ผลของ อินซูลินไลค์โกรทแฟคเตอร์ 1 ต่อการพัฒนา คุณภาพ และการแสดงออกของยีนของตัวอ่อนแมวบ้านที่ผลิตจากภายนอกและภายในร่างกายด้วยการเลี้ยงเดี่ยวและเลี้ยงเป็นกลุ่ม

นางสาวชมนาท ทองกิตติดิลก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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THE EFFECTS OF INSULIN-LIKE GROWTH FACTOR-1 (IGF-1) ON DEVELOPMENT, QUALITY AND GENE EXPRESSION OF *IN VITRO* AND *IN VIVO* PRODUCED DOMESTIC CAT EMBRYOS USING SINGLE AND GROUP CULTURE SYSTEMS

Miss Chommanart Thongkittidilok

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
Faculty of Veterinary Science
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การทดลองที่ 1 ศึกษาผลของอินซูลินไลค์โกรทแฟคเตอร์ 1 และการแสดงออกของยีนตัวรับของอินซูลินไลค์ โกรทแฟคเตอร์ 1 ในระหว่างการพัฒนาของตัวอ่อนแมวบ้านที่เลี้ยงเป็นกลุ่มและเลี้ยงเดี่ยว ผลการศึกษาพบว่าตัวอ่อนที่ได้จากการเลี้ยงเป็นกลุ่มมีอัตราการพัฒนาเป็นตัวอ่อนระยะบลาสโตซิสสูงกว่าตัวอ่อนที่เลี้ยงแบบเดี่ยวอย่างมีนัยสำคัญ ($P < 0.05$) ซึ่งสอดคล้องกับการแสดงออกของยีนตัวรับของ อินซูลินไลค์โกรทแฟคเตอร์ 1 ในตัวอ่อนระยะมอรูลาที่ได้จากการเลี้ยงเดี่ยวต่ำกว่าตัวอ่อนระยะมอรูลาที่ได้จากการเลี้ยงเป็นกลุ่มอย่างมีนัยสำคัญ ($P < 0.05$) การเติมอินซูลินไลค์โกรทแฟคเตอร์ 1 ที่ความเข้มข้น 25 และ 50 นาโนกรัมต่อมิลลิลิตรในตัวอ่อนที่เลี้ยงเดี่ยวช่วยเพิ่มอัตราการพัฒนาของตัวอ่อนโดยเฉพาะช่วงเปลี่ยนจากระยะมอรูลาไปเป็นตัวอ่อนระยะบลาสโตซิสได้ในระดับเดียวกับตัวอ่อนที่ได้จากการเลี้ยงเป็นกลุ่ม เนื่องจากมีการแสดงออกของยีนตัวรับอินซูลินไลค์โกรทแฟคเตอร์ 1 สูงขึ้นในตัวอ่อนระยะมอรูลาที่ได้จากการเลี้ยงเดี่ยวโดยพบว่ามีการแสดงออกในระดับเดียวกับตัวอ่อนระยะมอรูลาที่ได้จากการเลี้ยงเป็นกลุ่มที่ไม่ได้เติมอินซูลินไลค์โกรทแฟคเตอร์ 1

การทดลองที่ 2 ศึกษาผลของอิพิเดอร์มอลโกรทแฟคเตอร์และการออกฤทธิ์ร่วมกับอินซูลินไลค์โกรทแฟคเตอร์ 1 ต่อการพัฒนาของตัวอ่อนที่เลี้ยงเดี่ยว และศึกษาการแสดงออกของยีนตัวรับของอิพิเดอร์มอลโกรทแฟคเตอร์ในตัวอ่อนระยะต่างๆกัน ผลการศึกษาพบว่าการเติมอิพิเดอร์มอลโกรทแฟคเตอร์ในตัวอ่อนที่เลี้ยงเดี่ยวช่วยเพิ่มอัตราการเกิดตัวอ่อนระยะบลาสโตซิสในระดับที่ใกล้เคียงกับการเลี้ยงตัวอ่อนเป็นกลุ่ม โดยพบการแสดงออกของยีนตัวรับของอิพิเดอร์มอลโกรทแฟคเตอร์ ลดลงในตัวอ่อนระยะมอรูลาและบลาสโตซิสในกลุ่มที่ได้รับอิพิเดอร์มอลโกรทแฟคเตอร์ทั้งในตัวอ่อนที่เลี้ยงเป็นกลุ่มและเลี้ยงเดี่ยว

การทดลองที่ 3 ศึกษาความสัมพันธ์ระหว่างความเข้มข้นของอินซูลินไลค์โกรทแฟคเตอร์ 1 ที่ถูกหลั่งออกมาจากตัวอ่อนที่เลี้ยงเดี่ยวต่อผลการพัฒนาไปเป็นตัวอ่อนระยะบลาสโตซิส และศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับคุณภาพของตัวอ่อนระยะบลาสโตซิส (*OCT3/4 BAX* และ *BCL2*) ที่พัฒนาจากตัวอ่อนวันที่ 3 หลังจากการปฏิสนธิที่มีการหลั่งอินซูลินไลค์โกรทแฟคเตอร์ 1 ในระดับต่ำหรือสูงเปรียบเทียบกับตัวอ่อนที่ได้จากการเจริญภายในร่างกาย ผลการศึกษาพบว่าตัวอ่อนระยะ 4 ถึง 8 เซลล์และตัวอ่อนระยะมอรูลาที่สามารถพัฒนาไปเป็นตัวอ่อนระยะ บลาสโตซิสมีการหลั่งอินซูลินไลค์โกรทแฟคเตอร์ 1 มากกว่าตัวอ่อนที่ไม่พัฒนาเป็นระยะบลาสโตซิสอย่างมีนัยสำคัญ และพบว่ามีการแสดงออกของยีน *OCT3/4 BAX* และ *BCL2* เพิ่มขึ้นอย่างมีนัยสำคัญโดยเฉพาะในตัวอ่อนระยะบลาสโตซิสที่พัฒนามาจาก ตัวอ่อนในวันที่ 3 หลังจากการปฏิสนธิที่มีการหลั่งอินซูลินไลค์โกรทแฟคเตอร์ 1 ในระดับสูง

การทดลองที่ 4 ศึกษาบทบาทของอินซูลินไลค์โกรทแฟคเตอร์ 1 ต่อการสร้างอนุมูลอิสระและการแสดงออกของยีนต้านอนุมูลอิสระในตัวอ่อนแมวบ้านที่เลี้ยงเป็นกลุ่มและเลี้ยงเดี่ยว ผลการศึกษาพบว่าการเติมอินซูลินไลค์โกรทแฟคเตอร์ 1 เพิ่มอัตราการพัฒนาของตัวอ่อนไปเป็นตัวอ่อนระยะบลาสโตซิสได้เฉพาะในกลุ่มตัวอ่อนที่เลี้ยงเดี่ยว อย่างไรก็ตามพบว่าปริมาณอนุมูลอิสระที่วัดได้ในตัวอ่อนระยะบลาสโตซิสจากกลุ่มตัวอ่อนที่เลี้ยงเดี่ยวและมีการเติมอินซูลินไลค์โกรทแฟคเตอร์ 1 มีค่าสูงกว่ากลุ่มที่เลี้ยงเดี่ยวและไม่ได้เติมอินซูลินไลค์โกรทแฟคเตอร์ 1 อย่างมีนัยสำคัญ ($P < 0.05$) การเติม อินซูลินไลค์โกรทแฟคเตอร์ 1 ที่ความเข้มข้น 50 นาโนกรัมต่อมิลลิลิตรในตัวอ่อนที่เลี้ยงเดี่ยวมีผลลดการแสดงออกของยีน *GPX1* อย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มตัวอ่อนที่เลี้ยงเดี่ยวอื่นๆ แม้ว่า อินซูลินไลค์โกรทแฟคเตอร์ 1 จะไม่มีผลต่อการพัฒนาในตัวอ่อนที่เลี้ยงเป็นกลุ่ม แต่พบว่าการเติมอินซูลินไลค์โกรทแฟคเตอร์ 1 ที่ความเข้มข้น 50 นาโนกรัมต่อมิลลิลิตรสามารถลดระดับของอนุมูลอิสระได้อย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มตัวอ่อนที่เลี้ยงเป็นกลุ่มอื่นๆ ($P < 0.05$) และพบว่ามีการแสดงออกของยีน *GPX1 SOD1* และ *CATALASE* เพิ่มขึ้นอย่างมีนัยสำคัญในตัวอ่อนที่เลี้ยงเป็นกลุ่มร่วมกับการเติมอินซูลินไลค์โกรทแฟคเตอร์ 1 ที่ความเข้มข้น 50 นาโนกรัมต่อมิลลิลิตร ($P < 0.05$)

ภาควิชา สูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต .....

สาขาวิชา วิทยาการสืบพันธุ์สัตว์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5275951831 : MAJOR THERIOGENOLOGY
KEYWORDS: DOMESTIC CAT, GENE EXPRESSION, GROUP CULTURE, INSULIN-LIKE GROWTH FACTOR-1, IN VITRO PRODUCED EMBRYOS, IN VIVO PRODUCED EMBRYOS, SINGLE CULTURE

CHOMMANART THONGKITTIDILOK: THE EFFECTS OF INSULIN-LIKE GROWTH FACTOR-1 (IGF-1) ON DEVELOPMENT, QUALITY AND GENE EXPRESSION OF *IN VITRO* AND *IN VIVO* PRODUCED DOMESTIC CAT EMBRYOS USING SINGLE AND GROUP CULTURE SYSTEMS. ADVISOR: PROF. MONGKOL TECHAKUMPHU, D.V.M., Doctorate de 3e cycle, CO-ADVISOR: ASST. PROF. THEERAWAT THARASANIT, D.V.M., Ph.D., NUCHARIN SONGSASEN, D.V.M., M.Sc., Ph.D., pp.

EXP. 1 aimed to determine the effects of insulin-like growth factor-1 (IGF-1) and the mRNA expression of IGF-1 receptor (IGF-1R) during the *in vitro* development of cat embryos cultured in groups versus singly. Group embryo culture led to a significantly higher blastocyst development rate compared with single-embryo culture ($P<0.05$). The poor development of singly cultured embryos coincided with the significantly lower IGF-1R expression in morulae than in group-cultured morulae. IGF-1 (25 or 50 ng/ml) supplementation significantly improved the blastocyst formation rate of single embryos to a level similar to group culture by promoting the morula-to-blastocyst transition. IGF-1 supplementation (25 or 50 ng/ml) of singly cultured embryos upregulated the expression of IGF-1R mRNA in morula-stage embryos to the same level as that observed in group-cultured embryos (without IGF-1).

EXP. 2 aimed to examined the influence of EGF and its interaction with insulin liked growth factor-1 (IGF-1) on developmental competence of singly cultured embryos, expression of EGF receptor (EGFR) at various stage of development. EGF promoted blastocyst formation to the level comparable to that of group culture. Expression levels of EGFR were decreased in morulae and blastocysts cultured in the presence of EGF, irrespective culture regimens (group or individual culture).

EXP. 3 aimed to investigate the relationships of secreted IGF-1 concentrations on subsequent blastocyst formation and to determine the expression level of genes that related to blastocyst quality (*OCT3/4*, *BCL2*, and *BAX*) in blastocyst embryos which have low or high secreted IGF-1 level on Day 3 compared with *in vivo* produced blastocyst. Four to eight-cell and morula stage embryos that developed to blastocysts significantly secreted the IGF-1 at higher levels than those did not develop to blastocyst. The expression of *OCT3/4*, *BCL2*, and *BAX* significantly increased in the blastocyst especially from high IGF-1 secretion embryos.

EXP. 4 aimed to determine the roles of IGF-1 on the production of reactive oxygen species and the expression of anti-oxidative genes of domestic cat embryos cultured in group or singly. Supplementation of IGF-1 improved blastocyst formation only in singly culture. ROS level was higher in singly cultured embryos supplemented with IGF-1 than those without growth factor ($P<0.05$). In single culture, supplementation of IGF-1 at 50 ng/ml significantly decreased the expression of *GPX1* transcripts compared with others. In group culture, ROS level was decreased in group culture with 50 ng/ml of IGF-1 compared with other treatments ($P<0.05$). The expressions of *GPX1*, *SOD1* and *catalase* were also increased group culture with 50 ng/ml of IGF-1 compared with other treatment ($P<0.05$).

Department:	Obstetrics Gynaecology and Reproduction	Student's Signature	----------
Field of Study:	Theriogenology	Advisor's Signature	----------
Academic Year:	2014	Co-Advisor's Signature	----------
		Co-Advisor's Signature	----------

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°C	degree celcius
µl	microliter
µM	micromolar
AI	artificial insemination
Akt/PKB	v-Akt murine thymoma viral oncogene/Protein kinase B
ARTs	assisted reproductive technologies
ATP	adenosine triphosphate
BAD	Bcl-2-associated death promoter
BAX	Bcl2 associated X protein
BCL-2	B-cell lymphoma 2
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
COCs	cumulus-oocyte complexes
Cu, Zn-SOD/SOD1	copper, zinc superoxide dismutase
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
eCG	equine chorionic gonadotropin
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
ET	embryo transfer
FCS	fetal calf serum
FGF	fibroblast growth factor
FP	forward primer
GAPDH	glyceraldehydes 3 phosphatedehydrogenase
GJA1	gap junction proteinα 1
GLUT8	glucose transporter 8
GPX	glutathione peroxidase
GRB2	growth factor receptor-bound protein 2

h	hour
H	hydrogen
H_2O_2	hydrogen peroxide
hCG	human chorionic gonadotropin
HM	holding medium
H-SOF	Hepes modified synthetic oviductal fluid
i.m.	intramuscular
ICM	inner cell mass
IGF-1	insulin-like growth factor-1
IGF-1R	insulin-like growth factor-1 receptor
IGF-2	insulin like growth factor 2
IGF-2R	insulin like growth factor 2 receptor
IGFBP	insulin like growth factor binding proteins
IGFs	insulin like growth factor family
IgG	immunoglobulin G
IRS-1	insulin receptor substrate-1
IU	international unit
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* embryo production
IVV	*in vivo* produced embryo
K	Potassium
LIF	leukemia inhibitory factor
MAPK	mitogen-activated protein kinases
MEM	minimum essential media
mOsm	milliosmole
mg	milligram
min	minute
ml	milliliter
mM	millimolar
Mn-SOD/SOD2	manganese superoxide dismutase
mRNA	messenger ribonucleic acid
N/n	number
Na	sodium

NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4	monosodium phosphate
NEAA	nonessential amino acids
ng	nanogram
nm	nanometer
NT	nuclear transfer
$O^{\cdot-}_2$	superoxide anion
O_2	oxygen
OCT3/4	octamer binding protein-4
OCT3/4	octamer-binding transcription factor 4
$OH^{\cdot}$	hydroxyl
Paf	1-o-alkyl-2-acetyl-sn-glycero-3-phosphocholine
PBS	phosphate-buffered saline solution
PCD	programmed cell death
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDK-1	phosphoinositide-dependent kinase-1
PI3K	phosphoinositide 3-kinases
PPP	picropodophyllin
qPCR	quantitative polymerase chain reaction
rhFSH	recombinant human follicle-stimulating hormone
RNA	ribonucleic acid
ROS	reactive oxygen species
RP	reverse primer
RQ	relative quantitation
RT	reverse transcription
s/sec	second
SOF	synthetic oviductal fluid
SOX	SRY-like HMG-box gene
TBE	Tris-borate-EDTA
TNF	tumor necrosis factor
UV	ultraviolet

vol	volume
WOW	well of the well
wt	weight
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta

CHAPTER 1

Introduction

1.1 Importance and rationale

Assisted reproductive technologies (ARTs) such as artificial insemination (AI), *in vitro* embryo production (IVP), embryo transfer (ET), nuclear transfer (NT) and gamete cryopreservation play important roles in preserving biodiversity, basic research and also improvement of reproductive performances in wildlife conservation program (Pope, 2000). In the felidae family, 16 of 37 species are threatened by extinction due to habitat loss, persecution and infectious diseases (IUCN, 2013). The IVP technique has been developed in domestic cat and wild cat species during the past few decades. Although IVP is considered to be a powerful tool, it has not been routinely used in studying fertilization and preserving genetic materials of endangered species due to limited numbers of good quality gametes that would be available for *in vitro* maturation (IVM)/ *in vitro* fertilization (IVF). The use of domestic cat has, therefore, become as a model for understanding about reproduction and embryology that in turn efficiently aids the propagation of endangered felid species. At the present time, improving ability of oocytes/embryos to develop up to blastocyst stage *in vitro* is considered as a key factor for the implementation of IVP technique in wild felid species.

When the limited numbers of gamete is considered, the methods such as single embryo culture system might be developed in order to overcome this problem. Generally, embryos that are cultured under single embryo culture condition develop poorer than 'in group' embryo culture probably because of insufficiency of paracrine factors within the *in vitro* microenvironment (Paria and Dey, 1990). It has been demonstrated that supplementation of insulin-like growth factor-1 (IGF-1) or epidermal growth factor (EGF) beneficially affected the developmental competence of embryos in many species, including mouse, cow, rabbit, gerbil and sheep (Paria and Dey, 1990; Harvey and Kaye, 1992; Matsui et al., 1997; Palma et al., 1997; Herrler et al., 1998; Sirisathien et al., 2003; Yoshida et al., 2009; Neira et al.,

2010; Shabankareh and Zandi, 2010). This evidence was coincided with the presence of insulin-like growth factor-1 receptor (*IGF-1R*) and epidermal growth factor receptor (*EGFR*) in cumulus oocyte complex and also in the embryos (Kane et al., 1997; Peng et al., 2010; Waurich et al., 2010). Thus, these growth factors coupled with the expression of its receptor indicates that IGF-1 and EGF are key factors affecting the developmental competence especially in the *in vitro* culture system where several stress factors are markedly generated.

Due to the lack of suitable, objective standard in evaluation of embryo quality, studies have been focused on developing non-invasive technique to embryo quality assessment (Singh and Sinclair, 2007). Secretomic analysis is the one of non-invasive embryo quality assessment technique which helps in determining secreted factors that reflect viability and developmental competence. The enzyme linked immunosorbent assay (ELISA) technique has been widely used for identifying new biomarkers such as leptin (Gonzalez et al., 2000) and soluble human leukocyte antigen G (Noci et al., 2005; Sher et al., 2005). IGF-1 produced and secreted by preimplantation embryo is considered as survival factor, and it may be possible to use IGF-1 as biomarker, although this hypothesis has yet to be proven. The enzyme linked immunosorbent assay is preferable to other techniques such as high performance liquid chromatography with mass spectroscopy (HPLC-MS) or protron nuclear magnetic resonance (^{1}H-NMR) analysis (Marhuenda-Egea et al., 2010; Pudakalakatti et al., 2013) because IGF-1 level can be measured from individually produced embryo and the technique is simple, rapid and inexpensive.

Excessive generation of reactive oxygen species (ROS) during *in vitro* culture has been shown to negatively affect embryonic development (Agarwal et al., 2005). IGF-1 plays protective roles against embryonic stress via enhancing ROS elimination by upregulation of gluthathione peroxidase enzyme in the mitochondria (Jallali et al., 2007). It is, therefore, believed that IGF-1 as a cell survival factor may down regulate the oxidative-stress induced cellular program cell death and senescence. Thus, IGF supplementation may improve embryo development by protecting the cells against

oxidative stress. Evaluating the level of IGF-1 secreted from the embryos may also be used as a non-invasive tool for assessing embryo quality.

The aim of this dissertation was to elaborate the appropriate protocol for *in vitro* culture of a single feline embryo. To achieve this aim, the influence of IGF-1 and EGF on cat embryo development, gene expression and production of ROS were examined. The results obtained from the study will undoubtedly lead to improve the culture condition of feline embryos and can efficiently be applied for wild felid species.

1.2 Literature reviews

1.2.1 *In vitro* embryo production in feline species

Nowadays, the assisted reproductive technologies (ARTs) such as artificial insemination (AI), *in vitro* embryo production (IVP), embryo transfer (ET), nuclear transfer (NT) and gamete cryopreservation play roles in preserving biodiversity, developing basic research and also improvement of reproductive performances in wildlife conservation program. To date, more than half of extant wild felid species are threatened by extinction in their native range (IUCN, 2013). Thus, it is important to establish security populations of certain species *ex situ*, a process that can help retain the necessary genetic diversity to ensure species integrity. Within these threatened species collections, there are many genetically valuable females that have never reproduced or experiencing fertility issues as a result of advanced age. To propagate these individuals, efforts have been focused on developing 'assisted breeding' technologies originally established in livestock and humans, including *in vitro* embryo production to ensure that genetics of all individuals are represented in *ex situ* population (Pukazhenthi and Wildt, 2004; Comizzoli et al., 2010; Wildt et al., 2010). Thus far, live offspring have been produced after transferring *in vitro*-derived embryos into recipient females in wild felids species such as the Indian desert cat (*Felis silvestris ornata*), tiger (*Panthera tigris*), ocelot (*Leopardus pardalis*), sand cat (*Felis margarita*), black-footed cat (*Felis nigripes*), caracal (*Caracal caracal*), fishing cat (*Prionailurus viverrinus*) and African wild cat (*Felis silvestris libica*) (Pope, 2000; Pope

et al., 2012; Swanson, 2012). Despite the successes, there are still challenges associated with the limited numbers of good quality oocytes available for *in vitro* embryo production due to old age (Spindler and Wildt, 2002) or poor responses to ovarian stimulation protocols (Pelican et al., 2006).

In vitro embryo production has become the most promising tool for genetic preservation and distribution among domestic and endangered felid species (Pope, 2000). However, overall success in these species is still lower than other species (Farstad, 2000), principally because of limitations of knowledge and of the numbers of acquired gametes from endangered species. Developmental competence of *in vitro*-produced embryos depends on several factors, such as gamete quality and environmental factors (Díaz-Cueto and Gerton, 2001; Hardy and Spanos, 2002). Due to limited access to wild felid gametes, domestic cats are frequently used as animal models to develop reproductive technology for further applications in wild felid species because of their similar reproductive physiology. This approach can be used to understand about embryology and reproduction in wild felids and even aiding in propagation of endangered felid species (Pope, 2000). *In vitro* fertilization (IVF) is the technique that aims to mimic normal fertilization process outside the body. The sperm will be co-incubated with unfertilized oocytes in proper condition to achieve fertilization. Although cat embryos and offspring can be successfully produced by IVF (Goodrowe et al., 1989; Gomez et al., 2000), the blastocyst rate from IVF embryos is still lower than those fertilized *in vivo* (30-40% and 50-70%, respectively) (Farstad, 2000). After fertilization, many factors such as culture medium (Johnston et al., 1993; Herrick et al., 2007), embryo density (Spindler and Wildt, 2002), gas atmosphere (Johnston et al., 1991) and culture temperature (Johnston et al., 1991) are considered critically for the success of IVP. The fundamental knowledge about these factors remains to be elucidated in order to achieve acceptable blastocyst rates and high embryo quality.

1.2.2 Single embryo culture

Culture conditions during *in vitro* culture, such as medium composition (Johnston et al., 1993; Lane and Gardner, 1998; Gardner, 2007; Sananmuang et al., 2011), embryonic density (Lane and Gardner, 1992; Sananmuang et al., 2011; Vutyavanich et al., 2011), atmospheric gases, culture temperature (Johnston et al., 1991) are considered critical factors of successful *in vitro* embryo production. The paracrine effect from embryos cultured in groups is far superior to those cultured individually in a large volume of culture medium (Ebner et al., 2010; Sananmuang et al., 2011), but the single embryo culture system is still important for studying factors associated with embryo developmental competence and quality. A single embryo culture strategy would allow us to understand embryo physiology and to evaluate the metabolism of individual embryos, which, in turn, could be used to identify potential biomarkers for non-invasive selection of superior embryos (Goovaerts et al., 2010; Gardner et al., 2011; Gardner and Wale, 2013). Single embryos culture could also be used to culture small numbers of good quality gametes/embryos of valuable endangered species to improve developmental potential of embryos (Vajta et al., 2008; Goovaerts et al., 2009). However, development of this technology is hampered by the fact that the embryos cultured singly develop at a poorer rate (Goovaerts et al., 2010) than group-cultured embryos, presumably due to insufficient paracrine signaling (Paria and Dey, 1990; Lane and Gardner, 1992). It has been shown in the mouse and cow that small percentages of embryos cultured singly developed into blastocyst stage compared with those incubated in groups (mouse, 49% versus >80%; cow, 2.9% versus 30.7%) (Paria and Dey, 1990; Goovaerts et al., 2009). Furthermore, previous studies have also demonstrated that individually cultured cat (Spindler and Wildt, 2002) or cow embryos (Carolan et al., 1996) failed to develop to the compaction stage. As mentioned above, single embryo culture seems to be compromised by the lack of interaction among embryos within a culture droplet that leads to insufficient paracrine signaling. In order to overcome low blastocyst rates and impaired embryo quality, many strategies have been used in IVF protocols. Supplementation of growth factor or conditioned medium derived from group-embryo culture, and coculture embryo with autologous cumulus cells have been

shown to improve embryo development (Paria and Dey, 1990; Fujita et al., 2006; Goovaerts et al., 2010; Goovaerts et al., 2011). In pig, development of individual embryo improved in an atmosphere of 5% O_2 and 5% CO_2 compared to 2, 10, 20% O_2 and 2.5, 10% CO_2 (Berthelot and Terqui, 1996). Finally, the "well of the well" system (WOW) for *in vitro* culture (IVC) has been developed to obtain higher blastocyst rates than conventional group or single culture (Vajta et al., 2000; Vajta et al., 2008). In the present time, the study about feline single embryo culture for serving as a model for improving endangered feline embryo culture system is still not thoroughly explored.

1.2.3 The role of insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF) on preimplantation embryo development

The implantation and maintenance of pregnancy requires an effective maternal–embryonic dialogue mediated by growth factors (Hardy and Spanos, 2002). Growth factors play important roles in modulating embryonic and fetal growth and development (Watson et al., 1994). Their roles have been demonstrated in mouse and other species showing that preimplantation embryos and maternal reproductive tract are able to produce a range of polypeptide growth factor receptors, while many of their receptors can be detected on the cell's surface of embryo. The cellular responses of the embryo and maternal reproductive tract to maternally and embryonically derived growth factor was demonstrated in Figure 1, the interaction between the preimplantation embryo, itself (autocrine (green) and paracrine (dark brown)) and maternal environment (endocrine (light blue)) which involved in cell proliferation and differentiation, protein synthesis, endocytosis, glucose transport, embryo metabolism, gene expression and apoptosis.

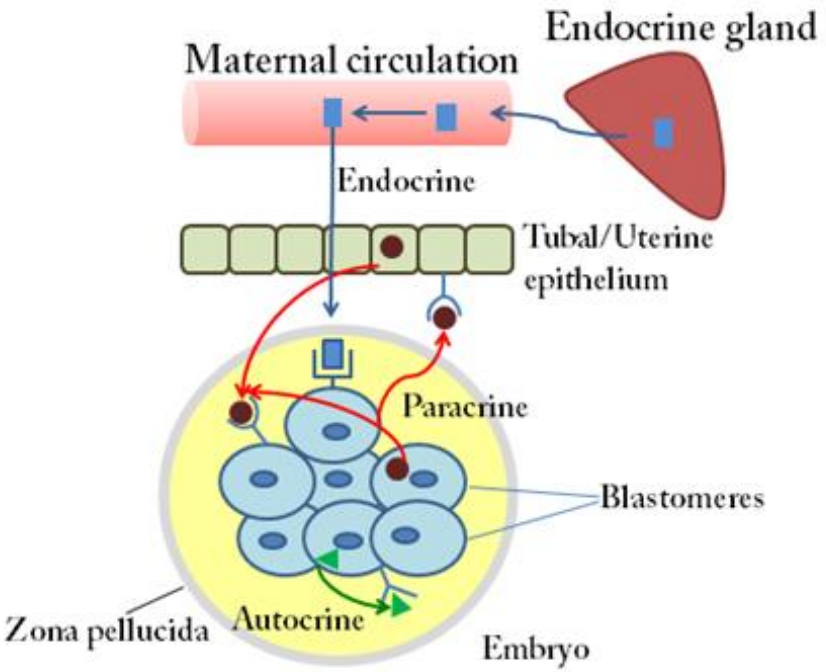

Figure 1. Cellular responses of the embryo and maternal reproductive tract to maternally and embryonically derived growth factor (modified from Kaye, 1997).

These growth factors include members of the insulin and insulin-like growth factor (IGF) family, the fibroblast growth factor (FGF) family, the epidermal growth factor (EGF) family, the tumor necrosis factor (TNF) family, and the platelet derived growth factor (PDGF) family (Kane et al., 1997). The physiological levels of several growth factors have been shown to affect murine development through increased synthetic rate of protein and RNA, cell division, cell number, blastocyst expansion and hatching (Mattson et al., 1988; Heyner et al., 1989).

Insulin and IGFs family are a group of structurally related polypeptides that regulate the growth of many types of mammalian cells (Froesch et al., 1985; Czech, 1989). The insulin and insulin-like growth factor family consists of insulin, IGF-1, and IGF-2. These proteins bind to their receptors (insulin receptor, IGF-1R, and IGF-2R) and six IGF binding proteins (IGFBP1–IGFBP6) (Heyner et al., 1989). IGF-1 and IGF-2 bind to their own specific receptors and are capable of binding to the heterologous receptors with lower affinities. IGFs are produced by many tissues within the body,

and therefore can act in a paracrine or autocrine fashion (D'Ercole et al., 1984). IGF-1 functions as a regulator of cell growth and differentiation. It is a well-documented stimulator of oocyte maturation and increases the proportion of blastocyst stage by stimulation of cell growth and prevention of apoptosis (Paria and Dey, 1990; Harvey and Kaye, 1992; Matsui et al., 1997; Palma et al., 1997; Herrler et al., 1998; Sirisathien et al., 2003; Yoshida et al., 2009; Neira et al., 2010; Shabankareh and Zandi, 2010). In mouse embryos, IGF-1 increases the glucose uptake by induction the translocation of glucose transporter 8 (GLUT8) (Carayannopoulos et al., 2000). A supplementation of IGF-1 in preimplantation embryo culture media decreased spontaneous apoptosis. It has been demonstrated that IGF-1 prevents apoptosis via Phosphoinositide 3-kinases (PI3K) and Protein kinase B (Akt/PKB) pathways (Vincent and Feldman, 2002) that in turn inactivate several downstream proteins involving in the programmed cell death (PCD). A primary target is the BAD which belongs to BCL-2 family member (Bai et al., 1999). In non-phosphorylated state, BAD locates at the mitochondrial membrane and interacts with BCL-2 to prevents BCL-2 from performing its anti-apoptotic functions (Vincent and Feldman, 2002). Tyrosine phosphorylation by the IGF-1R of insulin receptor substrate-1 (IRS-1) (Dudek et al., 1997), results in activate of PI3K. Activate of PI3K, in turn, leads to an increase in phosphatidylinositol 3,4,5-trisphosphate (PIP3) that binds to the Akt/PKB and phosphoinositide-dependent kinase-1 (PDK-1). PDK-1 then phosphorylates residue Threonine 308 on Akt/PKB resulting in an increase in activity of anti-apoptotic factor (Bcl-2 and BAX) and also inhibits pro-apoptotic factor (BAD and Caspase 9) (Vincent and Feldman, 2002). The IGF-1R also plays distinct roles in the regulation of PCD in different cell types via mitogen-activated protein kinases (MAPK) pathway (Vincent and Feldman, 2002). IGF-1R signaling via MAPK pathway has been most closely associated with an appropriate physiological response including cellular proliferation, differentiation, development and apoptosis in mammalian cells (Zhang and Liu, 2002). Upon IGF-1R autophosphorylation, the protein Shc is recruited to the receptor and becomes phosphorylated on tyrosine residues (Kim et al., 1998). Activated Shc binds the adaptor Grb2 in an IRS-1 independent manner, leading to an activation of the Ras/ERK pathway (Kim et al., 1998). Expression of *IGF-1* mRNA transcripts and its

receptor in preimplantation embryo has been detected in many species. *IGF-1* mRNA has been detected in *in vitro* produced bovine and ovine preimplantation embryos (Schultz et al., 1992; Watson, 1992; Yoshida et al., 1998; Lonergan et al., 2000), but others have concluded that the mRNA levels were below the detectable levels (Winger et al., 1997; Watson et al., 1998; Yaseen et al., 2001; Moore et al., 2007; Warzych et al., 2007; Wang et al., 2009). *IGF-1* mRNA was not observed in embryos of mouse (Rappolee et al., 1992), rat (Zhang et al., 1994), water buffalo (Daliri et al., 1999), and humans (Lighten et al., 1997). However, *IGF-1R* mRNA is found in mouse, rat, cow, pig (Kane et al., 1997), and cat embryos (Waurich et al., 2010) but not found in the human embryo (Lighten et al., 1997). While the effect of this IGF-1 supplementation has yet to be examined for feline embryos although the effect of IGF-1 has been demonstrated to improve developmental ability of cat immature oocytes (Kitiyanant et al., 2003).

EGF is considered as one of growth factors that promotes the development of preimplantation embryos by improving the rate of development and blastocoel expansion (Dardik and Schultz, 1991; Brice et al., 1993). EGF is a mitogenic polypeptide that is involved in regulating cell proliferation in many cell types, including granulosa cells of preantral follicles. This growth factor also has been shown to promote *in vitro* maturation (IVM) of mouse, cat, sheep and pig oocytes (Demeestere et al., 2005; Merlo et al., 2005; Peng et al., 2010; Procházka et al., 2011). Addition of EGF to IVM medium promotes cleavage and blastocyst development of *in vitro* matured gametes (Sirisathien et al., 2003; Neira et al., 2010; Shabankareh and Zandi, 2010; Song et al., 2011; Chandra et al., 2012). EGF binds with its receptor (EGFR) and stimulates MAP/ERK and Akt pathways that, in turn, enhances cell proliferation (Li et al., 2008). It has been shown that *EGFR* is expressed in 2-cell to blastocyst stage in the mouse, and from 8-cell embryos to blastocysts in human (Kane et al., 1997). It has been shown that EGF increases the rate of Na+/H+ exchange result in the influx of Na+ into rat hepatocytes (Moule and McGivan, 1990). Thus, it has been speculated that the binding of EGF with EGFR increases activity of Na+/H+ antiport and activates Na+/K+ ATPase-generated trans-trophectoderm ion

gradient, which involves in the influx of Na+ and water across the epithelium resulting in blastocoel expansion (Dardik and Schultz, 1991; Watson, 1992; Watson et al., 2004) (Figure 2). It is, therefore, speculated that supplementation of IGF-1 and/or EGF during embryo culture may promote the development of preimplantation feline embryos.

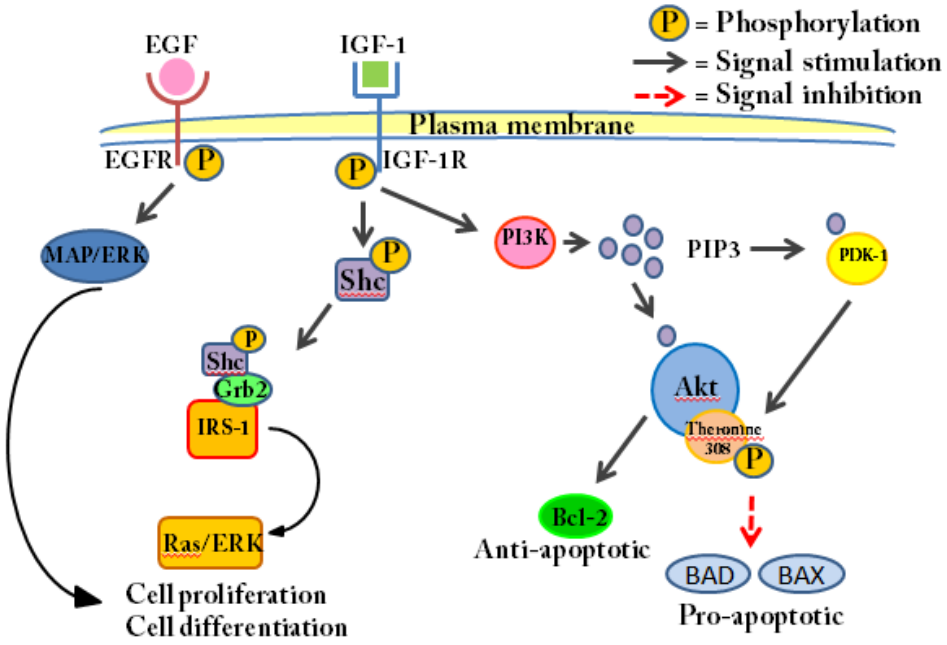

Figure 2. IGF-1 and EGF signaling pathway (modified from Vincent and Feldman, 2002; Kim et al., 1998; Li et al., 1998)

1.2.4 Non invasive embryo quality assessment

Embryo quality assessment is essential for improving the chance of obtaining healthy offspring. Normally, the assessment is performed by morphological criteria but this is hampered by a lack of suitable standard, subjective grading system, and harbor genetic or epigenetic defects (Singh and Sinclair, 2007). To avoid the limitation of morphological assessment, the quantitative techniques have been developed for the non-invasive assessment of embryo viability and metabolism. The non-invasive techniques include proteomics, metabolomics, and secretomics analysis. Secretomics analysis of secretome of mammalian embryo in preimplantation development may help in determining secreted factors that reflect viability and developmental

competence. Studies of secretomics in mammalian embryos have revealed that 1-o-alkyl-2-acetyl-sn-glycero-3-phosphocholine (paf) (O'Neill, 2005), leptin (Cervero et al., 2005), acrogranin (Díaz-Cueto and Gerton, 2001), soluble human leukocyte antigen G (Noci et al., 2005; Sher et al., 2005) and ubiquitin (Wang et al., 2004) are produced and secreted by mammalian preimplantation embryos. These secretomes correlated with survival factor during embryonic development, promoted the blastocyst formation, and involved in implantation process resulted in the use of these secretomes as biomarkers for embryo quality determination. The enzyme linked immunosorbent assay (ELISA) technique to identify/quantify secretome has been used for the findings of new biomarkers such as leptin (Gonzalez et al., 2000) and soluble human leukocyte antigen G (Noci et al., 2005; Sher et al., 2005). IGF-1 produced and secreted by preimplantation embryo is considered as survival factor and it may be possible to use IGF-1 as biomarker although this hypothesis has yet to be proven. The enzyme linked immunosorbent assay is preferable and used for measure IGF-1 level from individually produced embryo. The technique is simple, rapid and inexpensive. Moreover, it can cross reaction with IGF-1 in feline species. This secreted IGF-1 may quantitatively be used as a non-invasive biomarker for determination of embryonic quality.

1.2.5 Blastocyst quality determination: gene correlates to blastocyst quality

Determination of blastocyst quality can be performed by several techniques. In the past, morphology assessment is often used for selecting IVF derived embryo prior to embryo transfer. However, this technique is sometime difficult to differentiate between viable and fragmented cells (Van Royen et al., 2001). Until recently, several non-invasive scoring criteria have been available. The pronuclear morphology and number of nucleolar precursor bodies distributed in the pronuclei was used to predict embryo developmental capacity and chromosomal defect (Scott et al., 2000; Wittemer et al., 2000; Balaban et al., 2004). Morphological structures such as color of the blastomeres, kinetics of development and timing of blastocyst formation, the extent of compaction, expansion and diameter of the embryo at hatching can also be linked with embryo quality (Lonergan et al., 2006). However,

above mentioned techniques cannot precisely determine the blastocyst quality in terms of ability of embryos to express some specific genes related to the development. Gene expression has an important role during preimplantation period of development and its pattern can be used as a tool to assess the quality of IVP embryos. It has been shown that culture media, *in vitro* cultured condition and the presence of serum during culture period altered of gene expression pattern. The expression of pro-apoptotic (BAX) and anti-apoptotic (BCl-2) gene are closely related with the embryonic development. The study in bovine embryo shows that the expression of BAX was high in fragmented embryos, whereas Bcl-2 was high in good quality embryo or non-fragmented counterparts (Yang and Rajamahendran, 2002). The presence of serum in culture medium also cause elevation of transcripts for Mn-SOD, SOX, BAX, LIF and decrease in the GJA1 and interferon-**T** (Corcoran et al., 2005). A high embryo density (10 embryos: 12.5 µl medium volume; 1:1.25) often results in fragmentation of cell nuclei and low blastocyst formation rate due to upregulation of genes regulating stress response (HSP70) or apoptosis (BAX) (de Oliveira et al., 2005; Sananmuang et al., 2013). Despite the expression of apoptotic related gene that affects the embryonic quality, OCT3/4 is considered as an indicator of ICM which also reflects the quality of embryo. OCT3/4 plays an important role in regulating embryonic development (Filliers et al., 2012). Expression of *OCT3/4* in preimplantation embryos was demonstrated in several studies. In mouse and cow, the transcription of *OCT3/4* starts at 1-2 cells stage after zygotic genome activation until blastocyst that is found specifically in ICM (Madeja et al., 2013). In feline, *OCT3/4* transcription is found at low levels in germinal vesicle and early stage embryos but the transcripts gradually increased from eight-cell to blastocyst stages (Imsoonthornruksa et al., 2010; Waurich et al., 2010). Gomez et al. (2010) and Filliers et al. (2012) demonstrated that *OCT3/4* transcript was significantly reduced *in vitro* produced blastocyst compared with *in vivo* blastocyst. In bovine, determination of gene expression during preimplantation is now accepted as a suitable embryonic quality marker (Waurich et al., 2010).

1.2.6 Oxidative stress and anti-oxidative gene expression in preimplantation embryos

The oxygen metabolism is important for embryo development (Magnusson et al., 1986; Houghton et al., 1996; Thompson et al., 1996). After the metabolism process, Reactive oxygen species (ROS) is produced either endogenously or exogenously. In general, ROS and antioxidants are in balance. Its imbalance causes an overproduction and/or decrease the clearance of ROS by scavenging mechanisms leading to oxidative stress (Agarwal et al., 2005). ROS production in embryos is presented with three major types: superoxide ($O^{\cdot-}_2$), hydrogen peroxide (H_2O_2) and hydroxyl ($OH^{\cdot}$). Endogenously, ROS is produced by various metabolic pathways and enzymes, such as oxidative phosphorylation, NADPH oxidase and xanthine oxidase. For exogenous ROS production, there are many factors that elevate ROS production in both *in vivo* and *in vitro* culture system. In embryo culture system, the ROS is enhanced by culture condition such as oxygen concentration, metallic cations, visible light, amine oxidase, spermatozoa and even in freeze-thaw process, whereas *in vivo* condition ROS may occur from only metabolic pathway and enzymes. Oxidative stress reduces embryo quality, by compromising growth (Johnson and Nasresfahani, 1994; Tarin, 1996), increasing apoptosis (Salas-Vidal et al., 1998; Van Soom et al., 2002; Yuan et al., 2003), mitochondrial alterations and ATP depletion (Guerin et al., 2001). To overcome the detrimental effects of the oxidative stress, antioxidant has been intensively used during *in vitro* culture. Several antioxidant enzymes that defend oocytes and embryos against peroxidative damage are superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) (Li and Trush, 1993). Copper and zinc superoxide dismutase (Cu, Zn-SOD; SOD1) located in the cytosol as well as manganese-superoxide dismutase (Mn-SOD; SOD2) located in the mitochondria allow superoxide radicals to be scavenged. SOD2 transforms the toxic superoxide into hydrogen peroxide (H_2O_2), which is eliminated by catalase or GPX (Guerin et al., 2001). SOD1, SOD2, catalase, and GPX are presented in blastocyst stage embryo in mouse (El Mouatassim et al., 1999) and bovine (Arias et al., 2012). There are some studies about the protective effect of IGF-1 on mouse embryos that exposed to oxidative stress. The study demonstrates that the supplement of IGF-1 minimizes the negative influence of hydrogen peroxide and also promoted embryo development.

However, the mechanism by which IGF-1 counteracts hydrogen peroxide effect is still unclear. It may be possible that hydrogen peroxide induces apoptosis, whereas IGF-1 acts as anti-apoptosis leading to improve embryo development (Kurzawa et al., 2002; Kurzawa et al., 2004). Nowadays, the mechanism by which IGF-1 reduces oxidative stress is more clearly explained, IGF-1 plays the protective role against embryonic stress via enhance the ROS elimination by upregulation of gluthathione peroxidase enzyme in mitochondria (Jallali et al., 2007). It may be possible that supplement of IGF-1 can reduce ROS in *in vitro* culture condition.

1.3 Objectives of the thesis

1. To examine the effect of IGF-1 and EGF supplementation on developmental competence of cat embryos cultured singly.
2. To determine the relationship between the levels of secreted IGF-1 and expression of gene related to blastocyst quality and embryo development in *in vitro* and *in vivo* produced as a tool for non-invasive embryo quality evaluation.
3. To evaluated the effect of IGF-1 on stress response of cat embryos cultured *in vitro.*

1.4 Hypothesis

1. Supplementation of IGF-1 or EGF into singly cultured embryo enhances developmental capacity of domestic cat embryos cultured singly
2. The quantity of secreted IGF-1 correlates with embryo development and expression level of gene associated blastocyst quality
3. *In vitro* produced embryos possess higher oxidative stress and lower anti-oxidative gene expression than *in vivo* produced embryos and IGF-1 supplementation can reduce oxidative stress in *in vitro* produced embryos

1.5 Keywords: domestic cat, gene expression, group culture, insulin-like growth factor-1, in vitro produced embryos, in vivo produced embryos, single culture

1.6 Research merits:

1. The appropriate protocol for cat single embryo culture under the supplement of IGF-1
2. The application of secretomic study as a tool for non-invasive embryo quality evaluation
3. The knowledge about the effect of IGF-1 on oxidative stress and anti-oxidative gene expression altered in embryos cultured in specific conditions

CHAPTER 2

Insulin-like growth factor-1 (IGF-1) enhances developmental competence of cat embryos cultured singly by modulating the expression of its receptor (IGF-1R) and reducing developmental block

(Published in Growth Hormone and IGF Research 24:76-82)

2.1 Abstract

The aims of this study is to determine the effects of insulin-like growth factor-1 (IGF-1) and the mRNA expression of IGF-1 receptor (*IGF-1R*) during the *in vitro* development of cat embryos cultured in groups versus singly. Cumulus-oocyte complexes (COCs) were matured and fertilized *in vitro* with frozen-thawed semen. Cleaved embryos (48 h post-fertilization) were randomly assigned to one of the following treatments: 1) group embryo culture without IGF-1 (10 embryos per 50 µl droplet), 2) single-embryo culture without IGF-1, and 3) to 6) single-embryo culture (50 µl droplet per embryo) supplemented with different concentrations of IGF-1 (5, 25, 50 and 100 ng/ml, respectively). During *in vitro* culture, the embryos were analyzed for development to the morula, blastocyst and hatching blastocyst stage. Relative mRNA expression of *IGF-1R* was also examined by qPCR at the morula and blastocyst stages. In addition, the mRNA expression of *IGF-1R* in morula-stage embryos treated with IGF-1 was determined. The influence of IGF-1 to preimplantation embryo development was then explored by co-incubation with 0.5 µM IGF-1R inhibitor (Picropodophyllin; PPP). Group embryo culture led to a significantly higher blastocyst development rate compared with single-embryo culture ($P<0.05$). The poor development of singly cultured embryos coincided with the significantly lower *IGF-1R* expression in morulae than in group-cultured morulae. IGF-1 (25 or 50 ng/ml) supplementation significantly improved the blastocyst formation rate of single embryos to a level similar to group culture by promoting the morula-to-blastocyst transition. IGF-1 supplementation (25 or 50 ng/ml) of singly cultured embryos upregulated the expression of *IGF-1R* mRNA in morula-stage embryos to the same level as that observed in group-cultured embryos (without

IGF-1). The beneficial effects of IGF-1 on singly cultured embryo was ($P<0.05$) suppressed by PPP even in the group culture embryo without growth factor supplementation. IGF-1 supplementation improves the developmental competence of feline embryos cultured individually and also increases IGF-1R gene expression to levels similar to group-cultured embryos.

2.2 Introduction

In vitro embryo production has become the most promising tool for genetic preservation and distribution among domestic and endangered felid species (Pope, 2000). However, overall success in these species is still lower than other species (Farstad, 2000), principally because of limitations of knowledge and of the numbers of acquired gametes from endangered species. Developmental competence of *in vitro* produced embryos depends on several factors, such as gamete quality and environmental factors (Díaz-Cueto and Gerton, 2001; Hardy and Spanos, 2002). Due to limited access to wild felid gametes, domestic cats are frequently used as animal models to develop reproductive technology for further applications in wild felid species because of their similar reproductive physiology. In domestic cats, we previously found that the culture environment, such as the culture medium and culture density (embryos per culture volume), markedly influences gene expression and embryonic development capability (Sananmuang et al., 2011). When embryos are cultured in groups, each embryo secretes several factors that sufficiently stimulate the embryo development, possibly by autocrine and paracrine mechanisms (Díaz-Cueto and Gerton, 2001). Therefore, it is generally accepted that the developmental competence of group-cultured embryos at an optimal embryonic density is far superior to those cultured singly (Paria and Dey, 1990). Although the exact reasons for the poor development of singly cultured embryos is unclear, it has been hypothesized to be caused by insufficient paracrine (growth factors) secretion. Growth factors play important roles in the developmental capacity of oocytes and embryos in several species (Hardy and Spanos, 2002). Several growth factors and their ligands are present in developing mammalian embryos, such as insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), fibroblast growth factor (FGF),

platelet-derived growth factor (PDGF) and the tumor necrosis factor (TNF) family (Hardy and Spanos, 2002). IGF-1 plays distinct roles in the regulation of apoptosis in different cell types via the phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB/Akt) pathway (Vincent and Feldman, 2002). IGF-1 is also associated with appropriate physiological responses, including cellular proliferation, differentiation and development via the mitogen-activated protein kinase (MAPK) pathway (Zhang and Liu, 2002). IGF-1 promotes oocyte maturation by triggering mitotic division of granulosa cells (Shabankareh and Zandi, 2010), stimulating blastocyst growth, increasing glucose uptake by inducing glucose transporter 8 (GLUT8) translocation (Carayannopoulos et al., 2000), and preventing apoptosis (Paria and Dey, 1990; Neira et al., 2010; Shabankareh and Zandi, 2010). IGF-1 binds to both insulin and IGF-1 receptors (IGF-1R) due to their structural similarities, but its affinity for IGF-1R is approximately 100 times greater than for insulin receptor (Kaye, 1997). In domestic cats, IGF-1R mRNA is expressed throughout the preimplantation stage, with the highest expression during morula development (Waurich et al., 2010), the stage at which developmental block normally occur (Johnston et al., 1991; Pope et al., 1993) is different from other domestic species in that this developmental block usually occurs during an early stage of development (Jeong et al., 2006; Kobayashi et al., 2009; Asgari et al., 2012; Verma et al., 2012). Supplementation with growth factors such as EGF, transforming growth factor (TGF), and IGF-1 into culture medium has been applied to improve single-embryo culture systems (Paria and Dey, 1990). However, the benefits of IGF-1 in single-embryo culture system in relation to the expressions of its receptors remain unknown. The aims of this study were therefore to determine (1) the developmental competence, blastocyst quality, and *IGF-1R* mRNA expression between group and single-embryo cultures, (2) the effect of IGF-1 supplementation on developmental competence in single-embryo culture, (3) the effect of IGF-1 supplementation on the alteration of *IGF-1R* mRNA expression in feline embryos cultured singly and (4) the effect of IGF-1R inhibitor, picropodophyllin (PPP) supplementation on developmental competence in group or singly cultured embryos with or without IGF-1 supplementation.

2.3 Materials and Methods

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

2.3.1 Source of ovaries

Ovaries were obtained from domestic cats (*Felis catus*) following routine ovariohysterectomy at the Veterinary Public Health Division of the Bangkok Metropolitan Administration. The ovaries were transported at ambient temperature (approximately 30°C) to the laboratory within 2 h in 0.9% (wt/vol) normal saline solution containing 100 IU/ml penicillin and 100 µg/ml streptomycin.

2.3.2 Oocyte isolation

Upon arrival, the ovaries were washed twice in saline solution supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin, then placed in a holding medium (HM) (HEPES-buffered M199 supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, and 4 mg/ml bovine serum albumin (BSA; embryo tested)). Cumulus-oocyte complexes (COCs) were recovered after ovarian mincing in HM and then morphologically classified at ×40 magnification using a stereomicroscope (SMZ645; Nikon, Tokyo, Japan). Only grade I COCs fully surrounded by more than five layers of compacted cumulus cells with homogeneously dark cytoplasm were used (Figure 3a).

2.3.3 In vitro maturation and fertilization

In vitro oocyte maturation (IVM) and fertilization (IVF) were performed as previously described by Sananmuang et al. (2010). For IVM, groups of 25-40 COCs were cultured in 500 µl of IVM medium ($NaHCO_3$ buffered M199 with 1.0 mM sodium pyruvate, 2.0 mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml gentamicin, 4 mg/ml BSA and 0.05 IU/ml recombinant human follicle-stimulating hormone (rhFSH; Organon, Bangkok, Thailand) and 25 ng/ml EGF). After 24 h of *in vitro* maturation, groups of 10 oocytes (Figure 3b) were transferred to 50 µl droplets of IVF medium (Tyrode's balanced salt solution containing 1% (vol/vol) nonessential amino acids

(NEAA), 6 mg/ml BSA, 100 IU/ml penicillin and 50 µg/ml gentamicin). The semen used in this study was collected from a fertility-proven tom cat and then conventionally frozen. Before IVF, the frozen semen was thawed at 37°C for 30 sec and was subjectively evaluated. Only sperm with more than 50% progressive motility were used. The oocytes were co-cultured with the sperm at a final concentration of 5×10^5 sperm/ml. In all cases, the culture was performed at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

2.3.4 *In vitro* culture

After 18-24 h of IVF, cleaved embryos (Figure 3c) were washed and cultured for 24 h in 50 µl droplets of synthetic oviductal fluid (SOF) containing 4 mg/ml BSA and antibiotics (IVC-1 medium). Cleaved embryos without cumulus cells (48 h post-fertilization) were further cultured in IVC-2 medium (SOF supplemented with 10% vol/vol fetal calf serum (FCS, JR Scientific, Woodland, CA, USA). The culture medium was changed every other day. *In vitro* culture was performed at 38.5°C in a humidified atmosphere with 5% CO_2.

2.3.5 Assessment of embryo development

The embryos were morphologically observed under an inverted microscope (x40 magnification) to determine morula (Figure 3d) and blastocyst (Figure 3e) on day 5 and day 7, respectively (day 0=IVF). All blastocysts on day 7 embryos were fixed in 4% (wt/vol) paraformaldehyde and kept at 4 °C for 2 days before staining with 4',6-diamidino-2-phenylindole (DAPI, 0.1 µg/ml) in phosphate-buffered saline solution (PBS) for 10 min to count their nuclei. The fluorescently labeled embryos were then examined using an epifluorescence microscope (BX51 Olympus, Shinjuku, Japan) (Figure 3F). The percentages of morulae (>16 cells without blastocoels), blastocysts (>50 cells with blastocoel formation) and hatching blastocysts were evaluated on days 5, 6 and 7, respectively.

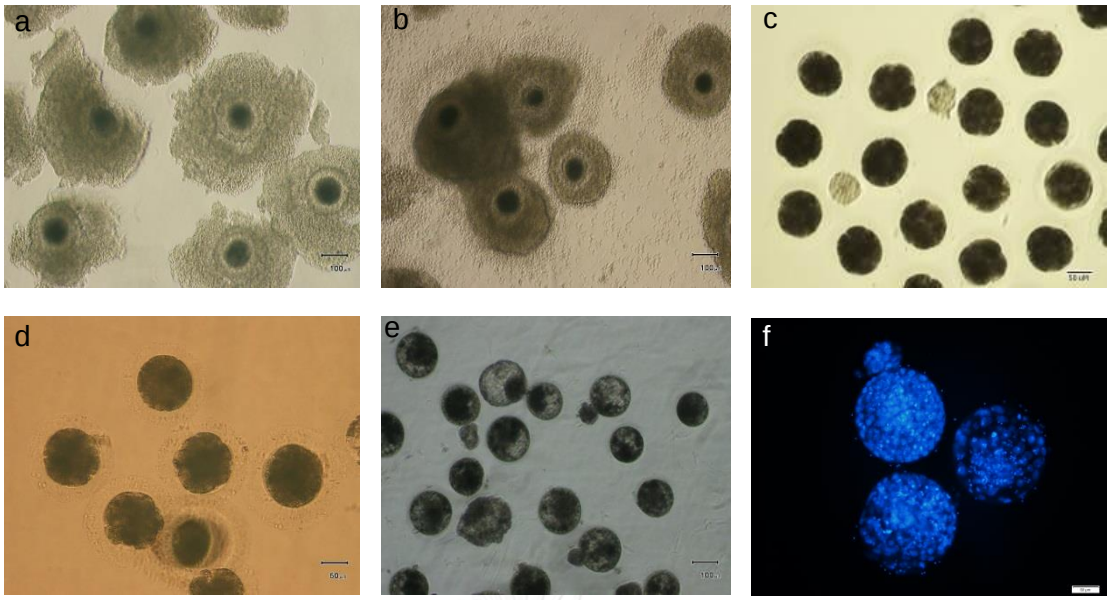

Figure 3. Oocytes and developmental stage of cat embryos. Cumulus oocyte complex (a), oocyte surrounded with expanded cumulus cells (b), 4-8 cells stage embryo (c), morula stage embryo (d), expanded blastocyst and hatching blastocyst (e), total cell of blastocyst determination with DAPI staining (f).

2.3.6 RNA extraction

In one replicate, total RNA was extracted from each stage of embryo development (pools of 10 morulae, and 5 blastocysts) using the Absolutely RNA Nanoprep Kit (Stratagene, San Diego, USA) following the manufacturer's instructions at room temperature. These pooled embryos were used to reduce individual variation and also to increase the amount of extracted RNA. The extracted RNA was assessed for quality and quantity using a spectrophotometer (ND-2000, NanoDrop, Wilmington, Delaware, USA) and immediately stored at -80 ℃ until further use.

2.3.7 Quantitative RT-PCR (qPCR)

Reverse transcription (RT) was performed using a First-Strand cDNA Synthesis Kit (SuperScript III Kit, Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The products were stored at -20 ℃ for further use in qPCR. The relative expression levels of IGF-1R were normalized to the endogenous control gene (*GAPDH*: glyceraldehydes 3-phosphatedehydrogenase) (Sano et al., 2005). Primer sequences of *GAPDH* was referenced from Sano et al. (Sano et al.,

2005) forward primer 5’ GGAGAAAGCTGCCAAATATG 3’ and reverse primer 5’ AGGAAATGAGCTTGACAAAGTGG 3’. *IGF-1R* primer sequences was obtained from Waurich et al. (Waurich et al., 2010): forward primer 5’ GCACAAGGAGCAGATGACATT 3’ and reverse primer 5’ CAGGTTCCGGCCACTTTAAA 3’. The amplicon size of GAPDH and IGF-1R were 191 and 255 bp, respectively. The qPCR using the ABI PRISM 7300 Real-time cycler (Applied Biosystems, Foster City, California, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Each PCR (total volume of 20 µl) consisted of 2 µl of reverse transcript and 18 µl of reaction mixture containing 10 µl of SYBR Green Master Mix, 1 µl of both forward and reverse primers (2.5 µM for *GAPDH*, 5 µM for *IGF-1R*) and 6 µl of diethylpyrocarbonate (DEPC)-treated water to reduce the chance of RNA degradation in the solutions. The thermal cycling conditions were as follows: 10 min at 95 ℃ to activate *Taq* DNA polymerase, and 40 cycles of 15 sec at 95 ℃ for denaturing, 30 sec at 55 ℃ for annealing and 60 sec at 72 ℃ for extension. The Sequence Detection System (SDS) Software (Version 1.4, Applied Biosystems, USA) was used to quantify and analyze the relative quantitation (RQ). Calculations of relative quantitation were performed by the comparative Cq method using morulae or blastocysts from singly culture as a control group. Data are reported as relative n-time difference in relation to the control group. PCR products were confirmed by melting curve analysis and gel electrophoresis. The amplified products were separated in 2% (wt/vol) agarose gel (Bio-Rad, California, USA) prepared in 1× TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8) containing 0.4 mg/ml ethidium bromide (Promega, Wisconsin, USA). The separated products in agarose gel were visualized under UV light (Syngene, Cambridge, UK).

2.3.8 Experimental design

Experiment 1: Developmental competence of feline embryos cultured in groups or singly

COCs were matured and fertilized *in vitro*. Cleaved embryos (day 2 of development, n = 110) were randomly assigned to either group culture (10 cleaved embryos, without IGF-1) or single-embryo culture (also without IGF-1) in 50 µl of IVC-2 medium. The embryos were assessed for morula development on day 5 and blastocyst formation on days 6 and 7.

Experiment 2: Expression of *IGF-1R* mRNA transcripts in group and single-embryo cultures

To compare the expression levels of *IGF-1R* in embryos cultured individually or in groups (no IGF-1 supplementation), morulae, and blastocysts were randomly collected and used to determine the expression levels of the *IGF-1R* gene relative to the endogenous control gene (*GAPDH*). The levels of *IGF-1R* mRNA transcript obtained from morulae or blastocyst from singly cultured were used as a control group. The relative expression levels assessed by qPCR are shown as RQ, and the size of each PCR product was further confirmed by gel electrophoresis.

Experiment 3: Effect of IGF-1 supplementation on developmental competence of feline embryos cultured singly

Experiment 1 indicated that the developmental competence of embryos cultured in groups developed to blastocysts at a higher rate than those cultured singly. This experiment was therefore designed to determine the effect of IGF-1 supplementation on developmental competence. Different concentrations of IGF-1 (0, 5, 25, 50, and 100 ng/ml) were added into embryo culture media from day 2 to day 7. The embryos were assessed for morula development on day 5 and the blastocyst formation rate on days 6 and 7.

Experiment 4: Effect of IGF-1 supplementation on *IGF-1R* mRNA expression in feline embryos cultured singly

This experiment determined the embryonic response, in the form of *IGF-1R* mRNA expression, after adding different concentrations of IGF-1 to embryos cultured individually. Day 2 (4-8 cells) embryos were cultured in media supplemented with different concentrations (25, 50, and 100 ng/ml) of IGF-1. The morula-stage embryos derived from each IGF-1 concentration were randomly collected and compared for *IGF-1R* gene expression. The *IGF-1R* expression was calculated in relation to the endogenous control gene (*GAPDH*). Morulae in singly culture without growth factor supplementation were used as a control group.

Experiment 5: Effect of PPP treatment on developmental competence of feline embryos cultured in group or singly with or without IGF-1 supplementation

Picropodophyllin (PPP) is selective inhibitor of IGF-1R, which does not interfere with insulin receptor or other related receptor (Strömberg et al., 2006). PPP (Santa Cruz Biotechnology, CA, USA) was prepared by dissolved in dimethylsulfoxide (DMSO) to a concentration of 0.5 mM. Day 2 (4-8 cells) embryos were randomly culture in the following treatments 1) group culture without IGF-1 supplementation, 2) group culture with 0.5 μM PPP, 3) single culture without IGF-1 supplementation, 4) single culture without IGF-1 supplementation with 0.1% DMSO, 5) single culture with 0.5 μM PPP, 6) single culture with IGF-1 25 ng/ml supplementation, 7) single culture with IGF-1 25 ng/ml and 0.5 μM PPP supplementation. The embryos were assessed for morula on day 5 and blastocyst development on day 7. IGF-1 was supplemented in culture medium from day 2 to day 7. PPP or DMSO was supplemented in culture medium during the transition stage of morula to blastocyst (day 5 to day 7).

2.3.9 Statistical analysis

Three to four independent replicates were performed in each experiment. Results are expressed as the mean ± standard error of the mean (SEM). The percentage of hatching blastocysts was calculated relative to the total number of blastocysts. Differences between groups in the mean percentage of morulae and different stages of blastocysts (day 6, day 7 and hatching blastocysts) were assessed by the chi-squared test using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, N.C., USA). The differences in cell numbers in each group were assessed by one-way ANOVA and Duncan's post hoc multiple range test. Differences with $P<0.05$ were considered statistically significant.

2.4 Results

2.4.1 Developmental competence of feline embryos cultured in groups or singly

In the absence of exogenous IGF-1 supplementation, the developmental competence of embryos to blastocysts at days 6 and 7 cultured in groups was significantly higher than those cultured individually ($P<0.05$; Table 1). However, the percentages of cleaved embryos that developed to morulae and hatching

blastocysts were not significantly different (P >0.05). The two culture systems had no effect on the cell number in blastocyst-stage embryos.

Table 1 Percentages (mean ± SEM) of developmental competence and embryo quality of cat embryos cultured in different culture conditions

Cultured conditions	No. of cleaved embryo	No. of cleaved embryo developed to			Hatching blastocyst (%)	Cell number
		Morula (%)	Day6 blastocyst (%)	Day7 blastocyst (%)		
Group w/o GF	60	41 (68.3±6.1)	29 (48.3±6.5)[a]	36 (60±6.4)[a]	12 (34.3±10.2)	155.3±11.2
Single w/o GF	50	32 (64±6.9)	13 (26±6.3)[b]	20 (40±7)[b]	6 (32.8±4.3)	141.6±9.3

[a, b] Within a column, different superscripts denote values that differ significantly (P<0.05).

w/o GF = without insulin-like growth factor-1

2.4.2 Expression of IGF-1R mRNA in group and single-embryo cultures

The expression of IGF-1R transcript in the morula stage of group embryo culture was significantly higher than that found in single embryo culture (P<0.05; Figure 4). However, the expression of *IGF-1R* was similar in blastocysts from either group culture or single embryo culture without IGF-1 (P>0.05; Figure 4). The PCR products of *GAPDH* and *IGF-1R* transcripts derived from qPCR confirmed the correct amplicon sizes (Figure 5)

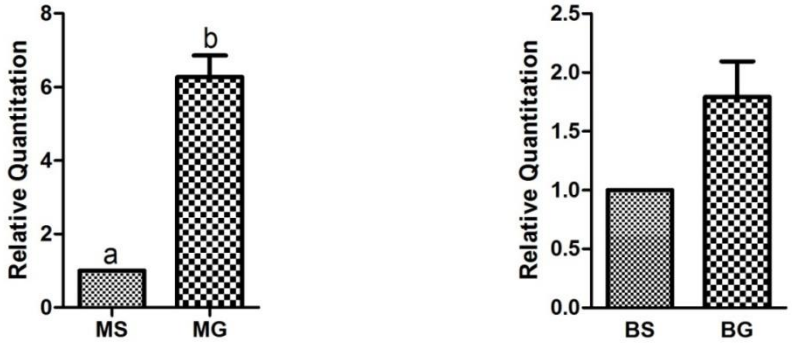

Figure 4. Relative expression (mean±SEM) of *IGF-1R* transcript in morulae and blastocysts acquired from embryos cultured singly (morulae: MS; blastocysts: BS) or in groups (morulae: MG; blastocysts: BG).

Relative quantitation was analyzed using real-time PCR. *GAPDH* was used to normalize each gene, and MS or BS embryos were used as calibrators.

[a, b] different letters on the bars indicate values that differ significantly (P<0.05).

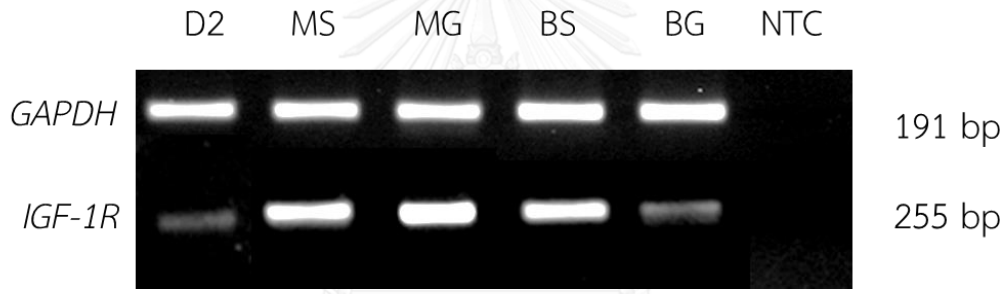

Figure 5. The amplicon size of PCR products of *GAPDH* and *IGF-1R* transcript in morulae and blastocysts acquired from embryos cultured singly or in groups

Embryos cultured singly (morulae: MS; blastocysts: BS) or in groups (morulae: MG; blastocysts: BG) acquired from realtime PCR were confirmed their correct sizes by gel electrophoresis. They were run on 2% agarose gel contained with ethidium bromide in 1xTBE buffer and were visualized under UV light. NTC= no template control.

2.4.3 Effect of IGF-1 supplementation on developmental competence of feline embryos cultured singly

IGF-1 supplementation generally improved the developmental competence of feline embryos cultured singly, except that 100 ng/ml IGF-1 was apparently excessive (Table 2). IGF-1 at 5 and 100 ng/ml had no significant benefit on embryo development in terms of morula and blastocyst formation rates compared with control (no IGF-1 added, P>0.05). By contrast, the 25 and 50 ng/ml IGF-1 doses

effectively promoted blastocyst development. On day 6 of development (day 0 = IVF), these two IGF-1 concentrations also accelerated the growth rates of blastocysts compared with other concentrations (5 and 100 ng/ml) and control ($P<0.05$). However, all IGF-1 concentrations had no significant effect on total cell number within blastocysts ($P>0.05$, Table 2). Although 25 and 50 ng/ml IGF-1 had similar effects on morula and blastocyst development rates, 25 ng/ml IGF-1 was best at stimulating hatching ($P<0.05$).

Table 2 Percentages (mean ± SEM) of developmental competence and embryo quality of cat embryos cultured singly with different concentrations of IGF-1 supplementation

Group	No. of cleaved embryo	No. of cleaved embryo developed to			Hatching blastocyst (%)	Cell number
		Morula (%)	Day6 blastocyst (%)	Day7 blastocyst (%)		
IGF-1 0 ng/ml	50	32 (64±6.9) [a,b]	13 (26±6.3)[b]	20 (40±7)[b]	6 (32.8±4.3)[a,b]	141.6±9.3
IGF-1 5 ng/ml	50	34 (68±6.7)[a,b]	13 (26.5±6.4)[b]	24 (49±7.2)[a,b]	5 (21.4±14.9)[b]	143.8±10
IGF-1 25 ng/ml	62	43 (69.4±5.9)[a,b]	27 (43.6±6.4)[a]	37 (59.7±6.3)[a]	19 (53±16.6)[a]	141.7±7.8
IGF-1 50 ng/ml	50	39 (78±5.9)[a]	22 (44±7.1)[a]	29 (58±7.1)[a]	5 (19.1±9.5)[b]	137.9±11.8
IGF-1 100 ng/ml	47	27 (57.5±7.3)[b]	11 (23.4±6.2)[b]	17 (36.2±7.1)[b]	4 (22.6±12.4)[b]	136.4±10.1

[a, b] Within a column, different superscripts denote values that differ significantly ($P<0.05$)

2.4.4 Effect of IGF-1 supplementation on IGF-1R mRNA expression in feline embryos cultured singly

IGF-1R was significantly upregulated during morula development under treatment of IGF-1 at 25 or 50 ng/ml compared to non-supplementation group ($P<0.05$; Figure 6). However, there was no significant difference between IGF-1 at 100 ng/ml and non-supplementation group. IGF-1 supplementation in the single embryo culture system increased *IGF-1R* gene expression to a level similar to that found in group culture (Figure 6). The PCR products of *GAPDH* and *IGF-1R* transcripts derived from qPCR confirmed the correct amplicon sizes (Figure 7).

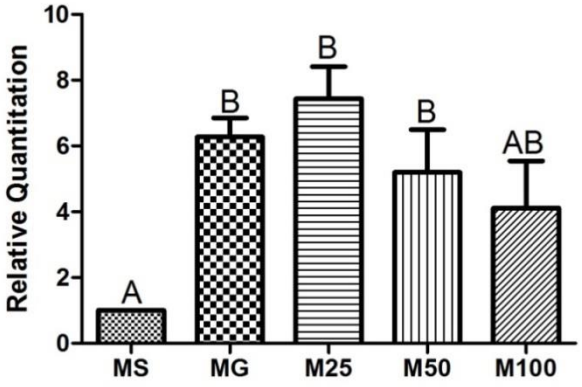

Figure 6. Relative expression (mean ± SEM) of *IGF-1R* transcript in morulae acquired from embryos cultured in group (MG) or singly (MS) supplemented with IGF-1 at 0, 25, 50 or 100 ng/ml (MS, M25, M50 and M100, respectively).

The expression levels, shown as relative quantitation, were analyzed using real-time PCR. *GAPDH* was used to normalize each gene, and MS embryos were used as calibrators.

[A, B] different letters on the bars indicate values that differ significantly (P<0.05).

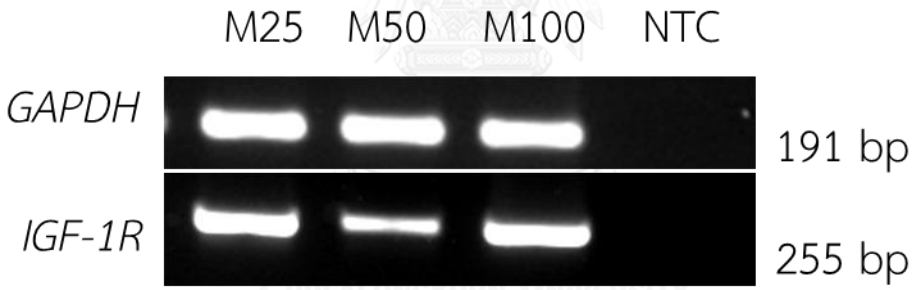

Figure 7. The amplicon size of qPCR products of *GAPDH* and *IGF-1R* transcript in morulae acquired from embryos cultured singly supplemented with IGF-1 at 25, 50 or 100 ng/ml (M25, M50 and M100, respectively)

They were run on 2% agarose gel contained with ethidium bromide in 1xTBE buffer and were visualized under UV light. NTC= No template control.

2.4.5 Effect of PPP treatment on developmental competence of feline embryos cultured in group or singly with or without IGF-1 supplementation

In the presence of PPP whether in group or singly culture, the percentages of blastocyst formation were significantly decreased compared with non PPP treated group. No blastocyst formation was observed in singly cultured embryo treated with PPP. Although IGF-1 supplementation at the concentration of 25 ng/ml significantly

promoted blastocyst formation of singly cultured embryo to the similar level of group culture embryos without IGF-1 supplementation, no blastocyst were observed in this condition after treated with PPP. The addition of 0.1% DMSO did not affect blastocyst formation (Table 3).

Table 3 Percentages (mean ± SEM) of developmental competence and embryo quality of cat embryos cultured in group or singly with different condition of PPP or IGF-1 supplementation

Group	No. of cleaved embryo	No. of cleaved embryos developed to		Cell numbers
		Morula (%)	Blastocyst (%)	
Group w/o GF	50	42(84±6)	29(58±5.8)[a]	97.1±7
Group w/o GF + PPP	50	42(84±3.3)	8(16±14.2)[b]	82±11
Single w/o GF	44	30(68.2±11.6)	21(47.7±2.7)[a]	114±9.5
Single w/o GF+DMSO	42	29(69.1±8.7)	24(57.1±8.9)[a]	99.9±14.1
Single w/o GF+PPP	44	31(70.5±6.1)	0(0±0)[c]	NA
IGF-1 25 ng/ml	43	35(81.4±7.1)	27(62.8±10)[a]	100.8±6.7
IGF-1 25 ng/ml + PPP	44	30(68.2±6)	0(0±0)[c]	NA

[a, b, c] Within a column, different superscripts denote values that differ significantly ($P<0.05$)

NA = Sample are not available

w/o GF = without insulin-like growth factor-1

2.5 Discussion

This study demonstrated the differences in developmental competence and *IGF-1R* gene expression between group and single-embryo culture systems. This study also confirmed that the developmental competence of feline embryos cultured with companion embryos enhanced their developmental potential compared with those cultured individually. However, IGF-1 supplementation in the culture medium improved the development of singly cultured embryos to a level that was similar to the group-cultured embryos (approximately 60% blastocyst formation rates), essentially via modulating *IGF-1R* expression. Culture conditions

during *in vitro* culture, such as medium composition (Johnston et al., 1993; Lane and Gardner, 1998; Gardner, 2007; Sananmuang et al., 2011), embryonic density (Lane and Gardner, 1992; Sananmuang et al., 2011; Vutyavanich et al., 2011), atmospheric gases, culture temperature (Johnston et al., 1991) and culturing in groups are considered critical factors of successful *in vitro* embryo production. The paracrine effect from embryos cultured in groups is far superior to those cultured individually but the single-embryo culture system is still important studying factors associated with embryo developmental competence and quality (Ebner et al., 2010; Sananmuang et al., 2011). A single-embryo culture strategy would allow us to understand embryo physiology and to evaluate the metabolism of individual embryos, which, in turn, could be used to identify potential biomarkers for non-invasive selection of superior embryos (Goovaerts et al., 2010; Gardner et al., 2011; Gardner and Wale, 2013). However, development of this technology is hampered by the fact that the embryos cultured singly develop at a poorer rate (Goovaerts et al., 2010) than group-cultured embryos, presumably due to insufficient paracrine signaling (Paria and Dey, 1990; Lane and Gardner, 1992). In this study, it was also clear that the superior development of the group cultured embryos was the result of the transition from morula to blastocyst, which occurs approximately 6 to 7 days after culturing. We therefore speculated that paracrine factor secreted from individual embryos were responsible for reducing the developmental block during the morula-to-blastocyst transition and that their effects were stage-specific. It is worth noting that feline embryos have unique physiology in that the developmental block usually occurs at the morula stage, although the maternal zygotic gene transition occurs around the 8-cell stage in domestic cats (Hoffert et al., 1997). The paracrine effects may come from a particular growth factor (IGF-1) that plays an essential role during embryo development in domestic cats. We examined *IGF-1R* gene expression (experiment 2) and the effect of IGF-1 on the developmental competence of feline embryos (experiment 3). The finding that the *IGF-1R* expression of morulae cultured singly was significantly lower than in morulae from group culture coincided with the poor development during the morula-blastocyst transition of singly cultured embryos. It appears that embryos in group culture secreted sufficient paracrine

factors (possibly IGF-1) to upregulate *IGF-1R* expression. On the other hand, this circuit may be insufficient in the case of single-embryo culture. This evidence suggests that the IGF-1 signaling pathways at the morula stage may play a central role in the morula-to-blastocyst transition. Interestingly, we found that supplementation of the culture medium with IGF-1 at the concentration of 25 or 50 ng/ml significantly improved the developmental competence of singly cultured embryos to a level comparable to that obtained from group culture. Meanwhile, IGF-1 at 5 or 100 ng/ml was not beneficial (Table 2). The positive effect of IGF-1 in modulating embryo development is in an agreement with the findings that supplementation with IGF-1 might be able to upregulate *IGF-1R* gene expression to a level that was similar to group culture (Figure 7). This result supports the previous finding that *IGF-1R* signaling in compacted preimplantation embryos (morula stage in mouse embryos) is activated by the stimulation of IGF-1 secretion (Kaye, 1997). The binding of IGF-1 to its receptors triggers several cellular cascades, in particular glucose metabolism pathways (Hogan et al., 1991), increasing glucose transport via GLUT1 and GLUT8 (Pantaleon and Kaye, 1996; Carayannopoulos et al., 2000). IGF-1 can also activate the AMP-activated protein kinase (AMPK) pathway, resulting in increased glucose uptake and enhancing embryo survival by decreasing apoptosis (Eng et al., 2007). This ligand-receptor binding is essential for cell growth and proliferation (Pantaleon and Kaye, 1996), which, in part, helps the embryos to overcome the developmental block at the morula stage. Here, the effects of IGF-1 on *IGF-1R* signaling and embryo development were dose-dependent. For example, while IGF-1 at 25 ng/ml promoted the highest *IGF-1R* expression, other higher concentrations (50 and 100 ng/ml) downregulated the gene. In the present study, IGF-1 supplementation at a concentration of 25 ng/ml was appropriate to stimulate blastocyst formation and hatching, while blastocyst cell number was not affected (experiment 3). The increased hatching rate may have resulted from an increase in blastocoel fluid via upregulation of the ATP1A1 gene (Bonilla et al., 2011). IGF-1 stimulates blastocyst development, increases glucose uptake via activation and translocation of glucose transporters (Carayannopoulos et al., 2000) and prevents apoptosis (Herrler et al., 1998; Kim et al., 2006). However, excessive glucose uptake

resulting from excessive binding of IGF-1 to its receptors can also inhibit hypoxanthine phosphoribosyltransferase (HPRT) activity, which, in turn, increases the production of reactive oxygen species via xanthine oxidase (Guerin and Menezo, 2011). Furthermore, high levels of IGF-1 could activate transforming growth factor beta type 3 (TGF-β type 3) and suppress the expression of p53, which are proteins that play critical roles in apoptosis and DNA repair, respectively (Velazquez et al., 2011). Finally, we found that a high concentration of IGF-1 (100 ng/ml IGF-1) led to downregulation of *IGF-1R*. This result suggests the existence of a homeostatic network controlling IGF-1 and its receptors in feline embryos. Further study would be required to characterize this mechanism. The important role of IGF-1 on cat embryo development was demonstrated in this study. Inhibition of *IGF-1R* ligand activation during embryo culture leads to reduction in blastocyst formation rate as well as blastocyst cell number of mouse blastocyst (Markham and Kaye, 2003; Inzunza et al., 2010). These results demonstrate the important role of IGF-1 on embryo development. Activation of IGF-1R by its ligand triggers the autophosphorylation of tyrosine kinase in β subunit. Selective inhibition of IGF-1R activity by PPP treatment resulted in significantly decreasing of blastocyst formation in group cultured embryos, and complete inhibition of blastocyst development in singly cultured embryo (Table 3). The reduction in blastocyst formation may due to the downregulation of IGF-1R tyrosine kinase activity and the increase in apoptotic rate by PPP (Strömberg et al., 2006). Effect of PPP during embryo culture of mouse embryo development has been demonstrated. Blastocyst formation rate was affected by blocking of IGF-1 by PPP (Inzunza et al., 2010). This finding supported our finding that IGF-1 is crucial for cat embryo development. In conclusion, poor feline embryo development was observed when embryos were cultured singly. This deficiency can be circumvented by IGF-1 supplementation, which can improve the developmental competence of feline embryos cultured individually and can also upregulate *IGF-1R* gene expression to a level similar to group-cultured embryos.

CHAPTER 3

Epidermal growth factor (EGF) improves developmental competence of singly cultured domestic cat embryos

(Submitted to Reproduction in domestic animals)

3.1 Abstract

Epidermal growth factor (EGF) improves embryonic development in low embryo density culture system in several mammalian species. Our study examined (1) the influence of EGF and its interaction with insulin liked growth factor-1 (IGF-1) on developmental competence of singly cultured embryos, (2) expression of EGF receptor (*EGFR*) at various stages of development, also (3) the influence of EGF on embryo quality in embryos cultured individually versus in group. Cat oocytes were *in vitro* matured, fertilized (IVM/IVF) and cleaved embryos (48 h post-fertilization) were randomly assigned to one of seven culture conditions: 1) to 2) group culture (50 µl droplet per 10 embryos) supplemented with EGF (0 or 5 ng/ml), 3) to 7) single embryo culture (50 µl droplet per 1 embryo) supplemented with EGF (0, 5, 25, 50 or 100 ng/ml), 8) to 11) single embryo cultured with EGF (5 or 100 ng/ml) and IGF-1 (25 or 50 ng/ml). Morulae as well as blastocysts and hatching blastocysts were assessed at Day 5 and 7 post-IVF, respectively. Embryo quality was assessed by total cell number, KI67 proliferative marker and the number of highly expressed OCT3/4 (pluripotency marker) cells within an inner cell mass. Relative mRNA expressions of *EGFR* of 2-4 cells, 8-16 cells, morulae, and blastocysts cultured in group or singly with or without EGF supplementation (Treatments 1 to 4) were examined using RT-qPCR. Localizations of OCT3/4 and KI67 in blastocysts derived from group or singly with or without EGF supplementation (Treatments 1 to 4) were examined. More embryos cultured in groups developed to the blastocyst stage than individually incubated cohorts (56.3 vs. 36.4%, $P < 0.05$). EGF promoted blastocyst formation to the level comparable to that of group culture. However, blastocyst formation rates of embryos cultured with EGF and IGF-1 were comparable to those incubated with EGF alone. Expression levels of *EGFR* were decreased in morulae and blastocysts

cultured in the presence of EGF, irrespective culture regimens (group or individual culture). Increasing in KI67 positive cells was observed in singly cultured embryos in the presence of EGF. However, there were no differences in highly expressed OCT3/4 cells among group or individual culture. Our findings indicated that embryo cultured singly have similar quality in terms of OCT3/4 positive cells to those group cultured embryo. In summary, our findings indicated that EGF enhanced developmental competence of cat embryos cultured singly via EGF/EGFR signaling that stimulating cell proliferation.

3.2 Introduction

In mammals, when only small numbers of good quality oocytes are available, it is necessary to culture gametes/embryos individually or in small groups (Vajta et al., 2008; Goovaerts et al., 2009). However, it has been shown in the mouse and cow that small percentages of embryos cultured singly developed into blastocyst stage compared with those incubated in groups (mouse, 49% vs. >80%; cow, 2.9% vs. 30.7%) (Paria and Dey, 1990; Goovaerts et al., 2009). Furthermore, previous studies have also demonstrated that individually cultured cat (Spindler and Wildt, 2002) and cow embryos (Carolan et al., 1996) failed to reach the compaction stage. To date, several strategies have been established to overcome the poor developmental rate of singly cultured embryos (Goovaerts et al., 2009). These include growth factor supplementation, *in vitro* culture in conditioned medium derived from group-embryo culture, and co-culture with autologous cumulus cells (Paria and Dey, 1990; Fujita et al., 2006; Goovaerts et al., 2009; Goovaerts et al., 2010; Goovaerts et al., 2011). Various growth factors, such as epidermal growth factor (EGF) and insulin like growth factor-1 (IGF-1) have been shown to enhance blastocyst development in single embryo culture system in the mouse and cow (Paria and Dey, 1990; Lim and Hansel, 1996; o'Neill, 1997). For the cat, we have recently shown that IGF-1 promote blastocyst development in single embryo culture system by modulating the expression of its receptor (*IGF-1R*) and thus developing beyond the developmental block (Thongkittidilok et al., 2014). Epidermal growth factor is a mitogenic polypeptide that is involved in regulating cell proliferation in many cell types, including granulosa cells of preantral follicles. This growth factor also has been

shown to promote *in vitro* maturation (IVM) of mouse, cat, sheep and pig oocytes (Lorenzo et al., 1994; Demeestere et al., 2005; Merlo et al., 2005; Conti et al., 2006; Peng et al., 2010; Procházka et al., 2011). Addition of EGF to IVM medium promotes cleavage and blastocyst development of *in vitro* matured gametes (Sirisathien et al., 2003; Neira et al., 2010; Shabankareh and Zandi, 2010; Song et al., 2011; Chandra et al., 2012). EGF binds with its receptor (EGFR) and stimulates MAP/ERK and Akt pathways that, in turn, enhance cell proliferation. It has been shown that *EGFR* is expressed in 2-cell to blastocyst stage in the mouse, and from the 8-cell stage to the blastocyst in the human (Kane et al., 1997). By using the domestic cat as model for wild felids, our laboratory has previously shown that IGF-1 at concentration of 25 or 50 ng/ml augmented the blastocyst formation rate of singly cultured embryo (in 50 µl) to the level comparable to that of group culture (10 embryos/50 µl) without growth factor supplementation via IGF-1 receptor modulation (Thongkittidilok et al., 2014). Similar to EGF, IGF-1 binds with its receptor and stimulate MAP/ERK and Akt pathways that, in turn, promote cell proliferation and differentiation (Vincent and Feldman, 2002; Conti et al., 2006). The synergic effect of EGF and IGF-1 on embryonic development was investigated in group and individually cultured embryos in the cow (Sirisathien et al., 2003) and mouse (Paria and Dey, 1990). In those studies, addition of both IGF-1 and EGF in a culture medium did not increase blastocyst development or blastocyst cell numbers when compared to EGF or IGF-1 supplementation alone (Paria and Dey, 1990; Sirisathien et al., 2003). However, some studies in cow demonstrated the synergic effect of IGF-1 and EGF on nuclear maturation and blastocyst development compared to IGF-1 or EGF supplementation alone (Lorenzo et al., 1994; Sakagami et al., 2012). To date, there have been no studies on the influence of EGF alone and/or in combination with IGF-1 on *in vitro* development of singly cultured cat embryos. As EGF involved cell proliferation of embryos. Detection of KI67 is considered as a good marker to determine cell proliferation in only active stage of the cell cycle (G1, S, G2 and M) in mouse embryo (Winking et al., 2004; Oh et al., 2009). Recently, singly embryo cultured has been studied in several species but the quality of blastocyst produced from this method have not clearly identified. The assessment of blastocyst quality for determination of cell proliferation marker

(KI67) and pluripotency protein (OCT3/4) which highly expressed in the inner cell mass as a transcription factors critical for embryogenesis and cell differentiation (Pesce et al., 1998; Wu and Scholer, 2014) is necessary for normal embryo development.

The objectives of this study were to determine (1) the effects of EGF alone or in combination with IGF-1 on developmental competence of cat single embryo culture (2) the *EGFR* expression at various developmental stages of cat embryos cultured in group versus singly (3) the effects of EGF on expressions of pluripotency marker protein (OCT3/4) and proliferation marker protein (KI67). We hypothesized that supplementation of EGF alone or in combination with IGF-1 to culture environment would promote embryonic development and *EGFR* expressed in cat preimplantation embryos.

3.3 Materials and Methods

All chemicals used in this study were purchased from Sigma Aldrich, St Louis, MO, USA, unless otherwise specified.

3.3.1 Oocyte collection, in vitro maturation, fertilization and culture

The techniques of oocyte collection, in vitro maturation, fertilization and culture have been previously described in chapter 2.

3.3.2 Assessment of embryo development

The embryos were classified as morula (having cell compaction without blastocoel formation) on Day 5 post-IVF, blastocyst (having a fully-expanded blastocoel, and two differentiated cell types; inner cell mass and trophectoderm), and hatching blastocyst (hatching of blastocyst cells from zona pellucida) on Day 7 post-IVF under an inverted microscope at 40x magnification (CKX41 Olympus, Shinjuku, Japan). All blastocysts observed on Day 7 were fixed in 4% (wt/vol) paraformaldehyde for overnight and washed twice in phosphate buffer saline (PBS) before permeabilization with 0.1% Triton X-100 in PBS for 15 min. Blastocysts were incubated with 1:50 anti-goat OCT3/4 polyclonal IgG antibodies (Santa Cruz

Biotechnology, California, USA) at 37 °C for 1 h and then rinsed in PBS and incubated for 2 h with 1:50 FITC labeled donkey anti-goat IgG (Santa Cruz Biotechnology, California, USA). After OCT3/4 staining, blastocysts were incubated with 1:50 anti-mouse KI67 monoclonal antibodies (Clone MIB-1, Dako Cytomation, Denmark) at 37°C for 1 h, then rinsed in PBS and incubated for 1 h with 1:100 TRITC labeled anti-mouse IgG (T5393) to determine the proliferating cells of the blastocyst. Blastocysts were stained with 0.1 µg/ml 4′, 6-Diamidino-2-phenylindole (DAPI) in PBS at 25°C for 10 min to assess total cell numbers. The immunofluorescently labelled were examined using epifluorescent microscope (BX51 Olympus, Shinjuku, Japan).

3.3.3 RNA extraction and reverse transcription (RT)

RNA extraction and reverse transcription were performed as previously described in chapter 2.

3.3.4 Quantitative RT-PCR (qPCR)

The relative expression levels of target gene (EGFR) were normalized to an endogenous control gene, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*), that has been previously shown to be stable during cat pre-implantation embryo development (Filliers et al., 2012): forward primer 5'GAAGAGTCCTACAAAGACAGCACGC 3' and reverse primer 5' AATTTTCCCCTCCTTCTCCTGC 3'. The amplicon size of *YWHAZ* was 115 bp. *EGFR* primer was designed from *Felis catus EGFR* mRNA, complete cds (Accession number: HQ185236.1) using the NCBI primer designing tool (Primer 3 software V 0.4.0): forward primer 5' AGATTGCGAAGGGCATGAAC 3' and reverse primer 5' GGCGTCTTCACCAGGACATT 3'. The amplicon size of *EGFR* was 118 bp. To confirm the amplified EGFR, the amplicon was extracted using Gel/PCR DNA Fragments Extraction Kit (Gene-aid Biotech, California, USA) and then was submitted for sequencing. The sequences were blasted in the NCBI GenBank to determine the nucleotide identity prior to being used in this study. The EGFR primer designed for this study contained 100% nucleotide identity of EGFR mRNA sequence found in *Felis catus* EGFR mRNA complete cds, HQ185236.1. The qPCR was performed using the ABI PRISM 7300 Real-time cycler (Applied Biosystems, Foster City, California, USA) with Luminaris Color

HiGreen High ROX qPCR Master Mix (Thermo scientific, California, USA). Each PCR reaction (total volume of 10 µl) consisted of 2.5 µl of DNA mixture which contained 2 µl of reverse transcription product and 0.5 µl of yellow sample buffer and 7.5 µl of reaction mixture which contained 5 µl of Luminaris Color HiGreen High ROX qPCR Master Mix, 0.3 µl of both forward and reverse primers (10 µM of YWHAZ, and 10 µM of EGFR) and 1.9 µl of nuclease free water. The thermal cycling conditions were as follows: 2 min at 50 °C to pre-treatment of urasil-DNA glycosylase (UDG), 10 min at 95 °C to activate Tag DNA polymerase, 40 cycles of 15 s at 95 °C for denaturing, 30 s at 55 °C for annealing and 60 s at 72 °C for extension. The relative quantitation (RQ) was qualified and analyzed by Sequence Detection System (SDS) Software Version 1.4 (Applied Biosystems, USA). Day 2 embryos were used as a control group for calculations of relative quantitation which performed by the comparative Cq method. Data was reported as relative n-times difference, in relation to the control group (Livark and Schmittgen, 2001). qPCR products were confirmed by melting curve analysis and run in gel electrophoresis. The qPCR products were analyzed on a 2% agarose gel (Bio-Rad, California, USA) in 1×TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH8) containing 1x/ml Redsafe nucleic acid staining solution (Intron Biotechnology, Gyeonggi-do, Korea), and then visualized under UV light (Syngene, Cambridge, UK).

3.3.5 Experimental design

Experiment 1: Effect of EGF alone or in combination with IGF-1 on development of embryos cultured singly compared with group cultured embryos without growth factor supplementation

This experiment determined the effect of EGF supplementation on developmental competence of singly cultured embryo compared with group cultured embryo without growth factor supplementation. Single embryos were cultured in IVC-2 medium supplemented with different concentrations of EGF (0, 5, 25, 50, and 100 ng/ml) from Day 2 to 7. We investigated the combination effect of EGF at 5 (low concentration) or 100 ng/ml (high concentration) with IGF-1 at 25 or 50 ng/ml (Thongkittidilok et al., 2014). Group embryos were served as a control and

were cultured in IVC-2 medium without growth factor supplementation from Day 2 to 7. The embryos were assessed for morulae on Day 5 and blastocyst formation rate on Day 6 and 7. Three or four replicates were performed in this experiment.

Experiment 2: Effect of EGF on development competence and the expressions of *EGFR*, OCT3/4 and KI67 of embryos cultured singly and in groups

Experiment 2.1: Effect of EGF on developmental competence of embryos cultured singly and in groups

From the result of experiment 1, we found that EGF supplementation even at the lowest dose (5 ng/ml) significantly promoted blastocyst formation of singly cultured embryo. This experiment aimed to investigate the effect of EGF (5 ng/ml) supplementation on developmental competence of either singly or group cultured embryos. Cleavage stage embryos were cultured individually or in groups in IVC-2 medium with or without 5 ng/ml EGF supplementation from Day 2 to 7. The control was group embryos cultured without EGF supplementation. The embryos were assessed for morula on Day 5 and blastocyst formation on Day 6 and 7. Four replicates were performed in this experiment.

Experiment 2.2: Effect of EGF on *EGFR* expression of embryos cultured singly and in groups

To evaluate the influence of EGF on EGFR expression at different developmental stages, Day 2 embryos (4-8 cells), Day 3 embryos (8-16 cells), morula (Day 5) and blastocyst (Day 7) stage embryos produced in experiment 2.1 were subjected to qPCR to assess mRNA expression. The relative mRNA expression levels of *EFGR* to the endogenous control gene (*YWHAZ*) at each developmental stage were determined and ΔCTs calculated (see above). Relative quantitation (RQ) of 8-16 cells, morulae and blastocysts were calculated based on ΔCT of the control (Day 2 embryos). The relative expression levels assessed by real-time PCR were shown as relative quantitation (RQ). The PCR product sizes were confirmed by gel electrophoresis. Three replications were performed in this experiment.

Experiment 2.3: Effect of EGF on OCT3/4 and KI67 expression in the blastocyst from singly and group culture

To determine the expression of OCT3/4 and Ki67, Day 7 blastocysts produced in experiment 2.1 were assessed for OCT3/4 and Ki67 positive cells using immunofluorescent staining. Forty blastocysts per culture treatment were used.

3.3.6 Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM). The percentage of cleavage was calculated relative to the number of oocytes that were subjected to IVF. Percentages of morula, Day 6 and Day 7 blastocyst were calculated relative to the number of cleaved embryos. Percentage of hatching blastocysts was calculated relative to the total number of blastocysts. Differences between groups in the mean percentage of each developmental stage were assessed by the Chi-squared test using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, N.C., USA). The differences in cell numbers in each group, the number of OCT3/4 and KI67 positive cells, and relative mRNA expression of *EGFR* were assessed by one-way ANOVA using general linear model method. Differences with $P \leq 0.05$ were considered statistically significant.

3.4 Results

3.4.1 Effect of EGF alone or in combination with IGF-1 on development of embryos cultured singly compared with group cultured embryos without growth factor supplementation

In the absence of EGF, more blastocysts were observed on Day 6 and 7 in group culture than individually incubated group ($P < 0.05$, Table 4). However, culture condition did not affect the numbers of morulae and hatching blastocysts produced as well as total cell number of blastocysts ($P > 0.05$). Addition of EGF, even at the low concentration of 5 ng/ml enhanced blastocyst production in single embryo culture system to the level comparable ($P > 0.05$) to group incubation (Table 4). The combination of EGF and IGF-1 did not increase ($P > 0.05$) blastocyst formation compared to the presence of EGF alone. Interestingly, embryos cultured in the low

dosage of EGF (5 ng/ml EGF) and the high dosage of IGF-1 (50 ng/ml) developed to blastocysts on Day 6 and Day 7 at similar rate to those cultured singly without EGF.

Table 4 Percentages (mean± SEM) of morulae, blastocysts (Day 6 and Day 7), hatching blastocysts and cell numbers of embryo cultured in group without EGF supplementation or singly without or with 5, 25, 50 or 100 ng/ml EGF and combination dose between 5 or 100 ng/ml EGF and 25 or 50 ng/ml IGF-1

Group	No. of cleaved embryo	No. of cleaved embryos developed to			Hatching blastocyst (%)	Cell number
		Morula (%)	Day 6 blastocyst (%)	Day 7 blastocyst (%)		
Group w/o GF	65	41 (64.1 ± 6.4)	29 (44.6 ± 3.4)[a]	36 (56.3 ± 2.9)[a]	14 (35.0 ± 7.2)	155.3 ± 11.2
Single w/o GF	55	36 (65.5 ± 5.8)	14 (25.5 ± 9.0)[b]	20 (36.4 ± 7.7)[b]	5 (26.3 ± 11.4)	146.3 ± 9.3
EGF 5 ng/ml	57	37 (64.9 ± 6.4)	31 (54.4 ± 6.7)[a]	32 (56.1 ± 6.6)[a]	14 (43.8 ± 16.0)	186.7 ± 9.1
EGF 25 ng/ml	61	35 (57.4 ± 6.4)	19 (31.2 ± 6.0)[a]	26 (42.6 ± 6.4)[a]	9 (34.6 ± 10.0)	161.4 ± 10.2
EGF 50 ng/ml	58	33 (56.9 ± 6.6)	21 (36.2 ± 6.4)[a]	30 (51.7 ± 6.6)[a]	13 (43.3 ± 6.2)	188.0 ± 12.7
EGF 100 ng/ml	66	45 (68.2 ± 5.8)	21 (31.8 ± 5.8)[a]	36 (56.3 ± 6.2)[a]	13 (36.1 ± 5.6)	166.6 ± 17.1
EGF 5 ng/ml+ IGF-1 25 ng/ml	68	46 (67.7 ± 5.7)	18 (26.5 ± 5.4)[b]	29 (42.7 ± 6.0)[a]	15 (51.7 ± 15.2)	122.1 ± 11.9
EGF 100 ng/ml+ IGF-1 25 ng/ml	62	44 (71.0 ± 5.8)	21 (33.9 ± 6.0)[a]	31 (50.0 ± 6.4)[a]	12(38.7 ± 17.9)	137.2 ± 10.9
EGF 5 ng/ml+ IGF-1 50 ng/ml	71	43 (60.6 ± 5.8)	12 (16.9 ± 4.5)[b]	23 (32.4 ± 5.6)[b]	7 (28.0 ± 6.1)	134.6 ± 10.7
EGF 100 ng/ml+ IGF-1 50 ng/ml	61	47 (77.1 ± 5.4)	17 (27.9 ± 5.8)[a]	38 (62.3 ± 6.3)[a]	7 (18.4 ± 9.6)	127.0 ± 6.8

[a,b] Within a column, different superscripts denote values that differ significantly ($P \leq 0.05$)

w/o GF = without growth factor

3.4.2 Effect of EGF on development competence and the expressions of EGFR, OCT3/4 and KI67 of embryos cultured singly and in groups

3.4.2.1: Effect of EGF on developmental competence of embryos cultured singly and in groups

EGF supplementation did not enhance embryonic development in group culture. However, the percentage of embryos developing to the morula stage was higher in group culture with EGF supplementation than single embryo culture

(P <0.05, Table 5). EGF enhanced blastocyst development in single embryo culture to the level comparable to group culture (Table 5). Culture conditions did not affect the number of hatching blastocyst and blastocyst cell numbers.

Table 5 Percentages (mean± SEM) of morulae, blastocysts (Day 6 and Day 7), hatching blastocysts and cell numbers of embryo cultured in group and singly without or with 5 ng/ml EGF supplementation.

Group	No. of cleaved embryos	No. of cleaved embryos developed to			No. of hatching blastocyst (%)	Cell number
		Morula (%)	Day 6 blastocyst (%)	Day 7 blastocyst (%)		
Group w/o GF	90	73 (81.3 ± 4.3)^{ab}	49 (51.7 ± 1.7)^{a}	65 (66.3 ± 3.8)^{a}	40 (69.1 ± 6.7)	157.6 ± 10.2
Group + EGF 5 ng/ml	90	79 (89.2 ± 7.1)^{a}	44 (45.4 ± 5.2)^{ab}	58 (60.4 ± 4.1)^{a}	32 (55.9 ± 5.1)	160.4 ± 11.5
Single w/o GF	88	66 (74.2 ± 5.7)^{b}	31 (34.4 ± 3.9)^{b}	41 (46.6 ± 2.4)^{b}	24 (58.5 ± 6.1)	172.9 ± 12
Single + EGF 5 ng/ml	86	63 (73.4 ± 4.2)^{b}	32 (35.7 ± 5.1)^{b}	55 (64 ± 8)^{a}	33 (60 ± 8)	160 ± 11

^{a,b} Within a column, different superscripts denote values that differ significantly (P ≤ 0.05)

w/o GF = without growth factor

3.4.2.2 Effect of EGF on EGFR expression of embryos cultured singly and in groups

When examining the influence of culture conditions on *EGFR* expression at a given developmental stage, EGF addition as well as culture density (group versus single) did not influence *EGFR* expression of 8-16 cells embryos (Figure 8a). However, *EGFR* transcripts was significantly higher in morula cultured singly in the absence of EGF than in others cultured condition (P <0.05; Figure 8b). In the blastocyst, EGF significantly decreased *EGFR* expression in blastocysts cultured singly or in group (Figure 8c).

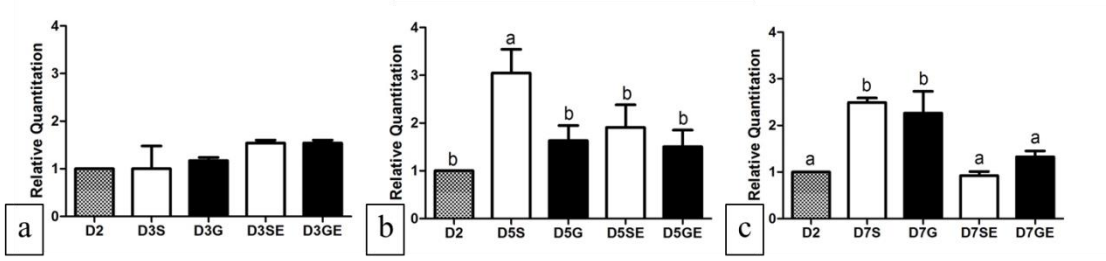

Figure 8. Relative expression (mean±SEM) of *EGFR* transcript among developmental stage

Relative expression (mean±SEM) of EGFR transcript in Day 3 embryos from singly or group cultured with (D3SE, D3GE) or without EGF supplementation (D3S, D3G) (a), Relative expression (mean±SEM) of EGFR transcript in Day 5 embryos from singly or group cultured with (D5SE, D5GE) or without EGF supplementation (D5S, D5G) (b), Relative expression (mean±SEM) of EGFR transcript in Day 7 embryos from singly or group cultured with (D7SE, D7GE) or without EGF supplementation (D7S, D7G) (c). Relative quantitation was analyzed using real-time PCR. YWHAZ was used to normalize each gene, and 4-8 cells embryos (D2) were used as calibrators.

a, b different letters on the bars indicate values that differ significantly ($P<0.05$).

3.4.2.3 Effect of EGF on OCT3/4 and KI67 expression in the blastocyst from singly and group culture

Culture condition did not influence the numbers of OCT3/4 positive cells in blastocyst in both single and group culture conditions (Figure 9a). However, EGF increased the numbers of KI67 positive cells in blastocysts in single culture conditions compared with group culture condition ($P <0.05$; Figure 9b). Immunofluorescent staining showed the presence of OCT3/4 protein in nuclei of both ICM and trophectoderm cells with a prominently observed in ICM (Figure 10a). KI67 protein expression was detected in nucleolus of both ICM and trophectoderm cells (Figure 10b).

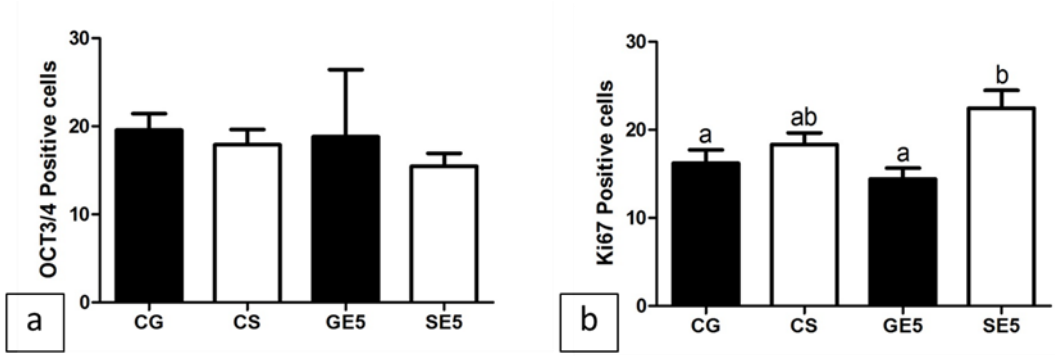

Figure 9. Number of OCT3/4 (A) and KI67 (B) positive cells among blastocysts derived from cultured singly and group with or without EGF supplementation

Blastocysts derived from cultured singly and group with 5 ng/ml EGF (SE5 or GE5, respectively) or without EGF supplementation (CS or CG, respectively).

[a, b] different letters on the bars indicate values that differ significantly (P<0.05).

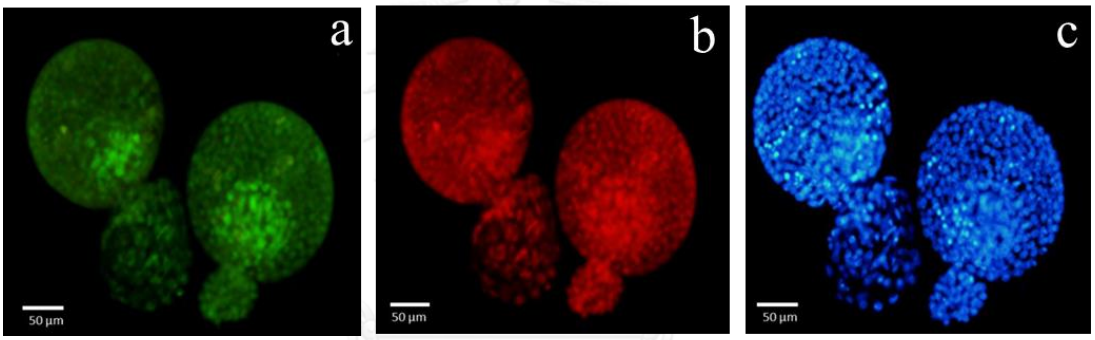

Figure 10. Immunofluorescent staining of blastocyst; OCT3/4 (a) and KI67 (b) of blastocysts. DAPI staining to show blastomere nuclei (c). Bar = 50 µm (x 200 magnification)

3.5 Discussion

Culturing a single embryo in a large volume droplet has been shown to impede embryonic development in many mammalian species, including the mouse, cow, and cat (Paria and Dey, 1990; Lane and Gardner, 1992; Keefer et al., 1994; Donnay et al., 1997; Pereira et al., 2005; Sananmuang et al., 2011). However, growth factor supplementation can circumvent the adverse effects of this low density embryo culture and thus, enhancing embryonic development *in vitro* (Paria and Dey, 1990; Cebrian-Serrano et al., 2014). In the present study, we demonstrated for the first time that (1) addition of EGF to culture medium enhanced the ability of singly

cultured embryos to form blastocysts, likely by promoting cell proliferation and (2) *EGFR* expression level at various developmental stages was influenced by culture density and growth factor supplementation.

During preimplantation development, mammalian embryos undergo cell proliferation, differentiation, and apoptosis, all of which are regulated by growth factors secreted from maternal reproductive tract or embryo themselves (Hardy and Spanos, 2002). The absence of growth factor or disruption of paracrine/autocrine signaling often results in growth retardation, chromosomal abnormalities, alteration of gene expression, metabolic disruption and increased apoptosis levels (Gopichandran and Leese, 2006; Riedemann et al., 2007). Growth factors exert their effects on preimplantation embryo development by binding to its receptor located within reproductive tract or embryo's plasma membrane that, in turn, stimulates downstream signaling pathways regulating cell proliferation and differentiation (Lim and Hansel, 1996). To date, the members of insulin and insulin like growth factor (IGF) family, epidermal growth factor (EGF) family, platelet derived growth factor (PDGF) family and tumor necrosis factor (TNF) family have been shown to regulate embryo development in the cat, mouse, cow, rat, human, pig, sheep, and rabbit (Hardy and Spanos, 2002; Richter, 2008; Thongkittidilok et al., 2014). EGF has been shown to be involved in blastocyst formation via stimulating protein synthesis, especially in the trophectodermal cells (Hardy and Spanos, 2002; Richter, 2008). Our finding that group embryo culture yielded more blastocysts than single embryo culture is consistent to previous studies in the cat (Spindler and Wildt, 2002), mouse (Ali, 2004), cow (Doherty et al., 1997), and human (Moessner and Dodson, 1995). Several studies have demonstrated that growth factors enhanced blastocyst formation in group cultured system by decreasing apoptotic rate, enhancing cell proliferation and protein synthesis (o'Neill, 1997; Sirisathien et al., 2003; Block et al., 2008; Ahumada et al., 2013). Specifically, supplementation of EGF at 5 ng/ml during 18 h post-insemination to 192 h post-insemination increased ($P < 0.05$) the percentage of bovine blastocyst cultured in group (20 embryos/50 µl) compared with non-supplemented group (50.0 vs. 36.5 %) (Sirisathien et al., 2003). In contrast, our study found that supplementation of EGF in group cultured system did not promote

blastocyst formation. This may be the difference in species (cat versus cow) or embryo density (10 embryos in 50 µl versus 20 embryos in 50 µl) which affected on blastocyst development during growth factor supplementation. It has been suggested that when embryos are cultured individually, most embryo derived autocrine growth factors were diluted in this system (Paria and Dey, 1990; o'Neill, 1997). Supplementation of growth factors could at least compensate for the low concentration of growth factors in this culture condition (Paria and Dey, 1990; o'Neill, 1997). In the present study, we found that addition of EGF even at a low concentration (5 ng/ml) in single embryo culture enhanced blastocyst development to the same level as group culture control. The synergistic effect of EGF and IGF-1 on bovine oocyte maturation has been demonstrated by Lorenzo et al. (1994). In that study, addition of 50 ng/ml EGF and 100 ng/ml IGF-1 significantly enhanced cumulus-cells enclosed oocyte to metaphase II compared to EGF or IGF-1 supplementation alone (61.4 vs. 52.1 and 49.5%, respectively). It has also been shown that the combination of EGF at 100 ng/ml and IGF-1 at 50 ng/ml significantly promotes bovine blastocyst formation compared to supplementation of IGF-1 or EGF alone (27.9 vs. 19.4 and 18.5%, respectively) (Sakagami et al., 2012). However, in the present study, the addition of both EGF and IGF-1 to a culture medium failed to enhance blastocyst development in cat embryos cultured singly compared to counterparts incubated with EGF alone. The findings observed in the present study were consistent to those previously reported in the cow and mouse (Paria and Dey, 1990; Sirisathien et al., 2003). The discrepancy in the effect of EGF and IGF-1 combination among studies may be due to the variations of EGF dosage used for *in vitro* culture. Specifically, Sakagami et al. (2012) demonstrated a positive effect of 100 ng/ml EGF plus 50 ng/ml IGF-1 on bovine blastocyst development, while Sirisathien et al. (2003) showed no additive effect of 5 ng/ml EGF plus 50 ng/ml IGF-1 when compared to EGF or IGF-1 alone. Furthermore, there may be species differences (cat and mouse versus cow) in the influence of growth factor supplementation on blastocyst development. Unexpectedly, the lowest EGF concentration (5 ng/ml) plus IGF-I either at 25 or 50 ng/ml reduced blastocyst formation compared to EGF addition alone. While the cause of this negative effect is unclear, it has been shown that IGFR can interact with

EGFR (Riedemann et al., 2007; Veeken et al., 2009). Thus, it may be that the addition of IGF-1 to the culture medium increased expression of *IGF-1R* (Thongkittidilok et al., 2014) that interacted with EGFR and mediated its availability to bind with EGF, especially when presence at a low dosage. Future studies should focus on the impact of IGF-1 on the expression of *EGFR* and EGF-EGFR signaling in cat embryos.

In this study, we also found that *EGFR* was expressed throughout cat preimplantation developmental stage (4-8 cells, 8-16 cells, morulae and blastocysts) in both group or singly cultured conditions. The influence of EGF on *EGFR* expression was assessed in our study. We found that EGF decreased the expression of *EGFR* transcript in morulae cultured singly and blastocysts cultured either individually or in groups. This finding is contrary to the previous study where EGF supplementation at 0.1 ng/ml significantly increased *EGFR* expression in pre-implantation mouse embryos compared with control and the higher concentration of EGF (1, 10 and 100 ng/ml) treatment group which *EGFR* expression tend to decrease in dose-dependent manner (Kim et al., 1999). Nevertheless, the down regulation of *EGFR* observed in the present study did not result in decreased embryonic development, as EGF increased morula development in group culture and blastocyst formation in those cultured singly. It has been previously shown in rat pleochromocytoma of adrenal medulla cell line (PC12 cells) and human squamous carcinoma cell line (A431) that after binding to its ligand and internalization, *EGFR* either recycles back to the cell surface or is transported to endosome/lysosome for degradation (Masui et al., 1993; Chen et al., 1998) of EGF to culture medium may result in excessive binding of *EGFR* and its ligand that leads to the reduction of *EGFR* on the cell surface. To date, the mechanisms by which EGF facilitating blastocyst formation has not been fully elucidated. In this study, we demonstrated that EGF enhanced cell proliferation as shown by the increase in KI67 positive cells in blastocyst. It has been shown that EGF/EGFR signaling stimulate the downstream MAPK pathway that in turn enhanced DNA synthesis and cell proliferation (Voldborg et al., 1997). Thus, the beneficial effect of EGF on singly cultured cat embryos observed in the present study is likely, in part, due to its cell cycle stimulating effect via MAPK signaling pathway. Future studies

that involve incubating cat embryos with EGF and specific inhibitors of EGFR or MAPK will confirm this hypothesis.

OCT3/4 known as pluripotency marker and expressed in inner cell mass (ICM) of blastocyst that could be used to determine blastocyst quality. The presence of OCT3/4 has been studied in cat embryos showing that OCT3/4 protein is present in nuclei of both trophectoderm and ICM but more prominently observed in ICM (Nestle et al., 2012). In the present study, we also observed OCT3/4 expression in both cell types. By using immunosurgery in mouse and sheep embryo, it has been demonstrated that EGF was limited in trophectoderm cells, suggests that EGFR are expressed in trophectoderm cells of the blastocyst during its formation from a morula (Wood and Kaye, 1989; Zhou et al., 2008). Although the number of OCT3/4 positive cells may not reflect the total cell number of ICM but OCT3/4 positive cells are essentially located in ICM (Kurosaka et al., 2004) which are required for embryonic patterning and ICM segregation.

In conclusion, our findings demonstrated, for the first time that EGF plays important role in cat embryo development. Furthermore, the finding that EGF enhanced development of domestic cat embryos cultured singly can be extrapolated to improve developmental potential of wild felid embryos, especially when the numbers of high quality oocytes are limited.

CHAPTER 4

Embryonically secreted insulin like growth factor-1 (IGF-1) predicts the developmental competence of in vitro derived cat embryos

4.1 Abstract

Embryonically secreted factors can be measured and used as a non-invasive method for evaluation of embryo quality. Insulin like growth factor-1 (IGF-1), produced and secreted by pre-implantation embryos, is one of key factors which plays a role in cell growth and differentiation. The aims of this study were to investigate the relationships of secreted IGF-1 concentrations on subsequent blastocyst formation and to determine the expression level of genes that related to blastocyst quality (Octamer binding transcription factor 4; *OCT3/4*, B-cell lymphoma protein 2; *BCL2*, and Bcl2 associated X protein; *BAX*) in blastocyst embryos which have low or high secreted IGF-1 level on Day 3 compared with *in vivo* produced blastocyst. Oocytes were matured and fertilized *in vitro*, and cleaved embryos (2 days after fertilization) were individually cultured in 50 µl droplet of synthetic oviductal fluid supplemented with 10% FCS for 7 days. To examine the secreted IGF-1 from 4-8 cell and morula stage embryos, the culture medium was collected for IGF-1 assay using IGF-1 ELISA kit on either Day 3 (N=61) or Day 5 (N=54) of culture. After the medium was collected, the embryos were subsequently cultured until Day 7 where the embryos were classified as blastocysts and non-blastocysts. The concentrations of secreted IGF-1 in the culture medium which collected on either Day 3 or Day 5 of culture were measured in the medium and then compared. Blastocyst embryos were assigned into 2 groups; 1) low IGF-1 secretion (the embryos that secreted IGF-1 levels between, 0.88-2.43 ng/ml) and 2) high IGF-1 secretion (the embryos that secreted IGF-1 levels between, 2.50-5.77 ng/ml). Relative mRNA expressions of *OCT3/4*, *BCL2*, and *BAX* were examined by qPCR among blastocyst embryos from *in vivo*, and group 1 and 2. Four to eight-cell and morula stage embryos that developed to blastocysts significantly secreted the IGF-1 at higher levels than those did not develop to blastocyst (2.70 vs. 1.86 ng/ml, 2.16 vs. 1.61

ng/ml, respectively; P <0.05). The expression of *OCT3/4*, *BCL2*, and *BAX* significantly increased in the blastocyst from high IGF-1 secretion embryos compared to *in vivo* or low IGF-1 secretion. Our findings demonstrated that secreted IGF-1 concentrations from early (4-8 cells) or morula stage of feline embryos closely related to the developmental capability of the embryos cultured singly and the expression of blastocyst quality related genes. This secreted IGF-1 may quantitatively be used as a non-invasive biomarker for determination of embryonic quality.

4.2 Introduction

Accurate embryo quality assessment is essential for improving the chance of obtaining healthy offspring and reducing the need for multiple embryo transfer (Bromer and Seli, 2008; Montag et al., 2013). While morphological evaluation is the primary method of embryo quality assessment, it is has been reported that morphologically normal embryos do not always develop into live offspring (Singh and Sinclair, 2007). This may be due to the abnormal epigenetic modification which leads to phenotypic abnormality (*i.e.* large offspring syndrome in ruminant (Fleming et al., 2004) or reduced in weight in mouse (Khosla et al., 2001) after preimplantation development (Bavister, 1995; Dean et al., 1998; Khosla et al., 2001). During the past decade, non-invasive assessments of embryo quality have been explored to identify a new biomarker for developmental potential of an embryo. Several studies focus on the changes in metabolic substances such as lactate, alanine and pyruvate in culture medium which are related to embryonic development by using high performance liquid chromatography with mass spectroscopy (HPLC-MS) or protron nuclear magnetic resonance (^{1}H-NMR) analysis (Marhuenda-Egea et al., 2010; Pudakalakatti et al., 2013). Alternatively, secretomic analysis of mammalian preimplantation embryos has shown a great potential in predicting viability and developmental competence of an embryo. The secretomic study revealed that 1-o-alkyl-2-acetyl-sn-glycero-3-phosphocholine (paf) (O'Neill, 2005), leptin (Cervero et al., 2005), acrogranin (Díaz-Cueto and Gerton, 2001), soluble human leukocyte antigen G (Noci et al., 2005; Sher et al., 2005) and ubiquitin (Wang et al., 2004) were produced and secreted by mammalian preimplantation embryos. The levels of these secretomes have been

found to be positively correlated with blastocyst formation and involved in implantation process. The same techniques that used for analysis of amino acid or other metabolites in embryo culture medium are used in secretomic study. However, the costs of equipment and maintenance are expensive compared with using enzyme linked immunosorbent assay. Insulin like growth factor-1 (IGF-1) produced and secreted by preimplantation embryo is considered as survival factor. It may be possible to use IGF-1 as biomarker, although this hypothesis has yet to be proven. To measure IGF-1 levels in culture medium, ELISA is preferable because it can measure IGF-1 level from individually produced embryo. In addition the technique is simple, rapid and inexpensive. Moreover, it can cross reaction with IGF-1 in feline species. To confirm that the levels of secreted IGF-1 correlate with the embryonic development, the expressions of genes related to blastocyst quality were examined in this study. *OCT3/4* is the transcription factor that regulates the formation of inner cell mass (ICM) and maintain pluripotency of ICM cells (Nichols et al., 1998; Pesce et al., 1998; Pesce and Scholer, 2001). OCT3/4 is also important for the primordial germ cells survival (Kehler et al., 2004). The expression of *OCT3/4* was found throughout stage of preimplantation embryo and is upregulated in the ICM of blastocyst (Kehler et al., 2004; Waurich et al., 2010). The expression of pro-apoptotic (*BAX*) and anti-apoptotic (*BCL2*) gene are closely related with the embryonic development. The study in bovine embryo shows that the expression of *BAX* was high in fragmented embryos, whereas *BCL2* was high in good quality embryo or non-fragmented counterparts (Yang and Rajamahendran, 2002).

The objectives of this study was to determine the relationship between the levels of secreted IGF-1 and the expression of genes related to blastocyst quality and embryo development in *in vitro* and *in vivo* produced cat embryos.

4.3 Materials and Methods

All chemicals used in this experiment were purchased from Sigma Aldrich, St Louis, Missouri, USA, unless otherwise specified.

4.3.1 Oocyte collection, *in vitro* maturation, fertilization and culture

The techniques of oocyte collection, in vitro maturation, fertilization and culture have been previously described in chapter 2.

4.3.2 Assessment of embryo development

The embryos were characterized as blastocyst (having a fully-expanded blastocoel, and two differentiate cell types; inner cell mass and trophectoderm) under an inverted microscope (x40 magnification) on day 7 (day 0 = IVF). All of blastocysts on day 7 were washed twice in Phosphate buffer saline (PBS) plus 0.1% Bovine serum albumin (BSA) and subsequently store at -80 ℃ for qPCR analysis.

4.3.3 Assessment of secreted IGF-1 level by Enzyme- linked immunosorbent assay (ELISA)

The human IGF-1 ELISA test kit (mediagnost®, Germany) was used in our study because the high specificity for IGF-1 and the precise measurement of very low IGF-1 levels at sensitivity of 0.09 ng/ml and the maximum detection level of IGF-1 is at the concentration of 50 ng/ml. This assay is also applicable with medium from embryo culture because it can be used with small sample volume (about 20 µl/sample). IGF-1 quantity detection by ELISA was performed according to human IGF-1-ELISA kit (Mediagnost®, Germany) protocol except that concentration of the standards were ranged from 0.5 to 30 ng/ml. Briefly, collected culture media from day 3 and day 5 of culture were thawed at 25˚C and diluted (1:2) with the dilution buffer included in the assay kit. Twenty microliters of diluted extracted samples were pipetted into each well of the coated biotinylated anti-human IGFBP-1 antibody microtiterplate and incubated for 1 hour at room temperature. Each sample was assessed in duplicates. After incubation, the contents were discarded and the wells were washed five times with washing buffer. A Horseradish-Peroxidase-labeled Streptavidin enzyme conjugate were added in each washed well and then incubated for 30 min at room temperature. After incubation, the wells were washed five times with washing buffer again. After washing, the horseradish-peroxidase-(HRP)-substrate was added in each well and incubated the plate for 15 min in the dark at room temperature. After incubation, the reaction was stopped by adding 0.2 M sulphuric acid. The IGF-1

concentrations were measured at the absorbance at 450 nm (with reference filter at 620 nm) within 30 min and then calculated by Magellan software version 7.1 via Sunrise™ Microplate reader (Tecan, Switzerland). The sensitivity of the assay was 0.09 ng/ml.

4.3.4 *In vivo* blastocyst production

Surgical collection of feline embryos was performed according to the Ethical Committees of Animal care and use, Faculty of Veterinary Science, Chulalongkorn University (Approval no. 12310048). Estrous induction and induce ovulation in ten queens were performed as previously described by Sananmuang et al. (2013). The embryos for transfer were produced by *in vitro* fertilization with frozen-thawed semen from one proven fertility tom cat. After 48 h of hCG injection, 30 cleaved embryos (4-8 cell stage) were transferred to the oviduct of each cat. On day 5 after embryo transfer, the recipients were ovariohysterectomized. The uterus was then flushed with Hepes modified synthetic oviductal fluid (H-SOF). The embryos classified as blastocysts were washed twice in PBS supplemented with 0.1% (wt/vol) BSA and immediately stored in -80°C until further use. Non blastocyst or unfertilized embryos were discarded.

4.3.5 RNA extraction and reverse transcription (RT)

RNA extraction and reverse transcription were performed as previously described in chapter 2.

4.3.6 Quantitative RT-PCR (qPCR)

The relative expression levels of individual target genes (*BCL2*, *BAX*, and *OCT3/4*) were normalized to the endogenous normalizer (*GAPDH*: glyceraldehydes 3-phosphatedehydrogenase), and run in separate wells. The PCR was performed using the ABI PRISM 7300 Real-time cycler (Applied Biosystems, Foster City, California, USA) with Power SYBR Green PCR Master Mix. Each PCR reaction (total volume of 15 µl) consisted of 2 µl of reverse transcription product (2.5 ng/µl of cDNA) and 13 µl of reaction mixture contained 7.5 µl of SYBR Green Master Mix, 1 µl of 2.5, 2.5, 5, and 4

μM forward and reverse primer were used for *GAPDH*, *BCL2*, *BAX*, and *OCT3/4*, respectively (Table 6) and fulfill with nuclease free water. The thermal cycling conditions area follows: 10 min at 95 ℃ to activate Tag DNA polymerase, 50 cycles of 15 sec at 95 ℃ for denaturing, 30 sec at 55 ℃ for annealing (except in *BCL2*, use 62℃ for annealing) and 60 sec at 72 ℃ for extension. The Sequence Detection System (SDS) Software Version 1.4 (Applied Biosystems, USA) was used to quantify and analyze the relative quantitation (RQ). Calculation of mRNA expression levels was performed by the comparative Cq method using the amplification efficiency of each gene as a correction factor. *In vivo* produced blastocysts were used as a control group for calculations of relative quantitation which performed by comparative cq method. Data was reported as n-times difference, in relation to the control group (Livak and Schmittgen, 2011). qPCR products were confirmed by melting curve analysis and run in gel electrophoresis. The amplified products were run in 2% agarose gel (BioRad, California, USA) prepared in 1×TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH8) containing 0.4 mg/ml ethidium bromide (Promega, Wisconsin, USA). The separated products in agarose gel were visualized under UV light (Syngene, Cambridge, UK).

Table 6 Description of forward (FP) and reverse (RP) primers used to assess the expression of target genes in blastocysts obtained from different culture conditions

Genes	Sequence (5' to 3' orientation)	Fragment length (bp)	References
GAPDH	FP: GGAGAAAGCTGCCAAATATG	191	AB038241
	RP: CAGGAAATGAGCTTGACAAAGTGG		
BCL2	FP: GGATGCCTTTGTGGAACTGT	222	AB096611
	RP: CGTTTCATGGGACATCACTG		
BAX	FP: CCGATGGCAACTTCAACTGG	244	NM_001009282
	RP: GTCAGCACTCCCGCCACAAA		
OCT3/4	FP: GAACATGTGTAAGCTGCGGC	282	GQ848232
	RP: CTTGATCGTTTGCCCTTCTG		

4.3.7 Experimental design

Experiment 1: The relationship between secreted IGF-1 levels from different stage of *in vitro* produced embryos, blastocyst formation, and blastocyst quality related gene expressions (*BCL2*, *BAX*, and *OCT3/4*).

To examine the secreted IGF-1 from 4-8 cell and morula stage embryos, 40 µl of culture medium was collected for IGF-1 assay using IGF-1 ELISA kit on either day 3 (N=61) or day 5 (N=54) of culture. After the medium was collected, the embryos were subsequently cultured until day 7 where the embryos were individually classified as blastocysts and non-blastocysts. The concentrations of secreted IGF-1 were measured in the medium and then compared. Culture medium without any embryos was identically incubated and used as negative control. Blastocysts from low and high IGF-1 secreted day 3 embryos were subsequently determined the expressions of blastocyst quality related genes (*BCL2*, *BAX*, *OCT3/4*). Day 3 embryos that secreted IGF-1 between 0.88 to 2.43 ng/ml were classified as low IGF-1 secreted embryos, while high IGF-1 secreted embryos were classified at 2.50-5.77 ng/ml IGF-1 secreted level. Low IGF-1 or high IGF-1 secreted embryos were classified by quartile range; IGF-1 secreted level in quartile 1 and 2 were classified as low IGF-1 secreted embryos and IGF-1 secreted level in quartile 3 and 4 were classified as high IGF-1 secreted embryos. Blastocyst embryos form either low or high IGF-1 secreted day 3 embryos were analyzed for the expression of *BCL2*, *BAX*, and *OCT3/4* by RT-qPCR analysis.

4.3.8 Statistical analysis

IGF-1 level in each sample was calculated by Magellan™ data analysis software (Männedorf, Switzerland) using four parameter marquardt analysis. Results are expressed as the mean±standard error of the mean (SEM). Differences between mean of secreted IGF-1 levels in 4-8 cells or morula stage in blastocyst and non blastocyst embryo were assessed by independent T test. Differences in relative mRNA expression of target genes were assessed by one-way ANOVA using general linear model method using the Statistical Analysis Systems software package (Version

9.0, SAS Institute Inc., 1996, N.C., USA). Differences with P<0.05 were considered statistically significant.

4.4 Results

4.4.1 The relationship between secreted IGF-1 levels from different stages of *in vitro* produced embryo, blastocyst formation, and blastocyst quality related gene expression (*BCL2*, *BAX*, and *OCT3/4*).

The IGF-1 levels that secreted from different stages of *in vitro* produced preimplantation embryos were observed in this study to determine the relationship between secreted IGF-1 levels and blastocyst formation. In this study, we found that the preimplantation embryos at both 4-8 cell or morula stage could produce IGF-1. Secreted IGF-1 from 4-8 cells and morula stage embryos ranged from 1.2 to 5.9 ng/ml and 0.7 to 8.3 ng/ml, respectively. Four to eight cell and morula stage embryos that developed to blastocysts significantly secreted the IGF-1 at higher levels than those did not develop to blastocyst (2.70 vs. 1.86 ng/ml, 2.16 vs. 1.61 ng/ml, respectively; P <0.05) (Figure 11a, 11b). The expression of *BCL2*, *BAX* and *OCT3/4* transcripts in blastocyst embryos between low and high secreted IGF-1 level of Day 3 embryo were observed in this study. Low and high IGF-1 groups exhibited higher expression levels of *BCL2* than *BAX*. *OCT3/4* expression was significantly lower in IVV and low IGF-1 than high IGF-1 group (Figure 12; P<0.05).

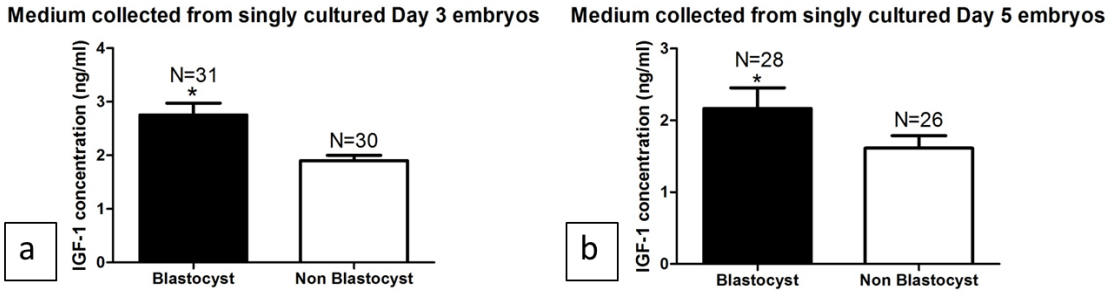

Figure 11. Secreted IGF-1 levels on Day 3 (a) and Day 5 (b) of development.
The embryos were further culture until day 7 after IVF to determine blastocyst development
Asterisk (*) indicate values that differ significantly (P<0.05).

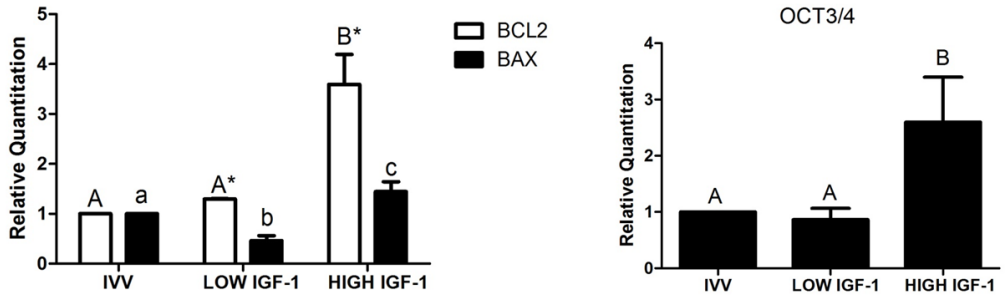

Figure 12. Relative expression (Mean±SEM) of *BCL2*, *BAX* and *OCT3/4* transcripts in blastocysts acquired from low and high secreted IGF-1 levels of Day 3 embryo.
Relative quantitation was analyzed using real-time PCR. GAPDH was used to normalize each gene, and in vivo blastocyst embryos (IVV) were used as calibrators. Different letters on the bars indicate values that differ significantly ($P<0.05$).
Asterisk (*) indicate values that differ significantly between BCL-2 and BAX within the same group ($P<0.05$).

4.5 Discussion

The relationship between embryonic secreted IGF-1, developmental capability of embryos, and gene expression related to apoptosis and blastocyst formation were investigated in this study. Four to eight cells or morula stage embryos which developed to blastocyst secreted IGF-1 at higher levels compared to those non blastocyst embryos. The transcription of gene that relate to blastocyst quality (*BCL2*, *BAX*, and *OCT3/4*) were significantly increased in embryos producing a high level of IGF-1 on day 3. Our result demonstrated that there were tight links between secreted IGF-1 level, blastocyst formation and gene expression related to blastocyst quality. Non invasive embryonic evaluation is considered as a tool for selecting the embryo prior to transfer without compromising embryo health. The study on secretomic analysis on the metabolites in which secreted from developing singly cultured embryo could be adapted for determining embryonic quality in bovine (Renard et al., 1980), mouse (Gardner and Leese, 1986) and human (Hardarson et al., 2012). Our study demonstrated that embryos that developed to blastocyst stage secreted IGF-1 at higher level than those did not develop to blastocyst. This finding suggested an important role of IGF-1 on blastocyst formation. IGF-1 enhances blastocyst formation by increasing cellular proliferation, differentiation and development via the mitogen-activated protein kinase (MAPK)

pathway (Zhang and Liu, 2002), preventing apoptosis (Paria and Dey, 1990; Neira et al., 2010; Shabankareh and Zandi, 2010) and also increasing glucose uptake (Carayannopoulos et al., 2000). IGF-1 has been shown to regulate glucose metabolism in preimplantation embryo by increasing glucose uptake via insulin or IGF-1 receptor signaling (Pantaleon and Kaye, 1996; Carayannopoulos et al., 2000). Glucose uptake and utilization are critical for embryo development and survival (Riley and Moley, 2006). The study of Spindler et al. (2000) demonstrated that oocytes having higher glucose metabolism had a higher blastocyst development compared to low glucose metabolism counterparts. This study also showed that grouping oocytes with similar rates of glucose metabolism also increased the overall blastocyst proportion compared to those randomly culture. Increasing in glucose uptake during the transitional stage of morula to blastocyst is important for blastocyst formation which is triggered by an increasing in oxygen consumption, proliferation and blastocoel formation (Leese, 2012). IGF-1/IGF-1R signaling leads to an increasing in glucose uptake and utilization via glucose transporter. Then ATPs from glucose metabolism activate Na/K ATPase at trophectoderm cells which leads to blastocyst formation (Pantaleon and Kaye, 1998). In our study, we also demonstrated that levels of secreted IGF-1 affect on expressions of apoptotic and pluripotency related genes. Significantly increased in *BCL2* expression in both low IGF-1 and high IGF-1 were found when compared to *BAX* expression. Several studies in porcine demonstrated that IGF-1 reduced the transcription of *BAX* gene (Kim et al., 2006; Wasielak et al., 2013) and IGF-1 also down regulated *BCL2* expression in porcine blastocyst (Wasielak et al., 2013). However, the study on the effect of IGF-1 on *BAX* and *BCL2* expression in bovine preimplantation embryo showed that IGF-1 did not affect transcript abundance of *BCL2* while the increase in expression of *BAX* was also found in IGF-1 treated group (Block et al., 2008).

Despite of the expression of apoptotic related genes that affect the embryonic quality, *OCT3/4* is considered as indicator of ICM numbers which also, in turn, reflect the quality of embryo as well. *OCT3/4* plays an important role in regulating of embryonic development (Filliers et al., 2012). Expression of *OCT3/4* in preimplantation embryos was demonstrated in several studies. In mouse and cow,

the transcription started at 1-2 cells stage after zygotic genome activation until blastocyst that was found specifically in inner cell mass (Madeja et al., 2013). In feline, *OCT3/4* transcription was found at a low level in germinal vesicle and early stage embryos but transcript was gradually increased from eight-cell to blastocyst stages (Imsoonthornruksa et al., 2010; Waurich et al., 2010). Gomez et al. (2010) and Filliers et al. (2012) demonstrated that *OCT3/4* transcripts were significantly lower in *in vitro* produced blastocysts compared with *in vivo* blastocysts. In the present study, we found that the levels of mRNA expression in blastocyst of *in vivo* and *in vitro* produced blastocyst from the low secreted IGF-1 were lower than from the high secreted IGF-1 counterparts. It might be due to the difference in culture conditions (*in vivo* and *in vitro* embryos) which have been found to induce the difference in gene expression pattern including epigenetic changes of imprinted genes (such as IGF-II, H19) which is responsible for embryonic and fetal growth (Niemann and Wrenzycki, 2000). The aberrant expressions of these genes have been involved in embryonic or fetal abnormalities (Moore and Reik, 1996). In addition, it might be possible that blastocysts from *in vivo* produced embryos are produced from *in vitro* before transfer back to *in vivo* condition at 4-8 cell stage. The *in vitro* culture before transfer might affect on the expression of *OCT3/4* level. Up regulation of *OCT3/4* in *in vitro* produced blastocysts from high IGF-1 appeared as a consequence of *in vitro* culture condition (Saenz-de-Juano et al., 2011). IGF-1 mediated IGF-1R through PI3K/Akt signaling to regulate pluripotent transcription factor such as *OCT3/4* (Huang et al., 2009). The presence of glucose in the response of high IGF-1 level related with *OCT3/4* expression (Saadeldin et al., 2011). The study in cell line reveals that the addition of IGF-1 into breast cancer cells enhances expression of *OCT3/4* (Castaño et al., 2013). Even our study demonstrated the relationship between IGF-1 level and blastocyst formation also the difference in gene related blastocyst quality, further study such as the differential staining to determine ICM/trophectoderm ratio or TUNEL assay in *in vitro* produced blastocyst from low or high secreted IGF-1 should be investigated.

Our findings demonstrated that secreted IGF-1 concentrations from early stage of feline embryos (4-8 cells) closely relate to the developmental potential of the embryos cultured singly and the expression of blastocyst quality related genes. This secreted IGF-1 may quantitatively be used as a non-invasive biomarker for determination of embryonic quality.

CHAPTER 5

The effects of IGF-1 supplementation on the production of reactive oxygen species and the expression of anti-oxidative gene of domestic cat embryos cultured in group or singly

5.1 Abstract

The production of reactive oxygen species (ROS) in *in vitro* culture condition is considered as a factor that impairs embryonic development. Insulin like growth factor-1 (IGF-1) is considered as a survival factor which plays a role in the utilization of ROS and enhanced embryo development. This study aimed to determine the roles of IGF-1 on the production of reactive oxygen species and the expression of anti-oxidative genes of domestic cat embryos cultured in group or singly. Oocytes were matured and fertilized *in vitro*, and cleaved embryos obtained 2 days after fertilization (D0) were randomly assigned into one of six conditions 1) to 3) group culture (50 µl droplet per 10 embryos) supplemented with IGF-1 (0, 25 or 50 ng/ml, respectively), 4) to 6) single embryo culture (50 µl droplet per 1 embryo) supplemented with IGF-1 (0, 25, 50 ng/ml, respectively). Morulae as well as blastocysts were assessed at Day 5 and 7 post-IVF, respectively. Amount of reactive oxygen species in blastocysts cultured in group or singly with or without IGF-1 supplementation (Treatments 1 to 6) were measured after H_2DFFDA staining using a confocal microscope. Relative mRNA expressions of anti-oxidative gene (*GPX1*, *SOD1* and *catalase*) in blastocysts cultured in group or singly with or without IGF-1 supplementation (Treatment 1 to 6) were examined using RT-qPCR. Supplementation of IGF-1 improved blastocyst formation only in singly culture. ROS level was higher in singly cultured embryos supplemented with IGF-1 than those without growth factor ($P < 0.05$). In single culture, supplementation of IGF-1 at 50 ng/ml significantly decreased the expression of *GPX1* transcripts compared with others. In group culture, ROS level was decreased in group culture with 50 ng/ml of IGF-1 compared with other treatments ($P < 0.05$). The expressions of *GPX1*, *SOD1* and *catalase* were also increased group culture with 50 ng/ml of IGF-1 compared with other treatment (P

<0.05). In conclusion, our findings demonstrated that IGF-1 involved in ROS production of *in vitro* embryo production and also influenced on the expression of anti-oxidative gene especially in group embryo culture.

5.2 Introduction

The oxygen metabolism is important for embryo development (Magnusson et al., 1986; Houghton et al., 1996; Thompson et al., 1996). After the metabolism process, Reactive oxygen species (ROS) is produced in endogenously (e.g. membrane NADPH oxidase, oxidative phosphorylation) or exogenously (e.g. light, oxygen tension, metallic ion, excessive of glucose, freeze-thaw process). In normal condition, ROS and antioxidants in cell metabolism are in balance. The imbalance causes an overproduction and/or a decrease the clearance of ROS by scavenging mechanisms leading to oxidative stress (Agarwal et al., 2005). In embryos, ROS production is presented with three major types: superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl ($OH^{\cdot}$). Endogenously, ROS is produced by various metabolic pathways and enzymes, such as oxidative phosphorylation, NADPH oxidase and xanthine oxidase while in exogenous ROS production, many factors elevate ROS production in both *in vivo* and *in vitro* culture system. In embryo culture system, the ROS is enhanced by culture condition such as oxygen concentration, metallic cations, visible light, amine oxidase, freeze-thaw process of spermatozoa. However under *in vivo* condition ROS may occur from only metabolic pathway and enzymes. Oxidative stress induces the embryo damage, such as retarded growth of embryo (Johnson and Nasresfahani, 1994; Tarin, 1996), apoptosis (Salas-Vidal et al., 1998; Van Soom et al., 2002; Yuan et al., 2003), mitochondrial alterations and ATP depletion (Guerin et al., 2001). To overcome the detrimental effect of the oxidative stress, the antioxidants have been intensively used. Several antioxidant enzymes that defend oocytes and embryos against peroxidative damage are superoxide dismutase (*SOD*), catalase and glutathione peroxidase (*GPX*) (Li and Trush, 1993). Copper, zinc superoxide dismutase (Cu, Zn-SOD; *SOD1*), located in the cytosol and manganese-superoxide dismutase (Mn-SOD; *SOD2*) located in the mitochondria allow superoxide radicals to be scavenged. SOD2 transforms the toxic superoxide into hydrogen peroxide (H_2O_2),

which is eliminated by catalase or GPX (Guerin et al., 2001). SOD1, SOD2, catalase, and GPX are presented in blastocyst stage embryo in mouse (El Mouatassim et al., 1999) and bovine (Arias et al., 2012). Recently, our study found that addition of IGF-1 to a culture medium enhanced the ability of singly cultured embryos to form blastocysts by modulating or IGF-1 receptor and reducing developmental block (Thongkittidilok et al., 2014). Despite of the role on enhancing of blastocyst formation, IGF-1 also plays the protective role against embryonic stress by enhancing ROS elimination via upregulation of gluthathione peroxidase enzyme in mitochondria (Jallali et al., 2007). It may be possible that supplement of IGF-1 is able to reduce ROS in *in vitro* culture condition.

The objectives of this study were to investigate (1) the oxidative stress and anti-oxidative gene expression in group and single culture of *in vitro* produced blastocyst compared with *in vivo* produced blastocyst and (2) the effect of IGF-1 on oxidative stress and anti-oxidative gene expression in group and single culture of IVF blastocyst embryos compared with *in vivo* produced blastocysts.

5.3 Materials and Methods

All chemicals used in this experiment were purchased from Sigma Aldrich, St Louis, Missouri, USA, unless otherwise specified.

5.3.1 Oocyte collection, *in vitro* maturation, fertilization and culture

The techniques of oocyte collection, in vitro maturation, fertilization and culture have been previously described in chapter 2.

5.3.2 Assessment of embryo development

The embryos were classified as morula (having cell compaction without blastocoel formation) on day 5 post-IVF, blastocyst (having a fully-expanded blastocoel, and two differentiate cell types; inner cell mass and trophectoderm) on day 7 post-IVF under an inverted microscope at 40x magnification (CKX41 Olympus, Shinjuku, Japan). All of blastocyst on day 7 were then collected for ROS

determination or washed twice in Phosphate buffer saline (PBS) plus 0.1% Bovine serum albumin (BSA) and subsequently store at -80 °C for qPCR analysis.

5.3.3 Quantification of Reactive oxygen species (ROS)

Reactive oxygen species were quantitatively examined as average fluorescent intensity using Reactive Oxygen Species (ROS) Detection Reagents (Invitrogen®, USA) as described by Arias et al. (Arias et al., 2012). In brief, the embryos were incubated in their specific treatments for 1 h in IVC-2 medium containing 20 µM of 5-(and-6)-carboxy-2,7'-dihydrofluorescein diacetate (H_2DFFDA). The embryos were washed two times with pre-equilibrate IVC-2 medium and instantly placed individually in 96 wells plate containing with 150 µl of IVC-2 medium and examined in controlled CO_2 and temperature chamber with a confocal microscope (FV1000, Olympus, Shinjuku, Japan) with FITC filter (488 nm). Total embryo height was evaluated and sections were made at 3 µm interval. The average fluorescence intensity (with relation to the total area) was determined using the image analysis software program (FV10-ASW 3.0 Viewer, Olympus) using the circle-drawing tool and selecting manually the total area nearby to the zona pullucida. Positive control was performed by incubating embryos before the assay with carboxy-(H_2DFFDA) with 2% of H_2O_2 and negative control by incubating with IVC-2 medium.

5.3.4 *In vivo* blastocyst production

The technique for *in vivo* blastocyst production was performed as previously described in chapter 4.

5.3.5 RNA extraction and reverse transcription (RT)

The technique for RNA extraction and reverse transcription was performed as previously described in chapter 2.

5.3.6 Quantitative RT-PCR (qPCR)

The relative expression levels of individual target genes (*SOD1*: Superoxide dismutase 1, *catalase*, and *GPX1*: Glutathione peroxidase) were normalized to the

endogenous normalizer (*GAPDH*: glyceraldehydes 3-phosphatedehydrogenase), and were run in separate wells (Table 7). The PCR was performed using the ABI PRISM 7300 Real-time cycler (Applied Biosystems, Foster City, California, USA) with Power SYBR Green PCR Master Mix. Each PCR reaction (total volume of 15 µl) consisted of 2 µl of reverse transcription product (2.5 ng/µl) and 13 µl of reaction mixture which contained 7.5 µl of SYBR Green Master Mix, 1 µl of both 10 µM forward and reverse primer were used for *GAPDH*, *SOD1*, *catalase*, and *GPX1* (Table 7) and fulfill with nuclease free water. The thermal cycling conditions area follows: 10 min at 95 °C to activate Tag DNA polymerase, 50 cycles of 15 sec at 95 °C for denaturing, 30 sec at 55 °C for annealing and 60 sec at 72 °C for extension. The Sequence Detection System (SDS) Software Version 1.4 (Applied Biosystems, USA) was used to quantify and analyze the relative quantitation (RQ). Calculation of mRNA expression levels was performed by the comparative Cq method using the amplification efficiency of each gene as a correction factor. *In vivo* produced blastocysts were used as a control group for calculations of relative quantitation which performed by comparative cq method. Data was reported as n-times difference, in relation to the control group (Livak and Schmittgen, 2011). qPCR products were confirmed by melting curve analysis and run in gel electrophoresis (Figure 16). The amplified products were run in 2% agarose gel (Bio-Rad, California, USA) prepared in 1×TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH8) containing 0.4 mg/ml ethidium bromide (Promega, Wisconsin, USA). The separated products in agarose gel were visualized under UV light (Syngene, Cambridge, UK).

Table 7 Description of forward (FP) and reverse (RP) primers used to assess the expression of target genes in blastocysts obtained from different culture conditions.

Genes	Sequence (5' to 3' orientation)	Fragment length (bp)	References
GAPDH	FP:GGAGAAAGCTGCCAAATATG	191	AB038241
	RP:CAGGAAATGAGCTTGACAAAGTGG		
SOD1	FP: GTTGGAGACCTGGGCAATGT	150	BC102432.1
	RP: CCACCTCTGCCCAAGTCATCT		NM_000454.4
			NM_011434.1
			NM_017050.1
			NM_001190422.1
Catalase	FP: CAGCTGACACAGTTCGTGACC	193	NM_001035386.1
	RP: CCAGAAGTCCCAGACCATGTC		NM_009804.2
			NM_012520.1
			NM_214301.1
GPX1	FP:CCCCTGCAACCAGTTTGG	253	NM_174076.3
	RP:CGCCTGGTCGGACGTACT		NM_008160.6
			NM_030826.3

5.3.7 Experimental design

Experiment 1: Effect of IGF-1 on development of embryos cultured in group

As we know from chapter 2 that the addition of IGF-1 at concentration of 25 and 50 ng/ml promoted development of single embryo culture, this experiment aimed to investigate the effect of IGF-1 supplementation on developmental competence of group cultured embryos. Group of embryos were cultured in IVC-2 medium supplemented with IGF-1 at concentrations of 25 ng/ml or 50 ng/ml from day 2 to 7 embryo cultured without IGF-1 served as a control group. The embryos were assessed for morulae on day 5 and blastocyst formation on day 7. Three replicates were performed in this experiment.

Experiment 2: Oxidative stress and the expression of anti-oxidative gene in *in vitro* cultured embryo from group and single culture with or without different concentrations of IGF-1 supplementation

Experiment 2.1: Oxidative stress in *in vitro* cultured embryo from group and single culture with or without different concentration of IGF-1 supplementation

To determine the ROS level in group or singly cultured under the different concentrations of IGF-1 supplementation. Blastocysts were collected from group or single embryo culture supplemented with or without IGF-1 at 25 or 50 ng/ml from day 2 to day 7. The culture medium was half changed on day 5. Blastocysts at day 7 from each group were collected for ROS detection. Total of 30-40 blastocysts were examined in each treatment.

Experiment 2.2: Anti-oxidative gene expression in *in vitro* cultured embryo from group and single culture with or without different concentration of IGF-1 supplementation compared with *in vivo* produced blastocyst

To examine the expressions of anti-oxidative genes which are related to blastocyst development (*SOD1*, *Catalase* and *GPX1*). Five blastocysts were collected from group and single cultured embryo supplemented with or without IGF-1 at 25 or 50 ng/ml from day 2 to day 7. The culture medium was half changed on day 5. Blastocysts at day 7 from each group and *in vivo* produced were collected for qPCR. Three replications were performed in this experiment.

5.3.8 Statistical analysis

Results were expressed as the mean±standard error of the mean (SEM). Differences between groups in the mean percentage of each developmental stage were assessed by the Chi-squared test using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, N.C., USA). Differences between mean of ROS level in each treatment group and relative mRNA expression of target genes were assessed by one-way ANOVA using general linear model method using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, N.C., USA). Differences with $P<0.05$ were considered statistically significant.

5.4 Results

5.4.1 Effect of IGF-1 on development of embryos cultured in group

High IGF-1 (50 ng/ml) decreased blastocyst formation in group cultured embryo compared with non IGF-1 supplemented group (P <0.05, Table 8). Morula formation was significantly increased in group culture embryo irrespective to IGF-1 supplementation (P<0.05) (Table 8).

Table 8 Percentages (mean ± SEM) of developmental competence and embryo quality of cat embryos cultured in group with different concentrations of IGF-1 supplementation

Group	No. of cleaved embryos	No. of cleaved embryos developed to	
		Morula (%)	Blastocyst (%)
Group w/o growth factor	60	53(88.3±6.3)[a]	35(58.3±6.3)[a]
Group + IGF-1 25 ng/ml	60	50(83.3±6.0)[a]	33(55±10.4)[a]
Group + IGF-1 50 ng/ml	60	49(81.7±9.1)[a]	24(40±8.7)[b]

[a, b] different letters within a column indicate values that differ significantly (P <0.05)

5.4.2 Oxidative stress in *in vitro* cultured embryo from group and single culture with or without different concentration of IGF-1 supplementation

In single embryo cultured system, supplementation of IGF-1 significantly increased ROS level in blastocysts (P <0.05; Figure 13a, Figure 14A, 14B, 14C). However IGF-1 supplementation in group cultured system at dose of 50 ng/ml reduced ROS production (P <0.05; Figure 13b, Figure 14G).

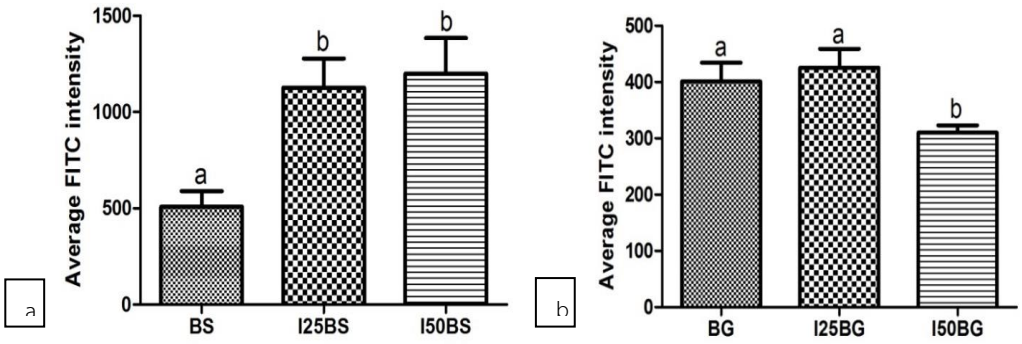

Figure 13. Average FITC intensity (mean ± SEM) of ROS detection in singly or group cultured blastocyst

(a) singly cultured blastocyst from non IGF-1 supplementation (BS), IGF-1 supplementation at 25 or 50 ng/ml (I25BS, I50BS, respectively) or group cultured blastocyst

(b) group cultured blastocyst from non IGF-1 supplementation (BG), IGF-1 supplementation at 25 or 50 ng/ml (I25BG, I50BG, respectively).

[a,b] Different letters on the bars indicate values that differ significantly ($P \leq 0.05$).

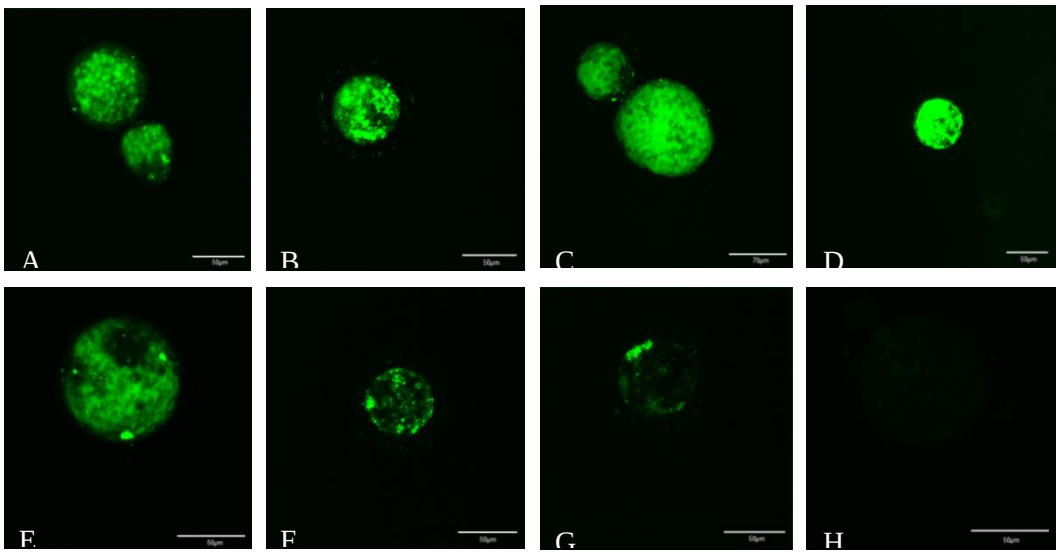

Figure 14. Reactive oxygen species in Day 7 blastocyst stained with H_2DFFDA. Bar = 50 µM (except figure 14C is bar = 70 µM)
A) blastocyst from singly cultured without IGF-1 supplementation,
(B) blastocyst from singly cultured supplemented with IGF-1 25 ng/ml,
(C) blastocyst from singly cultured supplemented with IGF-1 50 ng/ml,
(E) blastocyst from group cultured without IGF-1 supplementation,
(F) blastocyst from group cultured supplemented with IGF-1 25 ng/ml,
(G) blastocyst from group cultured supplemented with IGF-1 50 ng/ml,
(D) positive control; blastocyst incubated in 2% H_2O_2,
(H) negative control; blastocyst incubated in IVC-2 medium

5.4.3 Anti-oxidative gene expression in *in vitro* cultured embryo from group and single culture with or without different concentrations of IGF-1 supplementation compared with *in vivo* produced blastocysts

The expression of *GPX1*, *SOD1*, and *catalase* transcripts in blastocyst embryos between singly and group cultured embryos with or without IGF-1 supplementation were compared with *in vivo* produced embryos. As shown in Figure 16a, expression of *GPX1* was significantly higher in singly and group culture without IGF-1 supplementation than *in vivo* produced blastocyst, IGF-1 supplementation at 50 ng/ml in singly culture and IGF-1 at 25 ng/ml in group cultured embryos ($P < 0.05$). Moreover, IGF-1 supplement at 25 ng/ml in single embryo culture embryo or 50 ng/ml in group cultured embryos increased the expression level of *GPX1* compared with *in vivo* produced blastocysts ($P < 0.05$). The expression of *SOD1* was not significantly different from *in vivo* produced blastocyst in any treatment group except

that IGF-1 at dose 50 ng/ml supplemented group (P < 0.05; Figure 15b). Although the expression of *catalase* in *in vivo* produced blastocyst and IGF-1 at 25 ng/ml treated in group cultured embryos was not significantly different but the expression in other treatment groups were significantly higher compared with *in vivo* produced blastocyst (P <0.05; Figure 15c).

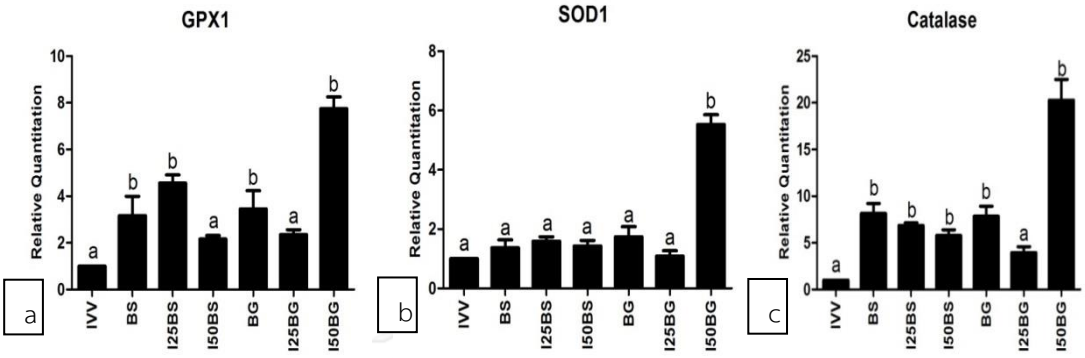

Figure 15. Relative expression (mean±SEM) of anti-oxidative gene transcripts GPX1 (a), SOD1 (b), catalase (c) transcript in in vivo produced blastocyst (IVV), blastocysts from embryos cultured singly without IGF-1 supplementation (BS) or with IGF-1 supplementation at 25 ng/ml and 50 ng/ml (I25BS, I50BS, respectively) and cultured in groups without IGF-1 supplementation (BG) or with IGF-1 supplementation at 25 ng/ml and 50 ng/ml (I25BG, I50BG, respectively) Relative quantitation was analyzed using real-time PCR. GAPDH was used to normalize each gene, and IVV embryos were used as calibrators.

[a, b] different letters on the bars indicate values that differ significantly (P<0.05).

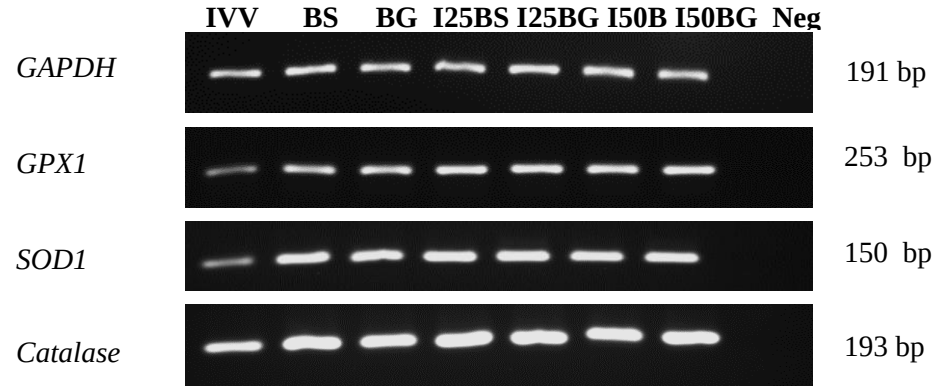

Figure 16. The amplicon size of PCR products of *GAPDH*, *GPX1*, *SOD1* and *Catalase* transcript of blastocysts acquired from embryos cultured singly or in group with or without IGF-1 supplementation

Blastocyst from singly cultured without IGF-1 supplementation (BS),
Blastocyst from singly cultured with IGF-1 supplementation at 25 ng/ml and 50 ng/ml (I25BS, I50BS, respectively),
Blastocyst from group cultured without IGF-1 supplementation (BG),
Blastocyst from group cultured with IGF-1 supplementation at 25 ng/ml and 50 ng/ml (I25BG, I50BG, respectively)
Neg = negative control

5.5 Discussion

IGF-1 is known as survival factor for embryo development which plays role in apoptosis inhibition, stimulation of cell proliferation and differentiation. Several studies demonstrated that supplementation of IGF-1 into culture medium enhanced blastocyst formation rate of both single and group embryo culture (Paria and Dey, 1990; Thongkittidilok et al., 2014). Despite of the role in apoptosis and cell proliferation, there have been reported that IGF-1 also involved in the production of ROS which is the one factor that affects embryo development (Jallali et al., 2007). In the present study, we demonstrated for the first time that ROS production and the expression of anti-oxidative genes in blastocyst were influenced by culture conditions and IGF-1 supplementation. During preimplantation development, mammalian embryos undergo cell proliferation, differentiation, and apoptosis, all of which are regulated by growth factors secreted from maternal reproductive tract or embryo themselves (Díaz-Cueto and Gerton, 2001; Hardy and Spanos, 2002). Oxidative stress is the one of main reason that why embryos are not developed well in culture condition. ROS in *in vitro* embryo production are generated from physical culture

environment such as oxygen tension, excessive of glucose, metallic ion, amine oxidase (presence in serum), and also light. The accumulation of ROS substances impaired embryo development in terms of retardation in growth, apoptosis, and mitochondrial alteration (Guerin et al., 2001). Our findings that supplementation of IGF-1 at 50 ng/ml reduced the level of ROS production in blastocyst from group culture embryo by upregulation of *GPX1*, *SOD1* and *catalase* that supported the protective role of IGF-1 on mouse embryos that exposed to oxidative stress (Kurzawa et al., 2002). They demonstrated that the supplement of IGF-1 minimized the negative influence of hydrogen peroxide and also promoted embryo development. However, the mechanism of IGF-1 in the reduction of hydrogen peroxide is still unclear. It may be possible that hydrogen peroxide induce apoptosis, whereas IGF-1 act as anti-apoptosis leading to improve embryo development (Kurzawa et al., 2002; Kurzawa et al., 2004). Although, our study showed that supplementation of IGF-1 at high dose (50 ng/ml) decrease ROS production by upregulation of anti-oxidative gene but it had negative effect on blastocyst formation. This might be due to the excessive of IGF-1 consumption of the embryonic cell leads to apoptosis which may relate to the excessive glucose metabolism (Chi et al., 2000). It could be noted that despite of IGF-1, the development of group culture embryo was control by other factors. Surprisingly, IGF-1 exerts positive effect on the reduction of ROS in group culture embryo but this effect was not observed in singly culture condition. Even if IGF-1 promote the development of singly culture embryo but the level of ROS production still increased.

It has been reported that IGF-1 stimulated ROS production in the aortic vascular smooth cells (Meng et al., 2007) and adipocytes (Fukuoka et al., 2010). It could be speculated that high ROS level in singly cultured embryo supplemented with IGF-1 related with the increased in mitochondrial metabolism. IGF-1 enhanced blastocyst formation in singly culture embryo by paracrine factors which reduce apoptosis, enhance cell proliferation and also stimulate glucose uptake (Paria and Dey, 1990; Carayannopoulos et al., 2000; Makarevich and Markkula, 2002). The increasing rate of glucose uptake turns to increase mitochondrial oxygen

consumption which result in the elevated in ROS level (Guerin et al., 2001). However in group culture condition, IGF-1 seems to have negative effect on blastocyst formation. This may cause by down regulation of IGF-1R or the excessive glucose metabolism that leads to apoptosis (Chi et al., 2000; Velazquez et al., 2011). Our results indicated that IGF-1 had an influence on the expression of anti-oxidative gene including *GPX1*, *SOD1* and *catalase*. In group culture condition, supplementation of IGF-1 at 50 ng/ml upregulated the expression of *GPX1*, *SOD1* and *catalase* transcript. In agreement with previous study by Fukuoka et al. (Fukuoka et al., 2010) which demonstrated that expression of *SOD* and *catalase* were increased in IGF-1 supplementation in 3T3-L1 adipocytes. These result suggested that IGF-1 plays role in the response to oxidative-stress.

Our findings demonstrated that IGF-1 involved in ROS production of *in vitro* embryo production and also influences on the expression of anti-oxidative gene especially in group embryo culture.

CHAPTER 6

General discussion and conclusions

6.1 *In vitro* embryo production in domestic cat and the optimization of single embryo culture system

To date, *in vitro* embryo production technologies have been successful in domestic cat. From the implementation of *in vitro* fertilization in 1989 that can produce cleaved embryos although embryos fail to develop beyond 16 cells stage (Johnston et al., 1989). Since then there have several studies aimed at overcoming the developmental block that occurs during morula to blastocyst transition by modification of culture environment such as gas atmosphere (Johnston et al., 1991) and culture medium type (Swanson et al., 1996). Nowadays, the efficiency of *in vitro* produced cat embryo has been variable among laboratories, ranging from 40 to 70% (Herrick et al., 2007; Tharasanit et al., 2014; Thongkittidilok et al., 2014). Not only of the success in IVP in domestic cat, but also the live offspring has been produced after transferring *in vitro* derived embryos into recipient females in wild felids (Pope, 2000; Pope et al., 2012; Swanson, 2012). Despite these successes, there are still challenges associated with the limited numbers of good quality oocytes available for *in vitro* embryo production. By considering in the low number of good quality embryos produced from limited good quality oocytes in wild felids, the study about single embryo culture is one of choices to manage with low number of embryos. However, the poor development was often observed in single embryo culture compared with group embryo culture by several studies (Paria and Dey, 1990; Doherty et al., 1997; Goovaerts et al., 2009).

Growth factor supplementation has been developed to improve the developmental capability of singly cultured embryos (Goovaerts et al., 2010). We developed this technique for improvement of the developmental competence of embryo produced by single culture using domestic cat as a model. This study also allows us to understand about the interaction via autocrine and paracrine signaling

between companion embryos and environment in culture medium and also the effect on gene regulation and ROS production. The requirement of paracrine or autocrine growth factors was elucidated by growth factor supplementation (IGF-1 or EGF) into single embryo culture system. We expected that the knowledge acquired from this study could provide the knowledge about developmental capability and the influences of growth factor supplementation (IGF-1 and EGF) on the developmental competence of group or single culture embryo. Moreover, our study could provide valuable data about embryonic secreted factors for applying as a biomarker for non invasive embryo quality assessment. This leads to understand about the effect of IGF-1 on oxidative stress and anti-oxidative gene expression altered in embryos cultured in specific conditions. The findings from our studies could help in the optimizing culture condition for wild felids propagation and conservation in the future.

When only small numbers of good quality oocytes are available, it is necessary to culture gametes/embryos individually or in small groups (Vajta et al., 2008; Goovaerts et al., 2009). However, it has been shown in the mouse and cow that small percentages of embryos cultured singly developed into blastocyst stage compared with those incubated in groups (mouse, 49% vs. >80%; cow, 2.9% vs. 30.7%) (Paria and Dey, 1990; Goovaerts et al., 2009). Various growth factors, such as EGF and IGF-1 have been shown to enhance blastocyst development in single embryo culture system in the mouse and cow (Paria and Dey, 1990; Lim and Hansel, 1996; o'Neill, 1997) by regulating cell proliferation and differentiation via MAP/ERK and Akt pathway (Zhang and Liu, 2002). We examined the effects of IGF-1 and/or EGF on singly embryo culture system. Our study demonstrated that IGF-1 and EGF enhanced blastocyst formation of singly cultured embryos to the same level as observed in group culture embryo without any growth factors supplementation by reducing developmental block during the transition from morula to blastocyst. We also demonstrated that the supplementation of IGF-1 or EGF modulated the expression of their receptors to regulate embryonic development. This evidence confirms the important role of IGF-1 and EGF as paracrine factors for embryonic growth in cat. However the synergic effect of IGF-1 and EGF was not found in our

study which is contradicted to the previous study in cow (Sakagami et al., 2012) where a positive effect of 100 ng/ml EGF plus 50 ng/ml IGF-1 on bovine blastocyst development was found. While the cause of this negative effect is unclear, it has been shown in human breast cancer cells that IGF-1R can interact with EGFR (Riedemann et al., 2007; Veeken et al., 2009). Thus, it may be that the addition of IGF-1 to the culture medium increased expression of IGF-1R (Thongkittidilok et al., 2014) that interacted with EGFR and mediated its availability to bind with EGF, especially at a low dosage. Future studies should focus on the impact of IGF-1 on the expression of EGFR and EGF-EGFR signaling in cat embryos.

As we learned from experiment 1 (Chapter 2) that IGF-1 promoted the development of single embryo culture. Then, we hypothesized that IGF-1 should be secreted from the embryo to stimulate and control their growth. There have been several studies showing that many kinds of protein were secreted from embryo to regulate the development. These secreted substances have been shown to correlate with blastocyst formation in preimplantation mammalian embryos. By using single embryo culture as a model for secretomics study, we determined the relationship between the level of secreted IGF-1 and blastocyst formation as well as the expression of gene that related with blastocyst quality. Our result revealed that embryos at 4-8 cells or morula stages secreted IGF-1 at higher level had higher chance to develop to blastocyst than those arrested embryos. We also found that secreted IGF-1 affected on the expression of gene related to blastocyst quality (*OCT3/4*, *BAX*, *BCL2*). In term of apoptosis regulated gene, we found that the expression of *BCL2* was significantly higher than the expression of *BAX* transcript in both low and high IGF-1 secreted embryo. In agreement with Hardy and Spanos (2002) and Paria and Dey (1990), our result also showed the anti-apoptotic effect of IGF-1 during the embryo development. Surprisingly, we found that *OCT3/4* transcripts were higher in high IGF-1 secreted embryos than *in vivo* produced or low IGF-1 secreted embryos. It might be due to the difference in culture conditions (*in vivo* and *in vitro* embryos) which have been found to induce the difference in gene expression pattern including epigenetic change of imprinted genes (such as IGF-II, H19). The reasons are responsible for embryonic and fetal growth (Niemann and Wrenzycki,

2000). The culture condition during *in vitro* culture may also affect the expression of *OCT3/4* level. Up regulation of *OCT3/4* in *in vitro* produced blastocyst from high IGF-1 appeared as a consequence of *in vitro* culture condition (Saenz-de-Juano et al., 2011). IGF-1 mediated IGF-1R through PI3K/Akt signaling to regulate pluripotent transcription factor such as *OCT3/4* (Huang et al., 2009). The presence of glucose in the response of high IGF-1 level related with Oct-4 expression (Saadeldin et al., 2011). The study in cell line revealed that the addition of IGF-1 into breast cancer cells enhanced *OCT3/4* expression (Castaño et al., 2013). From this previous study, it could be speculated that the secreted IGF-1 level from both 4-8 cell and morula stage embryos are closely related to the developmental capability of embryo cultured singly and the upregulation of gene related to blastocyst quality in response to stimulation of PI3K/Akt signaling.

Despite of the role in apoptosis and cell proliferation, there have been reported that IGF-1 also involved in the production of ROS that affects on the embryo development (Jallali et al., 2007) by regulating the expression of gluthathione peroxidase 1 (*GPX1*) as anti-oxidative gene. Our study is conducted to investigate the effect of IGF-1 on developmental competence of group and singly cultured embryo as well as the expression of anti-oxidative gene (*GPX1*, *SOD1* and *catalase*). The findings from our study showed that ROS production and the expression of anti-oxidative genes in blastocyst were influenced by culture conditions and IGF-1 supplementation. Supplementation of IGF-1 in group culture, as shown in chapter 5, did not enhance blastocyst formation which is contrast to the effects that found in singly cultured embryo. However, we found the significantly reduction in ROS producing and the upregulation of anti-oxidative gene in group culture supplemented with IGF-1 at 50 ng/ml. This finding brought us to understand that although IGF-1 reduces ROS production, there might be another growth factors from paracrine signaling that control the development in group culture embryos. Contradicted to the findings in group culture embryos, IGF-1 exerts positive effect on singly culture embryo by enhancing blastocyst formation with an increased in ROS production.

6.2 Suggestion for further investigations

At present we have developed single embryo culture system in domestic cat as a model for overcoming the problem of limited number of isolated gamete from endangered felids species. The knowledge from this study could provide the understanding about developmental capability and could be used as a model for study about embryo metabolism, secretions and gene expression pattern. In addition this could apply for using as a screening purpose before embryo transfer by measuring the growth factors to cytokines which are secreted from embryo as a biomarker for evaluation of embryo quality.

In Chapter 2, we demonstrated that supplementation of IGF-1 in singly cultured embryo promoted blastocyst development by modulation of IGF-1R and reducing developmental block. The further study about the roles of the Akt and ERK downstream pathways from the IGF-I receptor in singly cultured embryo will provide more mechanistic data on how IGF-1 stimulates these pathways. We also found that a high concentration of IGF-1 (100 ng/ml IGF-1) led to downregulation of *IGF-1R*. This result suggests the existence of a homeostatic network controlling IGF-1 and its receptors in feline embryos. Further study would be required to characterize this mechanism.

In Chapter 3, we revealed that supplementation of EGF in singly cultured embryo improved developmental competence of singly cultured domestic cat embryos by enhancing cell proliferation. Thus, the beneficial effect of EGF on singly cultured cat embryos observed in the present study are likely, in part, due to its cell cycle stimulating effect via MAPK signaling pathway. Future studies that involve incubating cat embryos with EGF and specific inhibitors of EGFR or MAPK will confirm this hypothesis. We have not found the synergic effect of IGF-1 and EGF on the embryo development in this study. Moreover, in some combination doses negatively affected on embryo development. The reason why the combination doses exert negative effect are still obscured. Future studies should focus on the impact of IGF-1 on the expression of *EGFR* and EGF-EGFR signaling in cat embryos.

In Chapter 4, our study demonstrated the relationship between IGF-1 level and blastocyst formation also the difference in gene related blastocyst quality. Further study such as the differential staining to determine ICM/trophectoderm ratio or TUNEL assay in *in vitro* produced blastocyst from low or high secreted IGF-1 should be investigated to confirm that the embryonic quality is truly related with the gene expression profile.

In Chapter 5, we have demonstrated that IGF-1 influence on ROS production and the expression of anti-oxidative gene in group and single embryo culture. Although IGF-1 improved the development of individually cultured embryo, it increased ROS production and downregulation of *GPX1*. We speculate that it could be due to the stimulation of ROS production in the response to IGF-1 by increasing glucose metabolism. Our study also demonstrated that supplementation of IGF-1 did not promote blastocyst formation in group cultured embryos. However, we found the significant reduction in ROS production and upregulation *GPX1*, *SOD1* and *catalase* mRNA transcript in response to IGF-1 supplementation. Further study such as the study about downstream signaling of IGF-1R that involves with glucose metabolism and ROS production and also transferring embryos produced from group culture with and without IGF-1 should be investigated to confirm blastocyst quality/developmental competence.

6.3 Conclusion

Single embryo culture system could be used for study in several aspects such as the response of embryos to paracrine growth factors, secretomic study. This leads to discover a new biomarker for non invasive embryo quality assessment before making a decision to select the best quality embryo for embryo transfer. The alteration of gene expression pattern in different culture environment can also be studied. In this study, effects of IGF-1 or EGF on developmental competence of group or singly cultured embryo and the expression of its receptor, secretomic study and expression of gene that relate with blastocyst quality, ROS production and the expression of anti-oxidative gene were observed and studied. These results introduced us several factors that could affect developmental competence of *in*

vitro produced embryos. Therefore the further studies are required for providing more information to deeply understand about the IGF-1 or EGF mechanism in cat embryos. The technique from our study could be applied to improve developmental competence of wild felids embryos for propagation and conservation of the species.

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APPENDIX

APPENDIX A

All media were prepared using MilliQ water, otherwise indicated. The pH of all media was adjusted to 7.3-7.4 for Hepes modified medium and 7.6 for culture media; the osmolarity was 270-290 mOsm/kg for culture medium. All media were sterilized by syringe filtration through 0.2 µm filter immediately after preparation then aliquoted and stored at 4 ºC, -20 ºC or -80 ºC

Stock solution

FSH	100 IU/ml diluent
EGF	25 ng/µl PBS+ 0.1% BSA
IGF-1	0.1 mg/ml PBS+0.1% BSA
hcG	300 IU/ml diluent
eCG	300 IU/ml diluent
DAPI	0.1 mg/ml PBS

Hepes modified medium (Oocyte holding medium)

Hepes	5.985 g/l
Medium M199 (M3769: Sigma)	1 bottle/l
BSA	1 g/l
L-glutamine	0.292 g/l
1 M Sodium pyruvate	1 ml/l
Penicillin	100 IU/ml
Streptomycin	100 µg/ml
Phenol red	500 µl/l

Oocyte maturation medium (Prepared in $NaHCO_3$-M199)

$NaHCO_3$-M199	10 ml
L-glutamine	0.00029 g/ml
BSA	0.004 g/ ml
1 M Sodium pyruvate	1 µl/ml
Penicillin	100 IU/ml
Gentamicin	50 µg/ml
rhFSH	0.05 IU/ml
EGF	25 ng/ml

***In vitro* fertilization medium (prepared in Tyrode's balance salt solution)**

BSA	0.006 g/ml
NEAA	1 % v/v
IVF 100x stock	1 % v/v
Penicillin	100 IU/ml
Gentamicin	50 µg/ml

***In vitro* fertilization medium (prepared in synthetic oviductal fluid solution)**

BSA	0.006 g/ml
Penicillin	100 IU/ml
Gentamicin	50 µg/ml

Synthetic oviductal fluid medium (SOF-IVC1)

NaCl	6.29 g/l
KCl	0.53 g/l
$NaHCO_3$	2.1 g/l
Na-lactate (60% w/w)	0.5969 g/l
$MgCl_2.6H_2O$	0.1 g/l

$CaCl_2.2H_2O$	0.25 g/l
KH_2PO_4	0.16 g/l
MEM NEAA	10 ml/l
NEAA	20 ml/l
Glucose	0.27 g/l
Penicillin	10,000 IU/l
Sodium pyruvate	0.0363 g/l
BSA	4 g/l
Phenol red	500 µl/l

Synthetic oviductal fluid medium (SOF-IVC2)

NaCl	6.29 g/l
KCl	0.53 g/l
$NaHCO_3$	2.1 g/l
Na-lactate (60% w/w)	0.5969 g/l
$MgCl_2.6H_2O$	0.1 g/l
$CaCl_2.2H_2O$	0.25 g/l
KH_2PO_4	0.16 g/l
MEM NEAA	10 ml/l
NEAA	20 ml/l
Glucose	0.27 g/l
Penicillin	10,000 IU/l
Sodium pyruvate	0.0363 g/l
FBS	10 % v/v
Phenol red	500 µl/l

10X TBE Buffer

Tris	108 g
Boric acid	55 g/l
0.5 M EDTA pH 8.0	40 ml/l

Adjust with distilled water up to 1 liter, then autoclaved and stored at 25 °C

APPENDIX B

List of publications and conference proceedings

1. Thongkittidilok C, Tharasanit T, Sananmuang T, Buarpung S and Techakumphu M 2014. Insulin-like growth factor-1 (IGF-1) enhances developmental competence of cat embryos cultured singly by modulating the expression of its receptor (IGF-1R) and reducing developmental block. **Growth Horm IGF Res.** 24: 76-82.
2. Tharasanit T, Buarpung S, Manee-In S, Thongkittidilok C, Tiptanavattana N, Comizzoli P and Techakumphu M 2012. Birth of kittens after the transfer of frozen–thawed embryos produced by intracytoplasmic sperm injection with spermatozoa collected from cryopreserved testicular tissue. 2012. **Reprod Dom Anim.** 47: 305-308.
3. Tiptanavattana N, Thongkittidilok C, Techakumphu M and Tharasanit T 2013. Characterization and *in vitro* culture of putative spermatogonial stem cells Derived from feline testicular tissue. **J Reprod Dev**. 59(2): 189-195.
4. Klincumhom N, Tharasanit T, Thongkittidilok C, Tiptanavattana N, Dinnyes A and Techakumphu M 2013. Modulating neurogenesis in embryoid body using a selective TGF beta1/ALK inhibitor affects gene expression of embryonic stem cell-derived motor neurons. **Thai J Vet Med** 43(1): 49-56.
5. Tharasanit T, Thongkittidilok C, Sananmuang T and Techakumphu M 2014. Recombinant human follicle stimulating hormone and growth factors improve the meiotic and developmental competence of cat oocytes. **Thai J Vet Med** 44(1): 107-115.
6. Thongkittidilok C, Sananmuang T, Tharasanit T and Techakumphu M 2011. The effect of growth factors on developmental competence of feline embryos cultured singly. **The 10th Chulalongkorn University Veterinary Annual Conference** 21-22 April 2011.

7. Thongpakdee A, Thongkittidilok C, Inthasri R, Tipkantha W, Tanpradit N, Kamolnorranath S, Siriaroonrat B and Techakumphu M 2011. Semen quality, sperm morphology and heterologous fertilizing capacity in Leopard cat. **The 10th Chulalongkorn University Veterinary Annual Conference** 21-22 April 2011.
8. Thongkittidilok C, Sananmuang T, Tharasanit T and Techakumphu M 2012. Insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF) improve the developmental competence of feline embryos cultured singly. **International Conference on Veterinary Science IICAB, APHIS, FAO Joint Symposiums** (37th Thailand ICVS) 29 February -2 March 2012.
9. Thongkittidilok C, Buarpung S, Tharasanit T and Techakumphu M 2012. The effects of oxygen tensions on *in vitro* embryo development in domestic cats. **RGJ Seminar Series LXXXVII "From life sciences to one health"**. 17 May 2012.
10. Thongpakdee A, Berg D, Tharasanit T, Thongtipsiridech S, Tipkantha W, Thongthainan D, Noimoon S, Tewnern J, Arsaithamkul V, Keawmai U, Punkong C, Suseridumrong J, Inthasri R, Kajornklin N, Thongkittidilok C, Kamoinorranath S, Comizzoli P and Siriaroonrat B 2012. Eld's deer embryos produced *in vitro* can develop to term after transfer into recipient females. The 6th **Zoo seminar "Fighting Extinction"**, Chiang Mai, Thailand. 18-20 July 2012.
11. Tharasanit T, Buarpung S, Manee-In S, Thongkittidilok C, Tiptanavattana N, Comizzoli P and Techakumphu M 2012. Kittens born following transfer of frozen-thawed embryos produced by intracytoplasmic sperm injection using sperm recovered from cryopreserved testicular tissue. **The 7th International Symposium on Canine and Feline Reproduction (ISCFR).** Whistler, British Columbia, Canada. 26-29 July 2012.
12. Buarpung S, Tharasanit T, Thongkittidilok C, Comizzoli P and Techakumphu M 2012. *In vitro* development of feline embryos after intracytoplasmic sperm injection with 7 day refrigerated testicular

sperm. **The 17th International Congress on Animal Reproduction (ICAR).** Vancauver, British Columbia, Canada. 29 July – 2 August, 2012.

13. Thongkittidilok C, Tharasanit T, Suwimonteerabutr J, Tiptanavattana N, Buarpung S and Techakumphu M 2013. Secreted insulin like growth factor-1 (IGF-1) predicts the developmental competence of *in vitro* derived cat embryos. **The SSR 46th Annual Meeting "Reproductive Health: Nano to Global"**, Montreal, Canada. 22-26 July, 2013.
14. Thongkittidilok C, Tharasanit T, Songsasen N, Sananmuang T, Buarpung S and Techakumphu M 2014. Insulin like growth factor-1 (IGF-1) and epidermal growth factor (EGF) enhance developmental competence of cat embryo cultured singly. **RGJ-Ph.D. Congress XV,** Jomtien Palm Beach Hotel and Resorts, Pattaya, Chonburi, Thailand, 28-30 May 2014.
15. Thongkittidilok C, Fujihara M, Tharasanit T, Techakumphu M and Songsasen N 2014. Donor age influences the responsiveness of intraovarian dog follicles to vascular endothelial growth factor (VEGF) and/or epidermal growth factor (EGF) in in vitro culture. **The SSR 47th Annual Meeting "Fertility: A Global challenge"**, Grand Rapids, Michigan, USA. 19-23 July, 2014.

VITA

Miss Chommanart Thongkittidilok was born on May 26th 1984 in Bangkok province. She graduated with Degree of Doctor of Veterinary Medicine (DVM) with 2nd honour from Faculty of Veterinary Science, Chulalongkorn University in 2009. In the year later, she recieved a scholarship from the Royal Golden Jubilee PhD program of Thailand Research Fund to conduct a PhD program of Theriogenelogy at the Department of Obstetrics, Gynaeccology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Her research is focusing on the role of Insulin like growth factor-1 (IGF-1) in in vitro embryo production in domestic cat using single and group embryo culture system in domestic cat as a model. This IGF-1 secretomics of from the in vitro produced embryo can future be applied as a biomarker for non-invasive embryonic quality evaluation.