INSULIN-LIKE GROWTH FACTOR-I PROMOTES MATURATION RATE OF THE DOMESTIC CAT OOCYTES *IN VITRO*

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The objectives of the present study were to determine the influence of insulin-like growth factor-I (IGF-I) on domestic cat oocyte maturation *in vitro* and to identify the optimal time for the *in vitro* maturation (IVM). In experiment I, a total of 879 oocytes were obtained from cats subjected to ovariohysterectomy and were classified into 4 grades using the criteria of the complement of cumulus cells and the morphology of oocyte cytoplasm: grade 1 with tightly compacted cumulus cells; grade 2 with less than 5 layers cumulus surrounding; grade 3 with partial cumulus surrounding; and grade 4 with denuded oocytes. Both grades 1 and 2 oocytes have uniformly dark ooplasm, whereas grades 3 and 4 have either dark or pale ooplasm. The oocytes in each grade were randomly divided and cultured in Dulbecco' s minimum essential medium (DMEM) containing 0.4% BSA; 1 μg/ml FSH, LH and estradiol with or without 100 ng/ml IGF-I at 38°C, 5% CO_2. After 32h, the assessment of oocyte maturation was performed. The immature oocytes were stained to identify the meiotic stages. The percentage of grades 1 and 2 mature oocytes (43.54%,46.41%) was markedly increased in DMEM + IGF-I compared to the control (22.40%, 29.50%). In addition, the percentage of mature oocytes in grades 1 and 2 were significantly higher than those in grades 3 and 4 ($p<0.05$) both in the control and experimental groups. The majority of immature oocytes both in DMEM and DMEM+IGF-I were arrested at germinal vesicle breakdown (GVBD) and metaphase I (MI). On the other hand, no effects of IGF-I on the maturation rate of grades 3 and 4 were observed. In experiment II, to determine the time required for IVM, grades 1 and 2 oocytes were cultured using the same protocol for 32, 36 and 48h. There were no significant differences ($p>0.05$) in maturation rate among the three incubation times. These results suggested that IGF-I was able to exert the stimulatory effect on only good quality domestic cat oocytes during maturation *in vitro*. This effect may be mediated via cumulus cells. In IVM of domestic cat oocytes, the 32-h culturing time is sufficient for resumption of meiotic maturation.

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ดำริ ดาราวิโรจน์ : ผลของ insulin-like growth factor-I ต่ออัตราการสุกของเซลล์ไข่ของแมวที่เพาะเลี้ยงภายนอกร่างกาย (INSULIN-LIKE GROWTH FACTOR-I PROMOTES MATURATION RATE OF THE DOMESTIC CAT OOCYTES IN VITRO) คณะกรรมการควบคุมวิทยานิพนธ์: กนก ภาวสุทธิไพศิฐ, ปร.ด., ยินดี กิติยานันท์, วท.ม., วิจิตรา เลิศกมลกาญจน์, Ph.D. 61 หน้า. ISBN 974-663-923-4

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อทดสอบผลของ insulin-like growth factor-I (IGF-I) ต่อการสุกของเซลล์ไข่ของแมวและตรวจหาเวลาที่เหมาะสมในการเพาะเลี้ยงเซลล์ไข่แมวภายนอกร่างกาย ทำการเก็บรังไข่แมวที่มารับการทำหมันจากโรงพยาบาลสัตว์ โดยแช่ในน้ำเกลือ (0.9% normal saline) ที่อุณหภูมิ 37 องศาเซลเซียส แล้วนำมาเจาะเก็บเซลล์ไข่อ่อนที่ห้องปฏิบัติการภายในเวลา 4 ชั่วโมง ในการทดลองที่หนึ่ง แบ่งเซลล์ไข่อ่อน เป็น 4 เกรด (เกรด 1 มีเซลล์หุ้มเซลล์ไข่หนาแน่นมากกว่า 5 ชั้น เกรด 2 มีเซลล์หุ้มเซลล์ไข่ไม่เกิน 5 ชั้น เกรด 3 มีเซลล์หุ้มเซลล์ไข่เพียงบางส่วน เกรด 4 ไม่มีเซลล์หุ้มเซลล์ไข่) แบ่งเซลล์ไข่แต่ละเกรดออกเป็นสองกลุ่ม กลุ่มแรกเลี้ยงในน้ำยาเพาะเลี้ยง Dulbecco's minimum essential medium (DMEM) ที่เติม 0.4 % BSA และ ฮอร์โมน FSH, LH และ estrogen 1 ไมโครกรัมต่อมิลลิลิตร กลุ่มที่สอง เลี้ยงใน DMEM ที่มี IGF-I 100 นาโนกรัมต่อมิลลิลิตร หลังจากเลี้ยงในตู้อบอุณหภูมิ 38 องศาเซลเซียส 5% CO_2 เป็นเวลา 32 ชั่วโมง นำเซลล์ไข่ทั้งสองกลุ่มไปตรวจการสุกโดยใช้กล้องจุลทรรศน์สเตอริโอ กำลังขยาย 20 เท่า และ ตรวจดูระยะของการเจริญแบบ meiosis ของเซลล์ไข่ โดยใช้การย้อมด้วย สี aceto-orcein พบว่า เซลล์ไข่เกรด 1 และ 2 มีอัตราการสุกมากกว่าในเกรด 3 และ 4 ทั้งในกลุ่มควบคุม และกลุ่มทดลอง และเซลล์ไข่ที่เลี้ยงใน DMEM ที่มี IGF-I มีอัตราการสุกสูงกว่าที่เลี้ยงใน DMEM อย่างเดียวเฉพาะในเกรด 1 และ 2 เท่านั้น การเสื่อมของเซลล์ไข่ พบมากในเกรด 3 และ 4 ในการทดลองที่สอง เพาะเลี้ยงเซลล์ไข่เกรด 1 และ 2 ใน DMEM และ DMEM ที่มี IGF-I เป็นเวลา 32, 36 และ 48 ชั่วโมง แล้วตรวจนับจำนวนไข่สุก และระยะแบ่งเซลล์แบบ meiosis ด้วยวิธีเดียวกันกับการทดลองแรก พบว่าอัตราการสุกของเซลล์ไข่แมว ที่เพาะเลี้ยงเป็นเวลา 32, 36 และ 48 ชั่วโมง ไม่แตกต่างกัน ทั้งในกลุ่มที่เลี้ยงในกลุ่มควบคุมและกลุ่มทดลอง จากการศึกษาครั้งนี้สรุปได้ว่า IGF-I สามารถเพิ่มอัตราการสุกของเซลล์ไข่แมว โดยผลของ IGF-I อาจเป็นการผ่านทางเซลล์หุ้มเซลล์ไข่ และเวลาที่ใช้ในการเลี้ยง 32 ชั่วโมงเพียงพอต่อการสุกของเซลล์ไข่ของแมวที่เพาะเลี้ยงภายนอกร่างกาย

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LIST OF ABBREVIATIONS

Ana.I	anaphase I
BSA	bovine serum albumin
°C	degree Celsius
DMEM	Dulbecco's minimum essential medium
FSH	follicular stimulating hormone
GV	germinal vesicle
GVBD	germinal vesicle breakdown
g	gram
IVM	*in vitro* maturation
IGF-I, -II	insulin-like growth factor-I, -II
IGFR-I,-II	insulin-like growth factor receptor I , II
IGFBPs	insulin-like growth factor binding proteins
h	hour
LH	luteinizing hormone
MI	metaphase I
MII	metaphase II
mg	milligram
ml	millilitre
mm	millimetre
ng	nanogram
Telo.I	telophase I
µg	microgram

CHAPTER I

INTRODUCTION

The habitat loss and the lack of nutrition sources lead to the rapid decreasing of wildlife population especially felid species such as tiger (*Panthera tigris*), clouded leopard (*Neofelis nebulosa*), flat-headed cat (*Prionailurus planiceps*), marbled cat (*Pardofelis marmorata*) and leopard (*Panthera pardus*). All the thirty-seven species of Felidae family have been considered either endangered or threatened by extinction except the domestic cat (*Felis catus*) (1). There may have 100,000 tigers at the end of the 19th century, but the maximum number is currently less than 7,700 individuals(2). In Thailand, the Royal Forest Department estimates the remaining number of tigers ranging from 400-600 individuals. However, tiger specialists suggested that the total number of tigers in the country is 150-248 individuals(2,3). Although there were several studies in the threatened felids (4-6) the biological information of these animals has been limited because ovaries from non-domestic cats are not readily available. Moreover, wild felids often fail to reproduce in captivity due to genetic incompatibility and behavioral or physical impairments. Thus, the domestic cat is commonly used as valuable animal model for comparative reproductive studies of non-domestic felids (1,6).

Reproductive physiology in female cats

Both domestic and wild cats are the members of the order Carnivora. Family Falidae consists of 3 genera ; Felinae, Pantherinae and Acinonychinae. These various species are classified into sub-species depending on various criteria. Even though there are differences among the genera, they share many similar characteristics which make them readily identifiable as cat, for instance large canine teeth and retractable claws as well as the territorial behavior(7). Similar to wild cats, the domestic cat is a reflex or induced ovulator. Ovulation occurs only after copulations which stimulates the releasing of sufficient amount of luteinizing hormone (LH) releasing from pituitary gland causing final maturation of follicular oocytes(1). It is likely that photoperiod plays a role in modulation of reproductive activity in tiger, snow leopard, cloud leopard and domestic cat. However, a seasonal impact on reproduction is not universal within the felid family. Although the reproductive hormonal pattern in different felid species is varied and species specific, the information integrating estrous cycles, seasonality and ovulatory response provides an essential database for studying reproductive physiology within this family (8,9).

The domestic cat (*Felis catus*) is placed in genera Felinae which mostly consists of the number of small felids. The domestic cat generally reaches sexual puberty at 6 to 9 months of age. However, the normal queens probably go through puberty and experience first estrous as early as 5 months old. The photoperiod has a major impact on the onset and duration of ovarian activity. The cat estrous cycle is separated into 4 major phases;

proestrous, estrous, diestrous and anestrous. However, the fifth phase, interestrous, may be found in the normal queen. Anestrous is the period of reproductive quiescence when the plasma estrogen and progesterone remain at basal level. The interestrous phase is termed for the brief periods of sexual or reproductive inactivity between repeated phases of estrus. The duration of the interestrous interval ranges from 2 to 19 days and averages 8 days. This period is characterized by an abrupt decline of plasma estrogen concentration to level below 20 pg/ml. Proestrous is the period of follicular function, estrogen synthesis and secretion. The duration of this phase is only 0.5 to 2 days. The abrupt rise in circulating estrogen is observed in the proestrous phase. The estrous phase associated with follicular estrogen synthesis and secretion is approximately 7 days in domestic cats. The peak of rising estrogen concentration above 50 pg/ml occurs on day 5. Diestrous is a phase of progesterone dominance in the queen. Plasma progesterone concentration above 2 ng/ml is associated with diestrus(1, 8, 9).

In the last decade, the developing biotechniques for assisted reproduction are extremely progress. *In vitro* maturation followed by *in vitro* fertilization (IVF) technique allows the rescue of female haploid genetic material from endangered species which unexpectedly die. These technologies are valuable for embryo production *in vitro* and cloning. Few data are available on the mechanism regulating oocyte maturation in carnivore including the domestic cat. Moreover, the proportion of the cultured oocyte achieving nuclear maturation in domestic cat has been lower than other species such as cows,mice and pigs (10).

In most mammals, the ovaries develop from gonadal ridges where germ cells originating from yolk sac migrate to. These primordial germ cells that migrate to developing ovary during embryogenesis become oogonia. The oogonia multiply mitotically several times and eventually differentiate into primary oocytes. These primary oocytes divide meiotically and arrest at diplotene stage of prophase I. The next stages of development are triggered by hormones during sexual maturity. The oocytes complete meiosis I and two haploid cells, the secondary oocyte and first polar body, are formed. Both of these cells undergo meiosis II to produce four haploid cells, but only one cell forms the mature oocyte or egg. The small polar bodies with little cytoplasm eventually degenerate. Each primary oocyte is surrounded by follicular cells in a cavity or sac called primary follicle or Graafian's follicle. The primordial follicle formed by flattened follicular cells surrounding primary oocyte serves as the pool of oocyte available for fertilization throughout the reproductive life of the female.

The mammalian oocytes will undergo meiotic maturation spontaneously after they are released from antral follicle and cultured *in vitro* (10, 11). The spontaneously maturing oocyte has been a widely model system for examining the factors regulating meiotic maturation. Many characteristics of oocyte maturation *in vivo* have been investigated in several species (12-14). Since the complexity of the follicular environment, numerous questions related to the mammalian oocyte maturation have not been possible to approach. For this reason, the process of oocyte maturation has been investigated under *in vitro* conditions. By applying culture method to study

oocyte maturation, it is possible to examine many factors influencing on maturation process and to compare maturation *in vitro* to that occurring *in vivo*. Consequently, many investigators have isolated mammalian oocytes and examined the process of oocyte maturation under the more available conditions to experimental manipulation *in vitro*. The mammalian oocyte maturation has been examined by using the following approaches; 1) the culture of oocytes *in vitro*, 2) the culture of isolated ovarian follicles and 3) the culture of ovaries or ovarian fragments (15). Nevertheless, using isolated mammalian oocytes to identify the culture conditions appropriate for successful maturation is common in various species (10). The knowledge of the requirement for oocyte maturation *in vitro* will offer unique opportunities for threatened or endangered wildlife. In family Felidae, *in vitro* techniques have been applied to investigate the optimal culture system for oocyte maturation. However, there is still a paucity of information for *in vitro* maturation requirement in these species (16-18).

The regulation and stimulation of oocyte growth require a number of various factors. These factors range from the simple small organic compounds to the complex macromolecular structures which require receptors and a network of subsequent messenger to execute their activities. The latter is a group of polypeptide hormones called growth factors. These growth regulating peptides were discovered in many tissues; for instance, liver , brain, and muscles. These growth factors are regulated by the more classical hormones. They are active in stimulating cell proliferation *in vitro* and act as proximal effectors of hormonal action on growth. Epidermal

growth factor (EGF), platelet derived growth factor (PDGF), nerve growth factor (NGF), fibroblast growth factor(FGF) and insulin-like growth factor (IGF) are the well- studied peptide growth factors whose actions are restricted to the highly specialized cell type (19,20). Insulin-like growth factor or somatomedin is structurally similar to human proinsulin and is mainly secreted by the liver. It has the growth regulating activity known as the sulfation factor in cartilage, insulin-like action in muscles and mitogenic property in the cell culture system. These growth factors regulated mainly ,but not absolutely, by somatotropin are classified into insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II). They are highly homogenous single chain proteins of 70 and 67 amino acid, respectively. IGF-I is a basic peptide with the greater growth hormone dependency and is significantly homology with human proinsulin whereas IGF-II is a neutral peptide with more potency in insulin-like activity. In addition, IGF also plays essential role in the growth hormone feedback mechanism. IGF systems are composed of two growth factors (IGF-I and IGF-II), two receptor types (IGFR-I and IGFR-II) and at least six different binding proteins (IGFBPs). (19, 21)

Receptors for both IGF-I and IGF-II are often found in the same tissue or cell type. In mammalian tissue, these receptors are highly homogenous displaying similar heterotetrameric transmembrane structures. They compose of disulfide-linked α subunit which is extracellular domain and β subunit which is the intracytoplasmic domain. The α subunit contains an extracellular domain binding to IGFs peptide and the transmembrane

β subunits display intrinsic, hormone-stimulated tyrosine kinase activity. IGFs receptors are classified into two types; the Type I and Type II receptors. Type I receptor binds IGF-I preferentially while Type II receptor has greater affinity to IGF-II. Both IGF-I and IGF-II can bind to both receptors with markedly different affinity. This binding potentiated autophosphorylation of receptor β subunit as well as phosphorylation of specific intracellular target proteins result in cell proliferation through paracrine and autocrine mechanism (19, 22). IGF-I and II cross-react with insulin receptor presented on most cells including theca and granulosa cells in ovaries. Receptors for insulin and IGF-I also have structural and functional similarities, including intracellular signaling involving tyrosine kinases.

Besides their receptors, IGFs are complexed with specific high affinity binding proteins (IGFBPs) that regulate the availability of IGFs to their target tissues and provide a mechanism whereby the bioactivity of IGFs can be controlled. IGFs and specific binding proteins are synthesized in a variety of sites. The binding proteins may either promote or inhibit the actions of IGFs (23, 24).

Role of IGF-I in ovaries

IGF-I was found in ovarian tissues in various species, for instance; human (25, 26), bovine(27), pig(28), rabbit(29), and rat (22). It has been implicated in ovarian physiology because it has significant trophic and

steroidogenic effect on granulosa cell in vitro. Granulosa and thecal cells seem to be the main sources of this peptide in ovaries. In pigs, granulosa cells were shown to both secrete and respond to IGF-I suggesting an autocrine function of this mitogenic peptide in the follicle (30). IGF-I had a direct effect on the cultured ovarian cell, these effects comprised of stimulation of granulosa cell mitogenesis, granulosa and luteal cells progesterone production and thecal cell androgen biosynthesis (31-33). In addition, granulosa, thecal and luteal cells contained IGF-I receptors which appeared to mediate the effect of this peptide. Adding to the complexity of the regulatory role of IGF-I is the presence of IGFBPs within the ovaries. These IGFBPs are produced by granulosa, thecal and luteal cells and their production is hormonally regulated (34).

Effects of IGF-I on the oocytes *in vivo* are probably regulated by IGFBPs because dramatic differences exist in IGFBPs in follicular fluid from follicle at different stages of development (35). IGFBPs might have a physiological inhibitory effect on the regulation of IGF-I actions. IGFBP-3 was the predominant binding protein detected in follicular fluid (33,36). But its expression was low or undetectable in follicular cells suggesting that IGFBP- 3 presented in follicular fluid would be derived from the circulatory pool (37). IGFBP-3 remained relatively constant among preovulatory and non-ovulatory follicles. Its concentration was also constant among follicles of different sizes and among follicles collected at the different time with the exception that this protein decreased in preovulatory follicular fluid and plasma during the final stages of the preovulatory period (37-39).

In contrast, IGF-binding protein-2, -4 and -5 in intrafollicular concentrations dramatically decreased during the terminal development of follicles due to a decrease in their synthesis by follicular cells and an increase in their degradation by the specific intrafollicular proteinase. This reduction brings about the increased IGF-I bioavailability in the large antral follicle. Moreover, the expression of IGFBP-2,-4 and -5 increased in atretic follicle indicating the decreasing of IGF-action (36). The role of IGF-I in ovarian system was investigated in many aspects, for instance, the relationship between IGF-I and gonadotrophin (27,33,39,40), the comparative studies of IGF-I and other growth factors in folliculogenesis (26,33,41,42) and the effect of IGF-I on different ovarian cells (22,31,43). Moreover, the effect of IGF-I on oocyte maturation *in vitro* was examined in bovine (44), buffalo (45), pig (46), and rabbit(47). Most of these studies indicated that IGF-I significantly stimulated nuclear maturation . However, in porcine oocytes, IGF-I had no positive effect on oocyte maturation despite the fact that it enhanced 15 folds of cumulus cell proliferation (28,48). Recently, impact of EGF, basic fibroblast growth factor (bFGF) and IGF-I on DNA synthesis of cat preantral follicles has been investigated (49,50). However, the effect of IGF-I on maturation rate of domestic cat oocytes *in vitro* has not been reported.

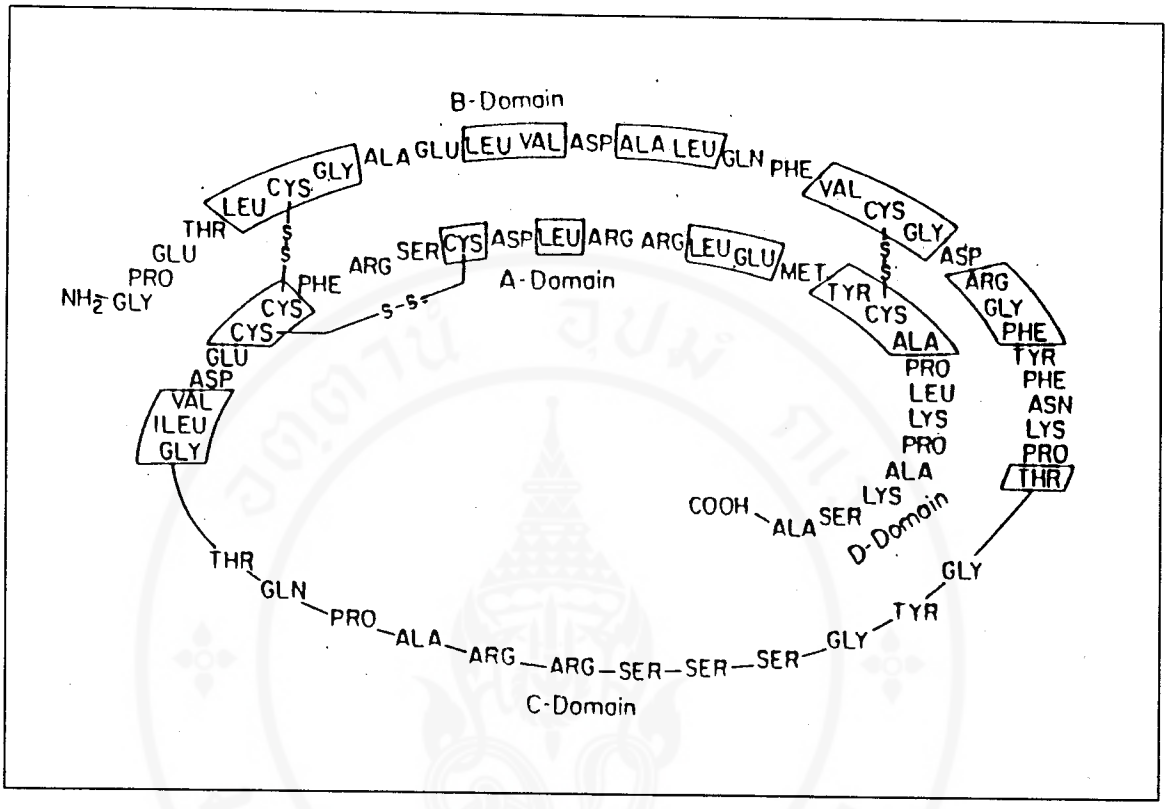

Figure 1. Primary structure of insulin-like growth factor-I. Residues enclosed in boxes in A and B domain of IGF-I are identical to amino acids in corresponding positions in human proinsulin molecule. C domain corresponding to C-peptide of proinsulin has no homology with the latter. The D domain is an eight residue extension at carboxy-terminus which does not exist in proinsulin. (Adopted from Underwood LE and Van Wyk JJ., 1985) (19)

CHAPTER II

OBJECTIVES

The aims of the present study were

1) to determine the effect of IGF-I on domestic cat oocytes of different qualities maturing *in vitro*.

2) to identify the optimum time of culture condition for cat oocyte maturation *in vitro*.

CHAPTER III

MATERIALS AND METHODS

1. Ovariohysterectomy (OVH) and ovary collection

Domestic cat ovaries were recovered from 60 randomly selected queens subjected to ovariohysterectomy at a small animal hospital and a local veterinary clinic. The ages of sexually matured cats were estimated by veterinarians (most of donors were over 8 months). Cats subjected to OVH were anesthetized with the combination of xylazine (1 mg/kg) and ketamine HCl (20 mg/kg). Following the induction of anesthesia, the hair around ventral abdominal wall was clipped and shaved. Cats were positioned in dorsal recumbency and sterile skin preparation was made in the middle third of caudal abdomen. The skin and subcutaneous tissues were incised approximately at 4 to 8 cm to expose the linea alba. Stab incision was made on linea alba and cut in the same line of skin incision. The ovarectomy hook was used to elevate the uterine horn. This uterine horn was pulled upward to observe the ovary and suspensory ligament at the proximal edge of ovarian pedicle. Two clamps were placed across the ovarian pedicle and a 2-0 absorbable suture was applied below the proximal clamp. Next, the second circumferential ligature was taken below the first to control hemorrhage. The ovarian pedicle was subsequently transected between the clamps and ovary. The similar procedure was concluded on the opposite side. After gently

tearing the broad ligament, the uterine horns were traced toward the uterine body to identify the cervix. Cranial traction was applied on the uterus, and the uterine body cranial to the cervix was tied. The transfixing suture was additionally applied to prevent the suture from being slippery. The uterine wall was grasped with forceps cranial to the ligature and the uterine body was transected. The uterine stump was replaced into the abdomen followed by the closure of all three layers of the abdominal wall. Immediately upon excision, ovaries were placed in 0.9% normal saline (NSS) containing 0.04 μg/ml gentamycin at 37 °C in a thermos and transported to the laboratory room within 2 to 4 h before oocyte recovery.

2. Oocyte recovery

Each pair of ovaries were washed 3 times in 0.9% NSS containing 0.04 mg/ml gentamycin, 65 μg/ml penicillin, 56 μg/ml streptomycin and 25 μg/ml amphotericin B (SQUIBB, Princeton, NJ). After being washed, oocytes were liberated from ovaries by repeatedly puncturing with a sterile needle. Follicular contents were aspirated into a 2.5 ml disposable syringe with 20-gauge needle containing 1 ml of Hepes buffered Tyrode's medium (TALP-Hepes) and placed in an evaporating dish. The dish was searched for oocytes which were transferred and washed three times in 75 μl droplets of equilibrated Dulbecco's Eagle minimum essential medium (DMEM; GIBCO Grand Island, NY) supplemented with 1 μg/ml follicle stimulating hormone (FSH), 1 μg/ml luteinizing hormone(LH), 1 μg/ml estradiol , 0.4 % bovine serum albumin (BSA; GIBCO Grand Island, NY) and 2.6 mg/ml pyruvate in 60x15 mm Falcon culture dish (Becton, Dickinson Labware, Lincoln Park,

NJ). The supplemented DMEM was also used in the experiment as maturation medium.

3. Grade classification of oocytes (Figure 2)

After being washed, collected oocytes were graded under a standard stereomicroscope by using the following criteria based on the uniformity of oocyte cytoplasm and cumulus cell complement.

Grade 1: oocytes with a full surrounding of more than 5 layers of compacted cumulus oophorus cells and uniform dark cytoplasm.

Grade 2: oocytes with a complete encircling of corona radiata cells but less than 5 layers of cumulus oophorus cells not as tightly compact as the higher grade and also uniform dark cytoplasm.

Grade 3: oocytes with nearly full surrounding of cumulus cells and lack of uniformity or express mosaic transparency of cytoplasm.

Grade 4: oocytes without surrounded cumulus oophorus cells and/or lighten cytoplasm.

4. Assessment of oocyte maturation

Assessment of oocyte maturation was conducted after 32-h culture. Cumulus cells were removed by a combination of exposure to 0.2% hyaluronidase for 10 minutes and mechanical displacement with a small bore, hand–pulled glass pipette. The oocytes displaying the first polar body were classified as mature , while the immature oocytes of each group were stained

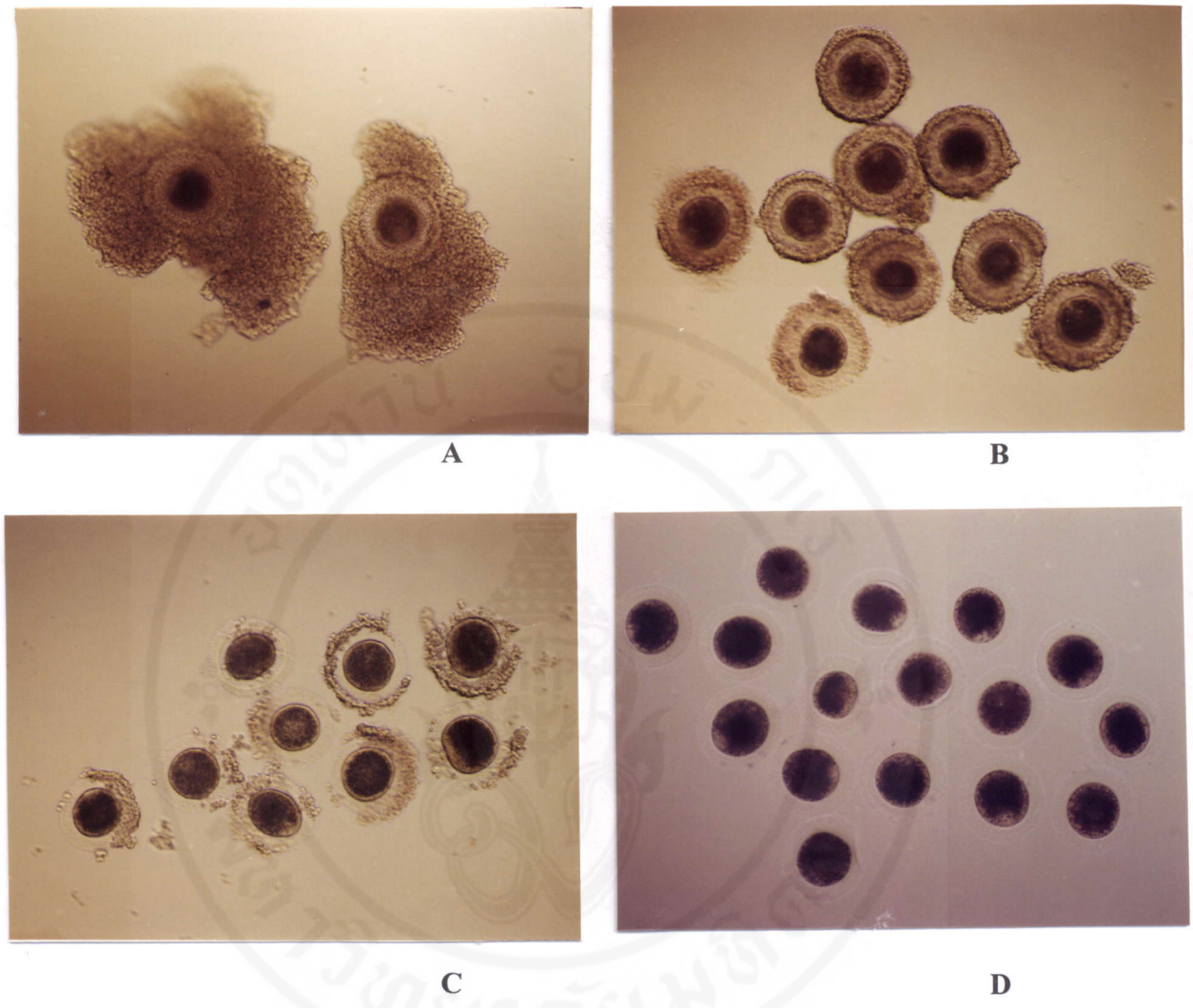

Figure 2. Grade classification of domestic cat oocytes (A) grade 1; compact cumulus surrounding oocytes, (B) grade 2; loosen complete cumulus enclosed oocytes, (C) grade 3; partial cumulus encircling oocytes and (D)grade 4; denuded oocytes . (x65)

by aceto-orcein to identify the stages of nuclear maturation. The degenerated oocytes were discarded.

5. Oocyte staining

One to six oocytes were placed on a microscopic slide with a minimal amount of medium and covered with a 22 mm^2 coverslip. A jelly paraffin wax was sparingly applied to the edges of the coverslip to allow gentle placement of the coverslip on the oocytes. The coverslip was gently compressed while observing the oocyte flattening under 30x stereomicroscope for avoiding oocyte rupturing then immersed the microscopic slides into the fixative (Glacial acetic acid : Ethanol, 3:1) in the jar. After 12 to 18 h fixation, all slides were stained with aceto-orcein dye and the meiotic stage of the oocytes was evaluated by microscope at 20x to 40x.

Chromosomal configurations (Figure 5) used for classification were categorized as follows :

The germinal vesicle (GV) stage consists of a round shaped nucleus containing a permanent nucleolus and fine filaments of chromatin.

The germinal vesicle breakdown (GVBD) / prophase stage was characterized by the appearance of condensing chromosome and the absence of nucleolus and nuclear membrane.

Metaphase I (MI) was identified by the presence of a single row of the pair chromosome.

Anaphase I was designated by the appearance of chromosomal separation and movement to the opposite pole.

Telophase I was identified by the appearance of two groups of chromosome.

Metaphase II (MII) was classified when the oocyte extruded the first polar body into perivitelline space.

Degenerative stage was classified when oocytes contained no identifiable chromosomal configuration.

Experiment I

The impact of the different qualities of oocytes and effect of IGF-I on meiotic stages of domestic cat oocytes were evaluated at 32 h of culturing time. Each grade of oocytes was divided into two groups and transferred the first one to 50 µl droplets of a maturation medium (supplemented DMEM) without 100 ng/ml insulin-like growth factor-1 (IGF-I), whereas another one was transferred to 50 µl maturation medium with IGF-I (2 to 10 oocytes/drop) under 3 to 4 ml of paraffin oil in 35 mm Falcon culture dishes. The pH and the osmolarity of maturation medium was 7.2 to 7.5 and 280 to 295 osmol/g, respectively. Both groups of oocytes were matured in a 38 ^{0}C humidified incubator containing 5% CO_2 in air for 32 h. The immature oocytes were stained and identified the nuclear meiotic stages by aceto-orcein dye.

Experiment II

The optimal time course for cat oocyte maturation *in vitro* and the effect of IGF-I at different time courses for cat oocyte maturation *in vitro* were examined by culturing the good quality oocytes for 32, 36 and 48 h. The grades 1 and 2 oocytes were recovered from ovariohysterectomized cats by using the protocol mentioned above. Each grade of oocytes was transferred to 50 µl droplets of a supplemented DMEM maturation medium with / without 100 ng/ml IGF-I supplementation (2 to 10 oocytes/drop) under 3 to 4 ml of paraffin oil in 35 mm Falcon culture dishes. The pH and the osmolarity of maturation medium were similar to that in Experiment I. Both groups of oocytes were matured in a 38 ^{0}C humidified incubator containing 5% CO_2 in air. After culturing for 32, 36 and 48 h, the percentage of matured oocytes were assessed.

Statistical Analysis

In Experiment I, data were subjected to least-squares analysis of covariance (ANCOVA) using the General Linear Models procedure of the Statistical Analysis System (SAS version 6.03). ANCOVA for a 2x4 factorial design was performed with IGF-I treatment as main effects and grade classification as a covariate. In Experiment II, analysis of variance (ANOVA) and subsequently unpair t-test were employed to analyze the differences in maturation rate among grades, maturation time and IGF-treatment. For all comparisons where significant differences were observed, the significant level (p) was less than 0.05 .

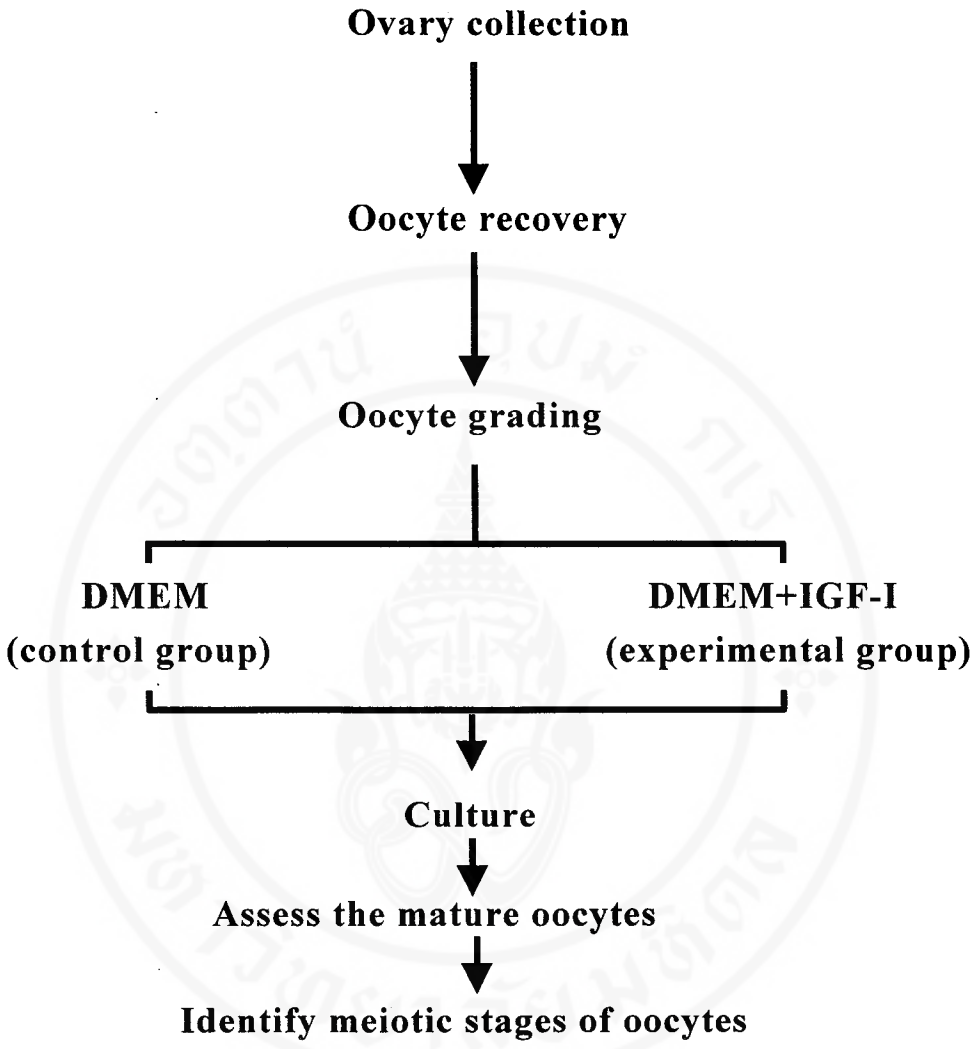

Figure 3. The diagram illustrating experimental plan. The cat oocytes were graded following recovery from ovaries. Oocytes in each grade were divided into control and experimental groups and were cultured at 38°C 5% CO_2 in DMEM and DMEM+IGF-I, respectively. After 32 h (including 36 and 48 h in experiment II) mature oocytes were identified by stereomicroscope whereas the immature oocytes were stained for meiotic stage evaluation.

CHAPTER IV

RESULTS

Experiment I

A total of 879 oocytes recovered from 120 ovaries of 60 domestic cat donors were randomly selected and classified for maturation procedure. Based on cumulus cell investment and characteristics of ooplasm, 124 (14.1%) oocytes met grade 1 criteria whereas 394 (44.82%) , 283 (32.20%) , and 78 (8.87%) were grades 2, 3, and 4 respectively. After 32 h culturing, mature oocytes reaching M II stage contained the first polar body within the perivitelline space as shown in Figure 8. It was found that the percentage of grades 1 and 2 matured oocytes were statistically higher than those of grades 3 and 4 both in the presence and absence of IGF-I supplementation. The comparison of maturation rates of all 4 grades of oocytes between being cultured in DMEM alone (control) and DMEM supplemented with IGF-I (treatment) were demonstrated in Figure 3. In the control group (without IGF-I supplement), the maturation rates of grade 1, 2, 3 and 4 were 21.57±11.8%, 32.3±8.8%, 15.03±2.8% and 9.13±4.07%, respectively. When 100 ng/ml IGF-I was added to the maturation medium before culturing, the maturation rates were significantly ($p < 0.05$) increased only in grade 1 (43.60±5.5%), and 2 (47.96±5%). However, there were no differences in maturation rates between grades 3 and 4 oocytes cultured in either DMEM or DMEM+IGF-I. The percentage of degenerated oocytes were significantly

lower in grades 1 (8.62% in DMEM, 3.03 % in DMEM+IGF-I) and 2 (9.00%, 9.27%) than those in grades 3 (20.41%, 21.32%) and 4 (55.88%, 59.09%) when being cultured in both control and experimental groups.

The meiotic stages of immature domestic cat oocytes following the aceto-orcein staining were demonstrated in Tables 1 and 2. In control group, the percentage of grades 1, 2, 3 and 4 immature oocytes were 68.96, 61.50, 64.62 and 38.18, respectively. Most of the immature oocytes were arrested at GV, GVBD and M I stages except to grade 1 immature oocytes that were significantly arrested at M I (29.31%). The percentage of grade 1 oocytes containing GV was significantly lower than those in the other grades. There were more oocytes reaching anaphase I and telophase I in grade 2. The percentage of oocytes with unidentifiable chromatin in all grades was 17 %. In IGF-I treated group, the percentage of immature oocytes from grades 1, 2, 3 and 4 were 53.03, 43.80, 64.71 and 38.62, respectively. The majority of these immature oocytes were arrested in GVBD and MI similar to the control group. Nevertheless, the proportion of each meiotic stages was not significantly different among grades. Such results were also not different from the control except the higher proportion of telophase oocytes.

Experiment II

A total of 149 in grade 1 and 357 in grade 2 oocytes were used in this experiment. After being cultured in complex medium DMEM for 32, 36 and 48 h , the percentage of grade 1 oocytes reaching MII stage was approximately 34.12% whereas 29.8 % was found in grade 2 . Although the maturation rate of grade 1 oocytes cultured for 48 h was higher than those

in 32 and 36 h , there were no significant differences in maturation rate at various times both in grades 1 and 2 oocytes. Most of the immature oocytes cultured for 36 and 48 h were arrested at GV whereas at 32 h of culture, the meiotic stage of both grades 1 and 2 oocytes were mostly arrested at GVBD and M I. As shown in Table 4, the average number of grades 1 and 2 mature oocytes maturated in IGF-supplemented DMEM was 47 and 45 % , respectively. It was found that the percentage of mature oocytes cultured for 36 h was higher than 32 and 48 h. However, there was no significant difference ($p > 0.05$) in maturation rate among these three culturing times. In addition, the proportion of the grades 1 and 2 mature oocytes was intensely increased after culturing in IGF-I supplemented medium in comparison to those matured in DMEM alone. The significant difference ($p<0.05$) was detected in both grades 1 and 2 cultured for 32 and 36 h. In addition, the cumulus expansion obviously occurred in 36 and 48 h comparing to 32 h (Figure 6).

Table 1. *In vitro* maturation in different grades of cat oocytes in DMEM after 32 h culturing.

Grade	Total No.	Meiotic stages of oocytes No. (%)							Degenerated No. (%)
		GV	GVBD	MI	Ana.I	Telo.I	MII	Unidentified	
1	58	1 (1.72)	10 (17.24)	17 (29.31)	- (0.00)	2 (3.45)	13 (22.40)	10 (17.24)	5 (8.62)
2	200	16 (8.00)	30 (15.00)	28 (14.00)	8 (4.00)	12 (6.00)	59 (29.50)	29 (14.50)	18 (9.00)
3	147	15 (10.20)	26 (17.69)	20 (13.60)	1 (0.68)	6 (4.08)	22 (14.96)	27 (18.37)	30 (20.41)
4	34	4 (11.76)	3 (8.82)	- (0.00)	- (0.00)	- (0.00)	2 (5.89)	6 (17.6)	19 (55.88)

GV: Germinal vesicle, GVBD: Germinal vesicle breakdown, MI: Metaphase I, Ana.I: Anaphase I, Telo.I : Telophase I and MII : Metaphase II

Table 2. *In vitro* maturation in different grades of cat oocytes in DMEM supplemented with IGF-I after 32 h culturing.

Grade	Total No.	Meiotic stages of oocytes No. (%)							Degenerated No. (%)
		GV	GVBD	MI	Ana.I	Telo.I	MII	Unidentified	
1	66	1 (1.51)	10 (15.15)	13 (19.70)	3 (4.55)	2 (3.03)	29 (43.94)	6 (9.09)	2 (3.03)
2	194	1 (0.51)	20 (10.30)	26 (13.40)	5 (2.58)	15 (7.73)	91 (46.91)	18 (9.28)	18 (9.27)
3	136	5 (3.68)	28 (20.59)	25 (18.38)	1 (0.74)	2 (1.47)	19 (13.97)	27 (19.85)	29 (21.32)
4	44	3 (6.81)	4 (9.09)	1 (2.27)	- (0.00)	- (0.00)	1 (2.27)	9 (20.45)	25 (59.09)

GV: Germinal vesicle, GVBD: Germinal vesicle breakdown, MI : Metaphase I, Ana.I: Anaphase I, Telo.I : Telophase I and MII : Metaphase II

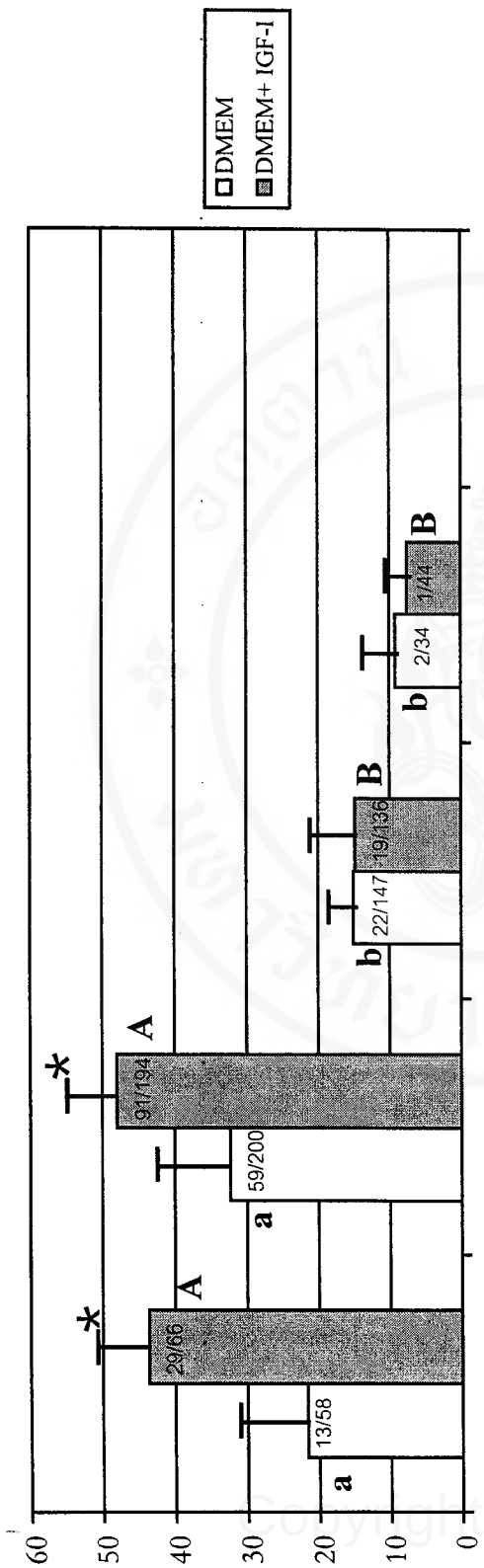

Figure 4. The effect of IGF-I on *in vitro* maturation in different grades of cat oocyte after 32 h of culture.

Value with a different superscript shows significant difference among grades ($p<0.05$) whereas * is significant difference within grade at $p < 0.05$

Table 3. The maturation rate and meiotic status of domestic cat oocytes cultured in DMEM for 32, 36 and 48 h.

Grade	Time (h)	Total No.	Meiotic stages of immature oocytes No. (%)							Mature No. (%)	Degenerate No. (%)
			GV	GVBD	MI	Ana.I	Telo.I	Unidentified	Total		
1	32	29	1 (3.45)	4 (13.79)	9 (31.03)	1 (3.45)	1 (3.45)	4 (13.79)	20 (68.97)	9 (31.03)	- (0.00)
	36	24	11 (45.83)	1 (4.17)	1 (4.17)	- (0.00)	- (0.00)	3 (12.50)	16 (66.67)	8 (33.33)	- (0.00)
	48	21	5 (23.80)	4 (19.05)	- (0.00)	- (0.00)	- (0.00)	4 (19.05)	13 (61.90)	8 (38.00)	- (0.00)
2	32	66	3 (4.55)	17 (25.76)	11 (16.67)	2 (3.03)	1 (1.51)	11 (16.67)	48 (72.72)	18 (27.27)	3 (4.55)
	36	55	19 (34.55)	7 (12.73)	1 (1.82)	- (0.00)	- (0.00)	8 (14.55)	36 (65.45)	19 (34.55)	1 (1.82)
	48	54	15 (27.78)	9 (16.67)	4 (7.41)	- (0.00)	- (0.00)	8 (14.81)	39 (72.22)	15 (27.78)	3 (5.55)

GV: Germinal vesicle, GVBD: Germinal vesicle breakdown, MI : Metaphase I, Ana.I: Anaphase I, Telo. I : Telophase I.

Table 4. The maturation rate and meiotic status of domestic cat oocytes cultured in DMEM+IGF-I for 32, 36 and 48 h.

Grade	Time (h)	Total No.	Meiotic stages of immature oocytes No. (%)							Mature No. (%)	Degenerate No. (%)
			GV	GVBD	MI	Ana. I	Telo. I	Unidentified	Total		
1	32	29	- (0.00)	4 (13.79)	8 (27.58)	1 (3.45)	1 (3.45)	3 (10.34)	17 (58.62)	12 (41.38)	- (0.00)
	36	25	7 (28.00)	2 (8.00)	1 (4.00)	- (0.00)	- (0.00)	- (0.00)	10 (40.00)	15 (60.00)	- (0.00)
	48	20	4 (20.00)	3 (15.00)	1 (5.00)	- (0.00)	- (0.00)	4 (20.00)	12 (60.00)	8 (40.00)	- (0.00)
2	32	70	2 (2.86)	7 (10.00)	12 (17.14)	- (0.00)	4 (5.71)	10 (14.24)	37 (52.86)	33 (47.14)	2 (2.86)
	36	56	14 (25.00)	6 (10.71)	- (0.00)	- (0.00)	- (0.00)	5 (8.93)	25 (44.64)	31 (55.36)	- (0.00)
	48	56	15 (26.79)	10 (17.86)	2 (3.57)	- (0.00)	- (0.00)	8 (14.28)	37 (66.07)	19 (33.92)	2 (3.57)

GV: Germinal vesicle, GVBD: Germinal vesicle breakdown, MI : Metaphase I, Ana.I: Anaphase I, Telo.I: Telophase I.

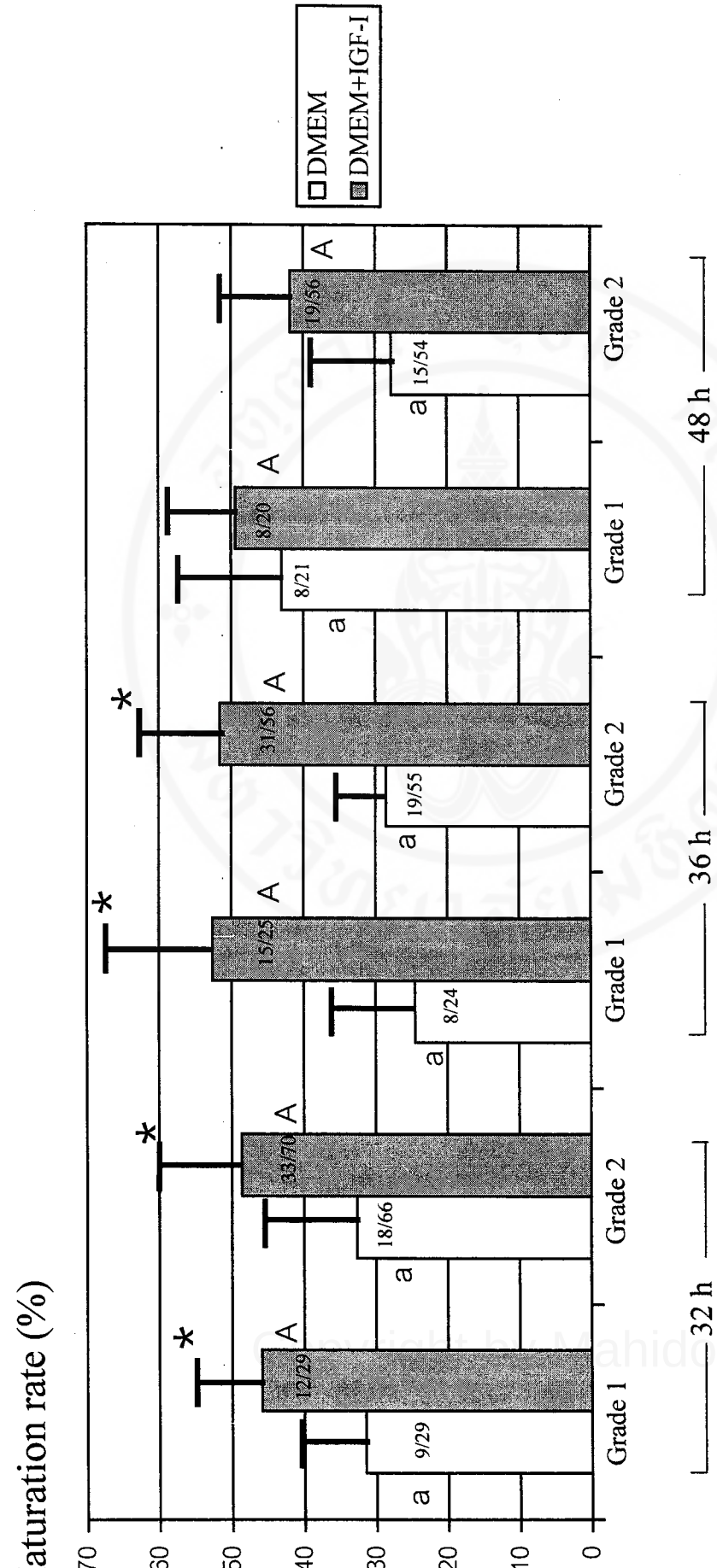

Figure 5. Maturation of cat oocytes cultured in DMEM with / without IGF-I for 32, 36 and 48 h.

* is a significant difference within grade

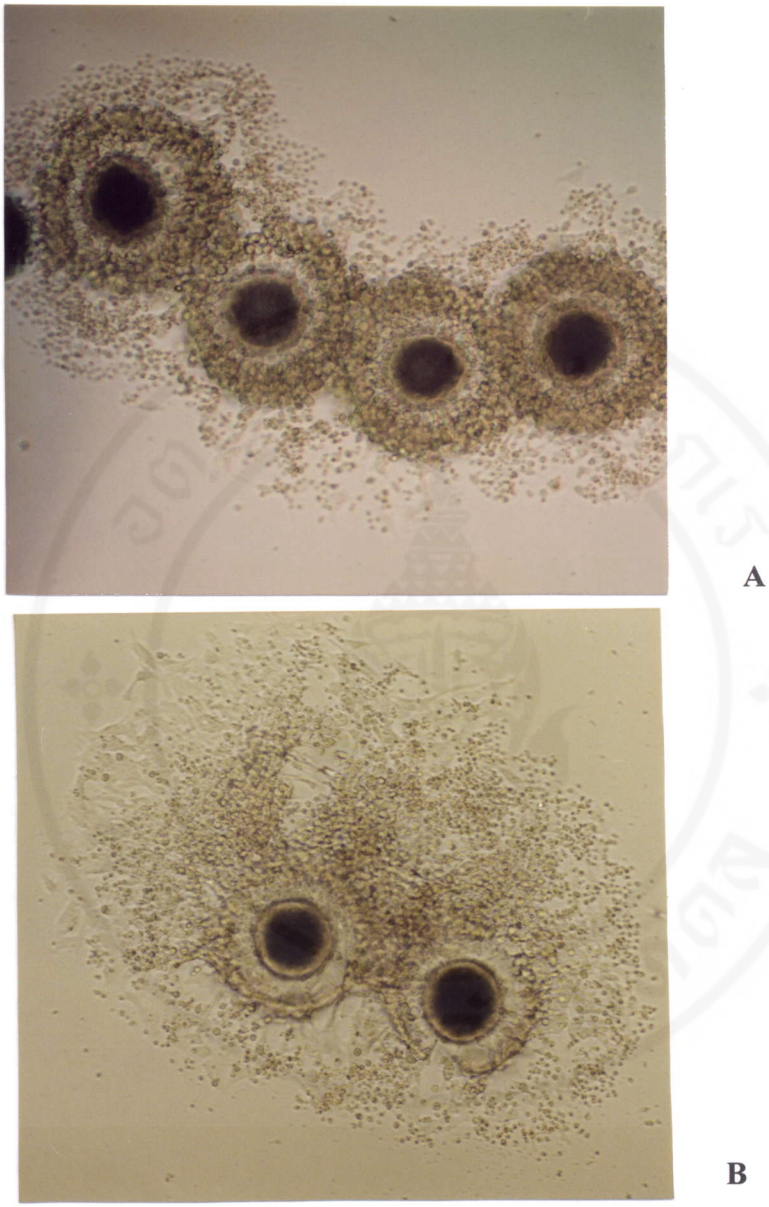

Figure 6. Domestic cat oocytes showing cumulus expansion after the culture in DMEM + IGF-I for 32 h (A) and 36 h (B) (x125)

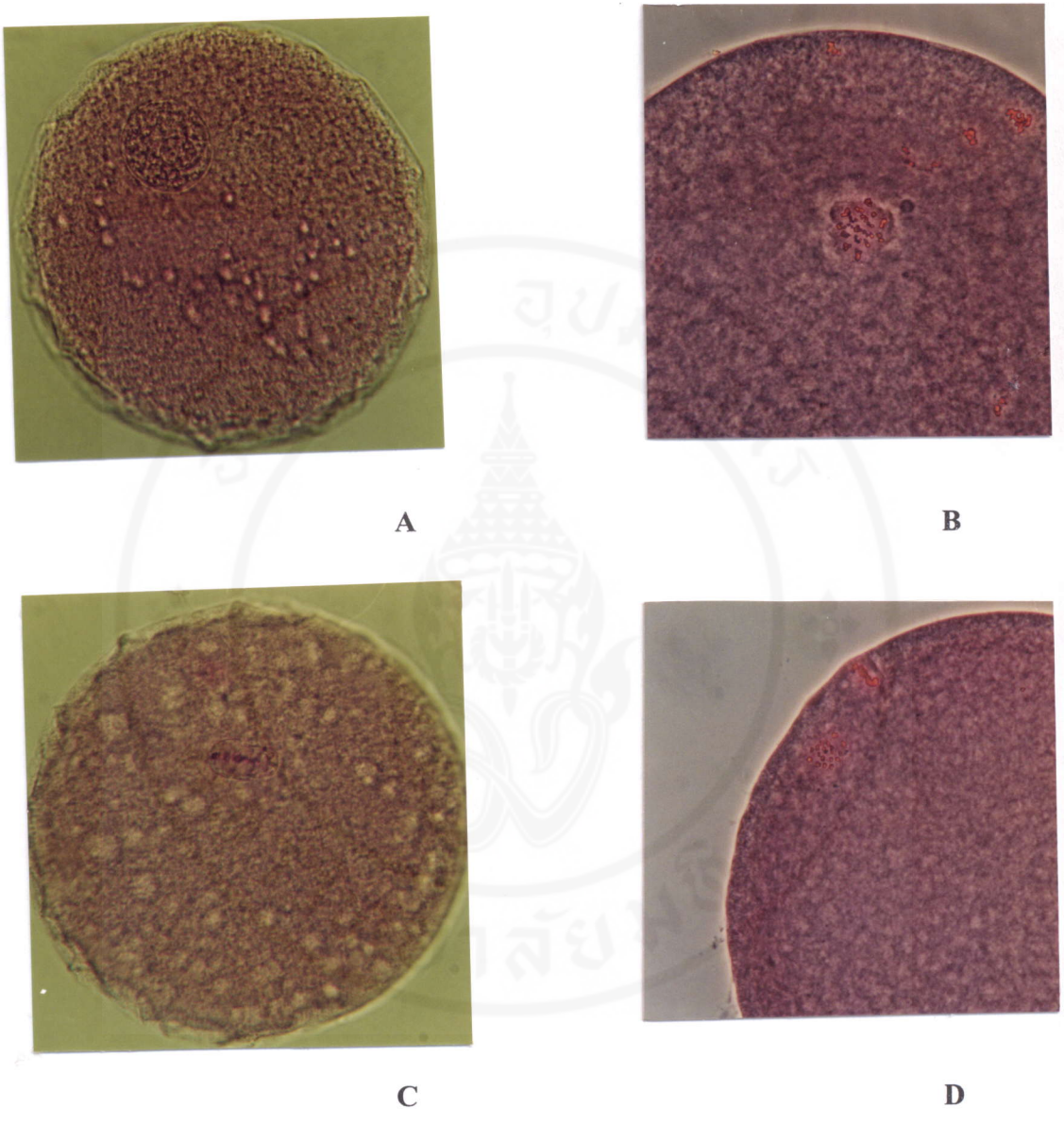

Figure 7. The nuclear meiotic stages of domestic cat oocytes stained with aceto-orcein after 32 h incubation *in vitro*. (A) GV, (B) GBVD (C) MI, and (D) MII oocytes. (A,C: x125; B,D: x250)

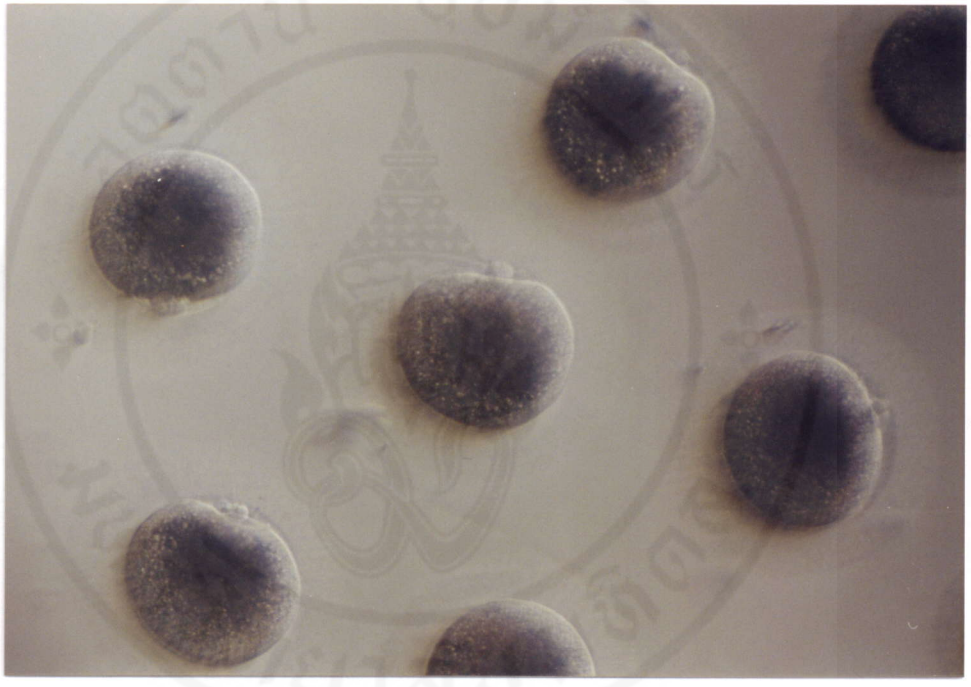

Figure 8 . Mature oocytes show the first polar body extrusion in the perivitelline space. (x250)

CHAPTER V

DISCUSSION

The present study demonstrated that quality of oocyte, IGF-I supplement and culturing time were important for the cat oocyte maturation *in vitro*. Many characteristics of oocyte maturation *in vivo*, including the chronology of meiotic stages, the influence of gonadotropins on maturation and the descriptive ultrastructure of maturing oocyte were carefully examined in felids (51-56). Due to the insufficient biological information of *in vitro* maturation of domestic cat oocytes, the effects of ovary collecting time, the different characteristics of oocytes and growth factor on cat maturation *in vitro* were concentrated in the present study. The domestic cat is an " induced ovulator " whose ovulation occurs only after multiple copulations. This event triggers the release of high LH concentration to cause final maturation of follicular oocytes (1,56). Thus the viability of cat intrafollicular oocytes probably sustains a longer period than other species which ovulated spontaneously. The factors affecting the viability and the integrity of oocytes *in vitro* are the maintenance temperature of the ovaries and the holding time until oocytes are recovered. In non-domestic felid species, for instance; tiger, puma and leopard , the immature oocytes were stimulated to mature *in vitro* even though the time from ovary excision to culture procedure was extended to 36 h (2). While in domestic cats, oocytes

liberated from ovaries stored in PBS at 4 °C for 72 h have an ability to achieve nuclear maturation after culture in Eagle's minimum essential medium (EMEM) for 24 h (57). Although the previous studies indicated that oocytes stored at 4 °C maintained the viability as long as 32 h and remained capable of maturing *in vitro* (58-61), the efficiency of maturation was not affected by either maintenance or transport temperature (4 °C and 22 °C) or delaying recovery of oocytes from antral follicle (2 to 8 h and 24 to 32 h) (57). In this experiment, we kept the ovaries during transportation in NSS at the body temperature (37°C) because the transportation time from a veterinary hospital to the laboratory was less than 2 h. This study demonstrated that cat ovaries stored at 37 °C and took less transportation time allowed the intrafollicular oocyte to mature *in vitro*.

Even though the mechanisms involving meiotic event seem similar among mammals, the time required for oocytes to achieve MII *in vitro* differs markedly among species. The appropriate time for *in vitro* culture of the bovine oocytes was approximately 18 to 24 h (62-64) whereas porcine oocytes required 35 to 48 h (65-66) to achieve meiotic maturation. In equine, 15 h of incubation period was sufficient for oocyte maturation *in vitro* (67). The time course for cat oocytes to reach the MII of meiosis *in vitro* in different experiments varies from 24 to 48 h (57-68). The different maturation time in the same species is probably due to the different factors employed in various experiments, for instance, maturation media, hormones and energy supplements. Naturally, female cats mating 3 times per day during estrous usually ovulate at 48 h or more after copulations and

induced LH-surge (51). Johnston et al.(1989) (57) reported that *in vitro* incubation of the domestic cat oocytes using EMEM have the ability to achieve MII at 24 h (12%) and eventually peaked at 48 h (62.5%). But the percentage of mature oocytes during 30 to 48 h of incubation were not significantly different. In contrast, Goodrowe et al. (1991) using modified Kreb-Ringer bicarbonate buffer (mKRB) showed that the peak of mature oocytes occurred at 24 h (33%) and remained stable through 48 h (68). This difference in maturation rate at the given time is probably due to the different types of maturation media and culture systems. Our result also showed that the maturation rates of domestic cat oocytes were not significantly different during 32 to 48 h. In addition, our preliminary study demonstrated that only a low number of cat oocytes were capable of reaching MII stage *in vitro* at 24 h. This outcome confirmed that 32 h of incubation was sufficient for maturing the cat oocytes *in vitro*.

The appropriate cultured medium for cat oocyte maturation has been previously investigated (16, 18, 58, 69, 70). Even though, simple defined medium or tissue culture medium-199 (TCM-199), generally used in bovine IVM was employed for *in vitro* maturation of cat oocytes (16, 18, 69). Lovoni and Oliva (1993) reported that this medium supplemented with gonadotropins allowed resumption of meiosis in only 4.3 to 18.7% of cat oocytes (18). In contrast, Pope et al. (1997) demonstrated that cat oocytes with good ooplasm appearance cultured in TCM-199 with gonadotropins reached M II stage around 50% (16). Goodrowe et al.(1991) found that cat oocytes cultured in mKRB oocytes could reach meiotic maturation. However,

the percentage of MII oocytes was never greater than immature oocytes (68). According to Byer et al.(1994) using Ham's F-10 resulted in higher percentage (76.5%) of mature oocytes (71) but these oocytes were obtained from gonadotropin treated cats, therefore, the efficiency of such medium on oocyte maturation probably needs further confirmation. Other media such as Waymouth MB753/1 medium (WAY) and EMEM were used to culture cat immature oocytes (58,68). On the other hand, Jewgenow (1998) demonstrated that Dulbecco's minimum essential medium (DMEM) had a slight superiority in cellular structure during maturation when compared with TCM 199. The author also suggested that DMEM without pyruvate and lactate was most suitable for culture cat follicles (69).

In addition to various types of media, protein and energy supplementation affected culture results. The information of essential energy for cat oocyte is rudimentary. Swanson et al.(1996) reported that glutamine-supplemented medium was ineffective in promoting greater blastocyst formation or increasing embryo cell number (70). Addition of pyruvate and/or lactate to culture media resulted in suppression of DNA-synthesis on preantral follicle of domestic cats (69). The influence of gonadotropins (67, 68, 72), serum (67, 72,73) and protein (74-77) supplementation on the ability of oocytes to resume and complete meiosis were reported. The percentage of frequencies of cat MII oocytes increased in medium supplemented with BSA and FSH/LH comparing with those without gonadotropins adding (54, 56). Wood et al. (1995) found that BSA appeared to enhance the incidence of maturation while fetal calf serum (FCS) inhibited

the ability of oocytes to reach MII stage (75). In contrast, Johnston et al. (1993) reported that BSA was not essential for nuclear maturation in the cat oocytes while FCS showed the negative effect similar to the earlier study (58). The stimulatory effect of BSA on oocyte maturation was also reported in other species. Eroglu (1993) stated that the BSA supplemented medium significantly increased incidence of maturation and improved the handling of porcine oocytes (76). The beneficial characteristics of BSA are : 1) the albumin-bound fatty acids are used as energy substrates 2) the ability to chelate toxic metallic ions contained in the medium and 3) the ability to stabilize membrane (77). From these advantages, BSA was added to maturation medium as the protein supplementation in our study.

It seems that supplementation of maturation media with FSH and LH is based on the presumptive involvement of these hormones in the *in vivo* maturation process. FSH and LH exert major effects on steroidogenesis in their respective follicle target cells through activating of membrane bound adenylate cyclase resulting in enhancing the rate of synthesis of cAMP from ATP and stimulate subsequent physiological responses of the cells (78). FSH has been demonstrated to be involved in proliferation and differentiation of preantral granulosa cell *in vitro* and thus, in growth and normal *in vitro* development of preantral follicle of many species including cows (79), pigs (80) and mice (81). Expression of the FSH receptor gene and FSH binding in granulosa cell and oocyte pointed out that the physiological action of this gonadotropin is on preantral follicles (78, 82). Johnston et al. (1989) stated that the addition of FSH into culture medium increased the incidence of cat

oocyte maturation *in vitro*(57). In addition, Goodrowe et al. (1991) reported that FSH support completion of nuclear maturation in the domestic cat. Although cat oocytes not supplemented with FSH could spontaneously resume meiosis , the integrity of the chromatin may have been compromised and subsequently meiotic development may have been attenuated (68). The effects of LH on the kinetics of oocyte maturation were investigated in several species. Nakagawa and Leibo (1997) reported that the bovine oocytes cultured in the presence of LH exhibited an increasing percentage of maturation rate (83). Wood et al. (1995) demonstrated that adding of combined gonadotropins (FSH+LH) and estradiol produced the highest incidence of domestic cat oocyte maturation *in vitro* whereas supplemented progesterone had no influence on IVM in cats (75). Moreover, Dominko and First (1997) stated that LH enhanced the higher speed of nuclear maturation in bovine oocyte when compared to FSH (82). Additionally, in bovine, LH may enhance IVM through modifying the nutritional environment to increase the energy available for the oocytes (84). The denuded oocytes did not respond to this hormone, thereby implicating the cumulus cell as the mediator of LH effect. Concentration of FSH and LH used in bovine IVM protocols varied in most studies between 1 to 10 μg/ml while physiological concentrations in the follicular fluid of preovulatory follicle at the time of the LH peak and thereafter are much lower than concentration used in IVM. In the present study as well as others (16, 61), 1 μg/ml of commercially available of FSH and LH were used. This concentration seems to be sufficient for germinal vesicle breakdown (GVBD) in cat oocytes. Although, the presence of estradiol in culture medium of *in vitro* maturation had no

effect on meiosis progression of oocyte in other species (85), there were several lines of evidence which suggest that estradiol supports cytoplasmic maturation assisting fertilization and early post fertilization development. In the present study a concentration of 1 μg/ml estradiol was added in cultured medium similar to other reports (56, 86). It was found that FSH and estradiol supplementation enhanced the maturation rate of cat oocytes (75).

The maturation rates of grades 1 and 2 at 32, 36 and 48 h in the present study were not significantly different. The meiotic stage indicated by aceto-orcein staining showed that the time course of cat IVM required at least 32 h. The oocytes remained immature after being cultured for 36 and 48 h mostly arrested in GV stage. The similar rate of maturation between grades 1 and 2 oocytes in this study indicated that the density of surrounding cumulus cells did not affect the achievement of meiotic maturation in domestic cat oocytes. Isobe et al.(1998) demonstrated that the disruption of gap junctions within cumulus cells induced the meiotic resumption in pig oocytes whereas disruption between the oocyte and cumulus cells which blocks the conduction of meiosis inhibitory signal from outer cumulus cells to the oocytes had no such induction (87). In addition, Zhou et al.(1991) demonstrated that IGF-I mRNA expression was detected only in granulosa cells closest to the growing oocytes. (29) This observation implied that the single layer granulosa cells in close proximity to the oocytes which are filled with fluid containing secreted protein from oocytes had an essential role during oocyte maturation.

The present study demonstrated an influence of IGF-I in the stimulation of domestic cat oocyte maturation *in vitro*. This promoting effect, however, occurred only in the oocytes with compact cumulus layers which are classified as good quality oocytes. In partial cumulus intact and denuded oocytes, the maturation rates were lower compared to cumulus enclosed oocytes in both DMEM and DMEM+ IGF-I. These morphological characteristics in cat oocytes reflect the different quality and ability to achieve nuclear maturation.

The essential role of cumulus cells in promoting normal cytoplasmic maturation was suggested by Vanderhyden and Armstrong (1989) (73). The presence of cumulus cells was necessary for oocyte maturation ,and frequencies significantly increased as shown in grades 1 and 2 comparing to grades 3 and 4 oocytes. The role of cumulus cells in oocyte maturation was reported in rats (73), cattles (88, 89), pigs (87), and cats (16, 86). The oocytes classified as partially denude were considered as intermediate between those with a complete and lacking investment. Either early degeneration changes or the aspiration procedure would account for the incompleteness of the investment. The latter possibility is unlikely because the diameter of the needle and pipette employed in the present experiment is much larger than the size of oocytes with their investment. The domestic cat oocytes with partial cumulus surrounding and denuded oocytes had a higher percentage of chromatin degeneration compared to oocytes with complete investment. The results of the present study clearly indicate that the meiotic competence of cat oocytes *in vitro* can be estimated by assessment of gross

morphology of the oocytes . The presence of an intact complement of cumulus cells surrounding the oocyte and a homogenous-appearance of ooplasm are the accurate indicators of immature oocyte ability to undergo maturation and embryo development (87, 89-91). Wood and Wildt (1997) reported that the most significant trait for classification of oocyte quality in the cat was the cytoplasmic quality indicated by translucent or mosaic pattern under macroscopic visualization (86). In cat oocytes, a great amount of lipid droplet or fatty yolk dramatically accumulates in ooplasm showing a dark appearance (1, 86) . The grades 3 and 4 oocyte appeared to reduce their ability to mature. This is probably because these oocytes were in the final growth phase that lacks of lipid yolk deposition and properly arranged organelles in cytoplasm. In addition to the high lipid contents, the cat oocytes exhibited other particular characteristics : its bilayered zona pellucida (92,93) and its lack of cortical-free domain after maturation (53). These observations were confirmed by the present study that most of the mature oocytes demonstrated the bilayered zona pellucida. However, the further ultrastructural investigation of the cortical granules was not performed. The present result pointed out that the presence of cellular investment and ooplasm feature may be the very essential determinants of the viability and ability of domestic cat oocytes to mature.

Considering the data reported here, exposure to IGF-I significantly stimulated the resumption of meiosis only in grades 1 and 2 oocytes but not in grades 3 and 4 oocytes. The results suggest that the effect of IGF-I seems to be involved with the surrounding cumulus cells. Based on the present

results , grades 1 and 2 oocytes might be the most appropriate oocytes for maturation and subsequent fertilization *in vitro*. Addition of IGF-I to culture medium has been successfully employed as a supplement of *in vitro* maturation in many species such as bovine (94), pig (95) and rabbit (47, 96). Adashi et al. (1991) demonstrated that rat granulosa cells expressed IGF-I receptor that was increased in number following treatment with FSH *in vitro* (90). Zhou and Bondy (1993) demonstrated the expression of IGF-I and IGF-I receptor in growing oocytes of infant and showed that this pattern of IGF-I mRNA distribution in human ovary (25) differed from that found in rat (29) where both IGF-I and IGF receptor mRNA were abundant and evenly distributed in granulosa cells. Yoshida et al. (1998) demonstrated that the transcripts for IGF-I occurred in bovine immature oocytes through mature oocytes (27). Samaras et al. (1992) demonstrated the expression of IGF-I and transcription of IGF binding proteins (IGFBP) in porcine ovary(97). Although Boomsma et al. (1994) reported the pattern of IGFBP-I in cat embryo implantation site(98). The presence of IGF-I receptor or the pattern of IGF-I gene expression in cat ovaries or oocytes has never been examined. The present study indicated that IGF-I may exist in cumulus cell of cat oocytes. However, the IGF-I expression should be further investigated. The stimulatory effect of IGF-I on oocyte maturation was studied in various species. Lorenzo et al. (1994) reported that IGF-I added in culture medium TCM-199 significantly enhanced the percentage of bovine mature oocytes from 35 to 45% , and this effect occurred only in the cumulus enclosed oocytes (44). Moreover, Lorenzo et al. (1995) showed that IGF-I increased the incidence of GV activation in various maturation periods from 6 to 30 h

(94). Sirothkin et al. (1998) demonstrated that IGF-I had a potency to stimulate meiotic maturation in porcine (95). Nevertheless, Reed et al. (1993) found that IGF-I had no positive effect on nuclear maturation of porcine oocytes (48). This lack of effect was confirmed by Grupen et al. (1997). The controversial results are probably due to the diversity of individual methodology (99). IGF-I used in the present study enhanced oocyte maturation in cumulus oocyte complexes but not in denuded oocytes. This result was in accordance with the results in bovine oocytes mentioned above. Rieger et al.(1998) reported that 100 ng/ml of IGF-I increased the maturation rate of bovine oocytes *in vitro*(100). Furthermore, Yoshimura et al.(1996) stated that IGF-I stimulated follicular growth and the resumption of rabbit follicular oocytes in a dose dependent manner (96). In addition, Lorenzo et al.(1996) demonstrated that the addition of IGF-I at various doses (50, 100 and 200 ng/ml) enhanced the incidence of MII rabbit oocytes and the maximum result occurred at the dose of 100 ng/ml (47). The effective concentration of IGF-I was also similar in porcine oocytes maturation (95). This concentration of IGF-I used in the present study also has a positive effect for cat oocyte mature *in vitro*. Several studies demonstrated that the stimulatory effect of IGF-I was enhanced by combination with EGF (42, 94, 100, 101). There have been several lines of evidence showing that IGF-I+EGF stimulated metabolism during oocyte maturation and enable cumulus expansion while IGF-I alone did not allow cumulus cell to expand (47, 100). Lorenzo et al. (1994 and 1996) showed that IGF-I did not affect cumulus expansion both in bovine and rabbit oocytes (44, 47). In contrast, Bever et al. (1997) reported that IGF-I has a stimulatory effect on cumulus

expansion in bovine (85). However, in porcine oocytes, IGF-I enabled cumulus expansion in response to FSH *in vitro.* (28) . In this study , IGF-I has a minimal effect on cumulus expansion at 32 h but this expansion increased after 36 and 48 h . Generally, cumulus cell expansion is one of the important features of oocyte maturation. Nevertheless, in the present study, IGF-I enhanced cat oocyte maturation *in vitro* and may benefit the rate of *in vitro* embryo production in this species.

CHAPTER VI

CONCLUSION

The present study demonstrated that IGF-I significantly enhanced follicular development and oocyte maturation in domestic cat. However, the stimulatory effect of IGF-I on cat oocyte maturation was exerted on only the cumulus-surrounded oocytes. This observation implies that the mechanism whereby IGF-I modulate resumption of meiosis in oocyte may be mediated via the cumulus cells. The cellular investment and ooplasm characteristics could be used as the indicator of oocyte quality and integrity to mature *in vitro*. The maturation rate of cat cumulus oocytes complex cultured at 32, 36 and 48 h were not significantly different both in the IGF-I supplemented and non-supplemented medium. This result indicates that the 32-h culturing time is sufficient for cat oocytes to reach the MII stage *in vitro*.

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