PRODUCTION OF CAPRINE AND BOVINE *IN VITRO*-FERTILISED AS WELL AS PARTHENOGENETIC EMBRYOS AND AN ATTEMPT TO VITRIFY *IN VIVO*- AND *IN VITRO*-DERIVED EMBRYOS

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE

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KUALA LUMPUR

2011

ABSTRACT

The main objective of this research was to determine the effects of oocyte grading and insemination duration on cleavage rate of embryos obtained from *in vitro* fertilisation (IVF) in bovine and caprine species. In addition, the effects of oocyte grading in bovine and caprine on production of parthenogenetic embryos as well as an attempt to cryopreserve embryos using vitrification technique were also evaluated. Bovine oocytes were obtained from abattoir while caprine oocytes were retrieved through laparoscopic oocyte pick-up (LOPU) or abattoir source. For laparoscopic oocyte pick-up goats, gonadotrophin injections were involved prior to surgery, which were Estrumate (125 µg), Pregnant Mare's Serum Gonadotrophin (PMSG) (1500 IU) and Ovidrel (250 IU). Following the washing in Phosphate Buffer Saline (PBS) (for laparoscopic oocyte pick-up oocytes) or TL-Hepes medium (for abattoir/ovariectomy oocytes), cumulus oocyte complexes (COCs) were washed with *in vitro* maturation (IVM) medium. Subsequently, the cumulus oocyte complexes were cultured according to the grades in the droplets of *in vitro* maturation medium which was pre-incubated overnight in carbon dioxide (5%) incubator at 38.5°C for 18 to 21, 24 to 27 and 22 to 24 hours, for laparoscopic oocyte pick-up caprine oocytes as well as abattoir/ovariectomy caprine oocytes and bovine oocytes, respectively. The grades of oocytes were based on the cumulus layers and the maturation of oocytes was based on the presence of the first polar body. For *in vitro* fertilisation (IVF), oocytes were partially denuded and co-incubated with post-thawed sperm ($1x10^6$ sperm/ml). *In vitro* culture (IVC) of presumptive zygotes was performed after 8 to 14 or 18 to 24 hours after fertilisation. The fertilisation rate was assessed by the presence of the second polar body. The cleavage rates of the embryos were then observed and recorded. For parthenogenetic activation (PA), matured oocytes were completely denuded and washed with 3 droplets of calcium ionophore, subsequently

incubated in it for 5 minutes. The oocytes were then washed with 3 droplets of 6-dimethylaminopyridine (6-DMAP) and incubated in it for 5 hours. After being washed with 3 droplets of preincubated *in vitro* culture droplets, the oocytes were cultured and the cleavage rates were recorded daily. In an attempt of vitrifying embryos, embryos were placed into holding medium (1 minute), followed by VS1 (3 minutes) and subsequently VS2 (45 seconds) before being plunged into liquid nitrogen. The vitrified embryos were devitrified by being immersed into TS (5 minutes), DS (5 minutes), and finally two holding medium (5 minutes each), stepwise. After being washed thrice in pre-incubated *in vitro* culture droplets, the oocytes were cultured and the survival rates were recorded daily. The data were analysed by using Analysis of Variance (ANOVA) and Duncan Multiple Range Test (DMRT). In bovine *in vitro* fertilisation, the maturation rates of Grade A (56.78±6.50%) and Mixed grade (74.69±6.68%) oocytes were significantly ($P<0.05$) higher than other grades of oocytes (Grades B: 46.58±5.92% and C: 31.29±7.11). The fertilisation rates of Grade A (70.49±6.47%) and Mixed grade (68.18±7.43%) oocytes and the insemination duration of 8 to 14 hours (77.37±6.52%) were significantly ($P<0.05$) higher. The cleavage rates of Grade A and Mixed grade oocytes were significantly ($P<0.05$) higher than that of other groups. However, no significant difference ($P>0.05$) was observed in both insemination durations of 8 to 14 and 18 to 24 hours. In caprine *in vitro* fertilisation, the maturation rate of Grade A (74.19±5.79%) oocytes was significantly higher than Grades B (54.66±7.42%) and C (42.50±7.20%) oocytes. The fertilisation rate of Grade A oocytes (40.54±8.23%) was significantly higher than that of Grade C (16.26±5.99%) oocytes. The cleavage rates of Grades A (38.39±8.89%) and B (35.90±9.23%) oocytes were significantly higher than Grade C (10.83±5.10%) oocytes; however, no significant differences ($P>0.05$) were found in the fertilisation and cleavage rates among all grades of oocytes as well as with both insemination durations. In parthenogenetic activation,

the cleavage rate of bovine was significantly ($P<0.05$) higher than that of caprine with the percentages of 63.90±7.30 and 26.77±9.75%, respectively. For the developmental competence of caprine embryos exposed to vitrification solution (toxicity screening) at blastocyst, 75.00% of survival rate for blastocyst up to hatched blastocyst was obtained. The survival rate of 33.33% was achieved in the vitrification of blastocyst. In conclusion, *in vitro* fertilisation protocols for both bovine and caprine species have been successfully developed, producing satisfactory development of embryos *in vitro*. However, intrinsic and extrinsic factors (especially those pertaining to specific laboratory situation of a country) that influence the developmental competence of embryos after *in vitro* fertilisation should be studied in detail, to ensure optimum outcomes of subsequent cleavage, pregnancy and birth.

ABSTRAK

Objektif utama penyelidikan ini adalah untuk menentukan kesan penggredan oosit dan tempoh inseminasi ke atas kadar pembelahan embrio yang diperolehi daripada persenyawaan *in vitro* (IVF) dalam spesies bovin dan kaprin. Selain itu, kesan penggredan oosit dalam bovin dan kaprin ke atas penghasilan embrio partenogenetik serta sebagai suatu percubaan untuk mengkrioawet embrio dengan menggunakan teknik vitrifikasi juga dinilai. Oosit bovin telah diperolehi daripada rumah sembelihan manakala oosit kaprin diperolehi melalui *laparoscopic oocyte pick-up* (LOPU) atau sumber rumah sembelihan. Bagi kambing *laparoscopic oocyte pick-up*, suntikan gonadotrofin terlibat sebelum surgeri iaitu *Estrumate* (125 µg), *Pregnant Mare's Serum Gonadotrophin* (PMSG) (1500 IU) and *Ovidrel* (250 IU). Selepas pembersihan dalam *Phosphate Buffer Saline* (PBS) (untuk oosit *laparoscopic oocyte pick-up*) atau medium TL-Hepes (bagi oosit dari sumber rumah sembelihan/ovariektomi), kompleks oosit kumulus (COCs) dibasuh dengan medium pematangan *in vitro* (IVM). Kemudian, kompleks oosit kumulus dikultur mengikut gred dalam titisan medium pematangan *in vitro* yang telah dipreinkubasi semalaman dalam inkubator karbon dioksida (5%) pada 38.5ºC selama 18 ke 21, 24 ke 27 dan 22 ke 24 jam, untuk oosit kaprin *laparoscopic oocyte pick-up* serta oosit kaprin dan bovin dari rumah sembelihan/ovariektomi, masing-masing. Gred oosit adalah berasaskan kepada lapisan kumulus dan pematangan oosit adalah berasaskan kepada kehadiran jasad kutub pertama. Bagi persenyawaan *in vitro* (IVF), oosit ditanggal sebahagian kumulusnya dan dieram bersama dengan sperma telah dinyahsejukbeku ($1x10^6$ sperma/ml). Pengkulturan *in vitro* (IVC) zigot andaian dilakukan 8 ke 14 atau 18 ke 24 jam selepas persenyawaan. Kadar persenyawaan telah dinilai dengan kehadiran jasad kutub kedua. Kadar pembelahan embrio seterusnya diperhati dan direkodkan. Bagi pengaktifan partenogenetik (PA), oosit matang ditanggal

kumulus sepenuhnya dan dibasuh dengan 3 titisan kalsium ionofor, dan seterusnya dieramkan di dalamnya selama 5 minit. Oosit tersebut kemudian dibasuh dengan 3 titisan 6-dimetilaminopiridin (6-DMAP) dan dieramkan di dalamnya selama 5 jam. Selepas dibasuh dengan 3 titisan daripada titisan kultur *in vitro* preinkubasi, oosit dikultur dan kadar pembelahan direkod setiap hari. Dalam suatu percubaan mengvitrifikasi embrio, embrio diletakkan ke dalam medium sementara (1 minit), diikuti dengan VS1 (3 minit) dan seterusnya VS2 (45 saat) sebelum dijunam ke dalam nitrogen cecair. Embrio divitrifikasi telah didevitrifikasi dengan cara rendaman ke dalam medium TS (5 minit), DS (5 minit), dan akhirnya dua medium sementara (5 minit setiap satu) secara berperingkat. Selepas dibasuh 3 kali dalam titisan kultur *in vitro* preinkubasi, oosit dikultur dan kadar hidup direkodkan setiap hari. Data dianalisis dengan menggunakan *Analysis of Variance* (ANOVA) dan *Duncan Multiple Range Test* (DMRT). Dalam persenyawaan *in vitro* bovin, kadar pematangan bagi oosit Gred A (56.78±6.50%) dan Gred Campuran (74.69±6.68%) adalah lebih tinggi dengan signifikan ($P<0.05$) berbanding dengan oosit gred lain (Gred B: 46.58±5.92% dan C: 31.29±7.11). Kadar persenyawaan oosit Gred A (70.49±6.47%) dan Gred Campuran (68.18±7.43%) serta tempoh inseminasi selama 8 ke 14 jam (77.37±6.52%) adalah lebih tinggi dengan signifikan ($P<0.05$). Kadar pembelahan bagi oosit Gred A dan Gred Campuran adalah lebih tinggi dengan signifikan ($P<0.05$) berbanding dengan kumpulan lain. Walau bagaimanapun, tiada perbezaan signifikan ($P>0.05$) diperhatikan dalam kedua-dua tempoh inseminasi selama 8 ke 14 dan 18 ke 24 jam. Dalam persenyawaan *in vitro* kaprin, kadar pematangan bagi oosit Gred A (74.19±5.79%) adalah lebih tinggi dengan signifikan berbanding dengan oosit Gred B (54.66±7.42%) dan C (42.50±7.20%). Kadar persenyawaan oosit Gred A (40.54±8.23%) adalah lebih tinggi dengan signifikan daripada oosit Gred C (16.26±5.99%). Kadar pembelahan oosit Gred A (38.39±8.89%) and B (35.90±9.23%) adalah lebih tinggi dengan signifikan daripada

oosit Gred C (10.83±5.10%); walau bagaimanapun, tiada perbezaan signifikan (P>0.05) yang ditemui dalam kadar persenyawaan dan pembelahan antara semua gred oosit serta bagi kedua-dua tempoh inseminasi. Dalam aktivasi partenogenetik, kadar pembelahan bovin adalah lebih tinggi dengan signifikan (P<0.05) daripada kaprin dengan peratusan 63.90±7.30 dan 26.77±9.75%, masing-masing. Bagi keupayaan untuk hidup untuk embrio kaprin yang telah didedahkan kepada larutan vitrifikasi (penskrinan ketoksikan) pada tahap blastosit, 75.00% kadar hidup bagi blastosis ke blastosis tetas telah dicapai. Kadar hidup sebanyak 33.33% telah dicapai dalam vitrifikasi blastosis. Kesimpulannya, protokol persenyawaan *in vitro* bagi kedua-dua spesies bovin dan kaprin telah berjaya dibangunkan, menghasilkan perkembangan embrio *in vitro* yang memuaskan. Walau bagaimanapun, faktor-faktor intrinsik dan ekstrinsik (terutamanya yang berkaitan dengan keadaan makmal-makmal khusus bagi sesebuah negara) yang mempengaruhi keupayaan perkembangan embrio selepas persenyawaan *in vitro* patut dikaji dengan terperinci, bagi memastikan penghasilan optimum pada pembelahan, kebuntingan dan kelahiran berikutnya.

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate	:	Tan Wei Lun	IC No.: 850528-07-5862
Matric No.	:	SGR 080107	
Name of Degree	:	Master of Science	
Title of Dissertation	:	Production of caprine and bovine *in vitro*-fertilised as well as parthenogenetic embryos and an attempt to vitrify *in vivo*- and *in vitro*-derived embryos	
Field of Study	:	Reproductive Biotechnology	

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ACKNOWLEDGEMENTS

I would like to express my utmost gratitude and appreciation to my supervisor Professor Dr. Ramli Abdullah for his cultivation, advice, patience, guidance and encouragement throughout the pursuit of this Master's degree. I am honoured to have the opportunity to study in his laboratory with challenging academic atmosphere that made me continually improving and striving for excellence. I would also like to thank him for his diligence and efficiency in all the oocyte-retrieval and embryo flushing surgeries as well as great effort in helping me to complete the dissertation write-up.

A great gratitude was to be given to Puan Edah Mohd. Aris who is my co-supervisor. I would like to thank her for the encouragement that she has given throughout my study. With her advice and encouragement, I was able to pull myself together again to work through the various difficulties.

I would like to express my heartfelt appreciation to Professor Dr. Wan Khadijah Wan Embong for her constructive comments and suggestions during the progress of this study as well as tremendous help in the oocyte-retrieval and embryo flushing surgeries. Sincere gratitude is expressed to Mr. Parani Baya for providing help in the ordering and purchasing of general laboratory supplies. My deep appreciations are also expressed to members and former members of the Animal Biotechnology-Embryo Laboratory (ABEL) who made this Master's study successful by giving encouragement and offering help in surgery, farm and laboratory. They are Mrs. Alice Phua Choon Yen, Mdm. Nor Fadillah Awang, Mdm. Sharrifah Nazari, Ms. Kong Sow Chan, Kwong Phek Jin, Mr. Shahrulzaman B. Shaharuddin, Mdm. Nor Farizah Abdul Hamid, Ms. Soh Hui Hui, Ms. Goh Siew Ying, Mdm. Azieatul Ashikin Bt. Abdul Aziz, Ms. Siti Khadijah Bt. Idris, Ms. Nor Azlina Bt. Kamaluding, Ms. Raja Ili Airina Bt. Raja Khalif, Mr. Mohd Nizam B. Abdul Rashid, Mr. Xiao Zhi Chao, Mr. Md. Rokibur Rahman, Ms. Asdiana Bt. Amri, Mr. Razali Jonit and Mr. Mohd Nor Azman B. Mat Nong.

I would like to thank Institute of Biological Sciences (ISB), Faculty of Science and Institute of Research Management and Monitoring (IPPP), University of Malaya for funding my research experiments under Postgraduate Research Grant (PJP) in the department. My gratitude is expressed to Institute of Graduate Studies (IPS), University of Malaya for providing me the 'University of Malaya Fellowship Scheme' to pursue my Master's degree. I would like to express my thanks to all the staff at the ISB Mini Farm for the constant help in animal management and handling as well as the staff at the Department of Veterinary Services and Abattoir Complex, Shah Alam and Senawang for allowing me to their abattoirs for getting the ovaries for my experiments as well as National Animal Biotechnology Institute, Jerantut, Pahang for the frozen bull semen.

Lastly, I would like to thank my family members. Their love and continuous support and encouragement have led me through the difficulties throughout the study. Not forgetting to thank those who have directly or indirectly contributed to the success of this project. Without their understanding and support, I would not have completed my Master's degree.

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AI	artificial insemination
ANOVA	analysis of variance
ART	assisted reproductive technology
BCB	Brilliant cresyl blue
BD	1, 3 butanediol
bFGF	basic fibroblast growth factor
BO	Brackett-Oliphant
BRL	buffalo rat liver
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CB	cytochalasin B
CCD	charge coupled devices
CHX	cycloheximide
CI	calcium ionophore
CIDR	Controlled Intravaginal Drug Release device
CO_2	carbon dioxide
COCs	cumulus oocyte complexes
CSF	cytostatic factor
CTC	chlortetracycline
6-DMAP	6-dimethylaminopurine
dDMAP	dimethylaminopurine
D-MRT	Duncan's multiple range tests
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E_2	oestradiol
eCG	equine chorionic gonadotrophin
EDI	electrodeionisation
EG	ethylene glycol
EGF	epidermal growth factor
EMiL	Embryo Micromanipulation Laboratory
ET	ethanol
FCS	foetal calf serum
FF	follicular fluid
FGA	flurogesterone acetate
FSH	follicle stimulating hormone
g	gramme(s)
g	Gravity, acceleration due to
G6PDH	glucose-6-phosphate dehydrogenase
GnRH	gonadotrophin releasing hormone
GOEC	goat oviduct epithelial cells
GSH	glutathione
GVBD	germinal vesicle breakdown
hCG	human chorionic gonadotrophin
HIS	high ionic strength
HM	holding medium
hr	hour
ICSI	intracytoplasmic sperm injection
IGF-I	insulin-like growth factor
i.m.	intramuscularly
IP3	inositol 1, 4, 5-triphosphate

IPPP	Institute of Research, Management and Consultancy
ISB	Institute of Biological Sciences
IU	international unit
I.V.	intravenous
IVM	*in vitro* maturation
IVMFC	*in vitro* maturation, fertilisation and culture
IVF	*in vitro* fertilisation
IVC	*in vitro* culture
IVEP	*in vitro* embryo production
IVP	*in vitro* embryo production
kg	kilogramme(s)
KSOM	Potassium Simplex Optimised Medium
LH	luteinising hormone
LN_2	liquid nitrogen
LOPU	laparoscopic oocyte pick-up
M	molar
MAP	methylacetoxyprogesterone
MAPK	mitogen activated protein kinase
mg	milligramme(s)
MGA	medroxyprogesterone acetate
MII	metaphase II
ml	millilitre(s)
mm	millimetre(s)
MOET	multiple ovulation and embryo transfer
MPF	maturation promoting factor
mtDNA	mitochondrial deoxyribonucleic acid
MTM	modified Tyrode's medium
n	number
NaTuRe	Nuclear Transfer and Reprogramming Laboratory
ng	nanogramme(s)
OGS	oestrus goat serum
OPS	open pulled-straw
OR	oocyte revovery
OSFs	oocyte secreted factors
PA	Parthenogenetic activation
PBS	Phosphate-buffered saline
PDGF	platelet derived growth factor
pg	picogramme(s)
PHE	penicillamine, hypotaurine and epinephrine
PKA	protein kinase A
pLH	porcine luteinising hormone
PMSG	pregnant mare's serum gonadotrophin
PVA	polyvinyl alcohol
PVP	polyvinylpyrrolidone
PZM-3	porcine zygote medium
RO	reverse-osmosis
ROS	reactive oxygen species
sAC	soluble adenylyl cyclase
SiO_2	silica
SM	sucrose medium
SOF	synthetic oviductal fluid
Sr	strontium

SSV	solid surface vitrification
TALP	Tyrode’s albumin-lactate-pyruvate
TGF β1β2	transforming growth factor
TS	thawing solution
UV	ultraviolet
vs.	versus
µg	microgramme(s)
µl	microlitre(s)
µM	micromolar(s)
%	percentage