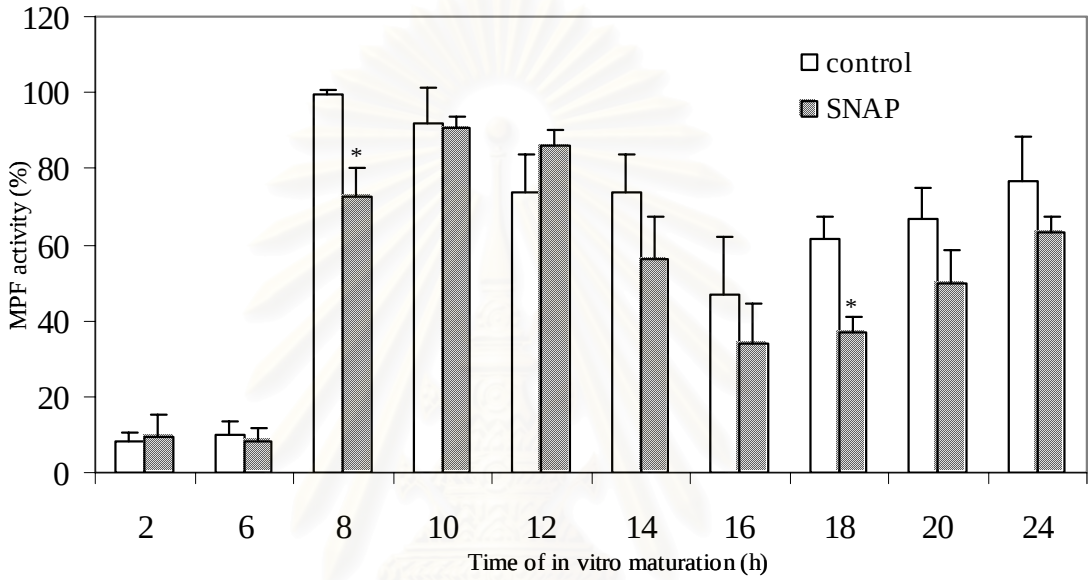


Figure 4-7 Comparisons of the effect of SNAP on MPF activity in COCs after cultured in basal medium alone or supplemented with 10^{-4} M SNAP for 24 h. COCs were collected at each time point and assayed for MPF activity after removal of cumulus cells. Eight COCs were used in each time point. Data are expressed as means ± S.E.M. of three replicates.

* significant difference compared with control at $p<0.05$

The effect of SNAP on MAPK activities

COCs were cultured in basal medium from 2 to 24 h, MAPK activity of COCs started to significantly increase at 8 h of culture and maintained high activity at 18 to 24 h (Figure 4-8).

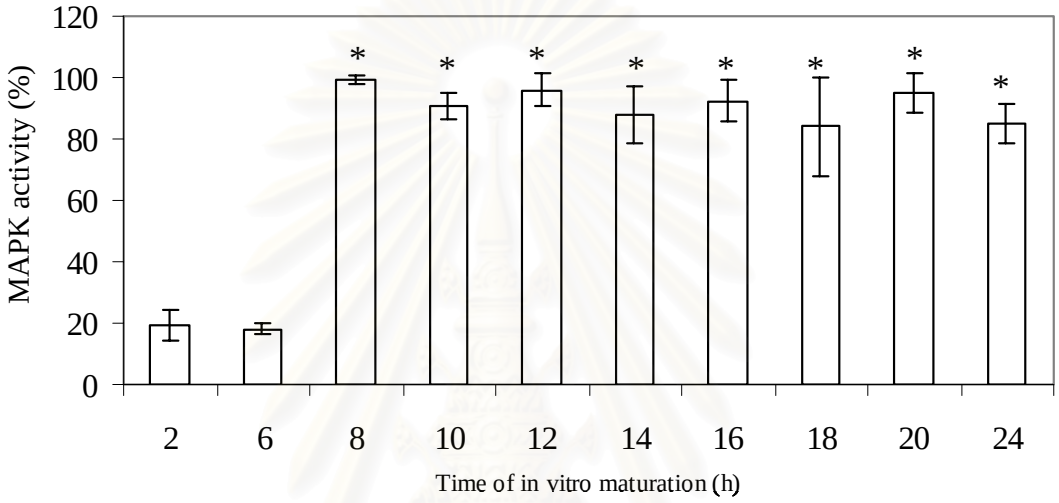


Figure 4-8 The MAPK activity in COCs after cultured in basal medium for 24 h. COCs were collected at each time point and assayed for MAPK activity after removal of cumulus cells. Eight COCs were used in each time point. Data are expressed as means ± S.E.M. of three replicates.

* significant difference compared with 2 h at $p<0.05$

In basal medium supplemented with 10^{-4} M SNAP, similar patterns of MAPK activity was shown, MAPK activity started to significantly increase at 8 h of culture and reached maximum activity at about 10 h and maintained high activity at 12 to 24 (Figure 4-9).

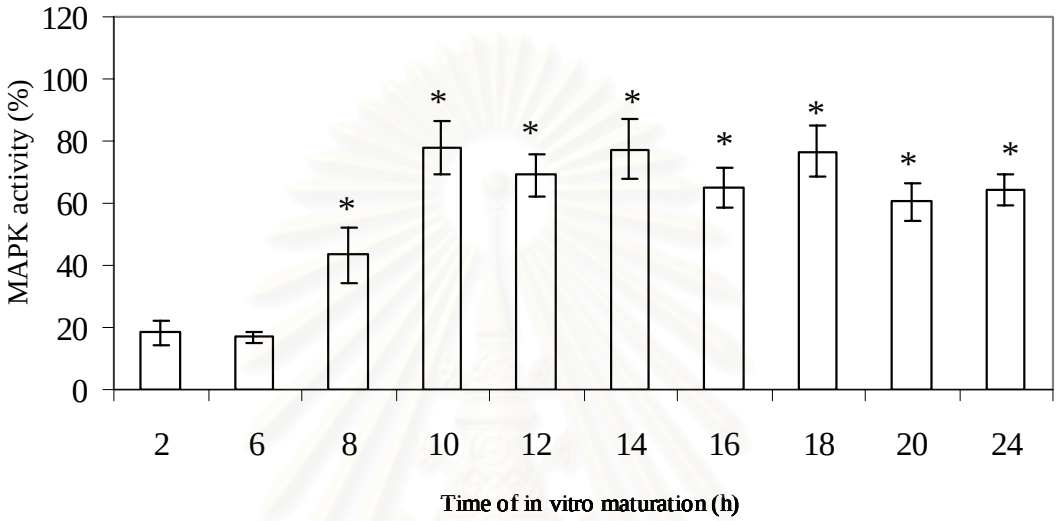


Figure 4-9 The MAPK activity COCs after cultured in basal medium supplemented with 10^{-4} M SNAP for 24 h. COCs were collected at each time point and assayed for MAPK activity after removal of cumulus cells. Eight COCs were used in each time point. Data are expressed as means ± S.E.M. of three replicates.

* significant difference compared with 2 h at $p<0.05$

Comparisons of the effect of SNAP on MAPK activity in COCs. The MAPK activity significantly higher in the control group at 8, 12, 16, 20 and 24 h. (Figure 4- 10).

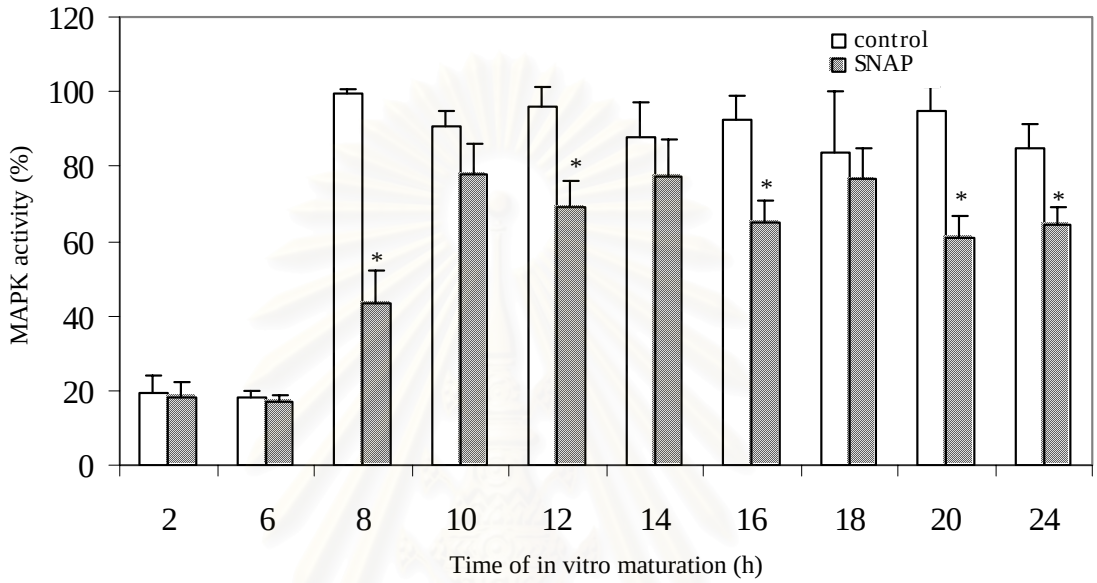


Figure 4-10 Comparisons of the effect of SNAP on MAPK activity in COCs after cultured in basal medium alone or supplement with 10^{-4} M SNAP for 24 h. COCs were collected at each time point and assayed for MAPK activity after removal of cumulus cells. Eight COCs were used in each time point. Data are expressed as means ± S.E.M. of three replicates.

* significant difference compared with control at $p<0.05$

The effect of SNAP on mitochondrial and cortical granule changes

Analysis of 3-5 randomly selected COCs obtained from the control and SNAP treated group. In both group, the ultrastructural features of the oocyte showed large numbers of microvilli were projected into the perivitelline space (PVS) after 24 h of culture. In the control group, the mitochondria were found mostly scattered, showed greater number than SNAP treated group. The cortical granules formed a line along the oolemma and were found the closer to the oolemma in both groups. However, some of SNAP treated showing a cluster of cortical granules and the numbers of cortical granules in the control were more numerous than the SNAP treated oocytes.

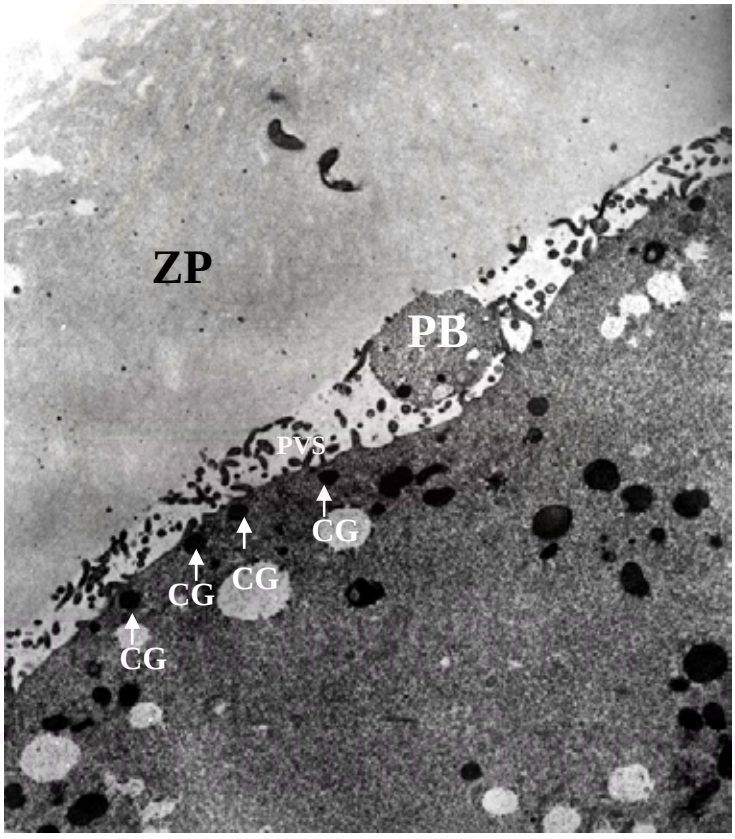


Figure 4-11 TEM micrograph of mature oocyte showing the first polar body (PB), zona pellucida (ZP), perivitelline space (PVS) and cortical granules(CG); (x 4,200: Bar = 1μm)

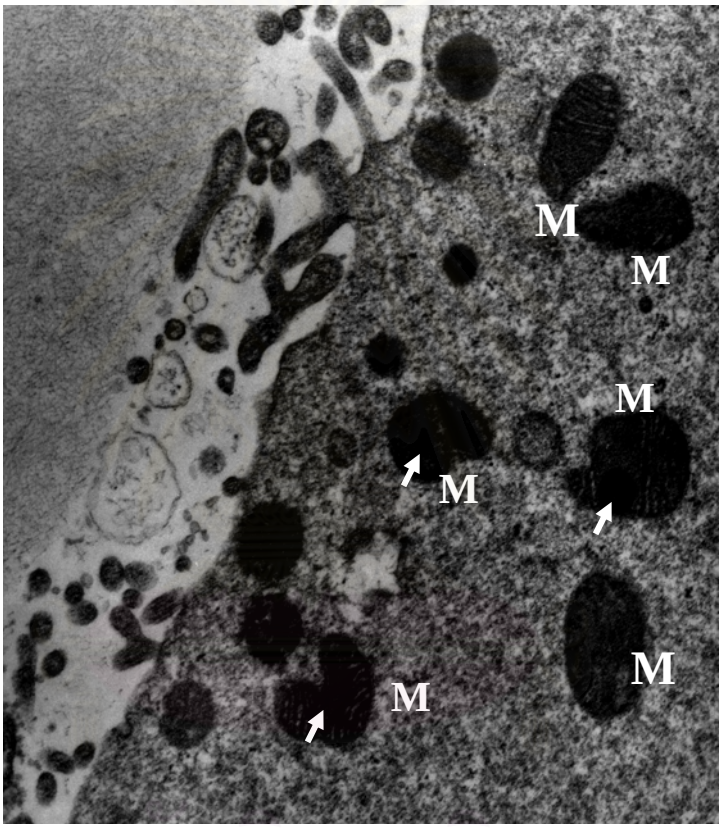


Figure 4-12 TEM micrograph of mature oocyte showing mitochondria (M) with electron dense matrix (arrow); (x 12,000; Bar = 0.5 μm)

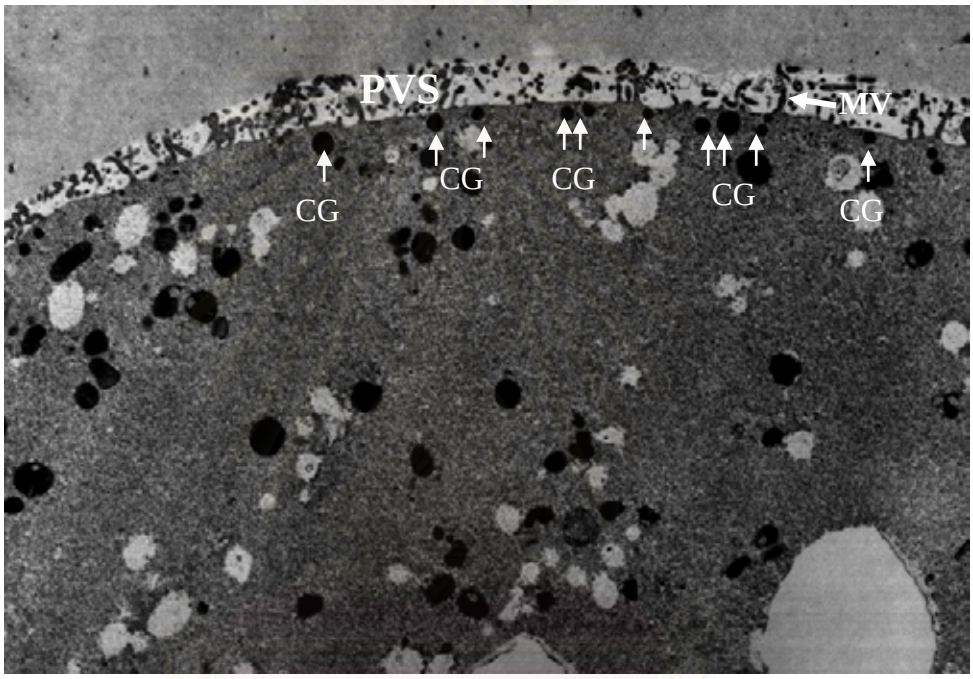


Figure 4-13 TEM micrograph of mature oocyte showing microvilli (MV) project to the perivitelline space (PVS) and electron dense cortical granules (CG) line along the oolemma; (x 4,200; Bar = 1 μm)

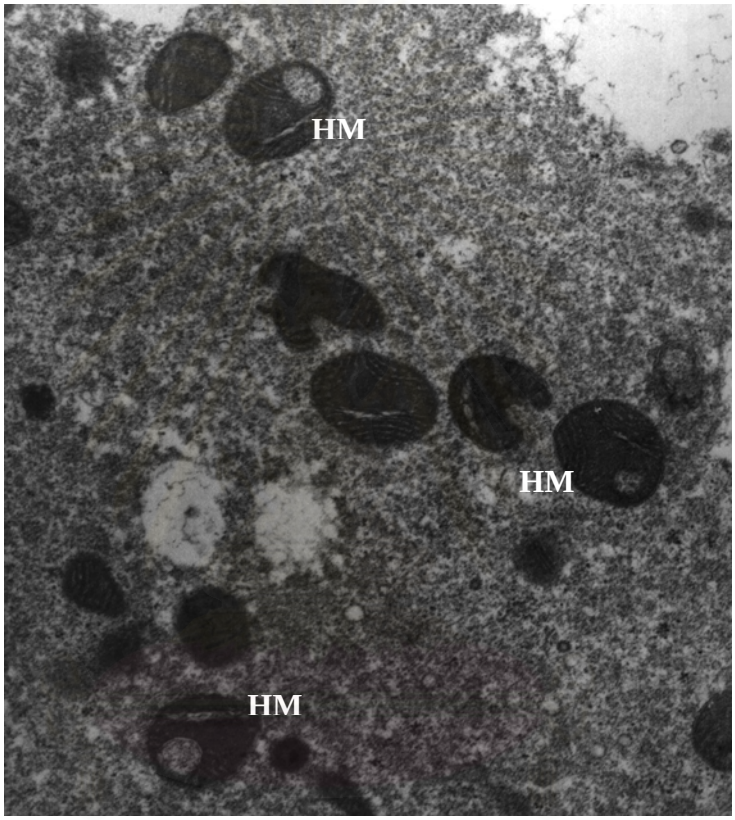


Figure 4-14 TEM micrograph of NO treated oocyte showing hooded mitochondria (HM); (x 10,000; Bar = 0.5 μm)

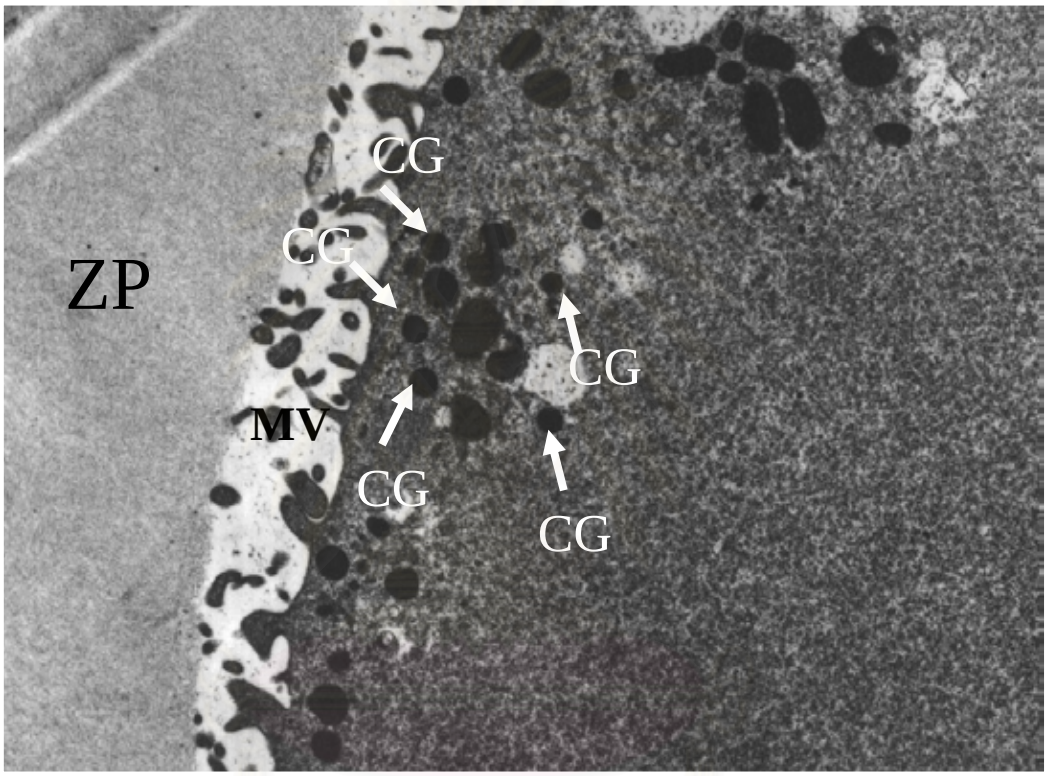


Figure 4-15 TEM micrograph of NO treated oocyte showing a cluster of cortical granules (CG) located near the periphery (x 4,200; Bar = 1 μm)

CHAPTER 5

DISCUSSION

The present study demonstrated that immature swamp buffalo oocytes cultured in M199 medium supplemented with 10% fetal bovine serum could undergo meiotic resumption and progression to GVBD and M II stage in 8 and 24 h. Supplementation with SNAP at 10^{-7} to 10^{-4} M decreased the maturation rate in the immature oocytes in both COCs and DOs. The percentages of GVBD in COCs and DOs after an incubation period of 8 h were significantly decreased compared with the control. The similar effects were also shown when the culture period was extended to 24 h, the percentages of M II stage were significantly decreased. It should be noted that in the study COCs and DOs cultured with SNAP were at the ANA I, TEL I and M II stage, indicating that they were in the process of nuclear maturation and not blocked at M I stage.

The inhibitory effect of SNAP on swamp buffalo oocyte meiotic maturation was a dose dependent manner. These findings were in good agreement with previous reports (Nakamura et al., 2002), where their data from *in vitro* studies provided evidence that SNAP dose-dependently inhibited GVBD in rat DOs. In addition, Goud et al. (2004) also showed the evidence that NO delayed oocyte aging, when the oocytes exposed to different concentrations of NO ($5 \times 10^{-6} - 3 \times 10^{-4}$ M SNAP); a significant diminution in ooplasmic microtubule dynamics was noted, significantly more oocytes exposed to NO had intact cortical granules and significantly lower zona pellucida dissolution time. These provided evidence that NO treated oocytes all eventually showed GVBD, they might require a significantly longer time to resume meiosis. From the present study, all concentrations of NO had only inhibitory effect on oocyte meiotic maturation which was inconsistent from Bu et al. (2003) who reported that NO exerted dual function (inhibiting or stimulating) dose-dependently on the mouse oocyte maturation; high concentrations of 10^{-3} M NO (sodium nitroprusside, SNP) inhibited first polar body extrusion of both COCs and DOs cultured in maturation medium. On the other hand, low concentrations of SNP (10^{-5} M) exhibited stimulatory effect on COCs in the presence of hypoxanthine, but at

this concentration NO had no effect on DOs. In the same way, 10^{-3} M SNP could complete prevention of GVBD in mouse oocytes for 5 h (Bu et al., 2004), in the present study NO could not completely block GVBD in swamp buffalo oocytes. The different effect of NO may depend on quality of the oocytes, species or the culture medium.

Low concentration of SNAP (10^{-7} M) had no significant effect on percentage of M II stage in COCs. On the other hand, this concentration had significant effect on the DOs. The results were similar to the previous report (Bu et al., 2003) which suggested that the inhibitory effect of SNAP on DOs was greater than that of COCs. The ability of NO to diffuse directly to the oocyte might allow it to remain higher activity than pass through the cumulus cells. The effect of cumulus cells on the maturation rate of COCs and DOs had been shown, although there was no significant effect but the percentages of GVBD and M II stage of COCs were higher than DOs. It is well known that the present of cumulus cells plays a very important role during oocyte growth and maturation. Cumulus cells are known to supply nutrients (Eppig,1982) and/or messenger molecules for the development of the oocyte (Buccione et al., 1990) and to mediate the effects of hormones on the COCs (Zuelke and Brackett, 1992). Previous study showed that the proportion of oocytes capable of *in vitro* maturation (IVM) was lower when cumulus cells were previously removed (Fukui and Sakuma, 1980; Zhang et al., 1995). In addition, the premature interruption of such communications was linked to low maturation (Luciano et al., 2004).

In the present study, NO was observed to inhibit oocyte meiotic maturation in spontaneous culture model in both COCs and DOs. The effect of NO was blocked by the ODQ which is a specific inhibitor of sGC (Garthwaite et al., 1995) in only COCs. The ODQ did not block the effect of NO in DOs. The result strongly supported the hypothesis that sGC was activated in the process of NO-mediated spontaneous oocyte resumption. However, regulation mechanism of NO on oocyte maturation might be complexes since the effect of ODQ on NO-inhibited oocyte meiotic maturation was different between COCs and DOs.

There are many mechanisms through which NO acts either intracellularly or in a paracrine fashion, diffusing through cell membranes. In several somatic cell systems, the effects of NO are mediated via activation of soluble guanylyl cyclase (sGC) and induction of cGMP synthesis. This intracellular transduction pathway is known to mediate the effects of NO, for instance, in vascular smooth muscle cell relaxation, platelet aggregation and neurotransmission (Beckman and Koppenol, 1996). A growing amount of experimental data indicated that NO induced its biological effects even via NO/cGMP-independent pathways (Gross and Wolin, 1995), for example, NO had an inhibitory action of NO on cytochrome P450 enzyme in granulosa cells (Van Voorhis et al., 1994) and hepatocytes (Stadler et al.,1994). However, the mechanisms through which NO influences oocyte maturation have only a few information.

Measurements of intraoocyte cGMP concentration showed that NO increased intraoocyte cGMP in COCs and ODQ partially reversed the inhibitory effect of NO in COCs, suggesting that the mechanism of action of NO involves activation of sGC in COCs, possibly cause to increase intraoocyte cGMP concentrations in cumulus cells.

Nitric oxide had no effect on intraoocyte cGMP production in DOs, thus intraoocyte cGMP increased in the oocyte was a result of cGMP diffusing from cumulus cells to the oocytes. The synthesis of cGMP is dependent on activity of guanylyl cyclase, which exists in both particulate and soluble forms. The particulate, membrane-associated forms as receptors, the enzyme activity of which is stimulated by binding of natriuretic peptides. In contrast, soluble guanylyl cyclase (sGC) is a heme-containing heterodimer, a major activator of sGC is NO, which binds the heme group of sGC and markedly stimulates activity of sGC, increasing cGMP production. Previous reports indicated that the treatment of cultured rat granulosa cells with NO donor or specific activator of sGC result in marked stimulation of cGMP production (Tafoya et al., 2004). The present results confirmed and extended these finding that NO donor stimulated cGMP production in the cumulus cells.

These observation suggested that NO/cGMP signaling pathway involved in NO-mediated oocyte maturation in COCs. Study of intraoocyte cGMP concentrations in the oocytes exposed to NO provided evidence for the source of intraoocyte cGMP in two sources: one, internally generated (within the oocyte), where levels were weakly stimulated by NO; and two, externally generated from cumulus cells, where levels were strongly stimulated by NO. In the present study, NO did not elevated the level of intraoocyte cGMP in DOs, possibly was due to an insufficient level of endogenous oocyte guanylyl cyclase activity.

The different effects of NO between COCs and DOs on spontaneous oocyte meiotic maturation suggested that cGMP was not the only signal molecule induced by NO and other signals might also be involved in the NO-mediated spontaneous oocyte maturation. Thus, NO might have NO/cGMP independent signaling pathway to inhibit spontaneous nuclear maturation in DOs.

The second messenger cGMP has an important role in the process of oocyte maturation. Tornell et al. (1990) reported that the levels of cGMP decreased in the oocyte during spontaneous maturation and microinjection of cGMP into isolated immature oocytes delayed the spontaneous maturation. The inhibitory action of cGMP on spontaneous maturation of hamster oocytes was dose and time dependent manner (Hubbard and Terranova, 1982). Cyclic GMP or 8-Br-cGMP, a cell permeating cGMP analogue, demonstrated a significant inhibitory effect on oocyte maturation in many species (Hubbard and Terranova, 1982; Meijer et al., 1989; Tornell et al., 1990; Bu et al., 2004).

Cyclic GMP may maintain meiotic arrest via the pathway involving sustenance in cAMP levels by inhibition of oocyte cAMP phosphodiesterase. An increase in cGMP could evoke a concomitant increase in cAMP by inhibiting its phosphodiesterase-3 (PDE3)-catalyzed hydrolysis to AMP in human platelets, vascular smooth muscle cells (Maurice and Haslam, 1990; Aizawa et al., 2003; Maurice, 2005) and sperm (Revelli et al., 2002). In addition, the inhibitor of PDE3 could prevent the oocyte meiotic resumption of mouse (Shitsukawa et al., 2001), rat (Richard et al., 2001), bovine (Mayes and Sirard, 2002; Thomas et al., 2004b), and macaque (Jensen et al., 2002).

It has also been reported that other pathway of NO may also directly modulate oocyte maturation in a cGMP-independent manner, such as, presumably through its ability to directly influence the activity of proteins, as has been demonstrated that NO inhibited cell cycle progression by inhibiting transcription of cyclin gene (Guo et al.,1998).

In addition, cGMP also maintained the meiotic arrest of the oocyte via another pathway: direct activating of cGMP-dependent kinase (protein kinase G, PKG) in the oocytes. Cyclic GMP/PKG signaling pathway has been reported to be involved in porcine oocyte maturation (Zhang et al., 2005).

The present study showed that swamp buffalo intraoocyte cAMP significantly dropped at 2 h of culture. This finding was consistent with previous reports that a drop in intraoocyte cAMP concentration resulted in meiotic resumption of mammalian oocytes (Vivarelli et al., 1983; Dekel et al., 1988; Yoshimura et al., 1992). In bovine, in the absence of hormonal stimulation, intraoocyte cAMP decreased after 3 h of culture (Luciano et al., 2004). A decrease in cAMP concentration in the oocyte has been associated with the resumption of meiosis and high levels of cAMP presents in oocytes resulted in meiotic arrest (Downs et al., 1989). Bilodeau et al. (1993) also reported that in bovine oocyte that high level of cAMP in the cumulus cells that surround the oocyte induced meiotic resumption, while high levels of cAMP inside the oocyte resulted in meiotic arrest. An intricate network of gap junction transmembrane channels facilitated direct communication and transfer of cAMP between the cumulus cells and the oocyte (Isobe et al., 1998). Gap junctions connect the cumulus cells to each other, and the innermost layer of cumulus cells surrounding the oocyte extend cytoplasmic processes through the zona pellucida, forming gap junctions with the oolemma. The level of communication between the two cell types dramatically decreased upon release of the COCs from the follicular environment, and the onset of GVBD occurred when the level of gap junctional communication had dropped to 40% of that initially, supporting the idea that GVBD occurred post- gap junctional communication breakdown (Thomas et al., 2004a).

There is a general consensus that a transient decrease in the cAMP level is an obligatory first step in the induction of oocyte maturation. The amount of cAMP present in the oocytes or cumulus cells depends primarily on the rate of synthesis by adenylyl cyclase and the rate of degradation by phosphodiesterases (PDE) that hydrolyze the 3' phosphorester bond of the 3', 5' purine ribose cyclic monophosphate cAMP and cGMP to generate AMP and GMP. A drop in intracellular cAMP levels followed by the decrease of the cAMP-dependent kinase A (PKA) activity is associated with resumption of meiosis. GVBD was blocked by addition of dibutyryl cyclic AMP (dbcAMP), a membrane-permeable cAMP analogue, isobutyl-methyl-xanthine (IBMX), a phosphodiesterase inhibitor, or forskolin, an adenylyl cyclase activator. Mechanism initiates GVBD after inactivation of PKA is still not clear. However, the decrease in intraoocyte cAMP is believed to cause a decline in the activity of cAMP dependent protein kinase A (PKA) and this in turn dephosphorylate and is associated with the activation of MPF activity (Matten et al., 1994).

Raising the levels of intraoocyte cGMP in COCs by NO, this effect did not maintain intraoocyte cAMP levels in the present study. The results were not in agreement with that of Bu et al. (2004) who reported that arresting effect of NO on GVBD mimicked that of the two cAMP-elevating reagents, forskolin (a specific stimulator of adenylyl cyclase) and rolipram (a specific pharmacological inhibitor of the cAMP-dependent PDE), especially to the effect of forskolin. However, cGMP decreased cAMP in *in vivo* study, FSH stimulated a rise in both cAMP and cGMP in COCs. While the increased in cAMP may be the initial meiotic trigger, cGMP may serve to subsequently lower cAMP by activating cAMP-PDE and thus permit the maturational process to continue (Hubbard and Price, 1988).

In a variety of cell types, NO has been reportedly that it altered the activity of adenylyl cyclase. The sum of the data indicated that NO specifically altered either a regulatory domain of the adenylyl cyclase itself or a distinct regulatory moiety. The effects of NO could be mediated by its inhibitory effect on cAMP production in neuroblastoma cell line (Tao et al., 1998). NO inhibitable isoforms of adenylate cyclase were expressed in mouse and human secretory colonic epithelia (Freeman and MacNaughton, 2004) and NO production decreased cAMP accumulation largely by

the cGMP-mediated activation of PDE2 (Gustafsson and Brunton, 2002), functionally linking the cAMP and NO pathways. However, in the present study NO stimulated intraoocyte cGMP production did not overcome the reduction of intraoocyte cAMP, thus the role of NO in delay meiotic maturation in swamp buffalo oocyte should not be via NO/cGMP/cAMP signaling pathway. Whereas, NO did not maintain intraoocyte cAMP concentration, but NO/cGMP might activate PKA to delay meiotic resumption. Because PKA contain specific cyclic nucleotide binding domains with significant homology to PKG, PKA might be activated by cGMP, although with a 50-fold lower selectivity than cAMP. Although the nucleotide binding sites of PKA and PKG are homologous (Pfeifer et al., 1999), differences between these sites exist, specifically in the substitution of key amino acid residues (Lohmann et al., 1997). NO/cGMP mediated PKA activation has been reported in smooth muscle cells (Cornwell et al., 1994; Algara-Suarez and Espinosa-Tanguma, 2004).

The effect of NO on cytoplasmic maturation was also studied. Cytoplasmic maturation is regulated by kinases involved in the initiation and progression of meiosis. The activation of MPF and MAPK, which share numerous substrates including proteins involved in nuclear membrane formation, chromatin condensation, and spindle assembly, results in resumption, progression, and arrest of meiosis at M II.

The present study showed that swamp buffalo oocytes incubated in M199 supplemented with 10 % fetal bovine serum exhibited GVBD at 8 h, and reach M I after 10-12 h, with the ANA I – TEL I transition occurring between 18 – 20 h and reached M II at 20-24 h. While, MPF activity started to increase at 8 h of culture and reached maximum activity at about 8 to 14 h and then declined between 16 to 18 h and increased activity again at 20-24 h. These observations, like other mammalian oocytes, the fluctuation of MPF activity matched well with oocyte nuclear progression reportedly previously. Increasing MPF activity was associated with GVBD in mouse oocytes (Hashimoto and Kishimoto, 1986), porcine oocytes (Meinecke and Krischek 2003), and bovine oocytes (Wehrend and Meinecke, 2001). After GVBD, a further increase of MPF activity is required for meiotic progression beyond the M I stage (Naito and Toyoda, 1991). Increasing MPF activity stimulates the ubiquitin proteolysis pathway that induces degradation of cyclin B, which triggers

a decrease in MPF activity; the reduction of its activity triggers the metaphase-anaphase transition (Hashimoto and Kishimoto, 1986).

MPF is a protein kinase whose activity triggers a series of reactions ultimately leading to nuclear membrane breakdown and chromosome condensation (Hunt, 1989). MPF is known to be a heterodimer, composed of two subunits: $p34^{cdc2}$ and cyclin B. Cyclin B is the regulatory component and $p34^{cdc2}$ is the catalytic component of MPF. The activation of MPF requires 1) new protein synthesis: the formation of the $p34^{cdc2}$-cyclin B complex, 2) phosphorylation of $p34^{cdc2}$ at T14, Y15 and T161, and subsequent dephosphorylation of $p34^{cdc2}$ at T14, Y15. Interference in any of these processes will inhibit MPF activation, prevent resumption of meiosis, and hold oocyte at the GV stage. The elevation of intraoocyte cAMP levels by treatment with dbcAMP (Funahashi et al., 1997) or hypoxanthine (Miyano et al., 1995), an inhibitor of phosphodiesterase, keep MPF in a hyperphosphorylated inactive form. Nonspecific inhibition of protein synthesis by cycloheximide has been shown to prevent activation of MPF (Meinecke et al., 2001). Nonspecific inhibition of protein phosphorylation with 6-dimethylaminopurine treatment also inhibited MPF activation (Fulka et al., 1991).

It has been shown that NO delayed GVBD in swamp buffalo oocyte. Furthermore, in the present study, NO has been shown to decrease the MPF activity at the time of GVBD but had no effect on the later stage of nuclear maturation. Previous study showed that the activation of MPF requires new protein synthesis, phosphorylation and subsequent dephosphorylation. Bovine oocytes required the synthesis of new protein during the first 6 h of culture to resume meiosis (Tatemoto and Horiuchi, 1995) and Cyclin B1 is required for autoamplification of MPF activity and meiotic resumption (Levesque and Sirard, 1996). NO might effect directly on the protein synthesis since it has been shown that NO acted as antiproliferative substance in a variety of cell types.

The present study showed that MAPK activity was activated around the time of GVBD and kept high activity through M II stage. The activation of MPF and MAPK activities appeared to occur simultaneously; their catalytic activities were low

during the first 6 h of culture and then increased around the time of GVBD. The similar pattern was shown in cattle (Fissore et al., 1996).

Mitogen-activated protein kinase belongs to a group of serine/threonine protein kinases. The most widely studied MAPK, the ubiquitously expressed 44-kDa and 42-kDa MAPK isoforms (ERK1 and ERK2, respectively), are rapidly activated in response to various growth factors and tumor promoters in mammalian cultured cells. It has been reported that an activation of MAPK occurred during the meiotic maturation of oocytes in several species, including marine invertebrates (Shibuya et al., 1992), *Xenopus* (Gotoh et al., 1995) and mammals (Verlhac et al., 1993; Fissore et al., 1996). Inoue et al. (1998) reported that in porcine oocytes, 1) inactive MAPK localized in the cytosol of immature GV oocytes, 2) part of the activated MAPK translocated into the GV just before GVBD, and 3) exogenous MAPK maintained its activity level in the GV and induced GVBD, indicating that MAPK mediated the maturation inducing signal from the cytoplasm into the nucleus and induced meiosis reinitiation. Furthermore, microtubule organization and chromatin condensation correlated with MAPK activity (Verlhac et al., 1994). MAPK acquires an active protein synthesis (Araki et al., 1996; Inoue et al., 1996; Verlhac et al., 1996) and is activated by a kinase cascade that includes several upstream activators; one of them is the product of the proto-oncogene mos. In bovine oocytes, injection of mos RNA elicited a rapid maximal activation of MAPK that resulted in accelerated resumption of meiosis and GVBD (Fissore et al., 1996). In contrary, Ohashi et al. (2003) reported that MAPK activation was not required for GVBD induction in porcine oocytes and that the major roles of MAPK during porcine oocyte maturation were to promote GVBD by increasing MPF activity and to arrest oocytes at M II stage. The relationship between MAPK and cAMP were probed, and the result showed that cAMP blocked GVBD by inhibiting MPF activation rather than inhibiting MAPK because MAPK activation was not the prerequisite of spontaneous meiotic maturation in mammalian oocytes (Sun et al., 1999).

In this study, NO delayed MAPK activity and suppressed it throughout the period of culture. However, the nuclear maturation was still capable in progressing to M II stage. The result was consistent with previous studies which showed that in

mammals the possible role of MAPK in MPF activation was ruled out by the observation that oocytes derived from mos knockout mice, which were unable to activate MAPK, displayed a normal pattern of MPF activation. Oocytes derived from these mutant mice were as expected, unable to activate MAPK, but they were able to proceed normally through early meiotic events, displaying a normal pattern of activation. The only default of these oocytes was their inability to arrest at M II; they underwent parthenogenic activation in the absence of sperm (Araki et al., 1996; Choi et al., 1996b). In consistency, pharmacological inhibitor of MAPK did not prevent the oocyte from the completing the first round of meiosis and emitting first polar body but did impair the ability to arrest at M II (Josefsberg et al., 2003). The effect of NO during the activation of mammalian oocytes was shown that NO was able to induce parthenogenetic activation of pig oocytes matured *in vitro*, however, it did not induce the exocytosis of cortical granules (Petr et al., 2005).

The ultrastructural of swamp buffalo oocytes showed that after 24 h of culture, the oocytes had possessed microvilli projected into a perivitelline space, intercellular communication between the cumulus cells and the oocyte was lost, cumulus cells projections had been retracted and a polar body was extruded, mitochondria distributed throughout the cytoplasm and cortical granules line along the oolemma. The present study showed that in control oocytes, mitochondria were distributed throughout the cytoplasm with active mitochondria showed electron dense matrix. Nitric oxide treated oocytes showed more immature mitochondria (poorly developed, peripheral cristae or a hood appearance) (Crosier et al., 2001). Previous study in pig oocytes indicated that 1) oocyte maturation, fertilization and early embryo development in pigs were associated with changes in active mitochondria distribution 2) mitochondria translocation was mediated by microtubules, but not by microfilaments and 3) incomplete movement of mitochondria to the inner cytoplasm and thus affect the cytoplasm maturation (Sun et al., 2001). In vivo study also indicated that there was a relationship between meiotic progression, cumulus expansion, mitochondria redistribution and their oxidative activity during final preovulatory maturation in pig oocytes (Torner et al., 2004).

Nitric oxide reduced the number of cortical granules forming a line along the oolemma were reduced and some of a cluster of cortical granules located near the periphery was also shown. This characteristic was similar to that in bovine immature oocytes (Hyttle et al., 1997). Incubation immature bovine oocytes in butyrolactone-I (BL-I) or roscovidine (ROS) which were selective inhibitors of the cyclin-dependent kinases caused degeneration of the cortical granules, effectively reducing the population of cortical granules (Lonergan et al., 2003). These appearances indicated that NO also delayed cytoplasmic maturation.

Nitric oxide delayed nuclear and cytoplasmic maturation of swamp buffalo oocytes may occur through several mechanisms. Previous study reported that NO inhibited the activity of cyclin dependent kinase and reduced mRNA levels of cyclin E and cyclin A in vascular smooth muscle cells (Ishida et al., 1997). NO induced cell cycle arrest in human breast cancer cells by down regulation of cyclin D1 and hypophosphorylation of retinoblastoma protein (Pervin et al., 2001). This action of NO in inhibiting protein synthesis was found to be guanylyl cyclase-dependent (Kim et al., 1998). Therefore, it is possible that NO may inhibit protein synthesis in the oocytes. In addition, NO generated from an NO donor resulted in p53 protein accumulation (Forrester et al., 1996) and the p53 played a role in the antiproliferative function of NO (Hemish et al., 2003). Increased expression of p53 is known to inhibit cyclin/cyclin dependent kinase complexes, thereby inhibiting cell cycle progression. Nitric oxide is known to signal through a cGMP-protein kinase A (PKA)-p53 pathway in aortic smooth muscle (D'Souza et al., 2003). Furthermore, NO has been shown to regulate mitochondrial respiration by competing with oxygen for mitochondrial cytochrome c oxidase, resulting in a reduce oxygen consumption (Brown and Cooper, 1994). The low level of oxygen consumed may possibly reflect a reduction in protein synthesis. Manser et al. (2004) proposed the role of NO in mouse preimplantation metabolism and development. One possibility was that NO might limit oxygen consumption at the blastocyst stage at the level of mitochondrial cytochrome c oxidase. NO is known to have an inhibitory effect on cell respiration of different cell types, including vascular endothelial cells (Beltran et al., 2000).

The major findings of the present study were the NO delayed both nuclear and cytoplasmic maturation of swamp buffalo oocytes via NO/cGMP dependent and independent signaling pathway. The oocyte maintained in meiotic arrest had beneficial effect in promoting development competence (Guixue et al., 2001). Furthermore, the oocytes from superior breeder could be transported conveniently in the meiotic arrested medium in stead of preservation in the cryoprotectants which are toxic to the oocytes.

The proposed pathway of NO mechanism of action was shown in Figure 5-1.

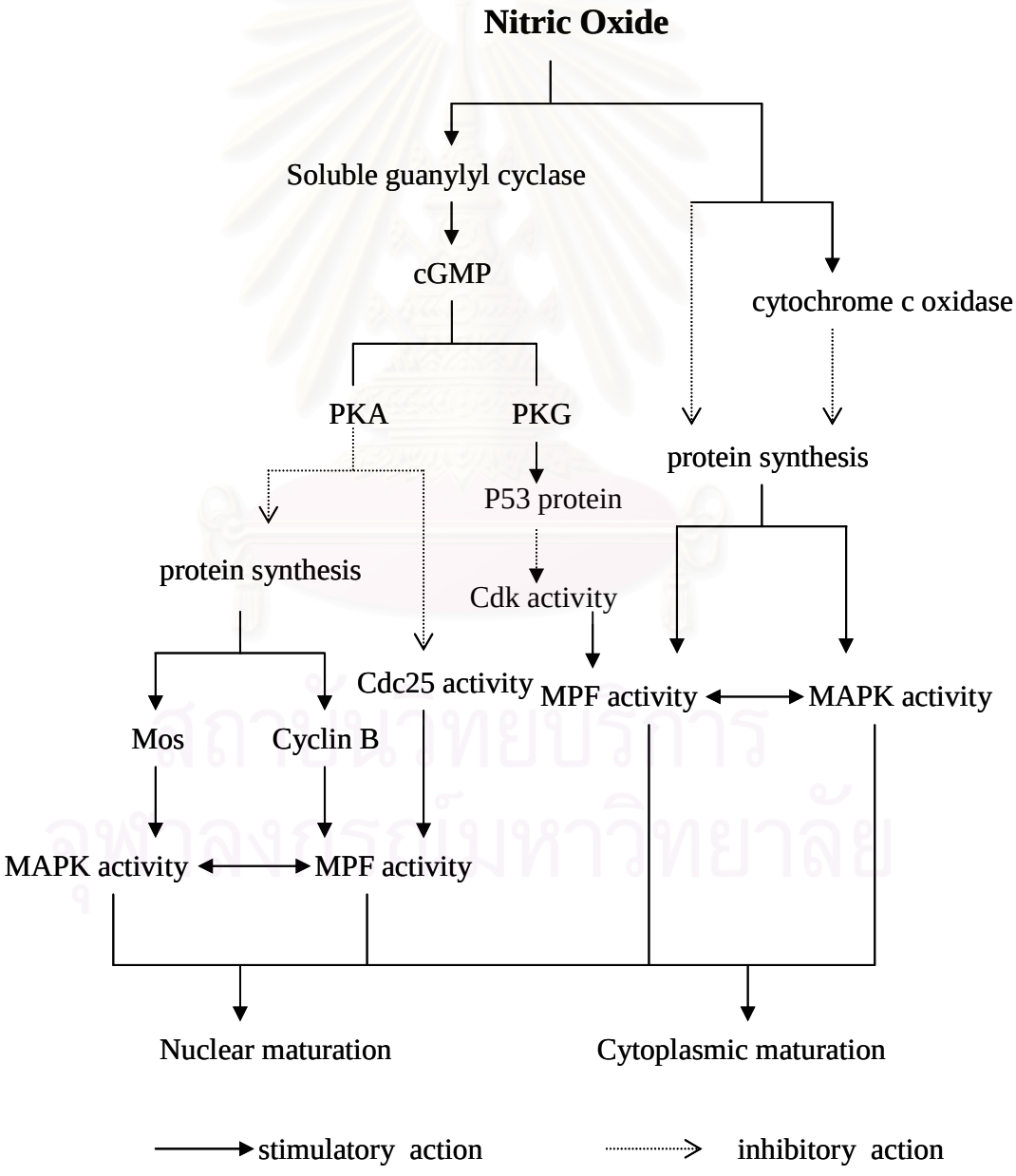


Figure 5-1 The proposed pathway of NO mechanism of action

CHAPTER 6

CONCLUSION

1. Nitric oxide played a role in the delay of oocyte maturation in both COCs and DOs. The inhibitory effect of NO on DOs was greater than that of COCs.
2. Nitric oxide delayed oocyte maturation via cGMP dependent signaling pathway and cGMP independent signaling pathway.
3. The inhibitory effect of NO on oocyte maturation did not seem to be mediated by cAMP.
4. MPF and MAPK activities occurred simultaneously around the time of GVBD in swamp buffalo oocyte.
5. Nitric oxide delayed cytoplasmic maturation in swamp buffalo oocyte. MPF and MAPK activities occurred simultaneously around the time of GVBD and were delayed in NO treated oocytes.
6. Mitochondria and cortical granules in NO treated oocytes were not capable in progressing to maturation stage at 24 h of culture.

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APPENDIX

APPENDIX

Buffers and Reagents

Preparation of stock solution for collection buffer

0.64 M EDTA

EDTA	2.382	g
DW	10	ml

1 M NaFl

NaFl	0.420	g
DW	10	ml

1 M Na_3VO_4

Na_3VO_4	1.839	g
DW	10	ml

0.1 M Phosphate Buffer Saline (PBS), pH 7.4

Na_2HPO_4	11.500	g
KH_2PO_4	2.584	g
NaCl	8	g
DW	1000	ml

Prepation of Collection Buffer

EDTA	100	µl
NaF	100	µl
Na_3VO_4	100	µl
PBS	9.7	ml

Preparation of stock solution for homogeneous buffer

4.5 M ß-glycerophosphate

ß-glycerophosphate	9.720	g
DW	10	ml

1.2 M *p*-nitrophenylphosphate

p-nitrophenylphosphate	4.346	g
DW	10	ml

2.0 M 3-[*N*-morpholino]-propanesulfonic acid (MOPS), pH 7.2

MOPS	4.186	g
DW	10	ml

2.4 M $MgCl_2$

$MgCl_2$	1.789	g
DW	10	ml

1.2 M ethylene glycol-*bis* (ß-aminoethylether) *N*,*N*,*N*1,*N*1-tetra-acetic acid (EGTA)

EGTA	0.56	g
DW	10	ml

4.0 M Dithiothreitol (DTT)

DTT	0.617	g
DW	10	ml

0.1 M Na_3VO_4

Na_3VO_4	0.184	g
DW	10	ml

Preparation of homogeneous buffer

ß-glycerophosphate	50	µl
p-nitrophenylphosphate	50	µl
MOPS	50	µl
$MgCl_2$	25	µl
EGTA	500	µl
DTT	10	µl
Na_3VO_4	5	µl
Leupeptin (5 mg/ml)	20	µl
Aprotinin (5 mg/ml)	40	µl
PBS	4.25	ml

Preparation of stock solution for kinase buffer

histone H1

histone H1	25	mg
DW	2.5	ml

Myelin Basic Protein

Myelin Basic Protein	10	mg
DW	500	µl

Protein kinase inhibitor

Protein kinase inhibitor	0.1	mg
DW	100	µl

Preparation of Kinase Buffer

histone H1	10	µl
Myelin Basic Protein	10	µl
Protein kinase inhibitor	1	µl
250 µCi/ml [γ-^{32}P]ATP	5	µl
Homogeneous Buffer	74	µl

SDS-PAGE

Preparation of SDS-PAGE reagents

1.5 M Tris, pH 8.8

Trizma Base	18.165	g
DW	100	ml

0.5 M Tris, pH 6.8

Trizma Base	6.055	g
DW	100	ml

10% SDS

SDS	10	g
DW	100	ml

30% N,N-Methylene-bis-acrylamide

acrylamide	60	g
bis-acrylamide	1.6	g
DW	200	ml

Resolving gel (10%)

1.5 M Tris pH 8.8	2.5	ml
10% SDS	0.1	ml
30% N,N-Methylene-bis-acrylamide	3.33	ml
DW	4.04	ml
TEMED (N,N,N',N'-tetramethylene-ethylenediamine)	5	µl
Ammonium persulphate (0.1 g/ml)	30	µl

Stacking gel (3%)

0.5 M Tris pH 6.8	1.25	ml
10% SDS	50	µl
30% N,N-Methylene-bis-acrylamide-acrylamide	0.5	ml
DW	3.2	ml
TEMED(N,N,N',N'-tetramethylene-ethylenediamine)	5	µl
Ammonium persulphate (0.1 g/ml)	50	µl

Sample buffer (dye marker)

Tris-HCL	0.0985	g
SDS	0.4	g
Glycerol	1	ml
2-Mercaptoethanol	0.5	g
Bromphenol blue	10	mg

Adjust to pH 6.8 using 1 N NaOH and add DW to 10 ml

Electrophosis buffer (running buffer)

Tris-base	1.2	g
Glycine	5.76	g
SDS	0.4	g
DW	400	ml

Coomassie Staining

Stain

Coomassie Brilliant Blue R	1	g
Acetic acid	35	ml
Methanol	250	ml
DW	215	ml

Destain

Acetic acid	35	ml
Methanol	125	ml
Glycerol	25	ml
DW	315	ml

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