

3-1-2014

Recombinant Human Follicle Stimulating Hormone and Growth Factors Improve the Meiotic and Developmental Competence of Cat Oocytes

Theerawat Tharasanit

Chommanart Thongkittidilok

Thanida Sananmuang

Mongkol Techakumphu

Follow this and additional works at: <https://digital.car.chula.ac.th/tjvm>



Part of the [Veterinary Medicine Commons](#)

Recommended Citation

Tharasanit, Theerawat; Thongkittidilok, Chommanart; Sananmuang, Thanida; and Techakumphu, Mongkol (2014) "Recombinant Human Follicle Stimulating Hormone and Growth Factors Improve the Meiotic and Developmental Competence of Cat Oocytes," *The Thai Journal of Veterinary Medicine*: Vol. 44: Iss. 1, Article 2.

Available at: <https://digital.car.chula.ac.th/tjvm/vol44/iss1/2>

This Article is brought to you for free and open access by the Chulalongkorn Journal Online (CUJO) at Chula Digital Collections. It has been accepted for inclusion in The Thai Journal of Veterinary Medicine by an authorized editor of Chula Digital Collections. For more information, please contact ChulaDC@car.chula.ac.th.

Recombinant Human Follicle Stimulating Hormone and Growth Factors Improve the Meiotic and Developmental Competence of Cat Oocytes

Theerawat Tharasanit^{1*} Chommanart Thongkittidilok¹ Thanida Sananmuang²
Mongkol Techakumphu¹

Abstract

In vitro embryo production has proven as potential tool for understanding factors involving developmental biology of embryos. This study aimed at examining the effects of recombinant human follicle stimulating hormone (rhFSH) on meiotic resumption and also on developmental competence of cat oocytes. Experiment 1 studied mRNA expression of luteinizing hormone (LH), follicle stimulating hormone (FSH), epidermal growth factor (EGF) and insulin like growth factor-1 (IGF-1) receptors (R) in cumulus cells. mRNA expression of LH receptor in cumulus cells after oocyte maturation was additionally examined. Experiment 2 examined the effect of different rhFSH concentrations (0.01, 0.05, 0.1 and 1 IU/ml) on meiotic competence. *In vitro* oocyte maturation without rhFSH served as the control group. The effects of 'chosen-dose' rhFSH in combination with rhLH and growth factors (IGF-1 and EGF) on developmental competence were tested in Experiment 3. Cumulus cells surrounding immature oocytes expressed mRNA of *FSHR*, *EGFR* and *IGF1R*, but not *LHR*. *LHR* was only expressed after *in vitro* maturation. All rhFSH concentrations significantly increased maturation rates of cat oocytes in a dose-dependent manner when compared with the control group ($p < 0.05$). The 0.1 IU/ml rhFSH was selected for Experiment 3 due to its efficacy. The developmental competence of cat oocytes was not affected by rhLH supplementation ($p > 0.05$), but IGF-1 and EGF significantly promoted cleavage and blastocyst formation rates compared with rhFSH and rhFSH plus rhLH ($p < 0.05$). It is concluded that cumulus cells express *FSHR*, *LHR*, *EGFR* and *IGF1R*. However, *LHR* mRNA was only expressed after *in vitro* maturation. Efficacy of FSH in meiotic resumption is in a dose-dependent manner. The combination of rhFSH and growth factors (IGF-1 or EGF) best improves cytoplasmic maturation of feline oocytes in terms of cleavage and blastocyst rates.

Keywords: cat, embryo development, growth factor, oocyte, recombinant follicle stimulating hormone

¹Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

²Faculty of Veterinary Medicine, Rajamangala University of Technology, Tawan-OK, Chonburi 20110, Thailand

* **Correspondence:** theerawat.t@chula.ac.th

Introduction

Fundamental and application study of reproductive biotechnologies including *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture of embryo (IVC) in domestic cat has become an increasingly important prospects for conservation of valuable/endangered felid species (Swanson, 2006) and also for biomedical research (O'Brien et al., 1999). However, these techniques have met only limited success, especially when developmental competence between *in vivo* and *in vitro* derived oocytes is compared (Roth et al., 1994; Swanson et al., 1994). Generally, *in vivo* follicle development, meiotic resumption and subsequent ovulation are centrally governed by luteinizing hormone (LH) and follicle stimulating hormone (FSH) as a part of gonadal-pituitary axis. The luteinizing hormone (LH) receptors express in correspondence with increased size of follicles (Xu et al., 1995; Bao et al., 1997). These receptors bind specifically to circulating LH and meiotic resumption is then initiated (Mehlmann, 2005; Tsafirri et al., 2005). As a result, an immature oocyte within a dominant follicle undergoes a series of nuclear and cytoplasmic changes in order to complete its competence. In contrast to *in vivo* situation, the LH as meiotic resumption inducer plays a lesser role in this process *in vitro* when compared with follicle stimulating hormone (FSH) (Eppig, 1991; Su et al., 1998). The FSH binds to the FSH receptors located at the membrane of cumulus-corona radiata cells and subsequently stimulates signals, via gap junctions between oocyte and its companion cells, to induce meiosis resumption (Matzuk et al., 2002).

Currently, a number of strategies have been developed, aiming specifically at improving the cytoplasmic competence of mammalian oocytes during oocyte maturation (Gilchrist and Thompson, 2007). These strategies revealed mechanisms insight the process of oocyte maturation, and also found potential markers that could reflect oocyte's developmental competence. Indeed, the study on the effects of individual gonadotropin (LH and FSH) have been restricted due primarily to the fact that most gonadotropins used for IVM are frequently contaminated with LH or other by-products (Törnell et al., 1995). It is therefore important to test the role of FSH without LH contamination. Recombinant FSH has been shown to stimulate oocyte maturation and subsequent embryo development in several species, including domestic cat (Sananmuang et al., 2010; Sananmuang et al., 2011; Tharasanit et al., 2011). However, optimal concentration and interaction with LH and other growth factors have not been reported.

While the FSH is known to stimulate both nuclear and cytoplasmic maturation of the oocytes, several studies also indicated that some growth factors such as epidermal growth factor (EGF) and insulin like growth factor-1 (IGF-1) improved the fertilization rate and embryo development in pig (Chen et al., 2008; Uhm et al., 2010), bovine (Lorenzo et al., 1994; Lonergan et al., 1996) and cat (Merloet et al., 2005). EGF and IGF-1 play a central role in cell proliferation and differentiation during embryogenesis (Lonergan et al.,

1996). In domestic cat, the effects of these growth factors on oocyte maturation and embryo development have been obscured. This study aimed at examining the effects of 'LH-free' gonadotropin (recombinant FSH) on nuclear and cytoplasmic maturation of cat oocytes. Furthermore, the effects of epidermal growth factor and insulin like growth factor-1 on developmental competence of *in vitro* matured oocytes were also studied.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich, St. Louis, USA, unless otherwise specified.

Oocyte collection and *in vitro* maturation of cumulus oocyte complexes (COCs): Feline ovaries from mixed breeds and various stages of estrous cycle were collected after routine ovariohysterectomy (OVH) from the Veterinary Public Health, Division of the Bangkok Metropolitan Administration and the Fertility and Neutering Clinic, Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. The ovaries were maintained and transported at room temperature (approximately 26-30°C) in a saline solution (0.9% (w/v) NaCl, Thai Otsuka Pharmaceutical Co, Ltd, Bangkok, Thailand) supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin (M&H Manufacturing Co, Ltd, Bangkok, Thailand). Within 6 hours after OVH, the ovaries were dissected from connective tissue and then washed twice in a holding medium (HM) (Hepes-buffered M199, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 4 mg/ml bovine serum albumin). Cumulus oocyte complexes (COCs) were isolated from the follicles by repeatedly slicing the ovaries using a surgical blade. The COCs were morphologically classified grading at a magnification x 40 under a stereomicroscope (SMZ645 Nikon, Tokyo, Japan). The COC quality was determined by the appearance of the ooplasm and the cumulus-corona radiata cells surrounding the oocytes. Oocytes with pale ooplasm as classified as non-fully grown immature oocytes were discarded from the study. The COCs with uniform size with darkened homogenous ooplasm and completely surrounded by at least 5 layers of cumulus cells were selected and randomly allocated according to the experimental design.

For *in vitro* maturation, groups of 35-40 COCs were cultured for 24 h in NaHCO₃ buffered M199 supplemented with 2 mM L-glutamine, 1 mM pyruvate, 100 IU/ml penicillin, 4 µg/ml gentamycin and 4 mg/ml BSA (embryo tested grade). The culture was performed at 38.5°C in a humidified condition with 5% CO₂ in air. Depending on experiment, this IVM medium was supplemented with different concentrations of recombinant human follicle stimulating hormone (rhFSH, Organon, The Netherlands), recombinant human luteinizing hormone (rhLH, Lutropin alpha, Bangkok, Thailand), insulin like growth factor-1 (IGF-1) and epidermal growth factor (EGF).

In vitro fertilization and embryo culture: After 24 h of *in vitro* maturation, *in vitro* fertilization was performed. In brief, groups of 10 *in vitro* matured oocytes were transferred to 50 μ l droplets of IVF medium (Tyrode's balanced salt solution containing 1% (v/v) MEM non-essential amino acids (NEAA), 6 mg/ml BSA, 100 IU/ml penicillin, 30 μ g/ml heparin, 1 mM L-glutamine, 0.36 mM sodium pyruvate and 0.11 mM calcium lactate). Frozen-thawed semen used in this study was collected from a proven-fertility tom cat using an electro-ejaculation as previously described by Wildt et al. (1986). The semen was cryopreserved by horizontally placing the straws at 4 cm above liquid nitrogen vapors for 10 min and then plunged into liquid nitrogen. The semen was thawed at 37°C for 30 sec and examined for progressive motility. Sperm suspension with more than 50% progressive motility were diluted with IVF medium. The sperm were co-incubated with oocytes at a final concentration of 0.5×10^6 sperm/ml.

After IVF for 18-24 h, cumulus cells were gently removed. Five to ten presumptive zygotes were then washed and cultured for 24 hours in 50 μ l droplets of Tyrode's medium containing 4 mg/ml BSA, 100 μ g/ml streptomycin and 100 IU/ml penicillin. On day 2 after IVF (IVF = day 0), cleaved embryos (4 to 8 cell stage) were further cultured in Tyrode's medium supplemented with 10% (v/v) FCS (Gibco®, Invitrogen, CA, USA). The culture medium was changed every second days. In all cases, *in vitro* culture was performed at 38.5°C in a humidified atmosphere with 5% CO₂.

Assessments of nuclear stages of in vitro matured oocytes and embryo development: To evaluate stage of nuclear maturation of cultured oocytes, cumulus cells were mechanically removed and the denuded oocytes were then fixed in 4% (w/v) paraformaldehyde (PF) overnight. The fixed oocytes were washed and then maintained in phosphate buffered saline (PBS) supplemented with 0.1% (w/v) BSA (PBS-BSA) until analysis. Fluorescent DNA probe (4',6-Diamidino-2-phenylindole: DAPI, 0.1 μ g/ml) was used to stain the oocyte nucleus. The fluorescently labelled oocytes were then mounted on a glass microscopic slide and

then visualized with an epifluorescent microscope (BX51 Olympus, Shinjuku, Japan). The nuclear status of IVM oocytes was classified according to chromatin morphology including germinal vesicle stage (GV), metaphase I (MI), metaphase II (MII) and degenerate oocytes.

Cytoplasmic maturation of the oocytes was determined by the capability of particular oocytes to develop to blastocysts. Percentages of cleavage and blastocyst (>60 blastomeres with blastocoelic formation) were evaluated under an inverted light microscope on days 2 and 7 of embryo culture, respectively (day 0=IVF). In order to examine embryonic quality, the embryos were fixed in 4% PF for 10 min and then maintained in PBS-BSA for immunolabelling against octamer-binding transcription factor 4 (Oct-4) and DNA in order to count cells within inner cell mass (ICM) and total cell number. This Oct-4 expression reflects the pluripotency of the ICM (Kirchhof et al., 2000). The embryos were first incubated at room temperature (26°C) for 15 min in a blocking solution containing 0.38% (w/v) glycine, 0.1% (v/v) Triton-X 100 and 3% (v/v) donkey serum. After washing twice, the embryos were then incubated at 37°C for 1 hour with goat polyclonal Oct-4 antibody (1: 100, Santa Cruz Biotechnology), followed by incubation for 1 h with donkey Alexa-488 conjugated anti-goat secondary antibody (1: 100, Santa Cruz Biotechnology, Dallas, USA). The embryos were finally labelled with DAPI and mounted on a slide with antifade mounting medium (Vectashield®, Vector laboratories, Burlingame, USA) to mitigate photo bleaching during examination with an epifluorescent microscope. The examination was carried out using 488 and 380 nm fluorescent filters for Oct-4 and DNA, respectively.

Reverse transcriptase polymerase chain reaction (RT-PCR): RNA was extracted from pooled of cumulus cells isolated before and after oocyte maturation (20 COCs for each maturation stage) using an Absolutely RNA Nanoprep Kit (Stratagene, San Diego, CA, USA) following the manufacturer's instructions.

Table 1 Description of forward (FP) and reverse (RP) primers used to assess expression of target genes in cumulus cells obtained from COCs before and after maturation

Gene	Sequence (5'-3'orientation)	Fragment length (bp)	Reference/NCBI Accession number
GAPDH	FP: GGAGAAAGCTGCCAAATATG RP: AGGAAATGAGCTTGACAAAGTGG	191	Sano et al., 2005
FSHR	FP: CATGCTGCTAGGCTGGATCTT RP: CTGGCGATCTTGGGTGCTACT	250	AY521181.1
LHR	FP: CTAATGCCTTTGACAACCTCCTC RP: CCCATTGAATGCATGACTTTGTGA	342	KF040100.1
EGFR	FP: AGATTGCGAAGGGCATGAAC RP: GGCGTCTTACCAGGACATT	118	HQ185236.1
IGF1R	FP: GCACAAGGAGCAGATGACATT RP: CAGGTCCGGCCACTTTAAA	255	Waurich et al., 2010

The quality and quantity of extracted RNA was determined using a spectrophotometer (Nanodrop ND-2000, Wilmington, Delaware, USA) and immediately stored at -80°C until further use. First-Strand cDNA Synthesis Kit (SuperScript III Kit, Invitrogen, Carlsbad, CA, USA) was used for reverse transcription (RT). The extracted RNA was incubated with random hexamers (50 ng/ μl) and 10 mM dNTP mix at 65°C for 5 min prior to being placed on ice for 1 min. The mastermix containing 2 μl of 10xRT buffer, 4 μl of 25 mM MgCl_2 , 2 μl of 0.1 M DTT, 1 μl of RNaseOut (40U/ μl) and 1 μl of SuperScript III RT (200 U/ μl) was added. The mixture was further incubated at 50°C for 50 min followed by 5 min incubation at 85°C . RNA template from the cDNA:RNA hybrid molecule was removed by digestion with 1 μl of RNase H (2U/ μl) after first-strand synthesis at 37°C for 20 min. The products were finally stored at -20°C for further use in polymerase chain reaction (PCR).

Polymerase chain reaction (PCR) was performed using a thermal-cycler. The primers used in this study are shown in Table 1. Each PCR reaction was consisted of reverse transcription product (1000 ng/ μl of cDNA) and reaction mixture containing GoTaq[®] Green Master Mix (Promega, USA), nuclease free water and primers (Table 1). Nuclease free water was used as a negative control. The thermal cycling conditions were as follows: 2 min at 95°C to activate Tag DNA polymerase, 45 cycles of 30 sec at 95°C for denaturing, 30 sec at 55°C for annealing and 30 sec at 72°C for extension. At the end of program, the amplified products were run in 2% agarose gel (Bio-Rad, CA, USA) prepared in 1xTBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8) containing 0.4 mg/ml ethidium bromide (Promega, WI, USA). The separated products in agarose gel were visualized under UV light using a gel documentation (Syngene, CB, UK). *EGFR* and *FSHR* primers were designed from *Felis catus* complete cds using NCBI primer designing tool. *LHR* primer was designed based on homologous sequences of *Bos taurus* luteinizing hormone choriogonadotropin receptor (*LHCGR*) mRNA (Accession number: NM_174381.1), *Homo sapiens* luteinizing hormone choriogonadotropin receptor (*LHCGR*) mRNA (Accession number: NM_000233.3), *Mus musculus* luteinizing hormone choriogonadotropin receptor (*LHCGR*) mRNA (Accession number: NM_013582.2), and *Rattus norvegicus* luteinizing hormone choriogonadotropin receptor (*LHCGR*) mRNA (Accession number: NM_012978.1). To confirm the amplified *LHR*, the amplicon was extracted using Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech, CA, USA) and then was sequenced. The sequences were blasted in NCBI GenBank to determine nucleotide identity to other species using NCBI BLAST. The resulted primers (*LHR*) were submitted to NCBI (Accession number: KF040100.1)

Experimental design

Experiment 1: mRNA expressions of LH, FSH, EGF and IGF-1 receptors in cumulus oocyte complexes (COCs): mRNA expressions of FSH, LH, epidermal growth factor and insulin like growth factor-1 receptors were performed. The cumulus cells of

immature oocytes (n=10-20) were collected and RNA was extracted for RT-PCR. *GAPDH* gene expression was used as an internal control. For LH, cumulus cells collected after maturation were additionally analysed. The transcript product of *LHR* was further sequenced and the nucleotide identity of the sequence was identified.

Experiment 2: effect of different rhFSH concentrations on meiotic competence of cat oocytes: A total of 292 COCs were used to test the effect of different rhFSH concentrations as follows: 1) 0.01 IU/ml (n=82), 2) 0.05 IU/ml (n=72), 3) 0.1IU/ml (n=68) and 1 IU/ml (n=70) on meiotic competence (nuclear maturation). COCs (n=103) cultured in IVM medium without rhFSH served as the control group.

Experiment 3: effect of rhFSH, LH and growth factors (IGF-1 and EGF) on developmental competence of cat oocytes: This study was designated to examine the effects of 'chosen dose' of rhFSH during IVM on developmental competence of oocytes in terms of cleavage, morula and blastocyst formation rates. Effect of growth factors (insulin like growth factor-1(n=105) and epidermal growth factor (n=164) and luteinizing hormone (n=100) during IVM was additionally examined.

Statistical analysis

Data were pooled from 4 independent replicates. SPSS program (version 17, IBM Corporation, Armonk, New York, USA) was used for statistical analysis. Differences among oocyte maturation, cleavage and blastocyst formation rates were compared using a Chi-Square statistical test. Total cell number and Oct-4 positive cells within blastocysts were compared by one-way ANOVA. In all cases, differences were considered significant when $p < 0.05$.

Results

Experiment 1 demonstrated that cumulus cells surrounding immature oocytes expressed mRNA of several genes including *FSHR*, *EGFR* and *IGF1R*. At immature stage, the cumulus cells did not express *LHR* gene (Fig 1A) but this receptor subsequently expressed after 24 hours *in vitro* maturation (Fig 1B). The *LHR* was fairly conserved among domestic species with nucleotide identity of 94%, 93%, 89% and 87% for bovine, human, rat and mouse, respectively. The expression of *FSHR* coincided with the findings that rhFSH supplementation in the oocyte maturation medium significantly increased the numbers of oocytes reaching metaphase II (MII) stage compared with the non-rhFSH control (MII rates: ~60-70 % vs. 36.9, $p < 0.05$; Table 2). Although the MII rates were not different among the rhFSH concentrations, the 0.01 IU rhFSH was inferior to the other rhFSH concentrations, in terms of the remaining GV stage oocytes after maturation (% GV: 34% vs. 13-14%, respectively; Table 2). According to results of Experiment 2, the 0.1 IU/ml rhFSH was selected to demonstrate the supportive ability of rhFSH on developmental competence by means of cleavage, morula and blastocyst rates (Experiment 3). Indeed, oocytes that

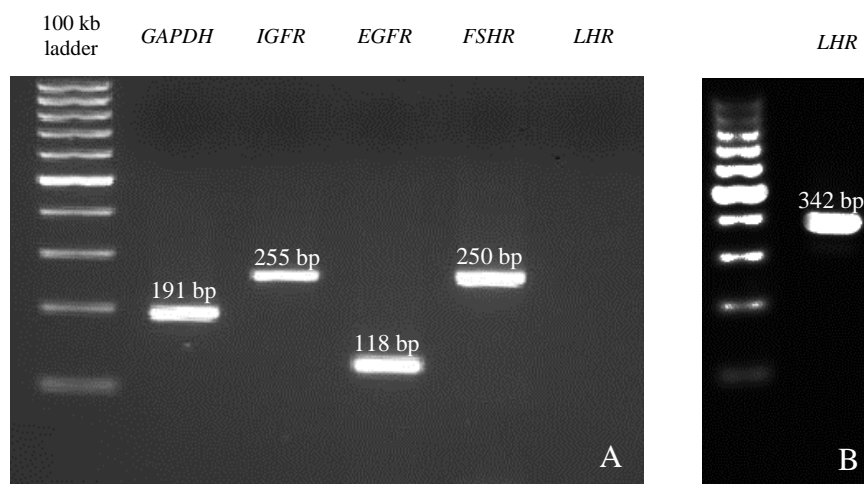


Figure 1 RT-PCR analysis of target genes in COCs. A: PCR products (*GAPDH*, *IGFR*, *EGFR*, *FSHR* and *LHR*) of cumulus cells surrounded immature oocytes. B: *LHR* mRNA expression was observed only in cumulus cells of *in vitro* matured oocytes.

were matured in the maturation medium supplemented with only rhFSH had significantly lower developmental capability compared with the oocytes matured with rhFSH and growth factors (EGF or IGF-1, Table 3). In contrast, the addition of LH into the maturation medium did not improve the developmental competence (Table 3). In all cases, the embryonic quality in terms of cell number and Oct-4 positive cells was not significantly different among the groups ($p > 0.05$).

Discussion

This study demonstrates that follicle stimulating hormone, insulin like growth factor-1 and epidermal growth factor synergistically exerted the meiotic and developmental competence of cat oocytes, while LH failed to stimulate embryo development. Experiment 1 indicated that cumulus cells expressed several genes including *FSHR*, *LHR*, *EGFR* and *IGF1R* but the expression was stage-of-maturation specificity. These genes play a central role in controlling meiotic and cytoplasmic maturation of mammalian oocytes. An action of a particular gene is mediated via its receptors located on the somatic cells surrounding the oocytes rather than directly to the oocytes since the oocytes seem to lack gonadotropin receptors (Amsterdam et al., 1975). The oocytes acquire their meiotic signals via transzonal process and gap junction communication between oocyte and cumulus-radiata cell complex (Matzuk et al., 2002). Although the mechanism by which the gonadotropin stimulates the meiotic resumption is entirely unclear, these gonadotropins are mediated through cyclic adenocine 3',5'-monophosphate (cAMP) (Downs et al., 1988; Eppig, 1989) and subsequent activation of mitogen activated protein kinase (MAPK). Our study did not observe *LHR* expression in cumulus cells of immature cat oocytes similar to other studies (Van Tol et al., 2006). It is postulated that the *LHR* expression in cat oocytes is controlled by some factors such as

FSH and maybe size of follicles. For example, *LHR* did not expressed in medium-size (2-6 mm) bovine follicles, but was highly expressed only in FSH-primed dominant follicle (Ireland and Roche, 1982). This study also found that the LH receptors were only expressed after *in vitro* maturation. This finding is coin contrast to a report indicating that *LHR* mRNA could not be detected in cumulus cells before or after IVM (Nuttinck et al., 2004). It is unclear about factors within IVM medium that stimulated the *LHR* expression in cumulus cells surrounding cat oocytes but it is possible that FSH can stimulate the *LHR* expression via increase in intracellular cyclic AMP (Downs et al., 1988; Eppig, 1989). Indeed, specific pathways for FSH primed *LHR* expression during *in vitro* maturation remain to be studied. The reasons mentioned above support our finding and other studies that adding recombinant LH during IVM did not improve cleavage and blastocyst formation rates (Choi et al., 2001; Ali and Sirard, 2002). In Experiment 2, rhFSH without any LH contamination significantly induced meiotic resumption in a dose- dependent manner. This finding indicated convincingly that FSH alone could stimulate meiosis *in vitro* as similar to other species. When FSH binds to its receptors, intracellular cAMP increases (Downs et al., 1988) along with the activation of mitogen-activated protein kinase (MAPK) (Shimada and Terada, 2002; Su et al., 2002). The activation of FSH coupled receptors can also be downstream via protein kinase A (PKA) (Su et al., 2002; Liang et al., 2005) and kinase C (PKC) (Fan et al., 2004; Jin et al., 2006). Indeed, FSH activates both PKAI and PKAII to increase levels of cAMP in cumulus cells (Downs and Hunzicker-Dunn, 1995). While activation of PKAI prevent meiosis resumption, PKAII is preferable for initiation of oocyte maturation (Newhall et al., 2006). Although FSH is known to stimulate both nuclear and cytoplasmic maturation (Table 2 and 3), we clearly demonstrated that the potential action of FSH on embryo development

Table 2 Meiotic competence of cat oocytes after *in vitro* oocyte maturation for 24 hours in a presence of different concentrations of recombinant human follicle stimulating hormone (rhFSH)

Group	Total oocyte	Oocytes that developed to			
		GV (%)	MI (%)	MII (%)	Degenerate (%)
Control (no rhFSH)	103	55 (53.4) ^a	3 (2.9) ^a	38 (36.9) ^a	7 (6.8) ^a
0.01 IU rhFSH	82	28 (34.1) ^b	2 (2.8) ^a	49 (59.8) ^b	3 (3.7) ^a
0.05 IU rhFSH	72	10 (13.9) ^c	6 (8.3) ^a	49 (68.1) ^b	7 (9.7) ^a
0.1 IU rhFSH	68	9 (13.2) ^c	3 (4.4) ^a	49 (72.1) ^b	7 (10.3) ^a
1.0 IU rhFSH	70	9 (12.9) ^c	6 (8.6) ^a	50 (71.4) ^b	5 (7.1) ^a

^{a, b and c} Within a column, different superscripts denote values that differ significantly.

Table 3 Developmental competence and embryonic quality of cat oocytes matured *in vitro* in maturation medium supplemented with rhFSH (0.1 IU/ml), rhLH, EGF and IGF-1

Group	No. oocyte	Embryo development		Cell number (mean±SD)	Oct-4 positive cells
		Cleavage (%)	Blastocyst* (%)		
rhFSH	160	65 (40.6) ^a	20 (30.8) ^a	80.7±16.7 ^a	8.3±7.2 ^a
rhFSH/rhLH	100	41 (41) ^a	11 (26.8) ^a	76.9±13.1 ^a	6.9±6.2 ^a
rhFSH/EGF	164	102 (62.2) ^b	38 (37.2) ^b	89±22.5 ^a	11±8.5 ^a
rhFSH/IGF-1	105	58 (55.2) ^b	23 (39.6) ^b	90.2±16.3 ^a	9.7±6.2 ^a

* blastocyst development in relation to cleaved embryos

^{a, b} within a column, different superscripts denote values that differ significantly

(cytoplasmic maturation) was limited. However, when IGF-1 and EGF were added into FSH containing IVM medium, the two growth factors synergistically improved the developmental competence of cat oocytes (Lonergan et al., 1996; Merlo et al., 2005). As similar to several reports, these two growth factors bind to its receptors and, in turn, activation PI3K and ERK pathways. While the mechanisms underlining the cytoplasmic maturation have yet to be further examined, the PI3K and ERK-MAPK pathways are essential for organelle remodeling, cell survival and proliferation (Li et al., 2008). This cellular activation during cytoplasmic maturation is critical for capability of embryos to develop to blastocyst and also to term because maternal mRNA stored within ooplasm will vanish during early development (2-16 cell stage) depending on species. Thus, premature reduction in the maternal mRNA will adversely affect maternal zygotic genome transition, resulting in embryonic block and death (Wang et al., 2006; Schier, 2007).

It is concluded that cumulus cells express several genes (*FSHR*, *LHR*, *EGFR* and *IGF1R*) necessary for oocyte maturation and embryo development. These receptor genes were present at an immature stage except that of *LHR* which was later on

expressed after *in vitro* maturation. FSH without other gonadotropins contamination can be used to improve nuclear and cytoplasmic maturation of cat oocytes in a dose- dependent manner. rhFSH at 0.1 IU/ml was the most appropriate dose for *in vitro* maturation and the effects in promoting embryo development is synergistic with IGF-1 and EGF.

Acknowledgements

This study was financially supported by Thailand Research Fund (RSA5680028). We would like to thank the Veterinary Public Health Division of the Bangkok Metropolitan Administration and the Fertility and Neutering Clinic, Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University for sample collection.

References

Ali A and Sirard MA 2002. The effects of 17β-estradiol and protein supplement on the response to purified and recombinant follicle stimulating hormone in bovine oocytes. *Zygote*. 10: 65-71.

- Amsterdam A, Koch Y, Lieberman ME and Lindner HR 1975. Distribution of binding sites for human chorion gonadotropin in the preovulatory follicle of the rat. *J Cell Biol.* 67: 874-900.
- Bao B, Garverick HA, Smith GW, Smith MF, Salfen BE and Youngquist RS 1997. Changes in messenger ribonucleic acid encoding luteinizing hormone receptor, cytochrome P450-side chain cleavage, and aromatase are associated with recruitment and selection of bovine ovarian follicles. *Biol Reprod.* 56: 1158-1168.
- Chen X, Zhou B, Yan J, Xu B, Tai P, Li J, Peng S, Zhang M and Xia G 2008. Epidermal growth factor receptor activation by protein kinase C is necessary for FSH-I induced meiotic resumption in porcine cumulus-oocyte complexes. *J Endocrinol.* 197(2): 409-419.
- Choi YH, Carnevale EM, Steidel GE, Squires J and Squires EL 2001. Effects of gonadotrophins on bovine oocytes matured in TCM-199. *Theriogenology* 56: 661-670.
- Downs SM, Daniel SA and Eppig JJ 1988. Induction of maturation in cumulus cell-enclosed mouse oocytes by follicle-stimulating hormone and epidermal growth factor: evidence for a positive stimulus of somatic cell origin. *J Exp Zool* 245: 86-96.
- Downs SM and Hunzicker-Dunn M 1995. Differential regulation of oocyte maturation and cumulus expansion in the mouse oocyte-cumulus cell complex by site-selective analogs of cyclic adenosine monophosphate. *Dev Biol.* 172: 72-85.
- Eppig JJ 1989. The participation of cyclic adenosine monophosphate (cAMP) in the regulation of meiotic maturation of oocytes in the laboratory mouse. *J Reprod Fertil.* 38: 3-8.
- Eppig JJ 1991. Maintenance of meiotic arrest and the induction of oocytes maturation in mouse oocytes-granulosa cell complexes developed *in vitro* from pre-antral follicles. *Biol Reprod.* 45: 824-830.
- Fan HY, Huo LJ, Chen DY, Schatten H and Sun QY 2004. Protein Kinase C and mitogen-activated protein kinase cascade in mouse cumulus cells: Cross talk and effect on meiotic resumption of oocyte. *Biol Reprod.* 70: 1178-1187.
- Gilchrist RB and Thompson JG 2007. Oocyte maturation: emerging concepts and technologies to improve developmental potential *in vitro*. *Theriogenology.* 67(1): 6-15.
- Ireland JJ and Roche JF 1982. Development of antral follicles in cattle after prostaglandin-induced luteolysis: Changes in serum hormones, steroids in follicular fluid, and gonadotropin receptors. *Endocrinology.* 111: 2077-2086.
- Jin S, Zhang M, Lei L, Wang C, Fu M, Ning G and Xia G 2006. Meiosis activating sterol (MAS) regulate FSH-induced meiotic resumption of cumulus cell-enclosed porcine oocytes via PKC pathway. *Mol Cell Endocrinol.* 249: 64-70.
- Kirchhof N, Carnwath JW, Lemme E, Anastassiadis K, Schöler H and Niemann H 2000. Expression pattern of Oct-4 in preimplantation embryos of different species. *Biol Reprod.* 63(6): 1698-1705.
- Lonergan P, Carolan C, Van Langendonck A, Donnay I, Khatir H and Mermillod P 1996. Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development *in vitro*. *Biol Reprod* 54: 1420-1429.
- Lorenzo PL, Illera MJ, Illera JC and Illera M 1994. Enhancement of cumulus expansion and nuclear maturation during bovine oocyte maturation *in vitro* by the addition of epidermal growth factor and insulin-like growth factor I. *J Reprod Fertil.* 101: 697-701.
- Matzuk MM, Burns KH, Viveiros MM and Eppig JJ 2002. Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science.* 296(5576): 2178-2180.
- Mehlmann LM 2005. Stops and starts in mammalian oocytes: Recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction.* 130: 791-799.
- Merlo B, Iacono E, Zambelli D, Prati F and Belluzzi S 2005. Effect of EGF on *in vitro* maturation of domestic cat oocytes. *Theriogenology.* 63(7): 2032-2039.
- Li M, Liang CG, Xiong B, Xu BZ, Lin SL, Hou Y, Chen DY, Schatten H and Sun QY 2008. PI3-kinase and mitogen-activated protein kinase in cumulus cells mediate EGF-induced meiotic resumption of porcine oocyte. *Domest Anim Endocrinol.* 34: 360-371.
- Liang CG, Huo LJ, Zhong ZS, Chen DY, Schatten H and Sun QY 2005. Cyclic adenosine 3',5'-monophosphate-dependent activation of mitogen-activated protein kinase in cumulus cells is essential for germinal vesicle breakdown of porcine cumulus-enclosed oocytes. *Endocrinology.* 146: 4437-4444.
- Newhall KJ, Criniti AR, Cheah CS, Smith KC, Kafer KE, Burkart AD and McKnight GS 2006. Dynamic anchoring of PKA is essential during oocyte maturation. *Curr Biol* 16: 321-327.
- Nuttinck F, Charpigny G, Mermillod P, Loosfelt H, Meduri G, Freret S, Grimard B and Heyman Y 2004. Expression of components of the insulin like growth factor system and gonadotropin receptors in bovine cumulus-oocyte complexes during oocyte maturation. *Domest Anim Endocrinol.* 27: 179-195.
- O'Brien SJ, Menotti-Raymond M, Murphy WJ, Nash WG, Wienberg J, Stanyon R, Copeland NG, Jenkins NA, Womack JE and Marshall Graves JA 1999. The promise of comparative

- genomics in mammals. *Science*. 286(5439): 458-462.
- Roth TL, Swanson WF and Wildt DE 1994. Developmental competence of domestic cat embryos fertilized in vivo versus in vitro. *Biol Reprod*. 51(3): 441-451.
- Sananmuang T, Techakumphu M and Tharasanit T 2010. The effects of roscovitine on cumulus cell apoptosis and the developmental competence of domestic cat oocytes. *Theriogenology*. 73(2): 199-207.
- Sananmuang T, Tharasanit T, Nguyen C, Phutikanit N and Techakumphu M 2011. Culture medium and embryo density influence on developmental competence and gene expression of cat embryos. *Theriogenology*. 75(9): 1708-17019.
- Schier AF 2007. The maternal-zygotic transition: death and birth of RNAs. *Science*. 316(5823): 406-407.
- Shimada M and Terada T 2002. Roles of cAMP in regulation of both MAP kinase and p34cdc2 kinase activity during meiotic progression, especially beyond the MI stage. *Mol Reprod Dev*. 62: 124-131.
- Su YQ, Wigglesworth K, Pendola F, O'Brien MJ and Eppig JJ 2002. Mitogen activated protein kinase activity in cumulus cells is essential for gonadotropin induced oocyte meiotic resumption and cumulus expansion in the mouse. *Endocrinology*. 143: 2221-2232.
- Su YQ, Xia GL, Byskov AG, He C, Fu GD and Yang CR 1998. Studies on the effects of gonadotropins on the meiotic resumption of cumulus-enclosed porcine oocytes in vitro. *Chin J Appl Physiol*. 14: 264-267.
- Swanson WF 2006. Application of assisted reproduction for population management in felids: the potential and reality for conservation of small cats. *Theriogenology*. 66(1): 49-58.
- Swanson WF, Roth TL and Wildt DE 1994. *In vivo* embryogenesis, embryo migration, and embryonic mortality in the domestic cat. *Biol Reprod*. 51(3): 452-464.
- Törnell J, Bergh C, Selleskog U and Hillensjo T 1995. Effect of recombinant human gonadotrophins on oocyte meiosis and steroidogenesis in isolated pre-ovulatory rat follicles. *Hum Reprod*. 10: 1619-1622.
- Tharasanit T, Manee-In S, Buarpung S, Chatdarong K, Lohachit C and Techakumphu M 2011. Successful pregnancy following transfer of feline embryos derived from vitrified immature cat oocytes using 'stepwise' cryoprotectant exposure technique. *Theriogenology*. 76(8): 1442-1449.
- Tsafiriri A, Cao X, Ashkenazi H, Motola S, Popliker M and Pomerantz SH 2005. Resumption of oocyte meiosis in mammals: on models, meiosis activating sterols, steroids and EGF-like factors. *Mol Cell Endocrinol*. 234(1-2): 37-45.
- Uhm SJ, Gupta MK, Yang JH, Chung HJ, Min TS and Lee HT 2010. Epidermal growth factor can be used in lieu of follicle-stimulating hormone for nuclear maturation of porcine oocytes *in vitro*. *Theriogenology*. 73(8): 1024-1036.
- Van Tol HTA, Van Eijk MJT and Mummery CL 2006. Influence of FSH and hCG on the meiosis of bovine oocytes surrounded by cumulus cells connected to membrane granulosa. *Mol Reprod Dev*. 45: 218-224.
- Wang H and Dey SK 2006. Roadmap to embryo implantation: clues from mouse models. *Nat Rev Genet*. 7(3): 185-199.
- Wildt DE, Howard JG, Hall LL and Bush M 1986. Reproductive physiology of the clouded leopard: I. Electroejaculates contain high proportions of pleiomorphic spermatozoa throughout the year. *Biol Reprod*. 34(5): 937-947.
- Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA and Youngquist RS 1995. Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. *Biol Reprod*. 53(4): 951-957.

บทคัดย่อ

ฮอร์โมนรีคอมบิแนนต์ ฟอลลิเคิล สติมูเลตติ้งและโกรทแพคเตอร์เพิ่มประสิทธิภาพการเจริญพร้อมปฏิสนธิของโอโอไซต์และตัวอ่อนแมว

ธีรวัฒน์ ธาราศานิต^{1*} ชมนาท ทองกิตติติก¹ ธนิดา สนั่นเมือง² มงคล เตชะกำฟู¹

การผลิตตัวอ่อนภายนอกร่างกายเป็นเทคนิคที่มีศักยภาพในด้านการศึกษาเพื่อให้เข้าใจปัจจัยที่เกี่ยวข้องกับชีววิทยาของตัวอ่อน การศึกษานี้มีวัตถุประสงค์เพื่อตรวจสอบผลของการเสริมฮอร์โมนรีคอมบิแนนต์ ฟอลลิเคิลสติมูเลตติ้ง (เอฟ เอส เอช) ต่อการพัฒนาพร้อมปฏิสนธิ และการเจริญของตัวอ่อนแมว การทดลองที่ 1 เป็นการศึกษาการแสดงออกยีนตัวรับฮอร์โมน ลูทีไนซิง (แอล เอช) เอฟ เอส เอช และ โกรทแพคเตอร์ชนิด อีพีเคอร์มอล (อี จี เอฟ) และ คล้ายอินซูลินที่ 1 (ไอจีเอฟที่ 1) ในเซลล์คิวมูลัสของโอโอไซต์ การทดลองที่ 2 เป็นการศึกษาผลของ เอฟ เอส เอช ในระดับความเข้มข้นต่างๆ กัน (0.01, 0.05, 0.1 และ 1 ใอยูต่อมล.) ต่อการเจริญพร้อมปฏิสนธิของโอโอไซต์ โดยใช้การเลี้ยงโอโอไซต์ในน้ำยาที่ไม่มีสารเสริม เอฟ เอส เอช เป็นกลุ่มควบคุม การทดลองที่ 3 เป็นการศึกษาผลของ เอฟ เอส เอช ร่วมกับ แอล เอช หรือ ร่วมกับโกรทแพคเตอร์ต่ออัตราการพัฒนาของตัวอ่อนการศึกษาครั้งนี้พบว่าเซลล์คิวมูลัส แสดงออกยีนของตัวรับ เอฟ เอส เอช อี จี เอฟ และไอ จี เอฟ ที่ 1 แต่ไม่พบการแสดงออกยีนตัวรับของฮอร์โมน แอล เอช เมื่อทำการตรวจในโอโอไซต์ระยะก่อนการเลี้ยงพร้อมปฏิสนธิ ตัวรับของฮอร์โมนนี้จะแสดงออกภายหลังการเลี้ยงโอโอไซต์ให้พร้อมปฏิสนธิ การเสริม เอฟ เอส เอช มีผลกระทบต่อการพัฒนาของโอโอไซต์ให้อยู่ในระยะพร้อมปฏิสนธิในลักษณะแปรผันตามปริมาณที่ใช้อย่างมีนัยสำคัญ ($p < 0.05$) เมื่อเทียบกับกลุ่มควบคุม ระดับ เอฟ เอส เอช ที่ความเข้มข้น 0.1 ใอยูต่อมล. ถูกนำมาใช้ในการศึกษาที่ 3 เนื่องจากให้ผลดีที่สุด โดยพบว่าเมื่อทำการเสริม เอฟ เอส เอช อย่างเดียว หรือใช้ร่วมกับ แอล เอช ให้ผลต่อร้อยละการพัฒนาของตัวอ่อนแมวระยะคลีเวจและบลาสโตซิสต์ ต่ำกว่ากลุ่มทดลองที่ใช้ เอฟ เอส เอช ร่วมกับ โกรทแพคเตอร์ อย่างมีนัยสำคัญ ($p < 0.05$) การศึกษานี้สรุปได้ว่า เซลล์คิวมูลัสที่อยู่ล้อมรอบโอโอไซต์แสดงออกยีนตัวรับฮอร์โมน เอฟ เอส เอช แอล เอช และโกรทแพคเตอร์ชนิด อี จี เอฟ และไอ จี เอฟ ที่ 1 ผลการเสริม ฮอร์โมน เอฟ เอส เอช ต่ออัตราการเจริญพร้อมปฏิสนธิเป็นไปในลักษณะที่แปรผันตามปริมาณฮอร์โมนที่ใช้ การเสริมฮอร์โมน เอฟ เอส เอช ร่วมกับโกรทแพคเตอร์ให้ผลดีที่สุดในการเจริญพัฒนาระดับไซโตพลาสซึมของโอโอไซต์ ที่ซึ่งเกี่ยวข้องกับการพัฒนาของตัวอ่อนระยะคลีเวจและบลาสโตซิสต์

คำสำคัญ: แมว การพัฒนาตัวอ่อน โกรทแพคเตอร์ โอโอไซต์ ฮอร์โมนรีคอมบิแนนต์ ฟอลลิเคิล สติมูเลตติ้ง

¹ภาควิชาสัตวศาสตร์ ภาควิชาสัตวบาลและวิทยาการสืบพันธุ์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กทม. 10330

²มหาวิทยาลัยเทคโนโลยีราชมงคลตะวันออก จ. ชลบุรี 20110

*ผู้รับผิดชอบบทความ E-mail: theerawat.t@chula.ac.th

