PRODUCTION OF PARTHENOGENETIC EMBRYOS USING CHEMICAL ACTIVATION OF OOCYTES IN MURINE, BOVINE AND CAPRINE SPECIES

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ABSTRACT

Since the crucial step in successful nuclear transfer protocols is the activation of recipient oocytes, incomplete oocyte activation may result in inability of pronuclear formation which leads to unsuccessful nuclear transfer. Therefore, it is important to establish an optimal method to activate caprine oocytes in order to proceed to the next step of cloning technique. The main aims of this study were to produce and evaluate the embryonic development of parthenogenetic murine, bovine and caprine embryos using various activation chemicals either by single or combination treatments as well as to evaluate the effect of post-hCG duration (in murine) and IVM duration (in caprine) on the subsequent parthenote development.

In Experiment 1, the effects of the different combinations of activation chemical on the production of parthenogenetic murine embryos as model animals were studied. Four different groups were compared which are: Group 1 was to evaluate the optimal $SrCl_2$ concentration (2, 4, 6, 8 and 10 mM) + 5 µg/ml CB; Group 2 was to evaluate the optimal duration incubation in 10 mM $SrCl_2$ (1, 2, 3, 4 and 5 hours) + 5 µg/ml CB; Group 3 was to compare the optimal combination agent (6-DMAP, CHX and CB) + 5 µM A23187; and Group 4 was to compare the optimal concentration of EtOH (7, 8 and 9%) + 2 mM 6-DMAP. Generally, the results showed that treatment of murine oocytes in combination of 10 mM $SrCl_2$ + 5 µg/ml CB for 3 hours was significantly ($P<0.05$) the highest when compared to the optimal treatments from each group.

In Experiment 2, even though there was insignificant difference ($P>0.05$) in the percent of murine oocytes with polar body between two groups of post-hCG duration [70.69±1.04% (13-15 hours) vs. 70.08±1.05% (16-18 hours)], 13-15 hours duration gave

significantly (P<0.05) the highest embryonic development at all stages [81.90±1.53% vs. 76.41±1.64% (2-cell); 67.87±2.02% vs. 60.20±1.92% (4-cell); 55.50±2.25% vs. 48.37±1.96% (8-cell); 43.68±2.42% vs. 35.19±2.01% (morula); and 34.36±2.34% vs. 26.04±1.88% (blastocyst), respectively] compared to those of 16-18 hours duration.

In Experiment 3, bovine was used as a model species of livestock animals and the effect of different combinations of activation chemical was evaluated. There were five treatments evaluated in this experiment included Treatment 1: A23187 + 6-DMAP; Treatment 2: Iono + 6-DMAP; Treatment 3: EtOH + 6-DMAP; Treatment 4: Iono + CHX; and Treatment 5: IVF control. Generally, there was no significant difference (P>0.05) in all treatments. However, treatment with combination of 10 µM Iono (5 minutes) + 2 mM 6-DMAP (4 hours) gave the highest embryonic cleavage rates compared to the other combination treatments.

In Experiment 4, effect of different combinations of activation chemical on the production of parthenogenetic caprine embryos obtained from LOPU procedure was studied. No significant difference (P>0.05) was observed in the comparison of activation by single chemical (Iono vs. A23187). For the activation by combination treatments, four group of combinations were evaluated included Group 1: 5 µM A23187 + 2 mM 6-DMAP (3, 4, 5 and 6 hours); Group 2: 10 µM Iono + 2 mM 6-DMAP (3, 4, 5 and 6 hours); Group 3: 10 µM Iono + 10 µg/ml CHX (3, 4, 5 and 6 hours); and Group 4: 10 µM Iono + 5 µg/ml CB (3, 4, 5 and 6 hours). There were insignificant differences (P>0.05) when comparing the parthenote development from the optimal treatments in each group with IVF control. However, treatment with 10 µM Iono + 2 mM 6-DMAP for 6 hours exhibited the highest cleavage (96.15±3.85%) and blastocyst (35.00±13.72%) rates. Cleavage rates from 8-cell to blastocyst were significantly higher (P<0.05) than IVF control.

In Experiment 5, two groups of IVM duration was compared (18-21 vs. 22-25 hours) for LOPU-derived oocytes. At 18-21 hours IVM duration, Grade D oocytes showed significantly lower ($P>0.05$) in maturation rate compared to Grades A and B oocytes. Similarly, at 22-25 hours IVM duration, the maturation rates of Grades A and B oocytes were significantly higher ($P<0.05$) than Grade D oocytes. In addition, maturation rate of Grades A and B oocytes from group of 18-21 hours IVM was significantly higher ($P<0.05$) than Grade D oocytes from 22-25 hours IVM group. No differences ($P>0.05$) were observed in cleavage and blastocyst rates for all oocyte grades from Group 18-21 hours IVM duration. In contrast, at 22-25 hours IVM duration, cleavage rate of Grade C oocytes was significantly lower ($P<0.05$) than Grade D oocytes (84.75±4.73% vs. 96.15±3.85%, respectively), whereas blastocyst rate of Grade A oocytes was significantly higher ($P<0.05$) than Grade D oocytes (23.22±6.36% vs. 7.69±5.21%, respectively).

In conclusion, for murine study, combination of 10 mM $SrCl_2$ + 5 μg/ml CB for 3 hours is the optimal way to produce parthenogenetic murine embryos and duration of 13-15 hours post-hCG injection was found to be a better choice to give higher percentage of oocytes with polar body and subsequent parthenote development. As for bovine study, treatment with combination of 10 μM Iono (5 minutes) + 2 mM 6-DMAP (4 hours) was the optimal method to produce parthenogenetic bovine embryos. As for caprine species, combination of 10 μM Iono (5 minutes) + 2 mM 6-DMAP (6 hours) is the optimal protocol to produce parthenogenetic caprine embryos and duration of 18-21 hours IVM is a better choice to give higher percentage in maturation rate and subsequent parthenote development. These findings are useful to be considered in future experiments involving nuclear transfer protocols and other advanced reproductive technologies in mammalian species.

ABSTRAK

Oleh kerana langkah penting dalam protokol pemindahan nukleus yang berjaya adalah pengaktifan oosit penerima, pengaktifan oosit yang tidak lengkap boleh mengakibatkan ketidakupayaan pembentukan pronukleus yang membawa kepada kegagalan pemindahan nukleus. Oleh itu, adalah penting untuk mewujudkan satu kaedah optimum untuk mengaktifkan oosit kaprin bagi meneruskan langkah teknik pengklonan. Matlamat utama kajian ini adalah untuk menghasilkan serta menilai perkembangan embrio murin, bovin dan kaprin secara aktivasi partenogenetik dengan menggunakan pelbagai kimia pengaktifan sama ada dengan perlakuan tunggal atau gabungan serta menilai kesan tempoh pasca-hCG (dalam murin) dan tempoh IVM (dalam kaprin) ke atas perkembangan partenot.

Dalam Eksperimen 1, untuk mengkaji kesan kombinasi kimia pengaktifan yang berlainan ke atas penghasilan embrio murin secara aktivasi partenogenetik sebagai model haiwan, empat kumpulan yang berbeza telah dibandingkan. Kumpulan 1 adalah untuk menilai kepekatan $SrCl_2$ yang optimum (2, 4, 6, 8 dan 10 mM) + 5 μg/ml CB; Kumpulan 2 adalah untuk menilai tempoh pengeraman dalam 10 mM $SrCl_2$ yang optimum (1, 2, 3, 4 dan 5 jam) + 5 μg/ml CB; Kumpulan 3 adalah untuk membandingkan kimia gabungan yang optimum (6-DMAP, CHX dan CB) + 5 μM A23187; dan Kumpulan 4 adalah untuk membandingkan kepekatan EtOH yang optimum (7, 8 dan 9%) + 2 mM 6-DMAP. Secara umumnya, hasil kajian menunjukkan bahawa perlakuan oosit murin dengan gabungan 10 mM $SrCl_2$ + 5 μg / ml CB untuk 3 jam adalah berbeza dengan signifikan ($P<0.05$) apabila di bandingkan dengan perlakuan terbaik dari setiap kumpulan.

Dalam Eksperimen 2, walaupun tiada perbezaan (P>0.05) dalam peratus oosit yang mempunyai jasad kutub antara dua kumpulan tempoh pasca-hCG [70.69±1.04% (13-15 jam) vs. 70.08±1.05% (16-18 jam)], tempoh 13-15 jam pasca-hCG menunjukkan perbezaan yang signifikan (P<0.05) di semua peringkat pembahagian embrio [81.90±1.53% vs. 76.41±1.64% (2-sel); 67.87±2.02% vs. 60.20±1.92% (4-sel); 55.50±2.25% vs. 48.37±1.96% (8-sel); 43.68±2.42% vs. 35.19±2.01% (morula); and 34.36±2.34% vs. 26.04±1.88% (blastosis), masing-masing] daripada Kumpulan 16-18 jam pasca-hCG.

Dalam Eksperimen 3, bovin telah digunakan sebagai rujukan untuk haiwan ternakan dan kesan kombinasi bahan kimia pengaktifan yang berlainan telah dinilai. Terdapat lima perlakuan dinilai dalam eksperimen ini temasuk Perlakuan 1: A23187 + 6-DMAP; Perlakuan 2: Iono + 6-DMAP; Perlakuan 3: EtOH + 6-DMAP; Perlakuan 4: Iono + CHX; dan Perlakuan 5: IVF sebagai kawalan. Secara umumnya, tiada perbezaan yang signifikan (P>0.05) diperhatikan dalam semua perlakuan. Walau bagaimanapun, perlakuan dengan gabungan 10 μM Iono (5 minit) + 2 mM 6-DMAP (4 jam) memberikan peratusan pembahagian embrio tertinggi berbanding kombinasi perlakuan lain.

Dalam Eksperimen 4, kesan kombinasi kimia pengaktifan yang berlainan ke atas penghasilan embrio kaprin secara aktivasi partenogenetik telah dikaji. Tiada perbezaan (P>0.05) diperhatikan dalam perbandingan pengaktifan secara tunggal (Iono vs. A23187). Untuk pengaktifan dengan rawatan gabungan, empat kumpulan telah dinilai termasuk Kumpulan 1: 5 μM A23187 + 2 mM 6-DMAP (3, 4, 5 dan 6 jam); Kumpulan 2: 10 μM Iono + 2 mM 6-DMAP (3, 4, 5 dan 6 jam); Kumpulan 3: 10 μM Iono + 10 μg / ml CHX (3, 4, 5 dan 6 jam); dan Kumpulan 4: 10 μM Iono + 5 μg / ml CB (3, 4, 5 dan 6 jam). Tiada perbezaan (P>0.05) apabila perkembangan partenot daripada rawatan yang terbaik dalam

setiap kumpulan dibandingkan dengan kawalan IVF. Walaubagaimanapun, rawatan dengan 10 μM Iono + 2 mM 6-DMAP selama 6 jam menunjukkan peratusan 2-sel (96.15±3.85%) dan blastosis (35.00±13.72%) tertinggi. Kadar pembahagian embrio dari 8-sel hingga blastosis adalah berbeza dengan signifikan ($P<0.05$) daripada kawalan IVF.

Dalam Eksperimen 5, terdapat dua kumpulan tempoh IVM dibandingkan (18-21 vs. 22-25 jam) bagi oosit LOPU. Pada 18-21 jam tempoh IVM, oosit Gred D menunjukkan perbezaan yang signifikan ($P>0.05$) dalam kadar kematangan oosit berbanding oosit Gred A dan B. begitu juga pada 22-25 jam tempoh IVM, kadar kematangan oosit Gred A dan B adalah berbeza dengan ketara ($P<0.05$) daripada oosit Gred D. Tiada perbezaan ($P>0.05$) dalam peratusan 2-sel dan blastosis untuk semua gred oosit dari Kumpulan 18-21 jam IVM. Sebaliknya, pada 22-25 jam tempoh IVM, peratusan 2-sel Gred C oosit adalah rendah ($P<0.05$) daripada Gred D oosit (84.75±4.73% vs. 96.15±3.85%, masing-masing) manakala peratusan blastosis Gred A oosit adalah tinggi ($P<0.05$) daripada Gred D oosit (23.22±6.36% vs. 7.69±5.21%, masing-masing).

Kesimpulannya, dalam kajian murin, gabungan 10 mM $SrCl_2$ + 5 μg/ml CB selama 3 jam adalah kaedah yang optimum untuk menghasilkan embrio kaprin secara aktivasi partenogenetik dan tempoh 13-15 jam suntikan pasca-hCG adalah pilihan terbaik untuk memberikan peratusan oosit dengan jasad kutub yang tinggi serta perkembangan partenot berikutnya. Untuk kajian bovin, perlakuan dengan gabungan 10 μM Iono (5 minit) + 2 mM 6-DMAP (4 jam) adalah kaedah yang optimum untuk menghasilkan embrio bovin secara aktivasi partenogenetik. Dalam kajian kaprin, perlakuan dengan gabungan 10 μM Iono (5 minit) + 2 mM 6-DMAP (6 jam) adalah kaedah yang optimum untuk menghasilkan embrio kaprin secara aktivasi partenogenetik dan tempoh 18-21 jam adalah pilihan terbaik untuk menghasilkan kadar kematangan yang tinggi serta perkembangan partenot berikutnya.

Penemuan ini adalah berguna untuk dipertimbangkan dalam eksperimen di masa hadapan yang melibatkan protokol pemindahan nukleus serta teknologi pembiakan mamalia yang lain.

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LIST OF PUBLICATIONS AND PRESENTATIONS

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Siti Khadijah I., R. B. Abdullah, W. E. Wan Khadijah and M. M. Rahman. 2013. Comparison between Different Combinations of Chemical Treatments on Parthenogenetic Activation of Mouse Oocytes and Its Subsequent Embryonic Development. Manuscript submitted to Molecular Reproduction and Development. Animal Cells and Systems. 17(3): 196-202. (ISI publication)

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%	percentage
ºC	degree Celcius
β	beta
µl	microlitre
µm	micrometer
µM	microMolar
µsec	microsecond
$(Ca^{2+})_i$	intracellular calcium
$[(Ca^{2+})_i]$	intracellular calcium concentration
6-DMAP	6-dimethylaminopurine
A23187	calcium ionophore A23187
ABEL	Animal Biotechnology-Embryo Laboratory
ABP	actin-binding proteins
AI	artificial insemination
AII	anaphase II
APC	anaphase promoting complex
ANOVA	analysis of variance
ARTs	assisted reproductive technologies
bFSH	bovine FSH
BME	basal medium eagle
BSA	bovine serum albumin
BSA-FAF	bovine serum albumin-fatty acid free
BSA-FV	bovine serum albumin-fraction V
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
$CaCl_2.2H_2O$	calcium chloride dehydrate
CaMK II	calcium/calmodulin dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CB	cytochalasin B
cdc2	gene that encode cdk
cdk	cyclin-dependent kinase 1

CG	corticol granule
CGE	cortical granule exocytosis
CHX	cycloheximide
CIDR	controlled intravaginal drug release device
CL	corpus luteum
cm	centimeter
CO_2	carbon dioxide
COCs	cumulus oocyte complexes
CSF	cytostatic factor
CZB	Chatot, Ziomek, Bavister medium
D-Glucose	Deoxy-Glucose
DAG	diacylglycerol
DMRT	Duncan‟s Multiple Range Tests
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	diphosphate buffered saline
e.g.	for example; *exempli gratia*
E_2	oestradiol
eCG	equine chorionic gonadotrophin
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EMiL	Embryo Micromanipulation Laboratory
EP	electric pulse
ER	endoplasmic reticulum
ESC	embryonic stem cell
ET	embryo transfer
EtOH	ethanol
et al.	et alii (and others)
F1	first generation
FBS	fetal bovine serum
FCS	fetal calf serum
FF	follicular fluid
FGA	fluorogestone acetate

FSH	follicle stimulating hormone
G	gauge (needle size)
g	Gravity
g	gramme
GV	germinal vesicle
GVBD	germinal vesicle breakdown
hCG	human chorionic ganadotrophin
hr	hour
HWM	Hepes Whitten‟s medium
i.e.	that is; *id est*
i.m.	intramuscular
i.p.	intraperitoneal
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IFAP	filament-associated protein
Iono	ionomycin
IP_3	inositol 1,4,5-triphosphate
IP_3R	phosphatidylinositol 4,5-biphosphate
IPPP	Institute of Research Management and Monitoring
IPS	Institute of Postgraduate Studies
ISB	Institute of Biological Science
ISBMF	ISB Mini Farm
IU	international unit
IVC	*in vitro* culture
IVF	*in vitro* fertilisation
IVF-TALP	*in vitro* fertilisation-Tyrode-Albumin-Lactate-Pyruvate
IVM	*in vitro* maturation
IVP	*in vitro* production
KCl	potassium chloride
kDa	kilo Dalton
KH_2PO_4	potassium phosphate monobasic
KSOM	Potassium Simplex Optimisation Medium
kV	kiloVolt

L-Glutamine	(Left)-glutamine
LH	luteinising hormone
LN_2	liquid nitrogen
LOPU	laparoscopic oocyte pick-up
M	molar
MI	metaphase I
MII	metaphase II
MAP	medroxyprogestrone acetate
MAP	microtubule associated proteins
MAPK	mitogen-activated protein kinase
mean±SEM	mean plus or minus standard error of means
MBP	myosin-binding protein
MEK 1	MAPK kinase
MEKK	MAPK kinase kinase
MEM	minimum essential medium
mg	milligramme
Mg^{2+}	magnesium ion
$MgCl_2.6H_2O$	magnesium chloride hexahydrate
$MgSO_4$	magnesium sulphate
$MgSO_4.7H_2O$	magnesium sulphate heptahydrate
min	minute
ml	millilitre
mm	millimeter
mM	millimolar
MOET	multiple ovulation and embryo transfer
mOsm	milliosmole
MPF	maturation promoting factor
mRNA	messenger ribonucleic acid
MTOC	microtubule-organising centre
n	number
NaTuRe	Nuclear Transfer and Reprogramming Laboratory
Na_2HPO_4	sodium pyrophosphate

NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
$NaH_2PO_4.H_2O$	sodium phosphate monobasic monohydrate
Na lactate	sodium lactate
Na pyruvate	sodium pyruvate
NT	nuclear transfer
O_2	oxygen
OAG	phorbol esters
OGS	oestrus goat serum
PA	parthenogenetic activation
PB	polar body
PBI	first polar body
PBII	second polar body
PBS	phosphate buffered saline
PE	phorbol ester
pFSH	porcine follicle stimulating hormone
pH	hydrogen potential
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PIP2	phosphatidylinositol 4,5- biphosphate
PLC	phospholipase C
PMSG	pregnant mare‟s serum gonadotrophin
PNI	pronucleus I
PNII	pronucleus II
PVP	polyvinylpyrrolidone
RO	reverse osmosis
rpm	rotation per minute
SCNT	somatic cell nuclear transfer
SEM	standard error of means
SOF	Synthetic Oviductal Fluid
SPSS	Statistical Package for Social Science
sp-TALP	sperm-Tyrode-Albumin-Lactate-Pyruvate
SS	steer serum

Sr^{2+}	strontium ion
$SrCl_2$	strontium chloride
TCM 199	tissue culture medium 199
TCM-Py	tissue culture media-pyruvate
TII	telophase II
UM	University of Malaya
UV	ultraviolet
vs.	versus
WM	Whitten‟s medium
ZP	zona pellucida

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Chapter 1

1.0 INTRODUCTION

Chapter 1

1.0 INTRODUCTION

1.1 BACKGROUND

Domestic animals, such as caprine, bovine, ovine and porcine undergo infertility or sub-fertility which results in reducing the number of offspring produced as well as lowering their economic productivity. Consequently, a lot of research activities have been focused worldwide on understanding the related reproductive processes and developing various assisted reproductive technologies (ARTs) to increase the reproductive efficiency of animals

Modern techniques of bioengineering of farm animals involve microinsemination; recombination of DNA; *in vitro* manipulation (Hafez and Hafez, 2000) of gametes and embryos such as *in vitro* maturation (IVM), *in vitro* fertilisation (IVF) and *in vitro* culture (IVC), which collectively known as *in vitro* production (IVP); intracytoplasmic sperm injection (ICSI); cryopreservation of sperm, oocytes as well as embryos; parthenogenetic activation (PA); embryo transfer (ET); nuclear transfer (NT); gene transfer as well as intra- and interspecies cloning and stem cell research.

In the process of normal fertilisation, interaction between a sperm cell and an oocyte triggers off a series of morphological and biochemical transformations, known as oocyte activation. The key fertilisation mechanism is a calcium signal as observed in most animal species. Several minutes after the penetration of a sperm cell into an oocyte, a quick and transitory drawing occurs from intracellular reserve of calcium. This is the calcium collected in endoplasmic reticulum (although extracellular calcium can also be used to supplement the reserve), thus enabling continuation of the calcium signal (Tosti *et al*., 2002). The activation of oocytes leads to meiosis resumption and extrusion of the second

polar body into the perivitalline space. Consequently, male and female pronuclei are formed, DNA synthesis begins and embryonic cleavage is initiated (Grabeic *et al*., 2007).

In contrast, parthenogenesis is the resumption of meiosis without sperm penetration into the ooplasm of oocytes (absence of sperm), which later results in formation of zygotes. The mechanism of parthenogenetic activation in oocytes is triggered by oscillation of intracellular free-calcium concentration and destruction of maturation promoting factor (MPF), resulting in activation of oocytes, similar as in normal fertilisation (Presicce and Yang, 1994b).

Parthenogenesis is the production of an embryo from a female gamete without the involvement of a male gamete and with or without eventual development into an adult (Beatty, 1957). This process is related to exogenous hormonal administration by controlling the ovulatory process and oocyte retrieval from donor ovaries before activating and culturing the parthenogenotes in appropriate culture media (Beatty, 1957).

In the metaphase stage of the second meiotic stage (MII), mammalian oocytes were arrested until fertilisation or artificial activation occurred (Fissore *et al.,* 2002a). As normal fertilisation by sperm cell or artificial activation took place, oocytes were being activated. Oocyte activation occurs when oocyte exits MII and oocyte starts to divide until it reaches embryonic development (Fissore *et al.,* 2002a. During activation, the oocyte would undergo a series of biochemical transformation and morphological changes (Navarro *et al*., 2005).

There are three main methods of oocyte activation, which are by chemical activation, electrical activation and physical activation (Liu *et al.,* 1998b; Meo *et al.,* 2004). The activation by chemical can be by using ethanol (EtOH), calcium ionophore (A23187), ionomycin (Iono), strontium chloride ($SrCl_2$), 6-dimetylaminopurine (6-

DMAP), cytochalasin B (CB) and cycloheximide (CHX) which can be used in order to induce embryonic development artificially. Electrical activation also plays the same role, such as using electrofusing pulse set, similar as in cloning protocol. As for physical activation, oocytes were being exposed to heat treatment or Sham injection to trigger the process of parthenogenesis.

1.2 JUSTIFICATION OF THE STUDY

The present research was undertaken to produce caprine embryos by parthenogenetic activation method to induce the oocytes and subsequently form embryos. However, the literature on activation protocols for caprine oocytes is scarce; therefore, this study was carried out to increase the basic parthenogenetic information as well as its application in this species. Murine model was used for learning curve because abundant murine oocytes are easily obtained and the murine management is simple compared to larger animals. Bovine oocytes obtained from abattoir were also used for comparison in parthenogenesis between the two ruminant species of livestock.

Several parthenogenetic protocols are normally used to activate the oocytes to produce pre-implantation embryos. It has been reported that different parthenogenetic activation treatments will result in different effects on the oocytes, thus affecting efficiency of pronuclear formation, cleavage rate and embryo size. The activation of oocytes depends on superovulation protocols such as types of hormone used and the duration between gonadotrophin treatment and oocytes retrieval. Aged oocytes are more prone to activation by each method of activation than younger oocytes, and some even underwent spontaneous activation without treatment and exhibited pronuclear formation and blastocyst development.

The methods of oocytes activation by parthenogenesis in this study, as recommended in the literature, involved various chemicals as activation agent, different activation protocol which was either single or combined activation as well as control in each activation protocol. The detailed approach of caprine oocytes activation by parthenogenesis involved considering the screening process of maturation, morphology of oocytes, testing on chemical treatments as well as the observation of subsequent embryonic development *in vitro* during the pre-implantation stage. These factors were often observed in producing embryos while carrying out the *in vitro* production of embryos.

1.3 SIGNIFICANCE OF THE STUDY

A phenomenon of parthenogenesis plays an important role in the production of large numbers of individual species in the case of unsuitable conditions or absence of male sex, in the production of oocytes to be used for many research experiments as well as in the aid for the assisted reproductive technologies (ARTs). Oocytes activation plays a crucial role and was also considered as key step in most assisted reproductive technologies, such as intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT) (Tian *et al.,* 2006; Meo *et. al.*, 2007; Heindryckx *et al.,* 2008).

Thus, parthenogenesis helps to provide more knowledge on oocyte activation by providing a greater understanding on the vital molecular components and morphological changes during the early stage of the activation of oocytes (Liu *et al.,* 1998b) as well as knowledge on the natural fertilisation. The changes and abnormalities during the spontaneous activation mechanisms and the entire chemical and the physical changes of the oocytes during the early stages of the embryonic development can also be observed and examined (Inoue *et al*., 2008). This valuable information would help to overcome the

limitation of cloning in nuclear transfer, besides increasing the efficiency of cloning in the nuclear transfer programme (Liu *et al.,* 1998a; Meo *et al.,* 2004). This is because, for successful nuclear transfer in cloning to be possible, full activation of the cytoplast in the recipient was required which was hardly achievable in nuclear transfer alone to induce sufficient activation for oocytes (Liu *et al.,* 1998a; Kishikawa *et al*., 1999). Besides, parthenogenetic activation could also provide better understanding in spontaneous activating mechanism.

Furthermore, since parthenogenetic embryos were easier to produce in high numbers compared to cloned embryos (De Sousa *et al*., 2002) or *in vitro* fertilised embryos (Kikuchi *et al*., 2002; Yoshioka *et al*., 2003), therefore parthenogenetic embryos which were artificially activated could be used in co-transfer experiments such as establishment of pregnancies in studies of somatic cell nuclear transfer (Fahrudin *et al.,* 2000). Moreover, reviewed studies showed that parthenogenetic activation combined with assisted reproductive technologies, including ICSI can help in pregnancies and the subsequent deliveries (Tejera *et al.,* 2008).

It was also reported that embryos produced by parthenogenetic activation could also be further used in cloning techniques, as well as in the production of embryonic stem cell (ESC) lines (Cevik *et al.,* 2009). In order to enhance the study on certain aspects in embryonic development, oocytes which were parthenogenetically activated could be used as a model (Liu *et al.,* 2002a). To date, parthenogenesis was used as tool for the production of stem cells, whereby stem cell lines were established via parthenogenesis (Winnerger, 2004). Since, oocytes which were artificially activated only have maternal genes in the cytoplasm, thus it acts as a very valuable tool in genomic imprinting studies (Liu *et al.,* 2002a; Meo *et al.,* 2007).

Because of all the functions of the artificial parthenogenesis, and how it is important to search for the optimum protocol, this study was conducted in order to obtain the best conditions for artificial activation protocols which later will provide the efficient results that can be used to improve the assisted reproductive techniques and other clinical and research purposes.

1.4 OBJECTIVES OF STUDY

The main objective of this study was to produce caprine embryos *in vitro* by parthenogenetic activation of oocytes. The specific objectives are shown as below:

a) To develop activation protocol for production of murine embryos by parthenogenesis as a learning curve.

b) To determine the effects of different activation protocols on the parthenogenetic murine oocytes and its subsequent *in vitro* embryonic development.

c) To determine the effect of post-hCG duration on the *in vitro* embryonic development of murine after parthenogenesis.

d) To develop activation protocol for production of bovine embryos *in vitro* by parthenogenetic activation of oocytes as a model species of livestock animals.

e) To determine the effects of different activation protocols on the parthenogenetic bovine oocytes and its subsequent *in vitro* embryonic development.

f) To develop activation protocol for production of caprine embryos *in vitro* by parthenogenetic activation of oocytes.

g) To determine the effects of different activation protocols on the parthenogenetic caprine oocytes and its subsequent *in vitro* embryonic development.

h) To determine the effect of IVM duration on the *in vitro* embryonic development of caprine after parthenogenesis.

Chapter 2

2.0 REVIEW OF LITERATURE

Chapter 2

2.0 REVIEW OF LITRATURE

2.1 HISTORICAL BACKGROUND

Parthenogenesis occurs naturally in some invertebrates and vertebrates animal species and it has been induced artificially in some fishes and amphibians. The beginning of venture of the parthenogenesis phenomenon was as early as 16th century in the 1690s by a Dutch biologist, where Anton van Leeuwenhoek who was the first to discovered parthenogenesis in the female aphids where it reproduced with absence of males. In the 1700s, Charles Bonnet, a Swiss naturalist discovered that aphids could be reproducing parthenogenetically following the finding of Anton van Leeuwenhoek (Mount and List, 1994). Moreover, observation on natural parthenogenesis which occurs on *Bombyx mori* also leads to the high interest in artificial study of artificial parthenogenesis. Later then in 1847, a French author, Bousier stated that he managed to obtained caterpillars when he laid eggs under the sunlight. His ideas later on lead to many further investigations on the possibilities of temperature as a factor of artificial parthenogenesis (Loeb, 1913).

Even so, in 1899, Jacques Loeb was the one who managed to achieved and accomplished a clear case in artificial parthenogenesis, where he puncture an unfertilised frog eggs with a needle, subsequently found that some embryonic development occurred. Later in the 19th century, the first parthenogenetically mammalian birth was engineered by Gregory Goodwin Pincus from United State where he activated the rabbit eggs by manipulating the temperature treatments in 1930s (Winnerger, 2004). Following the finding, in 1950s, he activated rabbit eggs by harvesting ovum from a female rabbit, then fertilised with hormones and salt solution *in vitro* and subsequently implanted the fertilised

ovum in the rabbit‟s uterus. The egg developed into an embryo and later formed foetus (Mount and List, 1994).

The molecular events that lead to oocyte activation has been studied extensively in invertebrates, amphibians and murine, which subsequently lead to the activation models involving the increased of intracellular calcium transients (Winnerger, 2004). Various activation agents have been applied to activate mammalian oocytes during the cloning of sheep, goats, pigs, cattle, and mice. However, activation protocols must be optimised for use in each species (Krivokharchenko *et al.,* 2003) as different species has different specific effect on the different kind of chemicals. Parthenogenetic activation of murine oocytes *in vivo* was first reported using ether anaesthesia (Braden and Austin, 1954a) followed by heat shock treatment (Braden and Austin, 1954b) and later on by electric stimulation of the oviduct (Tarkowski *et al.,* 1970; Witkowska, 1973). In the other hand, the first *in vitro* induction of murine oocytes artificially was done using hyaluronidase (Graham, 1970; Kaufman, 1973) and later trial was done by filtrates of sperm suspensions (Kaufman, 1973) as well as heat shock (Komar, 1973).

2.2 MILESTONE OF PARTHENOGENETIC ACTIVATION

Table 2.1: Timelines for the significant findings of parthenogenetic activation in murine species

Year	Author	Significant finding
1954a	Braden and Austin	First *in vivo* parthenogenetic activation using ether anaesthesia
1954b	Braden and Austin	First *in vivo* parthenogenetic activation using heat shock treatment

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1970	Graham	First *in vitro* induction artificially using hyaluronidase
1970	Tarkowski *et al.*	First *in vivo* parthenogenetic activation using electrical stimulation of the oviduct
1973	Kaufman	First *in vitro* induction artificially using filtrates of sperm suspensions
1973	Komar	First *in vitro* induction artificially using heat shock
1973	Witkowska	First *in vivo* parthenogenetic activation using electrical stimulation of the oviduct
1975	Kaufman	Oocyte activated using avertin anaesthesia
1978	Siracusa *et al.*	Activation response increases progressively with the concentration of protein synthesis inhibitors (CHX) more than 70%
1995b	Jones *et al.*	Fertilisation of CHX-treated oocytes revealed that continuous Ca^{2+} oscillations in response to sperm were observed after nuclear envelope breakdown but not during interphase which suggests that the ability of the sperm to trigger repetitive Ca^{2+} transients in oocytes is modulated in a cell cycle-dependent manner
1996	Moos *et al.*	Effects CHX-induced activation of mouse eggs on cdc2/cyclin B and MAPK activities
1996	Uranga *et al.*	Blastocyst rate of oocytes treated with A23187 + phorbol esters (OAG) significantly higher (42.1%) compared to treatment with A23187 (32.3%) or OAG (17.7%)
2002	Tong *et al.*	Egg activation by CHX + A23187 was suppressed by okadaic acid which partially reversed MAPK dephosphorylation
2004	Winnerger	Establishment of stem cell lines via parthenogenesis
2005	Navarro *et al.*	Study on spindle dynamics during mammalian oocyte activation

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2006	Rogers *et al.*	Absence of a Ca^{2+} signal not affect initial parthenogenetic events, but influence later gene expression and development as blastocyst CHX-activated embryos have a smaller ICM number and a greater rate of apoptosis than Sr^{2+}-activated embryos
2007	Kishigami and Wakayama	Addition of 2 mM EGTA with 5 mM $SrCl_2$ in CZB, M16 or KSOM activate more 80% oocytes comparable in Ca^{2+}-free medium
2007	Wang *et al.*	Treatment of oocytes with 10 mM $SrCl_2$ for 6 hours was the best condition for activation as well as in the development of mouse somatic cell cloned embryos (blastocyst rate: 76.9%)
2008	Ju *et al.*	ESC line establishment rate was higher from parthenogenetically activated oocytes (15.7%) than nuclear transferred (4.3%) or sham-manipulated oocytes (12.5%)
2008	Roh *et al.*	Microtube culture system enhances the in vitro development of parthenogenetic murine embryos activated by 10 mM $SrCl_2$ with 5 µg/mL CB in Ca^{2+}-free CZB for 5 hours with blastocyst rate 54.1% vs. 39.9% in droplet culture
2009	Xing *et al.*	Parthenogenetic mouse ESC line was confirmed propagated in an undifferentiated state for more than 30 passages and maintained a diploid karyotype to be used for autologous stem cell therapy
2012	Cheng *et al.*	Culturing oocytes in CZB medium induced spontaneous activation in F1 hybrid oocytes which indicates a dominant trait from C57BL/6

Table 2.2: Timelines for the significant findings of parthenogenetic activation in bovine species

Year	Author	Significant finding
1995	Landa and Kopeeny	DNA synthesis and distribution in parthenoenetic bovine embryos by ^{3}H-thymidine autoradiography
1996	White and Yue	Study of intracellular receptors and agents that induce activation in bovine oocytes showed that IP_3 receptor plays a role in early events associated with activation in bovine oocytes
1998	Lechniak *et al.*	Cytogenetic analysis of 24 bovine parthenotes after spontaneous activation revealed that 62.5% exhibited a normal, diploid chromosome complement, whereas 37.5% had various ploidy anomalies
1998a	Liu *et al.*	Effects of protein phosphorylation inhibitor using calcium ionophore and combination treatment with 6-DMAP towards histone H1 kinase and MAPK activities
1999	Chung	The cleavage rate of sham-injected control oocytes treated with Iono + 6-DMAP six times higher than oocytes treated with Iono alone (44.3 vs. 7.4%)
2000	Suttner *et al.*	Highest activation rates of ICSI bovine oocytes was obtained after treatment with Iono + 6-DMAP
2003	Galli *et al.*	Blastocyst rate of dithiothreitol-ICSI embryos were higher than Iono + CHX-ICSI embryos (24.3 vs. 16.7%)
2004	Meo *et al.*	Combination of EtOH + $SrCl_2$ and $SrCl_2$ + EtOH resulted in similar pronuclear formation (36.7-83.9% vs. 53.1-90.3%) and cleavage rates (31.3-81.3% vs. 65.6-80.7%) respectively
2005	Oikawa *et al.*	Pregnancy and birth rates for blastocysts of bovine embryos derived from the EtOH activation treatment (58.8 and 47.4%) were significantly higher than those of the Iono + 6-DMAP treatment (12.5 and 9.2%)
2006	Bhak *et al.*	Blastocyst rate of Iono + 6-DMAP treated SCNT bovine embryos were greater that Iono + CHX SCNT embryos (16.3% vs. 13.1%)

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2007	Talbot *et al.*	Comparative analysis of embryos created *in vivo* and by IVF, SCNT or PA in the establishment of a bovine blastocyst-derived cell line
2008	Wang *et al.*	Study of apoptosis in bovine parthenotes showed that EtOH + CHX + CB treatment (7.0%) significantly lower blastocyst apoptosis index compared to Iono + 6-DAMP treatment (9.1%)
2008	Hosseini *et al.*	Exposure of bovine oocytes to CaI + 6-DMAP or ET + $SrCl_2$ + 6-DMAP were found as the optimal artificial activation protocol for *in vitro* development for activation protocol in cloning procedure
2009	Abdalla *et al.*	ICSI oocytes activated with Iono + EtOH improved the blastocyst yield (29-30%) compared with that of non-treated oocytes (12%)
2009	Cevik *et al.*	Compare the effect of parthenogenetic activation and *in vitro* fertilisation of *in vitro* matured oocytes
2009	Hou *et al.*	Clevage and blastocyst rate of parthenogenetic vitrified-warmed bovine oocytes were optimal with 9% EtOH + 6-DMAP treatment (61.1 and 30.6%)
2010	Bevacqua *et al.*	Transgene expression rates were higher when ICSI-mediated gene transfer embryo were treated with Iono + 6-DMAP, Iono + Iono + 6-DMAP and Iono + $SrCl_2$

Table 2.3: Timelines for the significant findings of parthenogenetic activation in caprine species

Year	**Author**	**Significant finding**
2001	Ongeri *et al.*	Blastocyst development of embryos treated in Iono + 6-DMAP and EtOH + 6-DMAP id greater compared to IVF embryos (28.5, 27.4 and 10.3 %) respectively
2005	Jimenez-Macedo *et al.*	Prepubertal goat oocytes needed additional chemical stimulation (5 µM Iono for 5 minutes and 2 mM 6-DMAP for 4 hours) after conventional ICSI, to form zygotes with male and female pronuclei (2PN)

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2006	Shen *et al.*	Cleavage rate of the NT-embryos activated with 2.33 kV/cm EP + 6-DMAP was greater than that activated with 1.67 kV/cm EP + 6-DMAP (65.6% vs. 19.6%)
2011	Akshey *et al*.	Treatment of donor cells with roscovitine significantly increased the blastocyst rate and EP activation improves the production of handmade cloned goat embryos
2012	De *et al.*	Significant increase in cleavage rate and blastocyst yield when oocytes were activated by EP (76.29 and 19.07% respectively) than when A23187 was used for activation (63.45 and 14.09% respectively)
2012	Jena *et al.*	Cloned and parthenogenetic caprine embryonic development was higher in the RVCL medium in a comparison of embryo development in different culture media
2012	Kharche *et al.*	Activation of *in vitro* matured oocytes by 7% ethanol for 5 minutes in KSOM is most favorable for parthenogenetic caprine embryos production (cleavage rate: 40.32% and morula rate: 8.28%)

Table 2.4: Timelines for the significant findings of parthenogenetic activation in various animal species

Year	Author	Species	Significant finding
1690	Leeuwenhoek	Aphid	First to discover parthenogenesis in female aphids
1899	Loeb	Sea urchin	Found embryonic occurrence in unfertilised eggs
1930	Pincus	Rabbit	First parthenogenetically mammalian birth by activating the eggs in manipulating temperature treatments
1950	Pincus	Rabbit	Activated eggs, fertilised with hormones and salt solution and implanted the fertilised ovum in uterus where egg developed into embryo and foetus

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1990	Whitaker and Patel	Sea urchin	Oocyte activation was triggered by release of calcium ion
1992	Iwamatsu	Medaka	Influence of calcium in oocytes activation which were activated by sperm cell and artificial activation
1995	Hoshi *et al.*	Human	First pregnancy in ICSI using A23187 as treatment for oocyte activation
1997	Simili *et al.*	Chinese hamster	6-DMAP inhibition of early cell cycle events and induction of mitotic abnormalities
2002a	Liu *et al.*	Rabbit	Parthenogenetic activation using A23187, ethanol and thimerosal
2002	Takeuchi *et al.*	Rat	Study on the effect of Ca^{2+} and Mg^{2+}-free culture condition on spontaneous first cleavage in oocytes
2004	Gasparrini *et. al*	Buffalo	Aging negatively affects post-parthenogenetic (18-30 hours IVM) and post-fertilisation (18-30 hours IVM) development
2005	Yin *et al.*	Pig	Parthenogenetic activation in oocytes using A23187
2007	Lee *et al.*	Dog	Oocytes treated with 5 mM Ca-EDTA for 48 hours or 1 mM Ca-EDTA for 72 hours formed a parthenogenetic pronucleus
2008	Koh *et al.*	Crayfish	Parthenogenesis-derived multipotents stems cells adapted for tissue engineering applications
2008	Mishra *et al.*	Buffalo	Compare the effect of parthenogenetic activation and *in vitro* fertilised of oocytes
2008	Varga *et al.*	Pig	Parthenogenetic development of *in vitro* matured oocytes treated with chemical agents
2008	Wani	Camel	Protocol for chemical activation of dromedary camel oocytes was optimised with Iono + 6-DMAP combination

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2010	Bebbere *et al.*	Sheep	Study indicates that oocyte parthenogenetic activation does not impair *in vitro* pre-implantation development to the blastocyst stage, but affects the gene expression status of the embryo after the activation of its own genome

2.3 OESTRUS SYNCHRONISATION AND SUPEROVULATION

Oestrus synchronisation and superovulation is a crucial component of all the ART-protocols and has a major influence in the overall efficiencies of these programmes (Baldassarre and Karatzas, 2004). In small ruminants (e.g., caprine), induction and synchronisation of oestrus is usually achieved by insertion of progesterone-impregnated intravaginal devices combined with an injection of a luteolytic dose of prostaglandin (Holtz *et al.,* 2008). In murine, the pheromone phenomenon (odour of a male) was to stimulate the oestrous cycle and to synchonise the females in high percentage on the third day of pairing (Whitten, 1956). Superovulation was normally carried out through exogenous gonadotrophin treatment.

2.3.1 Oestrus Synchronisation

Exogenous progesterone or progestagen is a fundamental part of all superovulation protocols in small ruminants. These protocols enable the reliable timing of oestrus as well as producing increased numbers of follicles or embryos which are necessary for many assisted reproductive technologies (ARTs) (Ayres *et al.,* 2012). In most superovulation protocols, exogenous progesterone are used to control oestrus in combination with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), while FSH is used to increase the number of follicles that ovulate (Baldassarre and Karatzas, 2004; Gonzalez-Bulnes *et al.,* 2004). A single injection

of an appropriate dose (Greyling and Van Niekerk, 1986; Corteel and Leboeuf, 1990) of $PGF_{2\alpha}$ or one of its analogues is effective in inducing luteolysis. The exogenous progesterone supplements and replaces the endogenous progesterone that is normally produced by the corpus luteum (CL). Naturally occurring CL are then lysed by the administration of $PGF_{2\alpha}$, leaving only the exogenous source of progesterone which can then be removed at a precise interval, relative to the administration of follicle stimulating hormone (FSH) (Cognie, 1999; Gonzalez-Bulnes *et al.,* 2004).

There are several types of exogenous sources of progesterone including intravaginal sponges containing fluorogestone acetate (FGA) (Freitas *et al.,* 1997; Leboeuf *et al.,* 2003) or medroxyprogestrone acetate (MAP) (Fonseca *et al.,* 2005), subcutaneous implants containing norgestomet (such as ear/tail implants-Synchromate B, Crestar) (Freitas *et al.,* 1997) and controlled internal drug releasing devices containing progesterone (CIDR®-Eazi-breed) (Whitley and Jackson, 2004). Protocols using these exogenous sources of progesterone combined with FSH have then been successfully utilised by many researchers and breeders.

2.3.2 Ovarian Superovulation

The basis of superovulation is by manipulating the hormones which are associated with dominant follicle development, as well as regression of subordinate follicles (Meyers-Brown *et al.,* 2010). The action of FSH and luteinizing hormone (LH) on oocyte follicle can be stimulated by superovulation using exogenous gonadotrophin (Martin-Coello *et al.,* 2008).

The action of exogenous gonadotrophin is it helps to promote multiple follicle growth in a single cycle (Liu *et al.,* 2010). Studies shows that gonadotrophins hormones such as equine chorionic gonadotrophin (eCG), pregnant mare‟s serum gonadotrophin

(PMSG) and human chorionic gonadotrophin (hCG) can be administered during superovulation (Fowler and Edwards, 1957).

In caprine, superovulatory treatment typically consists of a combination of oestrus cycle control with an elevated dose of a gonadotropin, to induce the ovary to release more than the usual number of oocytes. FSH usually of porcine origin (pFSH), proved to be more efficacious than eCG (Armstrong *et al.,* 1983; Mahmood *et al.,* 1991; and Nowshari *et al.,* 1992), provided it contains an appropriate admixture of LH. Nowshari *et al.* (1995) proved that LH content in the range of 40% does not only provide the best superovulatory response but also superior embryo viability. Similarly as in caprine, in murine, superovulation is performed by administering exogenous gonadotrophin in order to obtain a large amount of fertilisable eggs (Vergara *et al.,* 1997).

PMSG is commonly used for superovulation because it is easy to be obtained, easy to used, effective and inexpensive (Goulding *et al.,* 1996). In addition, PMSG also has long half-life especially in bovine. The benefit of long half-life is it triggers extra follicular growth during the first follicular wave after ovulation (Goulding *et al.,* 1996). hCG hormone is also commonly being administered during superovulation since it induces ovulation and oestrus (Fowler and Edwards, 1957). Combination of two hormones such as PMSG/hCG and PMSG/LH are used to induce superovulation where hormonal treatments are found successful especially on rodent species (Martin-Coello *et al.,* 2008).

2.3.2.1 Factors influencing superovulation

The success rate of superovulation is variable and it can be due by many other factors such as type of donor breed/strain, age, weight and nutritional status, health as well as the housing conditions, the dosage of hormonal treatment and timing of hormonal injection (Fowler and Edwards, 1957; Edgar *et al.,* 1987a,b).

2.3.2.1 (a) *Donor breed/strain*

Number of oocytes produced varies among breed/strain, as some strain responses differently towards the hormonal treatment. This has been reported in murine (Gates and Bozarth, 1978; Hogan *et al*., 1986; Spearow, 1998, Zudova *et al.,* 2004, Byers *et al.,* 2006), ovine (Torres *et al.,* 1987; Vivanco *et al.,* 1994), caprine (Amoah and Gelaye, 1990; Mohd Noor Hisham, 2006) and bovine (Crister *at al.,* 1979; Holness *et al.,* 1980; Donaldson, 1984). Studies compared between outbred, inbred and hybrid murine strains shows that inbred murine are least responsive to hormonal treatment (Fowler and Edwards, 1957; Vergara *et al.,* 1997).

2.3.2.1 (b) *Age*

Another factor that which influences superovulation is age. A study by Lerner *et al.* (1986) shows that the number of oocytes collected in murine decrease with the increase in age. The poor superovulatory response in aged animal is probably due to the reduction in capability of number of follicles responding toward the gonadotrophin treatment. This finding was supported by a study conducted by Maurer and Foote (1971) on superovulation in rabbits and murine which shows low number of available oocytes in aged murine is due in low number in growing follicles (Lerner *et al.,* 1986). On the other hand, Hasler (1992) reported that the age of the animal was not considered to be main factor in superovulatory response whereby no significant age effects were shown on quantity and quality of oocytes obtained (Katska and Smorg, 1984; Wani *et al.,* 1999; Kong, 2010) as well as no similar effect on the total number of embryos obtained (Donaldson, 1984; Kong, 2010).

2.3.2.1 (c) *Weight and nutrition*

Body weight of animals and nutrition were significantly related to the reproductive performance. The nutritional and health status of animals may affect the body weight of the females. The optimal yield of oocytes through superovulated murine was from weight between 12.5 to 14.0 g. In murine, the low body weight may cause less superovulatory response as lower number of oocytes obtained after superovulation (Hogan *et al.,* 1986).

Different dietary regimes were shown to change the endocrine signaling pathways. However, the effect of these changes on fertility is unclear. O'Callaghan *et al*. (2000) reported that oocyte morphology, oocyte development and embryo production were affected by dietary intake. The spoil oocyte quality, embryo mortality and late embryo development *in vivo* (Mantovani *et al*., 1993; McEvoy *et al.*, 1995; Negrao *et al*., 1997) and *in vitro* (Papadopaulos *et al*., 2001) were affected by overfed animals. Overfeeding can lead to reduced pregnancy rates (Parr *et al*., 1987) and decreased the presence of embryos collected on Day-2 after fertilisation (Creed *et al.,* 1994). In contrast, embryo development was delayed during first two weeks after fertilisation (Parr *et al*., 1987; Abecia *et al.,* 1995) whereas embryo motality was higher during the first two weeks of pregnancy (Rhind *et al*., 1989; Abecia *et al*., 1995) during under-nutrition.

However, the variable responses and inconsistent outcomes in relation to nutrition and reproduction in ruminants are complex. In order to optimise the oocytes and embryo production, good nutritional management of donor caprine for superovulation programme and LOPU is required (Scaramuzzi and Murray, 1994).

2.3.2.1 (d) *Dosage of hormonal treatment*

Superovulation rate also affected by the concentration of hormone administered. A study by Fowler and Edwards (1957) shows that different concentration of hormone will result in

different number of oocyte retrieved. 3 IU PMSG and hCG produced more oocytes compared to 1 IU PMSG, followed by 2 IU hCG treatment. Moreover, excess stimulation will also suppress ovulation by the formation of atretic CL and atretic follicles. In addition, excess amount of hormone can reduced mating response (Fowler and Edwards, 1957; Edwards *et al.,* 1963). Though superovulation produce large number of oocytes in short time, however, if being administered excessively it will result to decrease in rate of fertilisation and increase in incidence of oocyte and embryo degeneration in murine. Besides, it will also increase the frequency of triploidy and sister chromatid exchange in early murine embryos (Edgar *et al.* 1987a).

2.3.2.1 (e) *Timing of hormonal injection*

Other factors that affect superovulation rate are time interval of hormone administration. Time interval between the administration of FSH (e.g., PMSG) and LH (e.g., hCG) plays an important role in both follicle and oocyte development. Edgar *et al.* (1987b) stated that by decreasing the time interval between the administration of constant doses of PMSG and hCG shows no significant different in 2-cell stage, but show a reduce in the frequency of blastocyst development. Both mice strain and post-hCG treatment significantly affect the superovulation rate where Hashlamoun and Killian (1985) showed that in a study on C57BL/6 and Swiss-Webster mice, 14 to 17 hours post-hCG produced the greatest rate of oocyte recovery (Vergara *et al.,* 1997). In addition, time interval of 36 to 48 hours between PMSG and hCG administration in C57BL/6 X CBA/Ca F1 hybrids shows no significant differences in 2-cell embryos; contradictorily, time interval of 60 hours shows significant increase in 2-cell embryos development up to blastocyst (Vergara *et al.,* 1997).

2.3.3 Adverse Effect of Superovulation

The superovulation protocol (synchronisation-hyperstimulation) was known influencing the variety in oocytes and embryos viability and quality (Leyva *et al.,* 1998). The progestagen treatment during synchronisation could contribute to alteration in endocrine (Scaramuzzi *et al.,* 1988) and follicular function (Leyva *et al.,* 1998) by lack of complete suppressive effect on LH secretion (Kojima *et al.,* 1992). Greve *et al.* (1995) shows that the alteration in follicular function led to the ovulation of oocytes with the abnormalities of their development competence diminished potential fertility and alteration in the normal process of early embryo development.

There are two important factors which impact on the superovulation response, which are the presence or absence of a dominant follicle as well as the presence or absence of CLs at the time of FSH administration. All follicles in a given wave have a potential to become dominant follicles and ovulate (Ginther *et al.,* 2003). However, it has been shown that if dominant follicles form, they have a detrimental effect upon the smaller follicles which later leads to an increase in non-ovulatory follicles (Veiga-Lopez *et al.,* 2006) as well as a decrease in the total number of embryos and number of viable embryos collected in caprine (Gonzalez-Bulnes *et al.,* 2003a) and ovine (Gonzalez-Bulnes *et al.,* 2003b; Veiga-Lopez *et al.,* 2005). Therefore, administration of FSH prior to the development of the dominant follicles would be expected to produce the maximum number of quality oocytes.

The uses of PMSG in superovulation regime treatment may cause a high number of non-ovulated follicles, early regression of CL, short or irregular oestrus cycle and potential risk of embryo expulsion (Amoah and Gelaye, 1990). Superovulation with PMSG-hCG combination has been claimed to lead the number of fragmented, degenerated and denuded

oocytes (Miller and Amstrong, 1981; Walton and Amstrong, 1981; Moor *et al.,* 1985; Lehtonen and Kankondi, 1987).

In addition, in a some caprine population, repeated use of eCG has been reported to result in poor fertility in fixed-time AI programs. These results have been attributed to the presence of anti-eCG antibodies developed as an immune response to previous treatments (Baril *et al.,* 1996; Roy *et al.,* 1999; Drion *et al.,* 2001; Herve *et al.,* 2004). The presence of these antibodies linked to a delay in the occurrence of oestrus, LH peak and ovulation in the synchronised animals which later on not fit with the timing of the fixed-time insemination which subsequently results in low fertility rate.

Moreover, it is well known that superovulatory treatments are followed by a shorter time period from the induction of luteolysis to ovulations. Consequently, the process of the oocyte maturation may be modified, and this may lead to ovulations of less mature oocytes which may have a reduced ability to undergo normal fertilisation and a normal embryonic development (Callesen *et al.,* 1987; Hyttel *et al.,* 1988; Assey *et al.,* 1994; Dominko and First, 1997).

2.4 RECOVERY OF OOCYTES

In order to achieve a successful *in vitro* production (IVP) system such as parthenogenetic activation and *in vitro* fertilisation, viable and continuous supply of adequate oocytes is important. Oocytes can be collected from both live and sacrificed donors. Caprine oocytes usually collected through laparoscopic oocyte pick-up (LOPU) and ovariectomy procedures from live donor or from slaughtered does at abattoir. Bovine oocytes usually obtained from the collected ovaries from the slaughterhouse. As for murine, oocytes were obtained from the sacrificed donor by laparotomy.

2.4.1 Laparoscopic Oocyte Pick-up (LOPU)

First described LOPU was in 1974 by Snyder and Dukelow. They aspirated 21 follicles and recovered 6 oocytes from an ovine by laparoscopy. However, the potential of LOPU was not fully appreciated until IVP technologies were developed (Baldassarre *et al.,* 1994, 2002; Graff *et al*., 1999). IVP of embryos using immature oocytes recovered by LOPU has the potential to overcome some of the problems associated with standard multiple ovulation and embryo transfer (MOET) techniques (Tervit, 1996; Baldassarre *et al.,* 2002; Cognié *et al.,* 2003).

Basically, the donor was given general anesthesia and oocytes were collected by the aspiration of ovarian follicles after surgical exposure of the ovary by laparotomy or through LOPU under laparoscopic observation (laparoscopy camera and monitor) using a 20 G aspiration needle connected to a collection tube and a vacuum line. In order to recover high number of good quality oocytes, the donor caprine were oestrus synchronised and stimulated with several doses of gonadotropins (Paramio, 2010). The procedure normally takes between 10 to 20 minutes per caprine, depending on the number of follicles to be aspirated. LOPU procedure can be repeated more times since it is less traumatic than other surgical methods (Baldassarre and Karatzas, 2004). This procedure was normally performed to retrieve the immature oocytes. Therefore, the oocytes obtained need to be *in vitro* matured in IVM medium before underwent the next experiment. Baldassarre and Karatzas (2004) reported that an average oocytes obtained is 13.5 oocytes per caprine.

2.4.2 Abattoir-derived Oocytes

Basically, oocytes from the slaughtered animal from abattoir were non-superovulated. Slaughterhouse ovaries are the other alternative source for oocyte retrieval as they are less expensive and most abundant source of immature oocytes for large scale (Kharche *et al.,*

2011). The main advantage of oocyte recovery by this technique is speed of operation, quality of oocytes and quantity of oocytes (Martino *et al.,* 1995a).

There are several approaches used for harvesting the oocytes from ovaries collected from slaughterhouse. These approaches includes oocyte liberation from aspiration of follicles, puncture of follicles and slicing or dissection of follicles (Martino *et al.,* 1995a; Wani *et al.,* 2000; Alm *et al.,* 2008; Rahman *et al.,* 2009; Hoque *et al.,* 2011). In adult caprine ovaries, oocytes are normally recovered by follicle aspiration by selecting follicles bigger than 3 mm diameter whereas for prepubertal caprine ovaries, ovary slicing or dissection allows collection of more oocytes per ovary than by follicle aspiration (1.27 vs. 6.05 oocytes), but the morphological quality of oocytes is lower (Martino *et al.,* 1994).

Ovary slicing was reported as simple and more efficient method compared to follicle aspiration (Martino *et al.,* 1994; Pawshe e*t al.,* 1994). However, recently, Majeed *et al.* (2011) reported that follicles aspiration technique showed higher recovery rate compared to the ovary slicing method (76.93% vs. 69.93%). This result was approved by the previous study in caprine and ovine (Sogorescu *et al.,* 2010). In contrast, in the previous studies in caprine (Wang *et al.,* 2007) and sheep (Wani *et al.,* 1999), they indicated that ovary slicing and puncture yielded significantly more oocytes per ovary than follicles aspiration.

High recovery rate with follicles aspiration might be due to aspiration was the most common techniques for obtaining good quality and quantity of oocytes while the presence of ovarian tissue debris in slicing or puncture technique interfere the searching of oocytes under the microscope (Kharche *et al.,* 2011) and also required more washing when compared to aspiration (Hoque *et al.,* 2011). As a result, a number of cumulus oocyte complexes (COCs) were denuded from cumulus cells due to repeated washing and

ultimately resulted in lower number of normal COCs when compared to aspiration (Majeed *et al.*, 2011).

2.5 *IN VITRO* MATURATION (IVM)

Every immature oocytes retrieved from any oocytes recovery procedures must be *in vitro* matured first before undergo the subsequent experiments. Embryo development is influenced by events occurring during oocyte maturation. For successful IVM, oocytes must undergo synchronically nuclear and cytoplasmic maturation (Paramio, 2010). In mammals, primary oocytes enter meiosis in early prenatal life and progress to the diplotene stage of prophase I (Geminal Vesicle; GV stage), and remain arrested shortly before the time of ovulation (Sharma *et al.,* 1996). Resumption of meiosis *in vivo* is assisted by hormonal stimulation (Zuckermann, 1960; Ireland and Roche, 1982; Dieleman *et al.,* 1983; Callesen *et al.,* 1986). However, in *in vitro* production of embryos, immature oocytes were cultured in IVM medium under suitable conditions for a particular period of time (Pincus and Enzyman, 1935; Edwards, 1965; Thibault and Gerard, 1973). Different species also require different period of IVM duration (Motlik *et al.,* 1986; Fukui *et al.,* 1988; Zhang *et al.,* 1992; Coonrod *et al.,* 1994).

During meiotic maturation, many intracellular changes occur including nuclear maturation, spindle formation and cytoplasmic distribution of organelles (Thibault *et al.,* 1987). At the same time, the nuclear membrane of oocytes disappears, germinal vesicle breakdown (GVBD) and followed by chromosome decondensation which happened at metaphase I (MI) stage. Subsequently, the presence of first polar body shows that the oocytes were entered metaphase I (MII) stage and was ready for the next IVP process. Maturation of oocytes was assessed by cumulus expansion and the extrusion of the first polar body.

Rajikin *et al*. (1994) described good quality of caprine oocytes will appear either golden, golden yellow or brownish in colour and have granulated appearance in the ooplasm. Size of oocytes also very important for the acquirement of maturation. De Smedt *et al*. (1992) mentioned that there are 86% of caprine oocytes with follicles 2 to 6 mm in diameter progressed to MII, whereas only 24% of oocytes with follicles 1 to 1.8 mm reached MII stage.

2.5.1 Events in Oocytes Maturation

Generally, oocytes of all species go through similar nuclear and chromosomal changes during meiosis. Development of mammalian oocytes is arrested at early prophase I (PI) of first meiotic division, shortly before birth (Habsah, 1996) and oocytes remain at the dormant stage, called a dictate nucleus. At this stage, nuclear material is enveloped and this structure known as GV. The oocytes remain at this stage until the onset of puberty. Oocytes then resume meiosis under influence of preovulatory surge of gonadotrophins and particularly in response to the LH surge (Tsafiri, 1978; Westergaard *et al.,* 1984; Downs *et al.,* 1988). At the same time, nuclear membrane of the oocytes disappears and germinal vesicle breakdown (GVBD), followed by chromosome condensation with the occurrence of the MI, anaphase I (AI) and telophase I (TI). Subsequently, oocytes reach MII stage with the extrusion of first polar body and remain at this stage until oocytes is activated either by sperm penetration or by parthenogenetic activation. Meiotic maturation involves two step processes which are: (i) oocytes resume the meiotic process evidenced by GVBD and (ii) completes this process and arresting at MII stage (Szybek, 1972; Tsafiri and Channing; 1975; Wassarman *et al.,* 1976).

Schmitt and Nebreda (2002) described that there are three steps involve in meiotic maturation in caprine which are: (i) resumption of meiosis I which includes GVBD,

chromosome condensation and spindle formation; (ii) the transition between meiosis I and meiosis II and (iii) arrest in MII because of cytostatic factor (CSF) activity.

Oocytes maturation involves the activation of signal transduction pathways that converge to activate maturation promoting factor (MPF) which is the key activity that catalyses entry into metaphase of meiosis I and meiosis II. MPF is activated after the resumption of meiotic division (Masui and Markert, 1971), then transiently inactivated and reactivated again to induce meiosis II. After GVBD, mitogen activated protein kinase (MAPK) is also activated and the activation of MAPK is required for a further increase and continuous in MPF activity and meiotic progression beyond the MI stage (Shimada and Terada, 2001; Shimada *et al.,* 2002).

2.5.2 Factors Affecting IVM

Embryonic development can be influenced by the incident occurring during oocyte maturation process (Rajikin *et al*., 1994, Rajikin, 1995; Teotia *et al.,* 2001). There were several factor has been reported affecting the process during the maturation process, such as, donor age (Izquierdo *et al.,* 2002), follicle size (Pavlok *et al.,* 1992; Blondin and Sirard, 1995), oocyte diameter (Hyttel *et al.,* 1997), oocyte developmental stage (Hagemann *et al.,* 1999), media composition (Lonergan *et al.,* 1997), hormones (Zuelke and Brackett, 1990) and serum (Avery *et al.,* 1998) as well as duration of maturation. Improper timing of maturation could lead impaired development (Marston and Chang, 1964), abnormal chromatin formation (Dominko and First, 1997) and oocyte aging (Hunter, 1989; Hunter and Greve, 1997).

2.5.2.1 Donor age

The developmental competence of IVM oocytes can be influenced by the oocyte‟s donor age which the oocytes are recovered. In bovine and porcine, oocytes from prepubertal donors display poor meiotic maturation and embryo development compared to oocytes from adult females (Marchal *et al.,* 2001; de Matos *et al.,* 2003; Sherrer *et al.,* 2004). Oocytes from sexually immature females display morphological, biochemical and physiologic alternations or anomalies that could partially account for their reduced competence, consistent with their lesser meiotic and developmental potential (Zheng, 2007).

In prepubertal calves, Presicce *et al.* (1996) demonstrated that the acquisition of oocyte‟s competence for normal embryo development associated with age and hormonal treatment. Earlier studies (Armstrong *et al.,* 1992; Duby *et al.,* 1995; Looney *et al.,* 1995; Presicce *et al.,* 1995) with bovine oocytes collected from prepubertal calves following *in vitro* maturation, fertilisation and culture (IVMFC) have shown that these oocytes can resume meiosis, undergo GVBD, reach MII and fertilised. The developmental ability for *in vitro* cleavage and blastocyst formation is still lower in prepubertal and pubertal calves than adult bovine even though the establishment of pregnancies and birth of live calves have been reported (Kajihara *et al.,* 1991; Palma *et al.,* 1993; Otio *et al.,* 1995; Revel *et al.,* 1995).

The reasons for low embryonic development of pre-pubertal calf oocytes are unknown and have been attributed to insufficient, delayed or abnormal nuclear maturation as well as cytoplasmic maturation (Damiani *et al.,* 1996; Khatir *et al.,* 1996). Nuclear morphology of calf oocytes was attributed to incomplete nuclear maturation, while some delay in organelle migration and redistribution such as cortical granules, mitochondria, and lipids was related to delayed cytoplasmic maturation. Differences in oocyte diameter,

protein synthesis and energy metabolism have been also suggested as reasons for poor *in vitro* development between calf and cow oocytes (Gandolfi *et al.,* 1988; Steeves *et al.,* 1999).

2.5.2.2 Follicle size and oocyte diameter

The relatively low level of efficiency achieved after *in vitro* maturation of oocytes, compared to those produced *in vivo*, is almost certainly related to the quality of the oocytes at the outset of IVM (Cognie *et al.,* 2003). The physical characteristics of follicles and oocytes before the onset of maturation are very important as the maturation is associated by the changes in the cumulus cells (Young, 1961). Oocytes without a cumulus investment or with a fragmented cytoplasm are typically considered to be low quality which later on will affect the ability to complete meiotic maturation *in vitro* (Warriach and Chohan, 2004).

When collecting oocytes for any IVP procedures, the size of the follicles where oocytes come from is also an important factor which affects oocytes maturation (Cognie, 1999). The relationship between follicular size and oocytes quality can be explained by some cause: (i) the larger follicles may provide a better environment to induce cytoplasmic maturation for the subsequent embryonic development; (ii) a factor inherent in the oocytes derived from smaller follicles limits their future development; (iii) the cytoplasm of oocytes derived from larger follicles may have some factors required for improved development (Qian *et al.,* 2001). Crozet *et al.* (1995) suggested that the acquisition of meiotic competence and the subsequent development of caprine oocytes occur progressively during follicular growth.

Zheng (2007) suggested that oocytes from larger follicles display a greater ability to complete meiotic maturation and its subsequent embryonic development than those from

smaller follicles in almost all mammalian species. The reason why oocytes from small follicles has lower maturation rate is may be due to the incomplete accumulation of a sufficient store of mRNAs and proteins (Pavlok *et al.,* 1992, 1993). Acquisition of developmental competence involves the synthesis and storage of a wide range of molecules during oocyte growth, including mRNA and proteins, followed by the reprogramming and ordered utilisation of these stored products during maturation, fertilisation and early embryogenesis (Moor *et al.,* 1998).

Caprine oocytes derived from small follicles (<0.5 mm) have been reported to be unable to matured *in vitro* and produced a significantly lower blastocyst rate compared to those obtained from large follicles (Cognie *et al.,* 1995a; Crozet *et al.,* 1995). The smallest follicles (2 to 3 mm) were only able to grow until preovulatory stages, however, not able to release viable oocytes (Gonzalez-Bulnes *et al.,* 2003a,b). Han *et al.* (2006) reported that majority of caprine oocytes acquire competence for development up to 8- to 16-cell stage in follicles >2 mm, but do not gain the ability to form morula or blastocyst until follicles larger than >3 mm in diameter. Zheng (2007) also suggested that follicles smaller than 3 mm have not yet attained the full RNA complement required for developing beyond the stage or time when embryonic transcription is initiated.

Martino *et al.* (1994) reported that large follicles (>3 mm) with more cumulus layers are tend to give better result when matured *in vitro* as caprine oocytes complete their growth and reach meiotic competence when the follicular diameter increase. The decrease in the developmental competence was suggested due to insufficient cytoplasmic maturity (Blondin and Sirard, 1995). Moreover, Crozet *et al.* (1993, 1995) reported that significantly higher number of blastocyst (26 vs. 9%) was obtained by the caprine oocytes from large follicles (>5 mm) compared to those from small to medium follicles (<5 mm).

In caprine oocytes, meiotic oocyte competence has been classified in term of oocyte diameter: (i) incompetent oocytes (<110 μm); (ii) partially competent oocytes (110 to 125 μm); (iii) competent oocytes (>125 μm) (De Smedt *et al.,* 1994; Martino *et al.,* 1995a; Anguita *et al.,* 2009). Increasing in size of oocyte occurs when it undergoes intensive synthesis of RNA during maturation which the length of oocyte diameter shows the level of oocyte growth (Lazzari *et al.,* 1994; Lonergan *et al.,* 1994). Lechniak *et al.* (2002) suggested that the smaller oocytes tend to follow the abnormal path of meiotic maturation and may cause disturbances in maturation process.

2.5.2.3 IVM duration

Edwards (1965) found that the rate of maturation *in vitro* and *in vivo* appears to be similar with some asynchrony as regards to the time taken to reach MII (Donahue, 1972). COCs required longer period to undergo GVBD in both BSA and serum supplemented media, i.e. an additional 2 to 4 hours as compared to the time taken by cumulus-free oocytes (Vanderhyden and Armstrong, 1989).

Researches from earlier study suggested that a wide range of time required for completion of meiosis in caprine, ranging from 24 to 32 hours (Crozet *et al.,* 1995). Such differences might be due to variety in culture conditions and recording procedure (Chan, 2008). Kong (2010) has shown that 22 to 25 hours of IVM culture is the best time for caprine oocytes derived from LOPU whereas the optimal maturation rate of abattoir-derived caprine oocytes for goat was when oocytes underwent maturation for 32 hours (Sharma *et al.,* 1996). The time required for maturation of ooeytes *in vitro* is slightly longer (27 hours) in caprine than in ovine and bovine (De Smedt *et al.,* 1994; Keskintepe *et al.* 1994; Martino *et al.,* 1994; Crozet *et al.,* 1995; Cognie, 1999).

Younis *et al.* (1991) found that, a five-time increase in maturation rate has been observed in the *in vivo* matured oocytes collected from gonadotrophin-treated donors compared to those from non-treated caprine. De Smedt *et al.* (1992) suggested that when follicles are stimulated by gonadotrophin treatment and oocytes are cultured in the medium containing oestrus serum, oestradiol and gonadotrophin, maturation peak was reported at 27 hours. However, El-Gayar and Holtz (2001) showed that oocytes from non-stimulated surface follicles obtained maturation peak at 32 hours of culture.

The selection of oocytes and optimal temperature, which ranging from 38.5 to 39˚C are the key of successes in maturation process at 27 hours in caprine (Crozet *et al.,* 1995). Crozet *et al.* (2000) also demonstrated that extending the culture period maybe necessary to promote full meiotic competence as well as to allow a large number of oocytes gain the ability to resume meiosis and complete meiotic maturation.

Some researchers (Shea *et al.,* 1976; McGaughey, 1977; Deb and Goswami, 1990) reported that during the later stages of maturation, there is an increase of oocytes with abnormal chromosome constitution, which probably due to the change in pH, osmolarity or accumulation of toxic materials in the media. Moreover, the differences in duration of oocyte maturation may be influenced by the medium used, medium concentration, serum percentage and the culture system (Samaké *et al.,* 2000; Teotia *et al.,* 2001; Cognie *et al*., 2003).

2.5.2.4 IVM culture media

Successful *in vitro* maturation (IVM) is more complex than simply ensuring an optimal culture environment that promotes nuclear maturation. However, oocyte maturation could be influenced by culture media components and culture conditions used for IVM (Cognie *et al.,* 2003). It is necessary to indicate that the selection of culture media is of extreme

importance, since it can condition the success of the oocyte maturation, fertilisation and later, the development of the embryos (Gliedt *et al.,* 1996).

The most commonly used culture system for maturation of oocytes *in vitro* is a culture medium supplemented with FSH, LH, oestradiol (E_2) and 10% fetal calf serum (FCS) (Moor and Trounson, 1977). For IVM of ovine and caprine oocytes, follicular fluid (FF) recovered from large follicles (>4 mm) can be used as a supplement in maturation medium containing TCM 199 and 100 ng of oFSH. This positive effect is enhanced when FF is recovered from non-atretic (Cognie *et al.,* 1995a) or gonadotropin-stimulated follicles (Cognie and Poulin, 2000). During *in vitro* maturation under these conditions, the extrusion of the first polar body at MII in ovine and caprine oocytes occurs between 16 and 24 hours after the start of maturation.

Gonadotropins are the primary regulators of nuclear maturation in mammalian oocyte *in vitro*. Saeki *et al.* (1996) and Totey *et al.* (1993) suggested that oocytes matured *in vitro* in the presence of gonadotropins and E_2 showed high maturation and fertilisation rates compared with those matured without hormones. Gonadotropins alter the metabolism of the cumulus cells and later induce resumption of meiosis (Salustri and Siracusa*,* 1983) by interrupting the mode of inhibitory substances through the gap junctions (Ball *et al.,* 1983). Pawshe *et al.* (1996) observed that gonadotropins and E_2 usually cause synergistic enhancement of nuclear maturation, depending on the type of serum supplement used in the maturation medium.

FSH was found to enhance early embryo development rather than meiotic maturation (Eyestone and De Boer, 1993). E_2 improves the completion of maturational changes, including the synthesis of presumed male pronucleus growth factor. E_2 is involved in oocyte maturation *in vitro* in numerous ungulates, including bovine (Younis *et al.,* 1989; Sirotkin, 1992; Choi *et al.,* 2001), ovine (Guler *et al.,* 2000), and caprine

(Pawshe *et al.,* 1996). Younis *et al.* (1989) demonstrated that in the bovine system, supplementing IVM medium with 1 μg/ml E_2 enhanced oocyte maturation rate in some cases, however, Beker-van Woudenberg *et al.* (2004) found that it was deleterious in others. Tesarik and Mendoza (1995) suggested that E_2 supported cytoplasmic maturation of human oocytes, thereby facilitating fertilisation and early embryonic development.

FCS was found to be necessary for FSH induced cumulus expansion. The maturation rate increase significantly with the media supplemented with 10% FCS (Pawshe *et al.,* 1996). Some studies (Younis *et al.,* 1989) have shown that oestrus serum has a beneficial effect on maturation and subsequent development whereas others (Totey *et al.,* 1993) have reported that FCS has the most favorable effect on IVM and subsequent development. Oestrus serum has been employed recently instead of FCS in the bovine (Lu *et al.,* 1987; Xu *et al.,* 1987) in caprine (Mogas *et al.,* 1992, 1993; Keskintepe *et al.,* 1994) and in buffalo (Totey *et al.,* 1992; Madan *et al.,* 1994). In caprine, the concentration of heat-activated serum in IVM media is 10 to 20%. Wani (2002) suggested that serum provides nutrients to cells in COC and prevents zona pelucida (ZP) hardening. Different kinds of serum were used in IVM medium in caprine including homologous and heterologous oestrus goat serum (OGS) (Agrawal *et al.,* 1995; Sharma *et al.,* 1996; Malik *et al*., 1999), steer serum (SS) (Jimenez-Macedo *et al.*, 2005; Jimenez-Macedo *et al.,* 2007), FCS (Crozet *et al.,* 1995; Gall *et al.,* 1996; Rho *et al.,* 2001) and FBS (Martino *et al.,* 1995b; Keskintepe *et al.,* 1997; Crozet *et al.,* 2000; Velilla *et al.,* 2002). Pawshe *et al.* (1994) reported that FCS has been successfully used for IVM of caprine oocytes.

Antibiotics such as penicillin and streptomycin (Zhou *et al.,* 2000) have been applied in IVM medium in order to avoid bacterial and fungal contamination. Schafer *et al.* (1972) suggested that gentamicin has a wider range of pH stability compared to penicillin

and streptomycin as well as it has an ability to maintain the biological activity in presence of serum.

2.6 OOCYTE ACTIVATION

Oocytes can be activated either by normal fertilisation with sperm or by artificial activation using chemical activation, physical activation or electrical activation (Liu *et al.,* 1998a). Activated oocytes will undergo a series of events such as sperm penetration, serial events in biochemical pathway and finally the resumption of meiosis which subsequently leads to the formation of zygote.

2.6.1 Normal Fertilisation

In the process of fertilisation, interaction between a sperm cell and a secondary oocytes triggers off a series of morphological and biochemical transformations which is known as oocyte activation. The key mechanism of oocyte activation in most animal species is a calcium signal (Whitaker and Patel, 1990). Once sperm cell penetrated into an oocyte, a quick and transitory drawing occurs from intracellular reserve of calcium which collected in endoplasmic reticulum, thus enabling continuation of the calcium signal (Tosti *et al*., 2002). Meiotic maturation of mammalian oocytes is spontaneously arrested at the MII stage and further nuclear progress depends on sperm-induced activation (Grabeic *et al*., 2007).

The early events of fertilisation by sperm cell include depolarisation of egg plasma membrane, sperm penetration, cortical granule exocytosis, perivitelline space formation and also resumption of meiosis (Iwamatsu, 1992). Fertilisation cause an increase in intracellular calcium; $(Ca^{2+})_i$ stored, which leads to oocyte activation (Kline, 1996; Meo *et*

al., 2004). There are two postulated theories of how normal fertilisation by sperm could trigger calcium release which are: (i) receptor release and (ii) fusion/sperm factor (Kline, 1996; Fissore *et al.,* 2002a).

The receptor theory suggested that the ligand for receptor of the plasma membrane of oocytes is sperm cell. This theory is based on the assumption that oocytes may behave as somatic cells, together with the addition of hormones which initiates the generation of $(Ca^{2+})_i$ oscillations following stimulation of specific surface receptors (Whitaker and Swann, 1993; Schultz and Kopf, 1995). There are two proposed receptor for sperm cell of receptor theory which is G-protein linked receptor (Miyazaki, 1988) and tyrosine kinase receptor (Carroll *et al.,* 1997; Jaffe *et al.,* 2001). In both of the proposed theory, the mechanism suggested is as sperm cells bind to the receptor (G-protein linked receptor or tyrosine kinase receptor) it will trigger the activation of phospholipase C (PLC) which can be either PLCβ or PLCγ (Carroll *et al.,* 1997) as shown in Figure 2.1. The activation of PLC will result to activation of inositol 1,4,5-triphosphate (IP_3) which will induce the release of intracellular stored calcium (Iwamatsu, 1992; Kline, 1996; Fissore *et al.,* 2002a).

Another theory is the fusion/sperm factor theory was shown in Figure 2.2. This theory suggested that after fertilisation of gametes, sperm cells will deliver sperm product into oocyte cytoplasm after gamete fusion which will subsequently trigger an increase in $(Ca^{2+})_i$ stored as well as responsible in initiating $(Ca^{2+})_i$ oscillation (Fissore *et al.,* 2002a). The theory is supported by studies where the initiation of calcium oscillations was monitored by monitoring transfer of fluorescent dyes between sperm and oocytes after fertilisation occur (Lawrence *et al.,* 1997; Jones *et al.,* 1998).

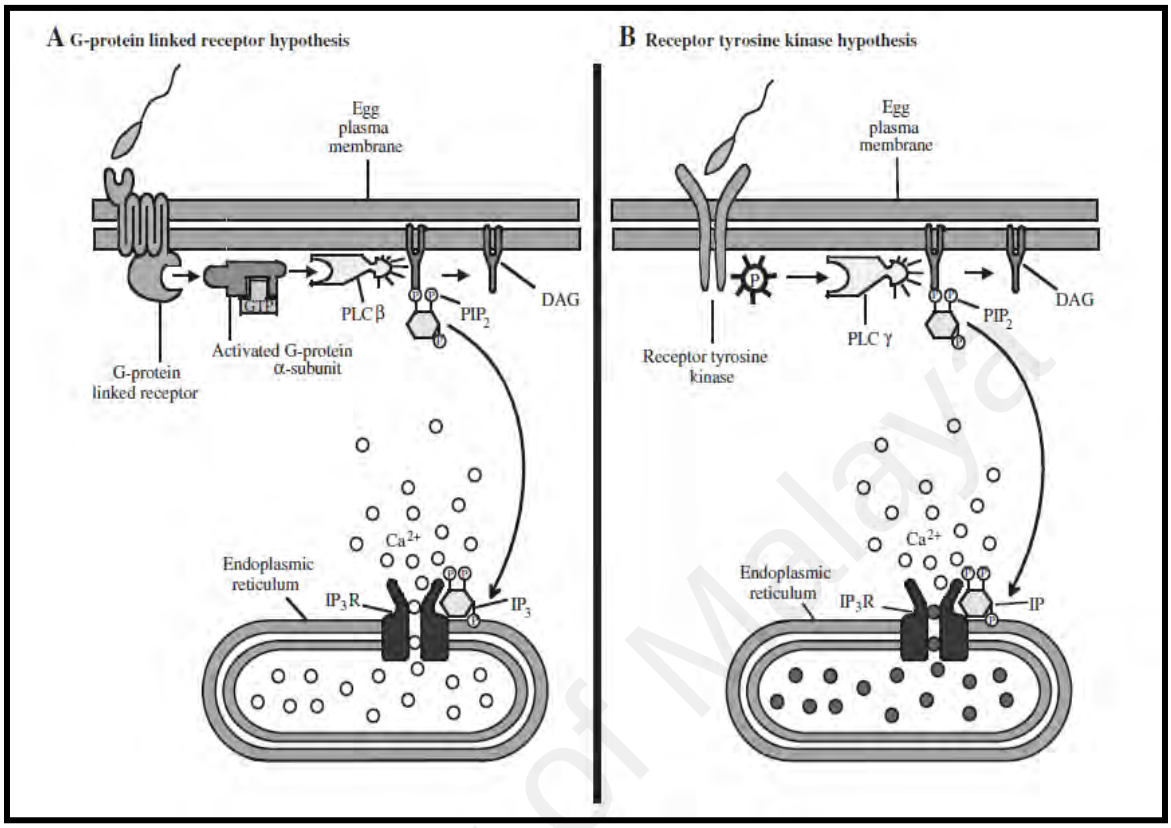

Figure 2.1: The receptor models which suggested the binding and activation of a surface receptor coupled by either: (A) G-protein or (B) tyrosine kinase signal transduction pathway which activate phospholipase C (PLC) and production of inositol 1,4,5-triphosphate (IP_3). PIP2: phosphatidylinositol 4,5-biphosphate, DAG: diacylglycerol, IP_3R: IP_3 receptor.
Adapted from Fissore *et al.* (2002a).

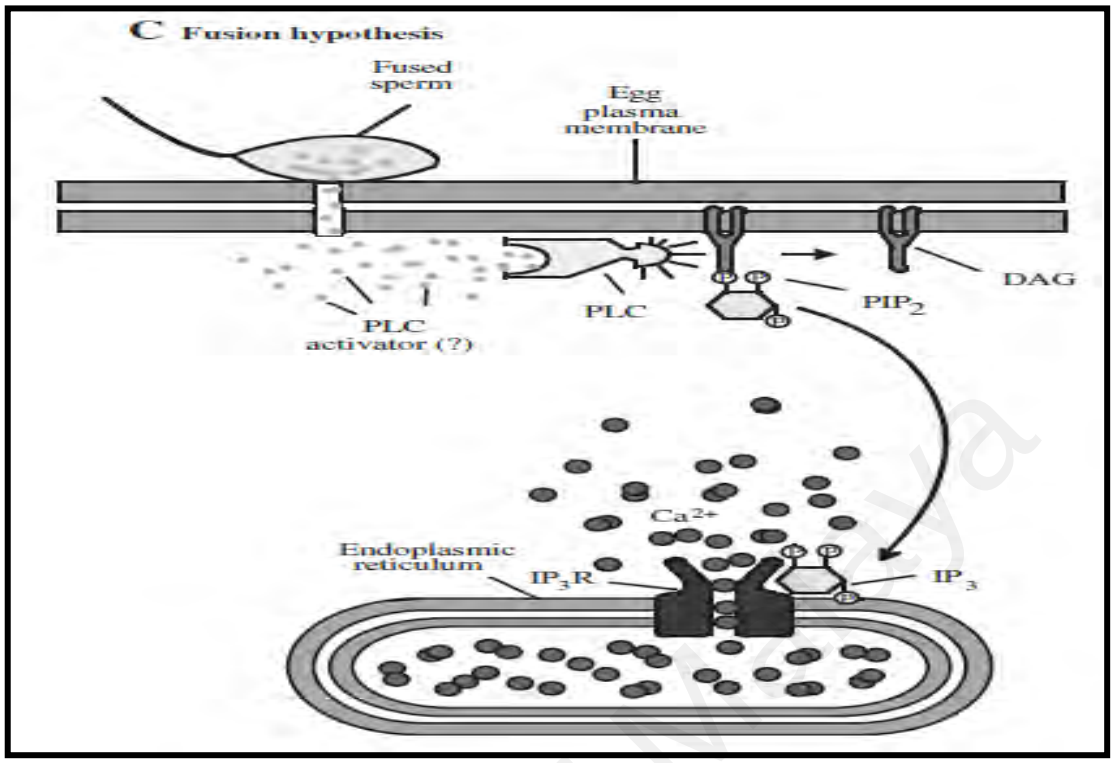

Figure 2.2: The mechanism of fusion/sperm factor theory where sperm releases factor into oocyte cytoplasm, though the sperm factor is unknown. Adapted from Fissore *et al.* (2002a).

2.6.2 Parthenogenetic Activation

Meiotic maturation of mammalian oocytes is spontaneously arrested at the MII stage and further nuclear progress depends on sperm-induced activation. Beatty (1957) described parthenogenesis as the production of an embryo from a female gamete without any genetic contribution from a male gamete (absence of sperm), and with or without eventual development into an adult. Embryos produced are known as uniparental embryos. The mechanism of parthenogenetic activation in oocytes is based on oscillation of intracellular calcium concentration; $[(Ca^{2+})_i]$. The activation leads to meiosis resumption and extrusion of the second polar body. Consequently, pronuclei are formed, DNA synthesis begins and embryonic cleavage is initiated of artificial activation, caused by external stimuli (Grabeic *et al*., 2007).

Unlike fertilisation, in which sperm penetration is sufficient to stimulate oocyte activation, both nuclear fusion and oocyte activation must be simultaneously induced by either chemical, mechanical or electrical pulse (Ongeri *et al*., 2000) Additional stimuli are often required to achieve adequate activation of the oocyte during IVP processes. Parthenogenetic activation often used as model in order to study biochemical changes and morphological events that involved during early embryonic development (Collas *et al.,* 1993b). Parthenogenetic activation has been conducted in order to mimic the action carried out by the sperm cells during fertilisation to activate oocytes which are arrested at MII of meiosis division (Liu *et al.,* 1998a; Meo *et al.,* 2007). Oocyte activation can be done artificially either by chemical, mechanical or electrical (Liu *et al.,* 1998a; Jilek *et al.,* 2000; Tian *et al.,* 2006). Regardless of the types of the parthenogenetic activation treatment, the main objective of parthenogenetic treatment is to allow resumption of meiosis for oocytes that are arrested at MII by increasing the level of intracellular calcium (Meo *et al.,* 2007). Increase in $(Ca^{2+})_i$ store will inactivate MPF and MAPK which later on allowing MII arrested oocyte to complete meiosis division (Meo *et al.,* 2007). Table 2.5 shows types of artificial oocyte activation methods.

Table 2.5: Types of artificial oocyte activation methods and reported cases in clinical research.

Types of artificial oocyte activation methods	Mechanism of oocyte activation	Cases of pregnancy and delivery in clinical research
Calcium ionophore†	Increase in Ca^{2+} permeability of cell membrane	+ (Hoshi *et al.* 1995 A23187[27])
Electrostimulation	Pore formation in cell membrane, entry of extracellular Ca^{2+}	+ (Yanagida *et al.* 1999[28])
Strontium	Release of endogenous Ca^{2+}	+ (Yanagida *et al.* 2006[29])
Ethanol	Increase in Ca^{2+} permeability of cell membrane	
Puromycin‡	Inhibition of protein synthesis	+ (Murase *et al.* 2004[30])
Cycloheximide	Inhibition of protein synthesis	

†Calcium ionophores include A23187 and ionomycin. ‡Puromycin is used in combination with A23187.

Adapted from Yanagida *et al.* (2008).

2.6.3 Mechanism of Oocyte Activation

Mammalian oocytes which are ovulated were arrested in second meiotic division at MII until oocytes were activated by means of normal fertilisation or artificial activation, subsequently resulting to embryonic development (Zernicka-Goetz *et al.,* 1995; Fissore *et al.,* 2002a). The process of activating the oocytes by releasing oocyte from MII is known as oocyte activation (Fissore *et. al.,* 2002a). Oocyte activation can be monitored by changes that occur at the cortical granules, zona proteins, cell cycle kinases and intracellular calcium stores (Ducibella, 1998). Oocytes which are arrested at MII stage are characterised by biochemical substances which are active MPF, mitogen activated protein kinase (MAPK) and cytostatic factor (CSF) (Navarro *et al.,* 2005). Thus, as the oocytes were activated either by means of fertilisation or artificial activations, it will trigger a biochemical cascade which allows the exit of oocytes from arrested at MII. Moreover, oocyte activation will also had an impact on the sequence of cellular changes which result to morphological changes in the oocytes, as well assured embryonic development (Fissore *et. al.,* 2002a; Navarro *et al.,* 2005).

During the oocyte activation, a series of events in the cellular changes take place where it can be organised into either early or late events as stated by Schultz and Kopf (Fissore *et al.,* 2002a; Iwamatsu, 1992). The early event during cellular change in oocyte activation include the initiation of $[(Ca^{2+})_i]$ oscillation or a series of spike in Ca^{2+} which will trigger other events of activation of oocyte. Increase in intracellular calcium concentration in the early events will result to: (i) cortical granule exocytosis to prevent polyspermy, (ii) recruitment of maternal mRNAs, and (iii) the resumption of meiosis with extrusion of the second polar body (Grabeic *et al*., 2007). As for the late cellular events in oocyte activation, there are three main cellular changes which include the: (i) formation of male and female pronuclei, (ii) synthesis of DNA, and (iii) first mitotic cleavage on

oocytes (Fissore *et al.,* 2002a; Navarro *et. al.,* 2005). The mechanism of oocyte activation will be sub-divided into two sub-topics which are: i) biochemical changes during oocyte activation, and ii) morphological changes during oocyte activation.

2.6.3.1 Biochemical changes during oocyte activation

Before matured oocytes are being activated, mammalian oocytes which are MII-arrested is characterised by the presence of CSF which contained other molecules such MPF, MAPK, *c-mos* and $p90^{rsk}$ which inhibits oocyte from resuming meiotic division (Navarro *et al.,* 2005; Nanassy *et al.,* 2007). In order for oocytes to continue the completion of meiotic division and to start mitotic cell cycles, MII-arrested oocytes must first be release from MII stage by oocyte activation (Swan and Jones, 2002). The activity of CSF is responsible in maintaining the matured oocytes from exiting MII stage (Kline, 1996; Heytens *et al.,* 2008). Besides, activity of CSF also stabilises and regulates MPF activity (Heytens *et al.,* 2008). MPF is a protein kinase which is the product of catalytic subunit (tyrosine-phosphorylated $p34^{cdc2}$) and cyclin subunit gene (cyclin B) which causes MII-arrested oocytes (Kline, 1996; Lee and Kirschner, 1996; Tatemoto and Terada, 1996; Wolgemuth *et al.,* 2002; Wun and Dittman, 2008). Besides CSF and MPF, presence of *c-mos* and MAPK also caused oocytes to be arrested in MII. This is because proto-oncogenes *c-mos* product prevents the degradation cyclins (Meo *et al.,* 2004; Navarro *et al.,* 2005; Nanassy *et al.,* 2007). MAPK which is serine/theorine kinases, helps oocytes to remain arrested at MII by maintaining the degradation of cyclin B (Liu *et al.,* 1998a; Meo *et al.,* 2004).

In order for biochemical changes to occur in oocyte activation, the concentration of calcium plays a significant role, as oocyte activation in mammalian is dependent on the increase of $[(Ca^{2+})_i]$ (Kline, 1996). This is because, Heytens *et al.* (2008) suggested that the

universal secondary messengers of cells is calcium (Ca^{2+}), where calcium plays a significant role in controlling a wide range of biological process including proliferation, differentiation, axis formation, transcriptional activation, as well as apoptosis. This is including the oocyte activation by normal fertilisation which calcium plays an important role where sperm cell triggers the increase in Ca^{2+}, besides transferring genetic material into the egg cell (Heytens *et al*., 2008). The increase in $[(Ca^{2+})_i]$ during oocyte activation allows exit of oocytes from MII-arrested stage, resulting to meiotic resumption until embryonic development (Iwamatsu, 1992; Swan and Jones, 2002; Heytes *et al.,* 2008).

Since oocyte activation is dependent on the oscillations of Ca^{2+}, thus increase of $[(Ca^{2+})_i]$ will also cause biochemical changes on MPF, MAPK and CSF by inactivating them (Kline, 1996; Meo *et al.,* 2004). In artificial activation, chemical stimuli such as ethanol, strontium chloride, calcium ionophore, ionomycin, and phorbol ester trigger the release of Ca^{2+} which results to increase in $[(Ca^{2+})_i]$ (Alberio *et al.,* 2001). The release of Ca^{2+} into the oocytes is controlled by Ca^{2+} release mechanisms which are extremely sensitive (Jones and Whittingham, 1996). The agents which trigger Ca^{2+} release mechanisms to release Ca^{2+} are sperm and IP_3 (Jones and Whittingham, 1996; Kline, 1996).

In early study, the mechanisms on how sperm promotes rise in Ca^{2+} level in still unclear, but date to 2008, study found that sperm induces rise in $[(Ca^{2+})_i]$ due to presence of sperm specific PLCδ in the oocyte cytoplasm (Jones and Whittingham, 1996; Kline, 1996; Heytens *et al.,* 2008). As sperm protein bind to oocyte receptor, activated PLCδ will produce IP_3 (Heytens *et al.,* 2008). IP_3 is known to stimulate increase in $[(Ca^{2+})_i]$ is produced from hydrolysis of phosphatidylinositol 4,5-biphosphate (IP_3R) where PLC act as an enzyme. After IP_3 is produced, it will bind to IP_3R which is a ligand-gated receptor. Since IP_3R is found in the endoplasmic reticulum (ER) which functions to store Ca^{2+}, IP_3

which is bind to IP_3R will trigger release of stored Ca^{2+}, allowing oocyte activation to take place (Fissore *et al.,* 2002a) as shown in Figure 2.3.

As the oocyte activation take place, biochemical substances (MPF, MAPK and CSF) changes which helps in the mechanism of oocyte activation can also be observed (Fissore *et al.,* 2002a; Navarro *et al.,* 2005; Nanassy *et al.,* 2007). CSF which held oocytes to be arrested at MII stage regulates the activity of MPF. In order for oocytes to remain in MII stage, CSF need to prevent MPF from being destructed which can be achieve by ensuring the anaphase promoting complex (APC) inactived (Heytens *et al.,* 2008). However, due to the increase in $[(Ca^{2+})_i]$ concentration, CSF will be inactivated, which will cause the degradation in cyclin B. Since cyclin B is a subunit of MPF, thus degradation of cyclin B will indirectly cause MPF to be inactivated. As MPF is being inactivated, dephosphorylation will reduce the level of MPF which later cause oocytes to release from MII stage (Nanassy *et al.,* 2007). Moreover, increase in $[(Ca^{2+})_i]$ can also be cause inactivation of MPF and MAPK by calcium/calmodulin dependent protein kinase II (CaMK II) (Zernicka-Goetz *et al.,* 1995; Johnson *et al.,* 1998).

As shown in Figure 2.4, increase in $[(Ca^{2+})_i]$ will cause Ca^{2+} to bind to calmodulin, producing Ca^{2+}/Calmodulin complex which subsequently activates CaMK II. CaMK II will result two effects which are activating APC directly and also promotes CSF degradation. As APC is being inactivated, it will then trigger the degradation of cyclin B by stimulating cyclin ubiquitination and also by activating proteasome degradation machinery (Johnson *et al.,* 1998). As cyclin B is being degraded, MPF is begun to be inactivated. Degraded CSF, however, will not activate the APC (Heytens *et al.,* 2008).

Enzymes present in MAPK are serine/theorine protein kinase which act together with their downstream substrate can stabilise MPF. MOS protein is the product for proto-oncogene *c-mos*. The function of MOS protein is a MAPK kinase kinase (MEKK) where it

will activate MAPK kinase (MEK 1). As MEK 1 is activated it will result to the activation of MAPK via phosphorylation. Activated MAPK will then phosphorylates, subsequently active serine/threonine kinase $p90^{rsk}$ causing Myt 1 inhibitory kinase to be inhibited. The cascade of MEKK/MEK 1/MAPK/ $p90^{rsk}$ will also decrease the activity of MPF, which will cause dephosphorylation resulting to degradation of MOS (Nanassy *et al.,* 2007).

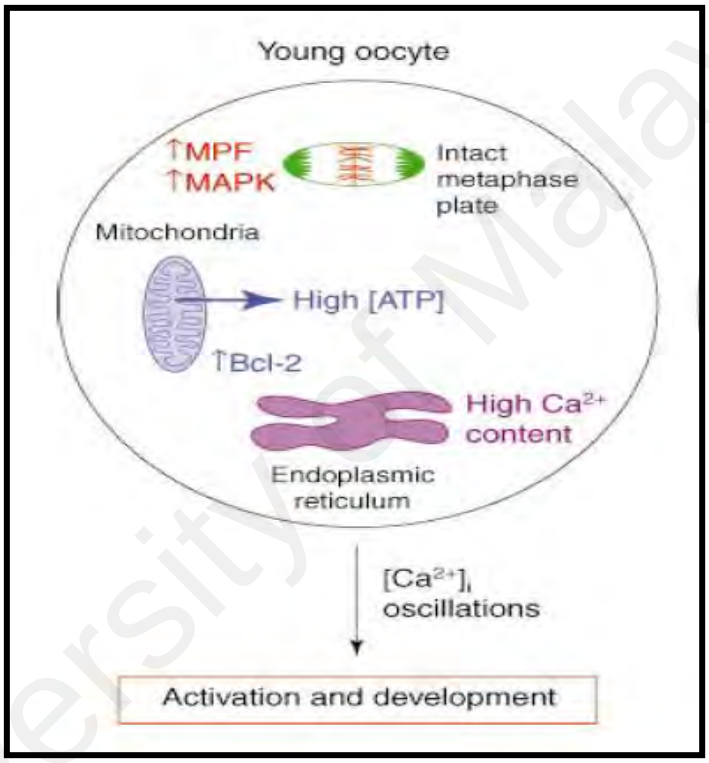

Figure 2.3: Cellular and molecular changes in mammalian oocytes that may underlie fragmentation or development arrest after activation or fertilisation. Adapted from Fissore *et al*. (2002b).

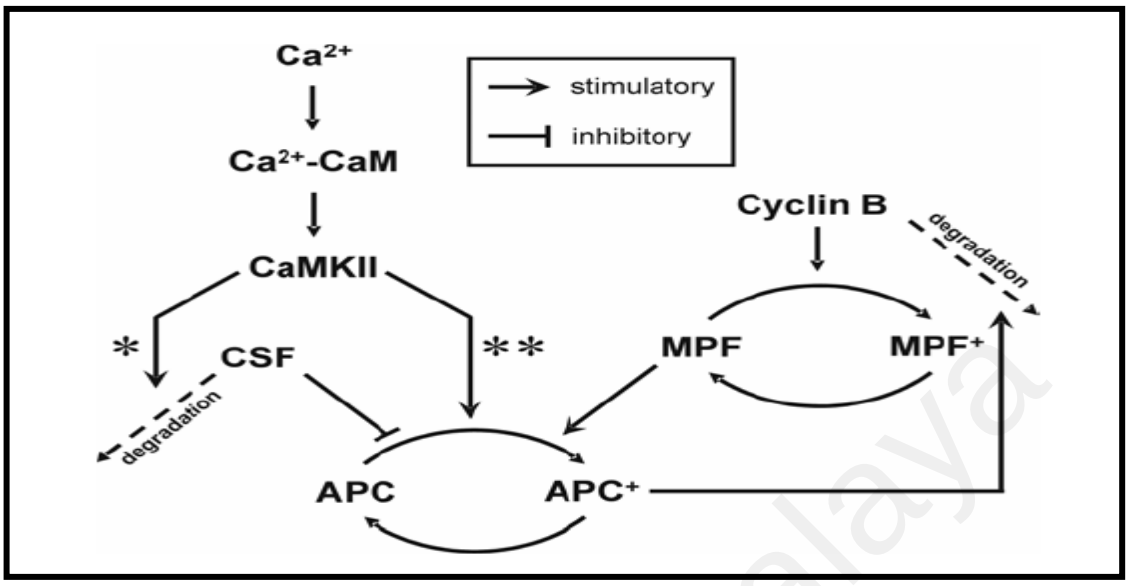

Figure 2.4: Schematic diagram illustrating the calcium-induced relief from metaphase II arrest and cell cycle resumption. The Ca^{2+}-CaM-CamKII axis directly and thus rapidly activates APC (**) and also promotes the degradation of CSF, a process with slower kinetics (*). CSF degradation releases the inhibition of APC. Activated APC (APC^{+}) promotes the degradation of Cyclin B and as a consequence, results in MPF deactivation. This culminates in the abolishment of MII arrest and resumption of the cell cycle.
Adapted from Heytens *et al.* (2008).

2.6.3.2 Morphological changes during oocyte activation

Once oocytes were activated, it not only causes changes in the biochemical components, but it also lead to some morphological changes in the oocytes cytoskeleton (Fissore *et. al.,* 2002a; Navarro *et al.,* 2005). The morphological changes in oocyte cytoskeleton during oocytes activation includes: (i) meiosis resumption, (ii) migration of the cortical granule which anchor at the cortex and exocytosis (iii) condensation of chromosome, (iv) formation and migration of spindle, (v) emission of polar body (PB), (vi) formation of pronuclear (PN), and (vii) syngamy (Ducibella *et al.,* 2002) as shown in Figure 2.5.

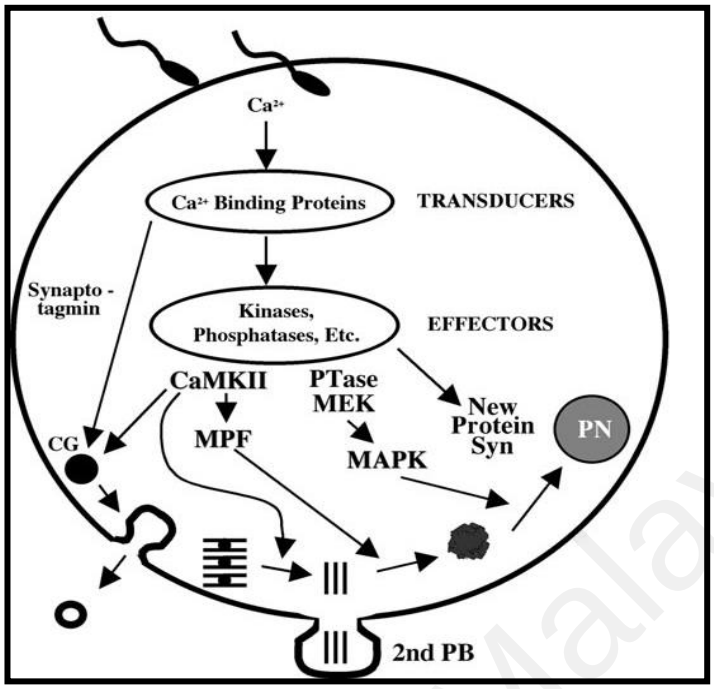

Figure 2.5: Summary of the mammalian egg activation events. Small vertical arrows indicate the activity of effector increases or decreases to promote the pathway with which it is associated. New protein synthesis (Syn) results from the maternal mRNAs; anaphase promoting complex; PTase phosphate; MAPK, MAP kinase; MEK, MAPK kinase, 2nd PB, second polar body; PN, pronucleus, CG, cortical granule; CAMKII, calmodulin dependent protein kinase II; MPK, maturating protein factor. Synaptotagmin is a calcium-sensitive protein which regulates secretion in other cells.
Adapted from Ducibella *et al.* (2002).

Changes in the cytoskeleton organisation after fertilisation includes changes in microtubules and microfilaments (Sun *et al.,* 2001). Alteration on the ZP glycoprotein occurred after the cortical granule exocytosis (CGE), where the alteration of ZP will subsequently prevent polyspermy in the oocyte (Wassarman *et al*., 2001; Talmor-Cohen *et al.,* 2002; Sun, 2003).The protein component present in ZP are ZP1, ZP2, ZP3 and ZP4 (Wassarman *et al.,* 2001; Conner *et al.,* 2005). Corticol granule (CG) will release its content into the perivitelline space of the oocytes which later will alter ZP2, ZP3 and ZP1 to prevent polysyngamy (Tsaadon *et al.,* 2006). Prevention of polyspermy is important in

order to prevent abnormal development. Syngamy is achieveable when zona and/or plasma membrane blocks to prevent other sperm from entering (Florman and Ducibella, 2006).

The formation of pronucleus also occurs after fertilisation takes place, forming two pronuclei during the S phase of first mitotic phase in order to produce two copies of each chromosome (Florman and Ducibella, 2006). The importance of pronuclear stage after fertilisation is, it allows identification of fertilised egg, prevent polyspermy, and disassemble of pronuclear envelopes as well as chromosome condensation which indicate the start of first mitotic division. The formation of pronuclei is dependent on the factors present in the egg cytoplasm where in mammals the starting point for pronuclei formation is the disassemble of sperm‟s nuclear envelope and inactivation of cell cycle kinases such as MAPK (Moos *et al.,* 1995). The formation of female pronucleus I (PNI) is indicated with the condensation of chromosomes and the start of ana-telophase II of second meiotic division, whereas the formation of pronucleus II (PNII) is indicated by the extrusion of second polar body and completion of the second meiosis stage. Also, chromosomes are begun to undergo decondensation and second polar body can be located near the pronucleus (Mogas *et al.,* 1997).

After sperm penetrate into the oocytes, the $[(Ca^{2+})_i]$ store increases which allow meiosis resumption and as well as CGE (Ducibella, 1998a,b; Florman and Ducibella, 2006; Tsaadon *et al.,* 2006). The origin of CG is from Golgi apparatus where during oocyte growth CG will increase in number and migrated towards the cortex. CG will continue to migrate until it reaches the periphery of oocyte, and forming a CG-free area which will surround the oocyte‟s spindle in MII stage during oocyte maturation (Tsaadon *et al.,* 2006). Due to the increase in $[(Ca^{2+})_i]$, CGE is triggered which caused alteration to the ZP gylcoproteins and subsequently allowing syngamy to be established by blocking

polyspermy (Ducibella, 1998a,b; Tsaadon *et al.,* 2006). One of the mechanisms that trigger CGE is by calmodulin 17 kDa and Ca2+/CaMK II where both are found at the MII stage spindle and at the cortex of egg (CaMKII) (Tsaadon *et al.,* 2006).

Calmodulin is a ubiquitious intracellular protein with stored Ca^{2+} while CaMKII is a multifunctional Ca^{2+} efector that can phosphorylate proteins such as synaptotagmin, synapsin, and synaptophysin (Tsaadon *et al.,* 2006). The action of Ca^{2+}/CaMKII triggers the CGE by inactivating CSF and MPF (Tsaadon *et al.,* 2006). Studies showed that by activating the activity of CaMKII by using artificial activation such as ethanol can trigger the CGE (Tsaadon *et al.,* 2006). Besides, increase in $[(Ca^{2+})_i]$ will also allow PKC (multigene of serine-threonine kinases) to carry out its activity (Tsaadon *et al.,* 2006). As fertilisation or artificial activation occur, phosphatidyl inositol 4,5-biphosphate (PIP2) will stimulate the release of second messengers such as IP_3 and diacyl glycerol (DAG) (Jones and Whittingham, 1996; Ducibella, 1998a,b; Kline, 1996; Fissore *et al.,* 2002a). Binding of IP_3 to IP_3R which is found in the endoplasmic reticulum subsequently trigger the release of $[(Ca^{2+})_i]$ where the increase in $[(Ca^{2+})_i]$ will result to CGE (Ducibella, 1998a,b; Tsaadon *et al.,* 2006).

In addition, cytoskeleton also undergoes some changes involving both microtubules and microfilament which are composed of actin, tubulin and intermediate filament proteins. In addition, cytoskeleton also has accessory proteins with different function such as microtubule associated proteins (MAP), actin-binding proteins (ABP), intermediate-filament-associated protein (IFAP) and myosin-binding protein (MBP). Presence of cytoskeleton in cytoplasm was discovered in the 1980s by Porter; Bershadsky and Vasiliev (Malhotra and Shnitka, 1996). Microfilaments are composed by thin filaments known as actin. Microtubules which are the largest filament in the cytoskeletal is made of tubulins and associated protein. The tubulin of microfilaments is composed of α-tubulin and β-

tubulin (Malhotra and Shnitka, 1996; Joshi, 1998). Tubulins play a significant in cytoskeleton during oocyte activation where it acts as a machinery of chromosomes in mitosis, locomotion of cell and cell shape maintenance. Besides, presence of cilia and flagella and mitotic spindle fibers form a more stable structure for microtubules (Gapco, 1996; Malhotra and Shnitka, 1996). Assembles of microtubule with tubulin in the cytosol by polymerisation and unidirectional elongation is regulated by factors such as ion concentrations, microtubule-associated proteins and microtubule-organising-centers (MTOC) (Malthotra and Shnitka, 1996; Tachibana, 2005). The assembles of tubulin can be inhibited by temperature and calcium ions (Malhotra and Shnitka, 1996).

The stability of microtubules is regulated by phosphorylation of the MAP which is catalysed by kinases such as calcium-calmodulin. Thus if MAP is inhibited, it reduce MAP ability to bind to tubulin, causing a decrease in microtubulins stability (Malhotra and Shnitka, 1996). Microtubule-organising centre (MTOC) plays an important role during cell division where duplicated centrioles that formed MTOC will migrate to spindle poles regions. Kinetochores which are formed at the centromic regions of chromotids and attached to spindle fiber during the early prophase stage. Kinotochore fibers will produce force causing chromosome to be at the equator in the metaphase stage. The kinetochore fibers are shortening in the anaphase stage accompanied elongation of the polar fibers, thus allowing the chromosome to move to the opposite poles during anaphase stage. Moreover, MPF also helps to regulate mitosis and meiosis division, where the activity of MPF increase as cells undergoes mitosis division (Malhotra and Shnitka, 1996).

2.6.4 Types of Artificial Oocyte Activation

Since increase in intracellular calcium level [$(Ca^{2+})_i$] is the key that permits resumption of meiosis division in fertilisation, numerous procedures on artificial activation has been

established including mechanical, chemical and electrical stimuli that elicit one or several Ca^{2+} transients in the oocyte (Alberio *et al.,* 2001). The efficiency and success of each mechanisms to activate oocyte are varies depending on the species involved.

2.6.4.1 Chemical activation

There are two ways to activate oocyte using chemical either by single or combined treatment. Chemicals such as calcium ionophore (A23187), strontium chloride ($SrCl_2$), phorbol ester (PE), ionomycin (Iono), ethanol (EtOH) and thimerosal help to generate calcium transient (Nakada and Mizuno, 1998). Calcium ionophore induces release of $[(Ca^{2+})_i]$ by facilitates influx of extracellular calcium ions (Kline and Kline, 1992). As for ionomycin, it depletes calcium store in order to mobilise $[(Ca^{2+})_i]$ (Cibelli *et al.,* 1998; Wells *et al.,* 1999). Study by Presicce and Yang (1994b) showed that treatment of oocytes using 7% of ethanol for 5 to 7 minutes allows pronuclear formation and successful activation. This is achievable as 7% ethanol promotes IP_3 formation and influx of extracellular calcium. These chemical generate a single Ca^{2+} rise in oocytes (Alberio *et al.,* 2001).

Various chemicals have been applied to activate oocytes. However, activation protocols must be optimised for use in each species (Krivokharchenko *et al.,* 2003) as different species have different specific effect on the different kinds of chemicals. $SrCl_2$ induces multiple Ca^{2+} transients by displacing bound Ca^{2+} in the oocyte (Whittingham and Siracussa, 1978) and also by inducing intracellular Ca^{2+} release (Kline and Kline, 1992). Earlier study conducted by Wakayama *et al.* (1998) had shown that $SrCl_2$ is the best chemical treatment which able to activate mouse oocytes after nuclear transfer successfully with higher mean cell numbers in blastocysts compared to the oocytes treated with ethanol. Moreover, $SrCl_2$ is the only parthenogenetic agent for mouse oocytes that induces

repetitive intracellular Ca^{2+} releases in a similar way to normal fertilisation process by sperm (Bos-Mikich *et al.,* 1995a; Kline, 1996). However, in bovine oocytes, study conducted by Meo *et al.* (2004) showed that there are no significant differences on pronuclear formation and cleavage rate when comparing the treatment using $SrCl_2$ and ethanol. Indeed, Fissore *et al.* (1999) also reported that $SrCl_2$ does not induce Ca^{2+} responses in bovine or porcine oocytes.

The action of endogenous DAG which activates phospholipid-dependent-protein kinase C is mimicked by PE in artificial activation (Nishizuka, 1984). Besides, PE also induces Ca^{2+} transients and formation of pronuclear in mouse oocytes (Cuthbertson and Cobbold, 1985). Since the activation rate of calcium ionophore is higher compared to PE (Uranga *et al.,* 1996), thus the latter is not used in other mammalian oocytes. Thimerosal which a sulfhydryl-oxidising agents produce repetitive Ca^{2+} oscillations though the peak and duration of Ca^{2+} oscillation are shorter compared to activation by sperm cell (Nakada and Mizuno, 1998).

While Fissore *et al.* (1995) demonstrated that thimerosal is found to be effective on bovine oocytes, Cheek *et al.* (1993) found that no oocyte activation was observed in mouse oocytes. However, combination treatment of thimerosal and its inhibitor allow successful embryonic development to blastocyst in pig oocytes (Macháty *et al.,* 1997). Despite the good side of thimerosal, Cheek *et al.* (1993) suggested that, after tubulin and chromatin were stained, destruction in meiotic spindle were detected.

In addition with treatment that increase the calcium level, inhibitors of protein synthesis, protein phosphorylation or histone kinase are used as secondary treatments as a combination during parthenogenetic activation in order to improve oocyte activation (Navara *et al.,* 1994; Susko-Parrish *et al.,* 1994). The parthenogenetic procedures were developed with the idea of combining the need for a rise in $[(Ca^{2+})_i]$, induced by a brief

exposure to a Ca^{2+} generator such as calcium ionophore, with persistent kinase inactivation, which was provided by 4 to 6 hours of exposure either to a kinase inhibitor such as 6-dimethylaminopurine (6-DMAP) (Susko-Parrish *et al.*, 1994) or to a protein synthesis inhibitor such as cycloheximide (CHX) (Presicce and Yang, 1994b).

Histone kinase inhibitor prevents reaccumulation of MPF. Parthenogenetic activation is made possible with 6-DMAP as 6-DMAP acts as a protein phosphorylation (serine threonine kinase) inhibitor by inhibiting protein kinase functions and promoting mitosis in both murine and bovine oocytes (Takeuchi *et al.,* 2002). Besides, 6-DMAP also enhances the formation of pronuclear. In the other hand, 6-DMAP can also inhibit extrusion of second polar body, similarly cause second meiotic spindle to disintegrate thus allowing oocyte to enter interphase stage directly with one diploid pronucleus (Ongeri *et al.,* 2001; Tian *et al.,* 2006).

Ongeri *et al.* (2001) also reported that another protein kinase inhibitor such as CHX and CD prevent the reaccumulation of maturating promoting factor (MPF). Studies showed that when treatment of oocytes using single treatment such as calcium ionophore, ethanol, 6-DMAP and CHX alone does not result to optimal parthenogenetic activation. In contrast, sequential combined treatments of calcium ionophore or ethanol with 6-DMAP or CHX plus CD resulted in a higher rate of pronuclear formation and significantly increased the rates of cleavage and blastocyst development (Ongeri *et al.,* 2001; Meo *et al.,* 2007). Another protein synthesis inhibitor i.e. CHX and puromycin are reported to be capable to induce oocyte activation in murine (Moses and Kline, 1995) and human oocytes (Balakier and Casper, 1993). However, these inhibitors are not sufficient to induce activation in rat (Zernicka-Goetz *et al.,* 1993) and porcine oocytes (Nussbaum and Prather, 1995).

The combination of a Ca^{2+} stimulating substance with a protein synthesis inhibitor has been widely used for activation in murine, caprine, ovine and bovine oocytes (Alberio

et al., 2001). Studies in murine showed that combination of calcium ionophore with CHX not only increase the embryonic development to blastocyst, but also gave higher rate in pronuclear formation (Hagemann *et al.,* 1995). In bovine, oocytes treated with ethanol and CHX showed success in producing several offspring in somatic NT (Presicce and Yang, 1994a). However, Soloy *et al.* (1997) found that CHX not only inhibit the proteins that maintaining MPF activity, but also inhibits proteins translations which are responsible for initiating DNA replication. Indeed, Alberio *et al.* (2001) also demonstrated that the initiation of DNA synthesis is delayed in NT embryos activated by ethanol plus CHX.

Combination treatment of calcium ionophore with 6-DMAP in ovine (Loi *et al.,* 1998) and bovine (Liu *et al.,* 1998b) oocytes also result to high activation rate, formation of pronuclear and successful embryonic development to blastocyst stage. However, mouse and bovine oocytes are not activated when incubated in 6-DMAP without previous Ca^{2+} release (Szöllösi *et al.,* 1993; Liu *et al.,* 1998b). Ongeri *et al.* (2001) and Hou *et al.* (2009) reported that development rate to blastocyst stage of bovine oocytes was higher in ionomycin + 6-DMAP than in ethanol + 6-DMAP treatment. However, oocytes activated with ionomycin + 6-DMAP displays some alterations in the DNA content, reflecting an abnormal pattern of karyokinesis during the first cell cycle (de la Fuente and King, 1998).

In addition, Liu *et al.* (1998b) reported that when bovine oocytes are activated using ethanol + 6-DMAP, it appeared that delayed blastocyst formation with no hatching blastocyst was observed. In contrast, cloned calves are successfully produced when combination treatment of calcium ionophore and 6-DMAP is applied after somatic NT (Alberio *et al.,* 2001). Alberio *et al.* (2001) stated that using protein kinase inhibitors is an efficient way to induce oocyte activation, however, it should be considered that these

inhibitors are not interfering with other kinase which involved in other cell functions, whose inhibition may be deleterious in subsequent cellular events after activation.

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2.6.4.2 Electroactivation

Activation by electrical pulse can also induce calcium oscillations by forming pores to the plasma membrane. The success rate of this activation treatment is very much dependent on the size of pores, the ionic content in the medium and also cell type (Zimmermann *et al.,* 1985). In this procedure, oocytes were placed between parallel electrodes plates and electric field is generated by direct current voltage. This process would cause protein to be charged in lipid bilayer of the cell membrane to move and pores were formed in the membrane (Zimmerman and Vienken, 1982). The activation oocytes occurred when the extracellular Ca^{2+} transient elevating the $[(Ca^{2+})_i]$ through the pores during generating the electric field. The process will take about 10 to 40 minutes at 37°C for pore to repair and will take longer duration if the temperature is lower (Bates *et al.,* 1987). This protocol has been used in activating porcine oocytes after nuclear transfer (Tian *et al.,* 2006).

Temperature, together with fluidity of protein and lipids in the membrane will determine the time require for restoring membrane integrity (Zimmermann *et al.,* 1985). When rabbit oocytes are pulsed in the presence of lithium (which prevents the production of IP_3) oocyte activation is inhibited (Ozil, 1990). This suggests that electrical stimulation induces the production of IP_3 that leads to intracellular Ca^{2+} release (Alberio *et al.,* 2001). Combined treatment of IP_3 electroporation in Ca^{2+} or Mg^{2+}-free medium followed by 6-DMAP had successfully activated parthenogenetic and NT rabbit oocytes (Mitalipov *et al.,* 1999).

2.6.4.3 Mechanical activation

Ca^{2+} level can be increased by mechanical disruption of oocyte plasma membrane using a fine needle. Study done by Kawamura (1939) proved that plasma membrane of frog oocytes that were disrupted mechanically could initiate development. Mechanical activation can also be done by inducing the oocytes with the injection needle such as during the ICSI procedure. The injection of sperm into oocyte during ICSI was found to lower the MPF activity of oocyte after ICSI. Other than that, using the Sham-injection or Piezo-drive injection is another alternative to increase the oocyte activation and cleavage rates. Previous research has been reported in murine (Kimura and Yanagimachi, 1995), caprine (Wang *et al.,* 2003) and bovine (Katayose *et al.,* 1999; Wei and Fukui, 2002).

Microinjection of Ca^{2+} is another way to increase $[(Ca^{2+})_i]$ using mechanical stimuli. Studies showed that microinjection of Ca^{2+} are effective in activating porcine oocytes where it shows similarity in event presence during fertilisation when injecting with $CaCl_2$ (Macháty *et al.,* 1996). In addition, temperature is also another type of physical stimuli where Stice *et al.* (1994) exposed oocytes to room temperature before nuclear transfer procedure to activate oocytes.

2.6.5 Constraints in Artificial Activation

Some drawbacks are cannot be avoided when activating oocytes artificially. One of the important contributing factors is the age of oocyte which related to the time of maturation (Presicce and Yang, 1994a; Bordignon and Smith, 1998). Activation was found to be easily achieved in aged oocytes due to the spontaneous reduction on MPF activity with aging of oocytes (Kikuchi *et al.,* 1995; Wu *et al.,* 1997) and changes in sensitivity of MII oocytes to the internal calcium perturbation provoked by an artificial stimulus (Kubiak, 1989).

However, aging also have a negative effects on oocyte itself. Negative cytoplasmic changes in oocyte causes alterations in components of the cytoskeleton (Collas and Robl, 1990; Kim *et al.,* 1996; Adenot *et al.,* 1997), impairs enucleation through changes in the location and organisation of the second meiotic spindle (Takano *et al.,* 1993; Dominko *et al.,* 2000), which later on affects the embryonic development after fusion (Collas and Robl, 1990; Adenot *et al.,* 1997; Wu *et al.,* 1997; Bordignon and Smith, 1998; Liu *et al.,* 1998b) and increases the frequency of fragmentation and caspases activation which responsible for cell apoptosis (Gordo *et al.,* 2000). Previous studies demonstrated that the dependence on oocyte age for parthenogenetic activation can be reduced by promoting multiple intracellular calcium pulses (Vitullo and Ozil, 1992) or by combining different artificial activation stimulus (Yazawa *et al.,* 1997; Liu *et al.,* 1998a).

Ware *et al.* (1989) and Tsunoda and Kato (1993) suggested that the response of young oocytes to parthenogenetic activation is low in certain species and aged oocytes are often used as recipient oocytes for nuclear transfer of embryonic nuclei (Takano *et al.,* 1993). The chance of aged NT oocytes develop into blastocyst is higher than that of young oocytes in mouse (Taniguchi *et al.,* 1996) and rabbit (Adenot *et al.,* 1997). Contradictorily, there are reports indicating the lower developmental potential of aged bovine (Takano *et al.,* 1996) and murine (Liu *et al.,* 2007) oocytes after parthenogenetic activation compared to the young oocytes.

2.7 *IN VITRO* CULTURE (IVC)

There are various factors that affect the performance of IVC which later on affect the success rate of oocyte and embryo developments either direct or indirect ways. These factors are donor strain, *in vitro* culture media and *in vitro* culture system which included

the pH, temperature, osmolarity, water quality, volume of the culture medium and embryo density, superovulation quality as well as the activation treatment used.

2.7.1 Donor Strains

Different strains of donor have proven to be crucial components in superovulation responses, *in vitro* fertilisation (IVF) as well as embryonic development of parthenotes. As for murine, it shows that 96% of hybrids of B6CB F1 oocytes recovered after 15 hours of post-hCG which are treated with 10 mM of $SrCl_2$ for two hours exhibit similar results with Kunming murine oocyte which was treated with the same activation procedure (Bos-Mikich *et al*, 1995a). This will then affect the blastocyst rate (Mizutani *et al.,* 2004).

Mizutani *et al.* (2004) reported that genetic background also can affect parthenogenetic activation of oocyte response *in vitro.* Though the activation treatment used is similar, different strain will result in different response towards the treatment (Kato *et al.* 2001). Similar findings were also reported on rat, where is shows that oocytes from Sprague-Dawley (SD) rats were more readily activated in 1.25 mM strontium for six hours treatment than Wistar strain (Kato *et al.,* 2001).

2.7.2 *In Vitro* Culture Media

van der Valk *et al*. (2010) reported that almost every cell type has its own requirements concerning medium supplements. Different cell types have different receptors involved in cell survival, growth and differentiation and release different factors to their environment. To achieve optimal experimental results, the composition of the culture medium is essential. The simplest medium is the classical Ringer‟s solution (Ringer and Buxton, 1887), which was developed as a solution with optimal concentrations of different salts to preserve frog heart muscle tissue. In order to maintain cells and tissues for longer periods

of time, the medium should contain components such as nutrients and pH buffering substances. This type of medium was later formulated by Harry Eagle, who developed Eagle‟s minimal essential medium (MEM) which also contained amino acids, glucose and vitamins (Eagle, 1955).

Mammalian preimplantation embryos have species-specific stages when they are very sensitive to the culture environment and will often fail to develop if exposed to inappropriate conditions (Bavister, 1995). Up to date, recent studies of preimplantation embryos have overcome this block phenomenon in many species, such as murine, hamster, rat, porcine, bovine and ovine, resulting in an increased number of blastocysts rate *in vitro* (Bavister, 1995).

There were various types of chemical semi-defined and defined media used as a basal culture medium depending on the species. For examples, Chatot-Ziomek-Bavister (CZB) (Chatot *et al.,* 1989; Ellington *et al.,* 1990), CR1aa and CR2 (Rosenkrans and First, 1991), potassium Simplex Optimisation Medium (KSOM) (Erbach *et al.,* 1994), G1.2 and G2.2 (Gardner, 1994), Synthetic Oviductal Fluid (SOF) (Tervit *et al.,* 1972; Krisher *et al.,* 1999), BECM (Dobrinsky *et al.,* 1996; Lim *et al.,*1999), G1 (Krisher *et al.,* 1999) and IVD101 (Abe and Hoshi, 2003).

Several reports has shown that in order to achieve successful embryonic development, oocytes which are treated in Ca^{2+}/ Mg^{2+}-free CZB medium can be used to activated enucleated oocytes (Takeuchi *et al.,* 2002). Such condition plays vital role commonly in oocytes treated with strontium, as to prevent excess amount of extracellular calcium ion from interfering the action of strontium in inducing $(Ca^{2+})_i$ (Takeuchi *et al.,* 2002).

Another important component in culture media is serum which the function is to maintain and proliferate cells. FBS and bovine serum albumin (BSA) commonly serve

most purposes of the standard cells necessity. FBS and BSA are a complex mixture of different factors and contains a large number of components which make it essential for the growth and maintenance of cells like growth factors, energy substrates, proteins, vitamins, trace elements, hormones and amino acids (van der Valk *et al*., 2010). In addition, it shows that presence of BSA and 0.5 mg/ml of hyaluronic acids in WM medium also improved the development to blastocyst stage in porcine oocytes with lower degenerated embryos observed (Miyano *et al.,* 1994).

The toxic heavy metal and free radical production may remove and prevent from IVC medium by using the antioxidant. The ethylene diaminetetraacetic acid (EDTA) could be used for this purpose (Nasr-Esfahani and Johnson, 1992). Moreover, study in ICR strain mouse oocytes culture shows that EDTA in WM medium also improved the success rate of development to blastocyst stage up to 70%. Similar results were observed in C57BL/6 mouse strain oocytes with 65-90% of oocytes successfully develop to blastocyst stage. However, without EDTA, ICR oocytes which developed to blastocysts stages are decreased to 15-30% (Abramczuk *et al.,* 1977). Other antioxidants could be used are thioredoxin and superoxide dismutase (Nonogaki *et al.,* 1991), catalase (Nasr-Esfahani and Johnson, 1992) and vitamin (C and E) (Vermeiden and Bast, 1995).

2.7.3 *In Vitro* Culture System

Culture medium is the place where all the oocytes/embryos get the needed substances, chemicals and ion concentrations from. Many oocyte damages and arresting stages effects the embryonic developments of the matured oocyte was due to the extreme exposure of some chemicals or pH levels which brought to the oocytes by the cultured medium (Hannoun *et al.,* 2010).

Mammalian cells are generally grown under well-established conditions in incubators, where the temperature is typically kept at 37°C with a controlled humidified gas mixture of 5% CO_2 and 95% O_2. The physical nature of embryo culture, temperature, pH, osmolarity, oxygen concentration, degree of humidification, oil used and embryo density are varied among laboratories.

2.7.3.1 Temperature

The suitable temperature was obtained in many previous research is 37°C in the IVF cases with follicular fluid temperatures between 36.4 to 36.9˚C during egg retrieval had higher rates of blastocyst development, rates of embryo implantation and live birth success rates compared to cases that had lower or higher or much lower temperatures. The well-established optimal temperature for culturing mammalian embryos is around 37°C (Lenz *et al.,* 1983). However, in bovine oocytes it showed that oocytes which are incubated at higher than 39°C temperature has higher viability compared than those being incubated at 35°C and 37°C. Besides, bovine oocytes which are incubated at 39°C also showed higher number of oocytes that develop from 1-cell to blastocysts stage (Staigmiller, 1988). As the temperatures of media were different between species, we use 37.5°C and 38.5°C as the optimal incubation temperature for murine and caprine/bovine respectively.

2.7.3.2 pH

Each organism has a specific range of pH levels they can survive. pH is an important parameter which effect on embryonic development. Any sudden changes in pH level can harm organisms. Due to the limited ability of embryos to regulate their internal pH, an optimal external pH of culture is vital during embryos culture (Swain, 2010). This was the reason why all the living cells, tissues and organs have constant level of pH along the life

and during the development processes. Studies showed that when bovine oocytes placed in acidic pH, the rate of blastocyst development decreases, which suggest a suitable pH is important for both laboratory procedures as well as for embryo quality (Swain, 2010 and Ocon and Hansen, 2010). The optimum pH for embryo culture as conducted by Iwamatsu and Chang is pH 6 to 7 (Iwamatsu and Chang, 1971). Furthermore, Ikuta *et al.* (2002) also reported that the optimal and suitable pH level for the living cell is 6 to 7. However, in our lab, the pH of IVC medium was maintained at range 7.2 to 7.4 for murine, caprine as well as bovine embryo cultivation.

2.7.3.3 Water quality

Water is the most crucial component in preparing culture medium for embryonic development (Fukuda *et al.,* 1987). Besides, the basis of all culture media is water (Loutradis *et al.,* 2006). Therefore, uncontrolled water quality will have a detrimental effect on embryonic development. A research was conducted on bovine oocytes where oocytes were cultured in four different types of water preparation which are tap water, deionized water, twice-distilled water and purified water using Milli-Q system. The result obtained shows that a higher embryonic development to blastocyst in media prepared with Milli-Q water compared to other three types of water preparation (Nagao *et al.,* 1995). In addition, the duration of storage of water also plays an important role. Study conducted by Nagao *et al.* (1995) shows that a higher frequency in embryos development to blastocyst when oocytes were cultured in media with freshly prepared Milli-Q compared to the oocytes cultured in media prepared by Milli-Q stored for one week. Also, fresh Milli-Q water has the lowest organic compounds concentration, total organic carbon, endotoxin concentrations and electrical conductivity (Nagao *et al.,* 1995; Elsheikh *et al.,* 2003).

2.7.3.4 Osmolarity

Although mammalian cells express a reasonable wide tolerance to osmolarity, osmolarity should always be carefully checked and coordinated properly in order to provide the optimal environment for development. The osmolarity of media was kept at 270 to 280 mOsm/kg (Tervit *et al.,* 1972; Younis *et al.,* 1991) or 280 to 300 mOsm/kg (Brackett and Oliphant, 1975; Takahashi and First, 1992).

2.7.3.5 Volume of the culture medium and embryo density

In order to support *in vitro* development, embryos from laboratory animals (murine, rats, hamsters, rabbits) and domestic animals (bovine, caprine, ovine) have been empirically cultured in drops of medium (e.g., 50 to 200 µl) covered with mineral oil. Recent studies in murine and ovine showed that the volume of medium and embryo density (number per unit volume) are important factors that influence early embryo development (Wiley *et al.,* 1986; Lane and Gardner, 1992; Gardner *et al.,* 1994; Kato and Tsunoda, 1994). Embryo development was found stimulated when zygotes or embryos were cultured in a small volume of medium (10 µl) with high embryo density in these species. Some investigators have interpreted this as indicating that embryos secrete a stimulators that acts on the embryos in an autocrine and/or paracrine manner. The concentration of the putative stimulator may be maintained high enough to affect embryo development in a small drop (Kite *et al.,* 1997).

2.7.3.6 Superovulation quality

Superovulation is a very important method in order to obtain large numbers of good quality oocytes. It is necessary to be done very carefully to successfully get the needed amount of oocytes which can later on proceed to the various experiment needed. Any

disturbance of superovulation process either directly or indirectly can negatively impact the quality of the oocytes obtained.

Chapter 3

3.0 MATERIALS AND METHODS

Chapter 3

3.0 MATERIALS AND METHODS

3.1 GENERAL INTRODUCTION

In this present research, there were 3 main laboratories used to conduct the experiments, namely Animal Biotechnology-Embryo Laboratory (ABEL), Embryo Micromanipulation Laboratory (EMiL) which both were located at the Institute of Biological Sciences (ISB) and Nuclear Transfer and Reprogramming Laboratory (NaTuRe) located at the Institute of Postgraduate Studies (IPS) at the University of Malaya. There were 3 species of experimental animals used in this study, which were murine, caprine and bovine species. Murine species, used as model animals, were bred at the Animal House, ISB Mini Farm (ISBMF). Caprine oocyte samples were obtained from the ISBMF whereas for bovine samples, the ovaries were collected abattoirs of the Department of Veterinary Services Abattoir Complex at Shah Alam and Senawang. This research was conducted from November 2009 to April 2012.

3.2 MATERIALS

The materials used in this present research include various facilities, experimental animals, equipment, instruments, labwares, glasswares, disposable materials, chemicals, reagents and media.

3.2.1 Facilities

The facilities used in this study included Animal House which supplied murine as experimental animals; Department of Veterinary Services Abattoir Complex at Shah Alam and Senawang as a source of ovary samples of bovine; ISBMF which was a source of live

experimental caprine; NaTuRe Laboratory for laparoscopic oocytes pick-up (LOPU) surgery and ovariectomy; ABEL and EMiL Laboratory for parthenogenetic experiments.

3.2.2 Experimental Animals

In this study, there were 3 experimental animals used which were murine, caprine and bovine species. For murine, there were 3 different strains used which were ICR strain (white), C57BL/6J strain (black) and CBA/ca strain (brown). Only murine females, aged between 6 to 8 weeks, were used as oocyte donors for the model experiments. The murine were kept in a clean cage with sawdust as bedding purpose and separated based on its gender, strain and age. Some were mixed between male (8 to 10 weeks) and female (6 to 8 weeks) with ratio of 2:3 male to female to allow mating in order to provide more offspring for experiments. The pregnant females were separated into a single cage until the pups were born and weaned about 1 month old, sorted according to gender. The condition of breeding area in the Animal House was maintained with suitable temperature with natural light and dark cycles. Natural light period was approximately 12 hours (0600 to 1800 hours) per day, while the natural dark period was approximately 12 hours (1800 to 0600 hours) per day. Besides, Animal House was also maintained with sufficient air supply as well as good ventilation. The murine were provided with commercial feed pellet and clean drinking water *ad libitum.* They were checked from time to time to ensure enough feed and clean drinking water.

Whereas for caprine, Jamnapari crossbred, Boer crossbred and local mixed breed does age ranging from 12 to 60 months old were used in this research. For LOPU, the does were treated for oestrus synchronisation and superovulation, according to specific regiment before surgery was performed. Oocytes were collected from 2 ovaries from each doe.

3.2.3 Equipment and Instruments

The model number, manufacturer`s and supplier`s name for all the equipment used in the present study are listed in Appendix Table 1.1. The commonly used equipment includes flushing and aspiration system (Cook Australia, Australia), laparoscopic system (Aesculap A.G. and Company, Germany), surgical set (Aesculap A.G. and Company, Germany), inverted microscope (Olympus Optical CO., Ltd., Japan) with micromanipulators (Narishige Co., Ltd., Japan), laminar flow (Gelman Sciences Pty. Ltd., Australia), stereomicroscope (Olympus Optical Co., Ltd., Japan), stage warmer (Linkam Scientific Instrument, England), La banana (self-assembled), and micropipette dispenser (Eppendorf, Germany), CO_2 incubator (Kendro Laboratory Products, Germany), autoclave (Hirayama Manufacturing Corporation, Tokyo, Japan), oven (Memmert GmbH, Germany), water bath (Memmert GmbH, Germany), ultrapure water system (Millipore, USA), pH meter (Hanna Instruments, Singapore), osmometer (Wescor, Inc., USA), centrifuge machine (Kendro Laboratory Products, Germany), liquid nitrogen tank (MVE, USA) and fluorescent microscope (Carl-Zeiss Inc., Germany).

3.2.4 Labwares, Glasswares and Disposable Materials

The model number, manufacturer`s and supplier`s name for each of the labwares, glassware and disposable materials used in this study are presented in the Appendix Table 1.2. Some of the common disposable materials used were graduated test tubes (14 ml, Becton Dickinson Labware, USA), Nunc 4-well dishes (Nunc, Denmark), small polysterene culture dish (11 mm; Becton Dickinson Labware, USA), medium polystyrene culture dishes (35 mm; Becton Dickinson Labware, USA), syringe (Terumo Corporation, Japan) and aluminium foil (Reynolds Consumer,USA).

3.2.5 Chemicals, Reagents and Media

All chemicals, reagents and media used were purchased mainly from Sigma-Aldrich Co., USA. The model number, manufacturer‟s and supplier‟s name for all the chemicals, reagents and media involved in this research are listed in Appendix Table 1.3. The pH for all the media prepared were adjusted to pH 7.2 to 7.4, while the osmolarity was adjusted to 280 to 285 mOsm.

3.3 METHODS

The methodology section was divided into 4 main sub-sections: (1) preparation of ambience for a successful *in vitro* production (IVP); (2) preparation of hormones, stocks and media; (3) preparation of microtools; and (4) experimental procedures. In order to provide easy description, the sub-sections were then further sub-divided into sub-sub-sections.

3.3.1 Preparation of Ambience for a Successful *In Vitro* Production (IVP)

Before conducting any IVP experiments involving *in vitro* maturation, *in vitro* fertilisation and parthenogenesis, it is very vital to ensure that the laboratory follows a strict cleanliness and sterile protocols throughout the experiments. In other words, to prevent contamination by bacteria and fungus on every culture, cleanliness and sterilisation is very important right from the commencement of the experiments. Besides, the laboratory must also be in accident-free environment to prevent untoward circumstances as well as to ensure successful outcomes in IVP. Moreover, it is also important to minimise potential infection and contamination from any sources. Every laboratory user is responsible to keep the laboratory and equipment inside in a very good condition and maintain it throughout the experimental duration, especially when handling embryo culture which can be easily

contaminated. In this research, all necessary preparations were taken before conducting IVP or parthenogenesis experiments as described below.

3.3.1.1 Water quality

All the media used in this study were self-prepared using ultrapure water as a based medium. Thus, it is important to have a reliable source of ultrapure water system. This is because ultrapure water is necessary to prevent bacterial contamination from surrounding environment. To prepare all the media, we used ultrapure water system (Milli-Q UF Plus) which included particular filtration, activated carbon cartridge filtration, reverse osmosis (RO) and electrodeionisation (EDI), ultraviolet oxidation system followed by Milli-Q UF Plus of at least 18 MΩ cm. The filtration of the ultrapure water system was through a membrane filter (0.22 μm) to eliminate trace particles as well as to prevent bacterial contamination in the environment from contaminating the media.

3.3.1.2 Air quality

Similar to water quality, air quality also plays an important role to ensure success in every IVP experiment. This is because the incubator used in the laboratory obtained air directly from the laboratory itself. Besides, since oocytes have no defense mechanism like other organism, whose first line of defense against any environmental hazards are vulnerable, thus it is very critical to maintain good air quality within the laboratory itself.

Some of the most common air pollutants as described by Elder and Dale (2000) include 1) volatile compounds which are released by cleaning procedures such as ethanol, besides which is being produced by laboratory instruments such as microscopes; 2) small inorganic molecules such as sulphur dioxide (SO_2) and carbon monoxide (CO); 3) substances from building materials such as aldehydes from flooring adhesives and 4) other

polluting compounds which may release from perfume or even propellant aerosols. Therefore, it is very important to minimise the level of air pollution in the laboratory. In our laboratory, we ensured that the air quality was conducive to embryo development.

3.3.1.3 General cleanliness and sterilisation of research laboratory

All the media and solutions used for experiments in this study were prepared in laminar flow work station (Gelman Sciences, Australia) under fluorescent light when it is necessary.

Before conducting any experiment in the laminar flow work station, the surface of the laminar flow work station, bench work, microscopes, microscopes stages and other equipment such as micropipettes were wiped with ethanol (70%; HmBG Chemicals, Germany). Any residual ethanol left was allowed to evaporate for at least 20 minutes before conducting experiments. The bottles containing reagents and media, and apparatus such as glass pipettes were flamed before used to make it more sterile. Any spillage were wiped with dry tissue and if necessary, with 70% ethanol. After completing experiment, the surface of laminar flow work station and microscopes stages were wiped once again with 70% ethanol. Water bath (Memmert GmBh, Germany) was also cleaned and the water was changed frequently.

All labwares and Pyrex glasswares used for medium preparation and surgical instruments were initially soaked in 1% v/v 7X detergent (Linbro, Flow Laboratories, Australia) for about half an hour before scrubbed thoroughly using a brush or sponge and then washed under running tap water. The glasswares and surgical instruments were then rinsed thoroughly with reverse-osmosis (RO) water (Milli-Q System, Millipore Products, USA). After that, the cap of glasswares were loosely placed and covered with a layer of aluminium foil. Autoclave tape was placed onto the aluminium cover for identification

later. On the other hand, conical tubes and micropipette tips were placed in an autoclave bag and sealed tightly, and a piece of autoclave tape was placed on the autoclave bag. All of these instruments and glasswares then were autoclaved (Hiclave Model HA-300M11, Hirayama, Japan) for 20 minutes at 121°C with pressure (15 lb/sq in) and later dried in an oven (60°C) before stored in a cool, dry plastic container at room temperature (25ºC). After autoclaving was finished, the glassware caps were tightened a little bit to prevent any contaminants from entering. As a precaution, the caps were not tightened completely until the glasswares had cooled to prevent a vacuum forming in the glasswares.

Pre-packed disposable and sterile Falcon tubes (Becton Dickinson, USA) plastic Petri dishes and pipettes were used for conventional tissue culture and manipulation. Rubber tubing of La banana, which was connected to the Pasteur pipette, was sterilised with alcohol (70%) first before leaving it in UV light for 15 to 30 minutes for sterilisation purposes.

3.3.1.4 Maintenance of carbon dioxide (CO_2) incubator

In vitro culture of embryos needs to be conducted in CO_2 (5%) incubator to maintain the pH at 7.2 to 7.4 in bicarbonate-buffered culture medium system. The CO_2 incubator (HERAcell 240; Kendro Laboratory Products, Germany) was carefully calibrated and controlled by monitoring it regularly and the LED display of temperature was checked with independent readings. It is vital to maintain the temperature at 38.5ºC for caprine and bovine samples whereas for murine sample the optimum temperature was 37ºC throughout the culture to obtain the highest cleavage rates. Besides CO_2 (5%) in humidified air, triple gas mixture of CO_2 (5%), O_2 (5%) and N_2 (90%) gas phase was needed to increase the optimum environment for embryos development *in vitro*.

Basically, the CO_2 incubator was cleaned at least once a month. The cleaning regime of CO_2 incubator includes wiping the inside walls, doors and racks with sterile RO water using sterile towel. The water in the incubator was changed and the tray was filled with sterile RO water during every cleaning. Moreover, the process of repeating opening and closing of the incubator door was kept to minimum, if possible, in order to stabilise the culture environment inside the incubator.

3.3.1.5 Mineral oil and silicone oil

It is very important to cover the microdroplets when culturing oocytes and embryos. There were various types of oil that were used to overlay the microdroplets which include mineral oil, paraffin oil and silicone oil. In this study, mineral oil (M8410; Sigma-Aldrich Co., USA) was used to overlay *in vitro* maturation, parthenogenetic activation culture and *in vitro* culture microdroplets for caprine and bovine. However, silicone oil (SI380-00; Systerm®, Malaysia) was used for parthenogenetic activation culture and *in vitro* culture microdroplets for murine.

The reason of using mineral oil and silicone oil is because they have a very clear appearance as well as less toxic to oocytes and embryos. Thus, usage of equilibrated mineral oil and silicone oil allow good results to be achieved, beside able to maintain the quality laboratory environment. The main purpose of covering the microdroplets by overlaying the equilibrated mineral oil or silicone oil is to prevent evaporation and delay gas diffusion as well as to stabilise the pH, temperature and osmolarity of the microenvironment surrounding the oocytes and embryos during taking outside the CO_2 incubator. Besides, these oils can serve as physical barrier by separating the microdroplets from atmosphere and also airborne particles or pathogens.

3.3.2 Preparation of Hormone

Each experiment conducted requires a large numbers of oocytes at one time to save time, energy, materials and also chemicals. Gonadotrophins such as pregnant mare`s serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) were the hormones used to induce the superovulation in caprine and murine donors. PMSG mimics the endogenous effect of follicle stimulating hormone (FSH), whereas hCG mimics the effect of luteinising hormone (LH) in the donor to increase the rate of ovulation.

The importance of administered the hormone treatment in two folds, as not only to increase the rate of ovulation in female donors, but similarly to control the timing of ovulation of female donors, so that it is independent from the natural oestrous cycle. Hormones prepared were filter-sterilised using Millipore filter (0.22 μm) to prevent contamination. Moreover, the hormones were kept frozen at -20˚C and thawed at room temperature prior used.

3.3.2.1 Preparation of pregnant mare's serum gonadotrophin (PMSG)

PMSG is a natural glycoprotein which consists of two sub-units (alpha and beta). PMSG (Folligon, Intervet International B.V., Boxmeer, Holland) has FSH-like and LH-like activities. Besides, PMSG also mimic the endogenous effect of FSH. PMSG was administered in order to increase number of follicles, as well as to stimulate the ovarian function and activity of the female donor. Furthermore, it also helped to improve fertility in oestrus-synchronised animals as well as to induce superovulation.

PMSG was presented as white freeze-dried crystalline powder (1000 IU per vial). As for the preparation of PMSG for caprine donor, 2 vials of PMSG (1000 IU x 2) were dissolved in sterile solvent (2 ml each vial) which comes along with PMSG packaging. The solution was mixed thoroughly by sucking in and out several times. 3 ml of the solution

was sucked into 5 ml syringe to make the concentration at 1500 IU and stored at -20˚C until used. The rest of the solution was sealed and kept frozen. As for the preparation of PMSG for murine, 1 vial of PMSG was mixed thoroughly with 20 ml of sterile solvent by sucking in and out few times to ensure all of the crystalline powder mixed perfectly. The PMSG solution then was sucked into 1 ml syringe 1 ml each, to make 20 syringes where the concentration now is 50 IU each syringe. All the syringes then kept frozen at -20˚C until used. PMSG can be stored up to 3 months without deterioration in quality of the hormone.

3.3.2.2 Preparation of human chorionic gonodotrophin (hCG)

hCG was used to increase the ovulation rate in the female murine donor. hCG (Chorulon, Intervet International B.V., Boxmeer, Holland) was administered to the female donor because it mimics the effect of LH. hCG was presented as white freeze-dried crystalline powder (5000 IU per vial). hCG was prepared by dissolving 1 vial of hCG into 100 ml of sterile solvent which comes along with hCG packaging. The solution was mixed thoroughly by sucking in and out few times to ensure all of the crystalline powder mixed perfectly. The hCG solution then was sucked into 1 ml syringe 1 ml each, to make 100 syringes where the concentration now is 50 IU each syringe. All the syringes then kept frozen at -20˚C until used. Similar with PMSG, hCG also can be stored in this form for three months without deterioration in its quality.

3.3.2.3 Preparation of ovidrel

Ovidrel™ (Merck Serono S.p.A., Switzerland) is a gonadotrophin ovulation stimulator which is used to increase the ovulation rate in the female caprine donor. It has a similar function as hCG. Ovidrel was administered to the female donor because it mimics

the effect of LH. Ovidrel was presented in a pre-filled syringe as a ready-made solution (250 µg/0.5 ml). Ovidrel was prepared by mixing 0.5 ml ready-made ovidrel into of Milli-Q water (25.5 ml) to make a total 26 ml solution. The solution was mixed thoroughly by sucking in and out few times to ensure all of the crystalline powder mixed perfectly. The ovidrel solution then was sucked into 1 ml syringe 1 ml each, to make 26 syringes where the concentration now is 250 IU each syringe. All the syringes then kept frozen at -20˚C until used. Similar with PMSG, ovidrel also can be stored in this form for 3 months without deterioration in its quality.

3.3.3 Preparation of Stock Solutions and Media

All of the media used this study were freshly prepared before each experiments in laminar flow work station, to ensure they were in a fresh and sterile condition. For sterility purpose, purified Milli-Q water was used as a base medium with the aim to prevent contamination. In order to measure the chemicals in powder form, digital analytical balance (Model AB204, Mettler Toledo, Switzerland) was used, whereas the chemicals in liquid form were measured using sterile disposable plastic pipette.

As for the pH of the medium, it was adjusted to pH 7.2-7.4 using pH meter (HI 8417, HANNA Instruments, Singapore). Besides keeping the chemical weight precise and also maintaining the pH of the chemical and medium, it is also important to maintain the osmolarity of the medium. The osmolarity of medium was adjusted to 280 to 285 mOsm using osmometer (Model 5500, WESCOR Instrument, USA). In order to prevent the medium from contamination, the medium was sterilised by filtration using disposable syringe-driven Millex©-GS filter (0.22 µm; Schleicher and Schuell, Germany). The entire medium prepared was either stored in bottles (100 to 1000 ml) or aliquot in microcentrifuge tubes (0.2 to 1 ml; Eppendorf GmbH, Germany) and they were kept in a

refrigerator at (4˚C) or freezer (-20˚C) as needed. Media without addition of bovine serum albumin (BSA) can be kept for 3 or 6 months. On the other hand, media with addition of BSA had to be used within a week, and was stored in a refrigerator at 4˚C.

3.3.3.1 Preparation of stocks and media for murine samples

3.3.3.1 (a) *Preparation of modified hepes Whitten's medium (HWM) stock solution*

Modified HWM medium was used as a holding medium in murine species for manipulation outside the incubator such as collecting oviduct, washing, rinsing oocytes after oocytes retrieval. Modified HWM medium was actually based on the HWM medium prepared by Whittingham (1971) which later was modified by Whitten (1971). Table 3.1 represents HWM medium stock preparation:

Table 3.1: Composition of modified HWM stock solution

Component	Sigma Cat. No.	g/100 ml
NaCl	S5886	0.5140
KCl	P5405	0.0356
KH_2PO_4	P5655	0.0162
$MgSO_4.7H_2O$	M8150	0.0294
$NaHCO_3$	S5761	0.0304
Na Hepes	H3784	0.6508
Ca Lactate	L4388	0.0338
Na Lactate (60% syrup)	L7900	0.3700 ml
Na Pyruvate	P4562	0.0029
D-Glucose	G6152	0.1000
Penicillin G	PENNA	0.0075
Streptomycin	S9137	0.0050
Phenol Red	P3532	0.0010
BSA	A7906	0.3000
Autoclaved Milli-Q water		100 ml
Total volume		100 ml

The stock solution was checked for pH (7.25±0.5) and osmolarity (255 to 270 mOsm). The stock was then filter-sterilised using syringe-driven Millex©-GS filter (0.22 µm) into sterile bottles (100 ml) and stored (4ºC) for 6 months. The stock medium needs to be supplemented with the following chemicals before being used:

Table 3.2: Composition of modified HWM working solution

Component	**Amount/10 ml**
HWM stock solution	10 ml
BSA (Sigma; A7906)	0.03 g
Total volume	10 ml

Modified HWM working solution was filter-sterilised by Millex©-GS filter (0.22 µm;) into centrifuge tube (15 ml; Becton Dickinson, USA) and warmed up in a water bath (37°C) before used. However, it only can be stored up to 1 week in a refrigerator (4°C).

3.3.3.1 (b) *Preparation of modified Whitten's medium (WM) stock solution*

Modified WM medium was prepared based on Whitten‟s medium (Whitten, 1971) with some modification. Modified WM medium was used as *in vitro* culture medium in murine species as well as a base medium in parthenogenetic chemical preparation. Table 3.3 represents WM medium stock preparation:

Table 3.3: Composition of modified WM stock solution

Component	**Sigma Cat. No.**	**g/100 ml**
NaCl	S5886	0.5140
KCl	P5405	0.0356
KH_2PO_4	P5655	0.0162
$MgSO_4.7H_2O$	M8150	0.0294
$NaHCO_3$	S5761	0.1900
Na Hepes	H3784	0.6508

(continued)

(continued)

Ca Lactate	L4388	0.0338
Na Lactate (60% syrup)	L7900	0.3700 ml
Na Pyruvate	P4562	0.0029
D-Glucose	G6152	0.1000
Penicillin G	PENNA	0.0075
Streptomycin	S9137	0.0050
L-Glutamine	G3126	0.0146
Taurine	T0625	0.0125
Phenol Red	15100-43	0.0010
BSA	A7906	0.3000
Autoclaved Milli-Q water		100 ml
Total volume		100 ml

The stock solution was checked for pH (7.25±0.5) and osmolarity (255 to 270 mOsm). The stock was then filter-sterilised using syringe-driven Millex©-GS filter (0.22 µm) into sterile bottles (100 ml) and stored (4ºC) for 6 months. Modified WM stock solution needs to be supplemented with the following chemicals before being used:

Table 3.4: Composition of modified WM working solution

Component	**Amount/10 ml**
WM stock solution	10 ml
BSA (Sigma; A7906)	0.03 g
Total volume	10 ml

Modified WM working solution was filter-sterilised by Millex©-GS filter (0.22 µm) into centrifuge tube (15 ml) and preincubated for at least 3 hours in CO_2 incubator (5%) at 37°C before used. However, it only can be stored up to 1 week in a refrigerator (4°C).

3.3.3.1 (c) *Preparation of Chatot, Ziomek and Bavier (CZB) Ca^{2+} free activation medium*

Basically, CZB Ca^{2+} free activation medium was formulated by Chatot *et al.* (1989). CZB Ca^{2+} free activation medium was used as a base medium in parthenogenetic chemical

preparation for strontium chloride ($SrCl_2$) and cytochalasin B (CB) combination in murine. Table 3.5 represents CZB Ca^{2+} free activation medium stock preparation:

Table 3.5: Composition of CZB Ca^{2+} free activation medium stock

Component	Sigma Cat. No.	g/100 ml
NaCl	S5886	0.4790
KCl	P5405	0.0360
KH_2PO_4	P5655	0.0160
$MgSO_4.7H_2O$	M8150	0.0290
$NaHCO_3$	S5761	0.2100
Na Lactate (60% syrup)	L7900	0.5300 ml
D-Glucose	G6152	0.1000
Phenol Red	15100-43	0.0010
BSA	A7906	0.5000
Autoclaved Milli-Q water		99 ml
Total volume		100 ml

The stock solution was checked for pH (7.25±0.5) and osmolarity (255 to 270 mOsm). The stock was then filter-sterilised using syringe-driven Millex©-GS filter (0.22 µm) into sterile bottles (100 ml) and stored (4ºC) for 3 months. CZB Ca^{2+} free activation medium stock can be straight away mixed with $SrCl_2$ and CB in a proper amount and preincubated for at least 3 hours in CO_2 incubator (5%) at 37°C before used.

3.3.3.1 (d) *Preparation of parthenogenetic activation medium*

There were several types of chemical used as an activation medium in order to activate murine oocytes. All the preparations of stock medium were sub-divided as below.

3.3.3.1 (d) (i) *Preparation of cytochalasin B (CB) stock solution*

CB (Sigma; C6762) was one of the several options used to activate murine oocytes. It was used along with calcium elevator such as calcium ionophore (A23187), ionomycin (Iono) and ethanol (EtOH) as a combination treatment. Whole content of CB vial (1 mg) was

dissolved into DMSO (2 ml; Sigma; D8779) to make the intial concentration as 500 µg/ml and labeled as „Stock A". „Stock A" was aliquot (10 µl) into microcentrifuge tubes (1.5 ml), wrapped in aluminium foil and stored up to 6 months in a freezer (-20°C). On the day of experiment, one tube of Stock A was taken out from the freezer and equilibrated CZB Ca^{2+}-free medium (890 µl) and $SrCl_2$ (100 µl) were added and mixed well. The final concentration of this working solution now was 5 µg/ml and can be immediately used to make a microdroplet culture.

3.3.3.1 (d) (ii) ***Preparation of strontium chloride ($SrCl_2$) stock solution***

$SrCl_2$ (Sigma; 255521) was one of the several options used to activate murine oocytes. It was used along with CB as a combination treatment. There were five concentrations of $SrCl_2$ used in this experiment. $SrCl_2$ powder (0.0053, 0.0107, 0.0160, 0.0213 and 0.0266 g) was mixed with Milli-Q water (1 ml) separately in microcentrifuge tubes (1.5 ml) to make the initial concentration (20, 40, 60, 80 and 100 µM) respectively and labeled as „Stock A". „Stock A" was aliquot (100 µl) into microcentrifuge tubes (1.5 ml), wrapped in aluminium foil and stored up to 6 months in a freezer (-20°C). On the day of experiment, one tube of „Stock A" was taken out from the freezer and CB stock (10 µl) along with CZB-Ca^{2+} free activation medium (890 µl) was added and mixed well (the final concentration: 2, 4, 6, 8 and 10 µM). These working solutions can be immediately used to make a microdroplet culture. The culture dishes were pre-incubated in CO_2 incubator (5%) at 37°C for at least 3 hours before used. Table 3.6 shows the preparation of $SrCl_2$+ CB in CZB-Ca^{2+} free activation medium:

Table 3.6: Composition of CZB-Ca^{2+} free working solution

Component	Concentration	Amount/1000 µl
CZB-Ca^{2+} free activation medium		890 µl
$SrCl_2$ stock	20, 40, 60, 80 or 100 µM	100 µl
CB stock	5 µg/ml	10 µl
Total volume		1000 µl

3.3.3.1 (d) (iii) ***Preparation of calcium ionophore (A23187) stock solution***

Calcium ionophore A23187 (Sigma; C7522) was used as one of the several options to activate murine oocytes right after denuding the cumulus cells after IVM culture. A whole content of calcium ionophore bottle (10 mg) was dissolved into dimethyl sulfoxide; DMSO (32.8 ml; Sigma; D8779) to make the initial concentration as 500 µM and labeled as „Stock A". „Stock A" was aliquot (10 µl) into microcentrifuge tubes (1.5 ml), wrapped in aluminium foil and stored up to 6 months in a freezer (-20°C). On the day of experiment, one tube of „Stock A" was taken out from the freezer and equilibrated *in vitro* culture medium; WM (990 µl) was added and mixed well. The final concentration of this working solution was 5 µM and can be immediately used to make a microdroplet culture.

3.3.3.1 (d) (iv) ***Preparation of ethanol (EtOH) stock solution***

Ethanol (System ChemAR®; ET 150-50) was used as one of the several options to activate murine oocytes right after denuding the cumulus cells after IVM culture. The initial concentration of commercial absolute EtOH is 100%. Absolute EtOH needs to be stored in a refrigerator (4°C) and took out prior oocytes activation in order ensure that the final concentration was not deviated as the absolute EtOH easy to evaporate. Absolute EtOH (70 µl, 80 µl and 90 µl) was respectively dispensed into microcentrifuge tubes (1.5 ml) and mixed with equilibrated *in vitro* culture medium (WM; 930 µl, 920 µl and 910 µl) to make

the final concentration of EtOH (7%, 8% and 9%). EtOH working solution can be immediately used to make a microdroplet culture.

3.3.3.1 (d) (v) ***Preparation of 6-dimethylaminopurine (6-DMAP) stock solution***

6-DMAP (Sigma; D2629) was one of the several options used to activate murine oocytes. It was used along with calcium elevator such as calcium ionophore, ionomycin or ethanol as a combination treatment. Whole content of 6-DMAP bottle (250 mg) was dissolved into Milli-Q water (7.7 ml) to make the intial concentration as 200 mM and labeled as „Stock A". „Stock A" was aliquot (10 µl) into microcentrifuge tubes (1.5 ml), wrapped in aluminium foil and stored up to 6 months in a freezer (-20°C). On the day of experiment, one tube of „Stock A" was taken out from the freezer and equilibrated *in vitro* culture medium; WM (990 µl) was added and mixed well. The final concentration of this working solution was 2 mM and can be immediately used to make a microdroplet culture.

3.3.3.1 (d) (vi) ***Preparation of cycloheximide (CHX) stock solution***

CHX (Sigma; C4859) was one of the several options used to activate murine oocytes. It was used along with calcium elevator such as calcium ionophore, ionomycin or ethanol as a combination treatment. The initial concentration of CHX ready-made solution is 100 mg/ml in 1 ml vial. Whole content of CHX stock was dissolved into DMSO (100 ml; Sigma; D8779) to make the intial concentration as 1 mg/ml and labeled as „Stock A". „Stock A" was aliquot (10 µl) into microcentrifuge tubes (1.5 ml), wrapped in aluminium foil and stored up to 6 months in a refrigerator (4°C). On the day of experiment, one tube of „Stock A" was taken out from the refrigerator and equilibrated *in vitro* culture medium; WM (990 µl) was added and mixed well. The final concentration of this working solution was 10 µg/ml and can be immediately used to make a microdroplet culture.

3.3.3.2 Preparation of stocks and media for caprine and bovine samples

Basically, the media used in caprine IVP were similar as the media used in bovine IVP. Therefore, the preparation of all of the media was done once for both caprine and bovine usage.

3.3.3.2 (a) *Preparation of normal saline*

Basically, normal saline was prepared in a 1 L bottle (Duran®, Germany) by adding up sodium chloride and heparin in 1 L of Milli-Q water as listed in Table 3.7 below. Normal saline was inserted into animal body as a replacement of body fluid lost during surgery. Normal saline was autoclaved to make it sterile and kept in the refrigerator (4ºC) up to 3 months for future use and warmed in a water bath (38.5ºC) prior used.

Table 3.7: Composition of normal saline

Components	**Volume/weight**
Autoclaved Milli-Q water	1 L
Sodium chloride (Sigma; S5886)	9 g
Heparin (Sigma; H0777)	0.1 g
Total volume	1 L

3.3.3.1 (b) *Preparation of flushing medium for laparoscopic oocyte pick-up (LOPU)*

Flushing medium was used for flushing microvolumes of fluid into the ovarian follicle, and aspirated the oocytes together with fluid from the follicle which connected to a flushing and aspiration system. Flushing medium was prepared a day before oocytes retrieval by LOPU. The aspirated medium was collected in a flushing tube to be searched for oocytes under a stereomicroscope later. The flushing medium consisted of Dulbecco‟s phosphate buffered saline (DPBS) supplemented with several chemicals as listed in Table 3.8.

Table 3.8: Composition of flushing medium

Components	Volume/weight
Autoclaved Milli-Q water	1 L
DPBS (Oxoid; Dulbecco A, BR0014G)	10 tablets
Polyvinylpyrrolidone (Sigma; PVP360)	1 g
Penicillin-streptomycin solution (Sigma; P0930)	1 ml
Total volume	1 L

The flushing medium was filter-sterilised using syringe-driven Millex©-GS filter (0.22 μm) and kept in room temperature (25ºC). Prior to oocytes retrieval (LOPU), flushing medium was warmed in a water bath (38.5ºC) and aliquot into syringe (50 ml; Terumo Corporation, Japan) and maintained at this temperature.

3.3.3.2 (c) *Preparation of ovary collection medium*

Ovary collection medium was prepared purposely for washing and collecting ovaries from abattoir. Ovary collection medium was prepared similar as normal saline, except without heparin instead supplemented with penicillin-streptomycin solution (P0930; Sigma-Aldrich Co., USA). Penicillin-streptomycin (1 ml) solution was added to normal saline (1 L) to make the concentration of antibacterial solution as (1 ml/L). The medium was prepared one day earlier and kept in the refrigerator (4ºC) and warmed in a water bath (38.5ºC) prior used (Table 3.9).

Table 3.9: Composition of ovary collection medium

Components	Volume/weight
Autoclaved Milli-Q water	1 L
Sodium chloride (Sigma; S5886)	9 g
Penicillin-streptomycin solution (Sigma; P0930)	1 ml
Total volume	1 L

3.3.3.2 (d) *Preparation of ovary slicing medium*

Ovary slicing medium also known as TL-Hepes was used for washing, rinsing and during slicing of ovaries collected from abattoir as well as during searching the oocytes before transferring into the maturation medium. The TL-Hepes stock solution was prepared as below (Table 3.10):

Table 3.10: Composition of TL-Hepes stock solution

Components	**Sigma Cat. No.**	**Final Conc. (mM)**	**g/1000 ml**
NaCl	S5886	114	6.6600
KCl	P5405	3.2	0.2400
$NaHCO_3$	S5761	2.0	0.1680
$NaH_2PO_4.H_2O$	S9638	0.4	0.0560
Na Lactate (60%Syrup)	L7900	10.0	1.8600 ml
$CaCl_2.2H_2O$	C3881	2.0	0.3000
$MgCl_2.6H_2O$	M2393	0.5	0.1000
Hepes $C_8H_{17}N_2O_4SNa$	H0763 or H3784	10.0	1.2000
Hepes $C_8H_{18}N_2O_4S$	H3375 or H6147	10.0	1.2000
Penicillin G	PENNA	100 IU/ml	0.0650
Phenol Red	P3532		0.0050
Autoclaved Milli-Q water			1000 ml
Total volume			1000 ml

The stock solution was checked for pH (7.4±0.5) and osmolarity (255 to 270 mOsm). The stock was then filter-sterilised using syringe-driven Millex©-GS filter (0.22 µm) into sterile bottles (1 L) and stored (4ºC) for 1 to 2 weeks. TL-Hepes stock solution need to be supplemented with the following chemicals before being used (Table 3.11):

Table 3.11: Composition of TL-Hepes working solution

Components	Volume/weight
TL-Hepes stock solution	100 ml
Gentamicin stock solution	50 µl
Sodium Pyruvate (Sigma; P4562)	0.0022 g
BSA-FV (Sigma; 7030)	0.1 g
Total volume	100 ml

Working solution was freshly prepared on the day of use, filter-sterilised using syringe-driven Millex©-GS filter (0.22 µm) and warmed in a water bath (38.5ºC) before used. However, TL-Hepes working solution can be stored up to 3 to 4 days in a refrigerator (4ºC).

3.3.3.2 (e) *Preparation of in vitro maturation (IVM) medium*

IVM medium was prepared a day before ovary slicing or oocytes retrieval session (LOPU). IVM needed to be equilibrated overnight in the CO_2 incubator (5%) at 38.5ºC. The base medium for maturation was TCM 199 (Sigma; M4530) supplemented with fetal bovine serum (FBS) or oestrus goat serum (OGS), bFSH, 17β-oestradiol, gentamicin and sodium pyruvate (Table 3.12).

Table 3.12: Composition of IVM medium

Components	Volume/weight
TCM 199	8.9 ml
FBS/OGS	1 ml
TCM-Pyruvate	100 µl
bFSH	10 µl
Gentamicin stock	5 µl
Total volume	10 ml

All chemicals above was added and filter-sterilised using syringe-driven Millex©-GS filter (0.22 µm) into a centrifuge tube (15 ml; Falcon™, USA). 17β-oestradiol was

added as (1 µl/ml) basis into the filtered solution and mixed thoroughly. Maturation medium can be stored up to 1 week in a refrigerator (4^{o}C).

The components used in maturation medium were prepared as stock solution and can be stored until certain duration accordingly. The following sub-divisions show the details to prepare stock of components used in maturation medium.

3.3.3.2 (e) (i) ***Preparation of TCM-pyruvate stock***

Sodium pyruvate (0.0022 g; Sigma; P4562) and cystein (0.0085 g; Sigma; C2529) were weighed and added to TCM 199 (1 ml; Sigma; M4530) in microcentrifuge tube (1.5 ml), and slowly vortexed for 10 seconds. This stock was stored in a refrigerator (4^{o}C) for 2-3 days.

3.3.3.2 (e) (ii) ***Preparation of bFSH stock***

Folltropin-V^{R}, (0.0050 g; Vetripharm; L032-B053) was dissolved into a solution (1 ml) provided, aliquot into microcentrifuge tubes (1.5 ml), covered with aluminium foil and stored in a freezer (-20^{o}C).

3.3.3.2 (e) (iii) ***Preparation of gentamicin stock***

Gentamicin (1 g; Sigma; G3632) was weighed and added into DPBS (20 ml; Gibco; 21600-010). Gentamicin solution was filter-sterilised using syringe-driven Millex$^{©}$-GS filter (0.22 µm), aliquot into microcentrifuge tubes (1.5 ml), covered with aluminium foil and stored in a refrigerator (4^{o}C).

3.3.3.2 (e) (iv) ***Preparation of 17β-oestradiol stock***

17β-oestradiol (0.001g; Sigma; E8875) was weighed and added into ethanol (1 ml; GR grade: filter-sterilised using syringe-driven Millex©-GS filter (0.22 μm), aliquot into microcentrifuge tubes (1.5 ml), covered with aluminium foil and stored in a refrigerator (4°C) up to 6 months.

3.3.3.2 (e) (v) ***Preparation of foetal bovine serum (FBS) stock***

FBS (Gibco; 16000-044) was heat-inactivated at 56°C for 30 minutes, filter-sterilised (0.45 μm), aliquot (1 ml) into microcentrifuge tubes (1.5 ml), covered with aluminium foil and stored in a freezer (-20°C) for 6 months.

3.3.3.2 (e) (vi) ***Preparation of oestrus goat serum (OGS) stock***

OGS has a similar function as FBS and can be used in maturation medium instead of FBS. It provides additional growth factors and hormones needed by oocytes/embryos development.

The caprine blood samples were collected aseptically through jugular vein of oestrus caprine by puncturing using vacutainer tubes (without heparin) with a needle (21 G). The freshly collected blood in vacutainer tubes was centrifuged (3000 rpm) for 10 minutes at room temperature (25ºC) to let the sediment (clotted blood) separated from the supernatant (serum). The serum was aspirated out from the vacutainer tubes into sterile centrifuge tubes (15 ml) and heat-inactivated in a water bath for 30 minutes at 56ºC to destroy components that might lead to cell lysis by antibody binding.

The serum was removed from the water bath after 30 minutes and was cooled at room temperature (25ºC). Prolonged heat treatment could cause deterioration of some

components of the serum. The serum was centrifuged (1000 rpm) again for 10 minutes at room temperature (25ºC) to let the residual erythrocytes sediment to the bottom of the tube in order to get more pure serum. The supernatant (serum) was aliquot (1 ml) into microcentrifuge tubes (1.5 ml), covered with aluminium foil and stored in a freezer (-20^{o}C) for 6 months.

3.3.3.2 (f) *Preparation of hyaluronidase solution*

Hyaluronidase solution was purposely used to denude cumulus cells surrounding cumulus oocytes complexes (COC) after maturation. A type IV-S hyaluronidase from bovine testes (Sigma; H4272) was used to prepare hyaluronidase solution. The preparation of hyaluronidase stock solution is depicted as in a Table 3.13 below:

Table 3.13: Composition of hyaluronidase stock solution

Components	Volume/weight
Autoclaved Milli-Q water	1 ml
Type IV-S Hyaluronidase (Sigma; H4272)	1 mg

Hyaluronidase stock solution was filter-sterilised by Millex©-GS filter (0.22 μm) and aliquot (1 ml) into microcentrifuge tubes (1.5 ml), covered with aluminium foil and stored at -20^{o}C for 6 months. The concentration of stock solution was 1 mg/ml. To remove the cumulus cells, hyaluronidase solution (0.1%) was needed. The dilution of hyaluronidase is shown in Table 3.14 below:

Table 3.14: Composition of hyaluronidase working solution (0.1%)

Components	Volume/weight
Hyaluronidase stock solution	100 μl
Hepes TCM 199 (Gibco; 12340-030)	900 μl
Total volume	1000 μl

The prepared solution was filter-sterilised by Millex©-GS filter (0.22 µm) and aliquot (100 µl) into microcentrifuge tubes (1.5 ml), and stored in a freezer (-20°C) for 6 months.

3.3.3.2 (g) *Preparation of potassium simplex optimisation medium (KSOM) stock solution*

KSOM was used as *in vitro* culture medium in caprine and bovine species as well as a base medium in parthenogenetic chemical preparation. KSOM was actually originated from simplex optimisation medium (SOM) developed by Lawitts and Biggers (1991) and later on was modified to KSOM (Lawitts and Biggers, 1993). Table 3.15 represents KSOM stock preparation:

Table 3.15: Composition of KSOM stock solution

Component	Sigma Cat. No.	Final conc. (mmol/l)	g/100 ml
NaCl	S5886	95	0.5553
KCl	P5405	2.5	0.0186
KH_2PO_4	P5655	0.35	0.0048
$MgSO_4$	M7506	0.2	0.0024
Lactate	L7900	10	0.1860 ml
Pyruvate	P4562	0.2	0.0022
D-Glucose	G6152	0.2	0.0036
$NaHCO_3$	S5761	25	0.2101
$CaCl_2$	C5670	1.71	0.0190
L-Glutamine	G3126	1	0.0146
EDTA	E9884	0.01	0.0004
Autoclaved Milli-Q water			100 ml
Total volume			100 ml

KSOM stock solution was stirred well with magnetic stirrer and filter-sterilised by Millex©-GS filter (0.22 µm) into stock bottle (100 ml) and stored in a refrigerator (4ºC) up to 4 weeks. Before KSOM can be used for culture medium, KSOM stock solution needed to be prepared freshly as presented in Table 3.16.

Table 3.16: Composition of KSOM working solution

Components	Amount/10 ml
KSOM stock solution	9.85 ml
BSA (Sigma; A6003)	0.0400 g
MEM (Sigma; M7145)	50 µl
BME (Sigma; B6766)	100 µl
Total volume	10 ml

KSOM working solution was filter-sterilised by Millex©-GS filter (0.22 µm) into centrifuge tube (15 ml) and preincubated for at least 3 hours in CO_2 incubator (5%) at 38.5°C before used. D-glucose (0.004 g; Sigma; G6152) was added into KSOM working solution (10 ml) for culturing later stage of bovine and caprine embryos (Kwong, P.J., personal communication, 2011).

3.3.3.2 (h) *Preparation of parthenogenetic activation medium*

There were several types of chemical used as an activation medium in order to activate caprine and bovine oocytes. All the preparations of stock medium were sub-divided as below.

3.3.3.2 (h) (i) *Preparation of calcium ionophore (A23187) stock solution*

Preparation of calcium ionophore stock solution for caprine and bovine was similar as in calcium ionophore preparation for murine activation medium. [Sub-section 3.3.3.1 (d) (iii)]

3.3.3.2 (h) (ii) *Preparation of ionomycin (Iono) stock solution*

Ionomycin (Sigma; I3909) was used as one of the several options to activate caprine and bovine oocytes right after denuding the cumulus cells after IVM culture. The initial concentration of ionomycin ready-made solution (1 ml) is 1 mM in DMSO. Subsequently,

ionomycin solution was aliqout (10 µl) into microcentrifuge tubes (1.5 ml), wrapped in aluminium foil and stored up to 6 months in a freezer (-20°C) and labeled as „Stock A". On the day of experiment, one tube of „Stock A" was taken out from the freezer and equilibrated *in vitro* culture medium; KSOM (990 µl) was added and mixed well. The final concentration of this working solution was 10 µM and can be immediately used to make a microdroplet culture.

3.3.3.2 (h) (iii) ***Preparation of ethanol (EtOH) stock solution***

Preparation of ethanol stock solution for caprine and bovine was similar as in ethanol preparation for murine activation medium. [Sub-section 3.3.3.1 (d) (iv)]

3.3.3.2 (h) (iv) ***Preparation of 6-dimethylaminopurine (6-DMAP) stock solution***

Preparation of 6-DMAP stock solution for caprine and bovine was similar as in 6-DMAP preparation for murine activation medium. [Sub-section 3.3.3.1 (d) (v)]

3.3.3.2 (h) (v) ***Preparation of cycloheximide (CHX) stock solution***

Preparation of CHX stock solution for caprine and bovine was similar as in CHX preparation for murine activation medium. [Sub-section 3.3.3.1 (d) (vi)]

3.3.3.2 (h) (vi) ***Preparation of cytochalasin B (CB) stock solution***

Preparation of CB stock solution for caprine and bovine was similar as in CB preparation for murine activation medium. [Sub-section 3.3.3.1 (d) (i)]

3.3.3.2 (i) *Preparation of sperm capacitation medium (Sp-TALP) stock solution*

Sp-TALP was used as a holding medium for caprine and bovine sperm as well as a sperm capacitation medium while capacitating it for several hours in CO_2 (5%) incubator. Sp-TALP stock solution preparation was portrayed as in Table 3.17.

Table 3.17: Composition of Sp-TALP stock solution

Component	Sigma Cat. No.	g/100 ml
NaCl	S5886	0.584
KCl	P5405	0.0023
$NaHCO_3$	S5761	0.21
$NaH_2PO_4.H_2O$	S9638	0.004
Na Lactate (60%Syrup)	L7900	0.368 ml
$CaCl_2.2H_2O$	C3881	0.031
$MgCl_2.6H_2O$	M2393	0.008
HEPES $C_8H_{17}N_2O_4SNa$	H0763 or H3784	0.119
$C_8H_{18}N_2O_4S$	H3375 or H6147	0.119
Phenol Red	P3532	0.001
Total volume		100 ml

Sp-TALP stock solution was mixed well and filter-sterilised by Millex©-GS filter (0.22 µm) into stock bottle (100 ml) and stored at 4ºC up to 4 weeks. Before Sp-TALP can be used for holding and capacitation medium, Sp-TALP stock solution needed to be freshly prepared and added by these chemicals below (Table 3.18):

Table 3.18: Composition of Sp-TALP working solution

Component	Amount/10 ml
Sp-TALP stock solution	10 ml
BSA (Sigma; A6003)	0.06 g
Sodium Pyruvate (Sigma; P4562)	0.0011 g
Gentamicin stock solution	5 µl
Total volume	10 ml

Sp-TALP working solution was filter-sterilised by Millex©-GS filter (0.22 µm) into centrifuge tube (15 ml) and preincubated for at least 3 hours in CO_2 incubator (5%) at 38.5°C before used.

3.3.3.2 (j) *Preparation of in vitro fertilisation medium (IVF-TALP) stock solution*

IVF-TALP was used as a holding medium and equilibration medium for caprine and bovine oocyte in CO_2 (5%) incubator as well as *in vitro* fertilisation medium. IVF-TALP stock solution preparation is depicted as shown in Table 3.19.

Table 3.19: Composition of IVF-TALP stock solution

Component	**Sigma Cat. No.**	**g/100 ml**
NaCl	S5886	0.6660
KCl	P5405	0.0235
$NaHCO_3$	S5761	0.2104
$NaH_2PO_4.H_2O$	S9638	0.0055
Na Lactate (60% syrup)	L7900	0.368 ml
$CaCl_2.2H_2O$	C3881	0.0300
$MgCl_2.6H_2O$	M2393	0.0100
Penicillin G	PENNA	0.0065
Phenol Red	P3532	0.001
Total volume		100 ml

IVF-TALP stock solution was mixed well and filter-sterilised by Millex©-GS filter (0.22 µm) into stock bottle (100 ml) and stored at 4ºC up to 4 weeks. Before IVF-TALP can be used, IVF-TALP stock solution needs to prepared fresh and added by these chemicals below (Table 3.20):

Table 3.20: Composition of IVF-TALP working solution

Component	Amount/10 ml
IVF-TALP stock solution	10 ml
BSA (Sigma; A6003)	0.06 g
*IVF-Pyruvate	100 µl
Gentamicin stock solution	5 µl
Total volume	10 ml

(*IVF-Pyruvate was prepared by mixing sodium pyruvate (0.0022 g; Sigma; P4562) into IVF-TALP stock solution (1 ml) in microcentrifuge tube (1.5 ml), and slowly vortexed for around 10 seconds. IVF-Pyruvate can be stored for 2-3 days at 4^{o}C).

IVF-TALP working solution was filter-sterilised by Millex©-GS filter (0.22 µm) into centrifuge tube (15 ml) and preincubated for at least 3 hours in CO_2 incubator (5%) at 38.5°C before used.

3.3.4 Preparation of Microtools and Accessories

Microtools that used in this study were pick-up glass pipette and mouth-controlled pipette. All the preparation of microtools was done in the laboratory.

3.3.4.1 Preparation of mouth-controlled pipette

Mouth-controlled pipette was purposely used for handling the oocytes and embryos such as picking up, transfer from one droplet to another and denuding. This type of pipette was functioned based on the sucking air in and out controlled by mouth which results in sucking medium in and out through the glass Pasteur pipette. Mouth-controlled pipette was assembled with a two parts of long silicone tube, syringe filter (Millex©-GS filter; 0.22 µm; Schleicher and Schuell, Germany), 1000 µl microtip and glass Pasteur pipette. One part of silicone tube was attached to a 1000 µl microtip while the other end was attached to one end of syringe filter. The other end of syringe filter was attached to the second part of

silicone tube and the other end of silicone tube was attached to 1000 µl microtip which this end will later attached to the glass Pasteur pipette. All the assembled part was sealed with Parafilm® (Pechiney Plastic Packaging, USA) tightly to avoid disjunction.

3.3.4.2 Preparation of glass Pasteur pipettes

Glass Pasteur pipettes (Paul Marienfeld GmbH, Germany) were used in handling oocytes and embryos along with mouth-controlled pipette. Before glass Pasteur pipettes can be used, it needs to be soaked overnight in RO water and rinsed thoroughly before sealed in autoclave bag for sterilisation. The sterile Pasteur pipettes were dried in oven (56°C) and can be straight away used each time during experiments. The glass Pasteur pipettes was softened by rotating it in a fine flame until the glass became soft and the glass was immediately withdrawn from the heat and both ends were quickly pulled away smoothly from each other to produce a tube with an internal diameter of approximately 200 to 400 µm for oocytes or embryos and cumulus oocyte complexes accordingly.

During the pulling of the glass, it should not be pulled when the glass was still in flame. Besides, a special care was taken during the pulling of the glass Pasteur pipette where the glass was pulled straight. This is important, as not to produce two resulting pipette which will bend in the area where the glass had been melted. For a neat break, the glass tube was scored using a diamond stone and the tip was broken by gently bent the stone to snap on it. It is also important to produce an evenly broken straight tip, as it is difficult to handle embryo, if the capillary end was jagged. Besides, uneven tip, chipped tip and also tip which was not exactly at 90°C angle cannot be used.

The tip of the pipette was fire polished by touching it on the flame quickly to produce a blunt end, in order to minimise the damage of zona pellucida during manipulation. Sharp edges of pipette were easily caught by the plastic surface of the culture dish and causing the pipette to break, consequently will tend to collect more debris during the oocytes transfer. Besides, the diameter of the micropipette opening should be sufficient to hold the embryos, but not too large.

3.3.5 Experimental Procedures

Following are different experimental procedures to obtain caprine, bovine and murine embryos derived from different manipulation in chemicals via parthenogenetic activation.

3.3.5.1 Procedures in murine species

Murine used in the present study were self-bred and sourced from Animal House at ISB Mini Farm, University of Malaya.

3.3.5.1 (a) *Superovulation of female murine as oocytes donor*

To obtain the optimum quality and quantity of murine oocytes to conduct the experimental procedures, mature females murine aged 8 to 12 weeks were superovulated by intraperitoneal (i.p.) injection of PMSG (10 IU; 0.2 ml) on day 1 at 1800 hours followed by i.p. injection of hCG (10 IU; 0.2 ml) at 48 hours apart. Murine was picked up by the scruff of its neck as close as possible to the ears. Extra care must be taken not to suffocate or hurt the murine in the process by grabbing enough skin but not squeezing the murine neck. The murine tail was held firmly by twisting it around the little finger to properly hold it to

prevent the murine struggled and move while being injected. Hormones were injected intraperitoneally at the centre point between the 4 tits of the ventral abdomen by sterile hypodermic needles (26 G). The needle was stayed for a few seconds after injected before withdrawn out to prevent the hormone from seeping out.

3.3.5.1 (b) *Oocytes retrieval and collection in murine*

The superovulated female murine was humanely sacrificed by cervical dislocation around 13 to 18 hours post-hCG injection and the body wall (peritoneum) was cut open. Coil of guts were push out of the way and two horns of the uterus, the oviducts and the ovaries were located. The upper end of the uterine horns was grasped with fine forceps and the uterus, oviduct, ovary and fat pad was gently pulled away from the body cavity to reveal a fine membrane (the mesometrium) which connects the reproductive tract to the body wall and carries prominent blood vessels. A hole was poked in the membrane close to the oviduct with the closed tips of a pair of fine forceps or scissors. The oviduct, ovary and fat pad were pulled with fine scissors and a cut between the oviduct and ovary was made with fine scissors. The forceps was repositioned to cut the uterus near the oviduct. The oviduct was transferred into the HWM microdroplet in Petri dish.

Newly ovulated oocyte which was surrounded with cumulus cell can be found in the upper part of the oviduct (ampulla) which was much enlarged after 12 hours of post-ovulation. The fimbriae end of the oviduct (infundibulum) was also swollen during ovulation and can be easily located under 20x magnifications under the stereomicroscope. A pair of fine forceps was used to grasp the oviduct next to the swollen infundibulum and was held firmly on the bottom of the dish while a pair of needle (26 G) was used to tear the ampulla, releasing clutch of cumulus mass (eggs surrounded with cumulus cells). If the oocytes do not flow out by themselves, the needle was used to push them out by gently

squeezing the oviduct. If the oocytes stick the needle, the needle was lifted out of the Petri dish and they will retained by the surface tension of the medium and will fall back to the bottom of the dish. The COCs was straight away placed in hyaluronidase solution (0.1%; 100 µl) and denuded using micropipettor (100 µl) to removed the cumulus cells. The naked oocytes were rinsed in HWM medium three times and only oocytes with the presence of first extruding polar body were chosen for the subsequent experiments (Figure 3.1).

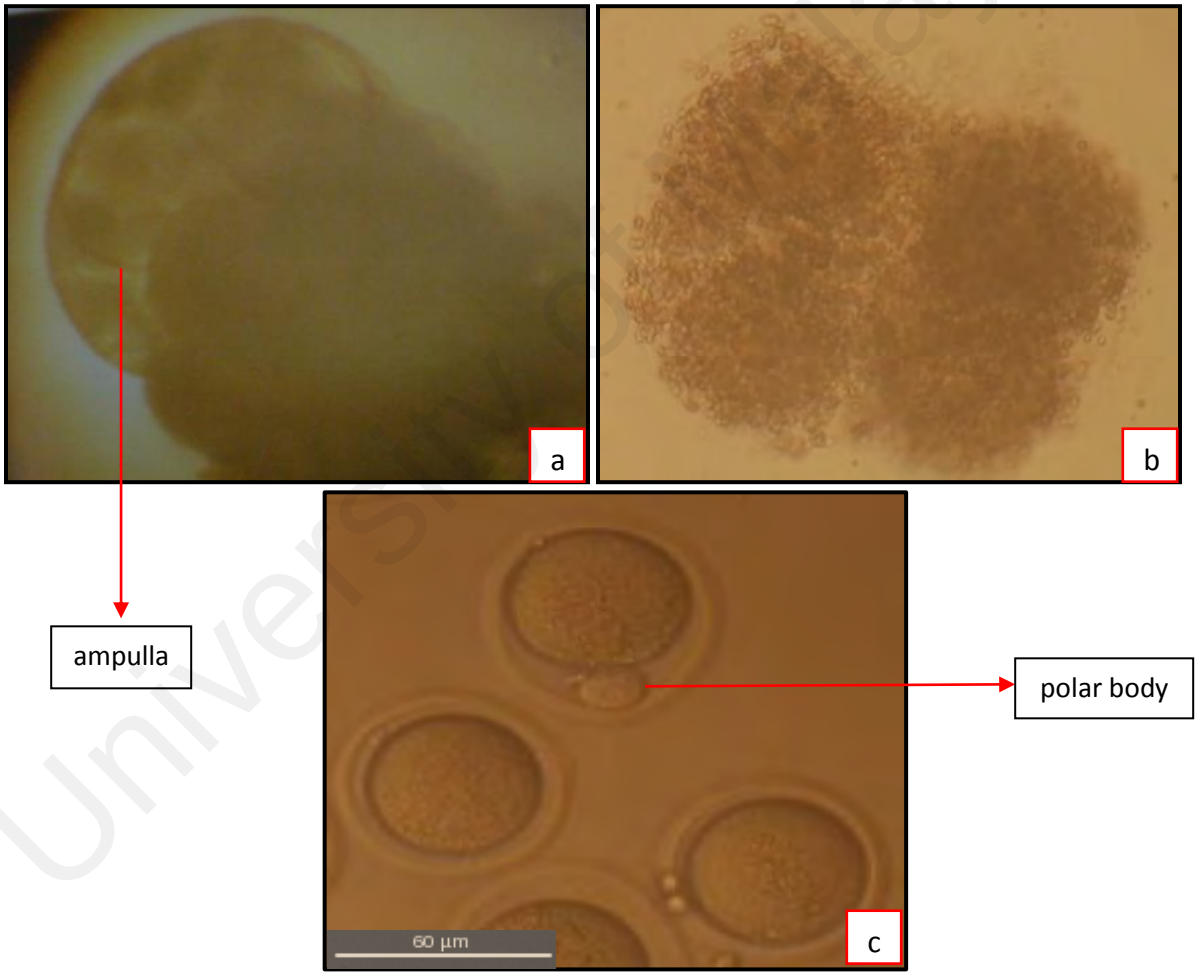

Figure 3.1: (a) Swollen ampulla at murine oviduct that contains cumulus oocyte complexes (COCs). (b) COCs which contain oocytes. (c) Naked oocyte with the presence of polar body.

3.3.5.1 (c) *Activation of murine oocytes*

3.3.5.1 (c) (i) *Activation of murine oocytes by $SrCl_2$ and CB in CZB Ca^{2+} free medium*

Basically, a culture dish was made for activation medium, contain the mixture on $SrCl_2$ and CB in CZB Ca^{2+} free medium. Each culture dish (35 mm; Nunc, Denmark) contains 7 microdroplets (100 µl/microdroplet) of activation medium overlaid with silicone oil and equilibrated in CO_2 incubator (5%; 37ºC) overnight. The naked oocytes were treated in activation medium microdroplet for 1 to 5 hours according to the experiment and cultured inside CO_2 incubator (5%; 37ºC) before transferred into IVC medium.

3.3.5.1 (c) (ii) *Activation of murine oocytes by A23187and 6-DMAP*

Basically, two culture dishes were made for activation medium, one for A23187 and the other one for 6-DMAP. Each culture dish (35 mm) contains 7 microdroplets (100 µl/microdroplet) of activation medium overlaid with silicone oil and equilibrated in CO_2 incubator (5%; 37ºC) overnight. The naked oocytes were first treated in A23187 microdroplet for 5 minutes in CO_2 incubator (5%; 37ºC) and rinsed in modified HWM three times followed by equilibrated modified WM three times to wash off the A23187 residue before transferred into 6-DMAP microdroplet. The culture dish were culture inside CO_2 incubator (5%; 37ºC) 4 hours before transferred into IVC medium.

3.3.5.1 (c) (iii) *Activation of murine oocytes by A23187 and CHX*

Basically, two culture dishes were made for activation medium, one for A23187 and the other one for CHX. Each culture dish (35 mm) contains 7 microdroplets (100

µl/microdroplet) of activation medium overlaid with silicone oil and equilibrated in CO_2 incubator (5%; 37ºC) overnight. The naked oocytes were first treated in A23187 microdroplet for 5 minutes in CO_2 incubator (5%; 37ºC) and rinsed in modified HWM three times followed by equilibrated modified WM three times to wash off the A23187 residue before transferred into CHX microdroplet. The culture dish were culture inside CO_2 incubator (5%; 37ºC) 4 hours before transferred into IVC medium.

3.3.5.1 (c) (iv) ***Activation of murine oocytes by A23187 and CB***

Basically, two culture dishes were made for activation medium, one for A23187 and the other one for CB. Each culture dish (35 mm) contains 7 microdroplets (100 µl/microdroplet) of activation medium overlaid with silicone oil and equilibrated in CO_2 incubator (5%; 37ºC) overnight. The naked oocytes were first treated in A23187 microdroplet for 5 minutes in CO_2 incubator (5%; 37ºC) and rinsed in modified HWM three times followed by equilibrated modified WM three times to wash off the A23187 residue before transferred into CB microdroplet. The culture dish were culture inside CO_2 incubator (5%; 37ºC) 4 hours before transferred into IVC medium.

3.3.5.1 (c) (v) ***Activation of murine oocytes by EtOH and 6-DMAP***

Basically, two culture dishes were made for activation medium, one for EtOH and the other one for 6-DMAP. Each culture dish (35 mm) contains 7 microdroplets (100 µl/microdroplet) of activation medium overlaid with silicone oil. Only 6-DMAP culture dish was equilibrated in CO_2 incubator (5%; 38.5ºC) overnight while EtOH culture dish was prepared fresh prior experiment to ensure that the concentration of the EtOH will not

deviated from the required concentration as EtOH was easy to evaporate. The naked oocytes were first treated in EtOH microdroplet for 5 minutes inside the laminar flow and rinsed in modified HWM three times followed by equilibrated modified WM three times to wash off the EtOH residue before transferred into 6-DMAP microdroplet. The culture dish were culture inside CO_2 incubator (5%; 37ºC) 4 hours before transferred into IVC medium.

3.3.5.1 (d) *In vitro culture (IVC) of activated murine oocytes*

Culture dish (35 mm) contains 7 microdroplets (80 µl/microdroplet) of modified WM working solution was overlaid with silicone oil and equilibrated in CO_2 incubator (5%; 37ºC) overnight. All the activated oocytes were washed thrice respectively in modified HWM followed by equilibrated modified WM after the few hours of the respective treatments in order to wash off the residue of activation medium (PA). Activated oocytes were then transferred into WM microdroplet (10-15 oocytes/microdroplet) respectively, and subsequently cultured in CO_2 incubator (5%; 37ºC) for later embryos development. The embryos were checked at Day-2, -4 and -6 accordingly and their development was recorded.

3.3.5.2 Procedures in Caprine and Bovine Species

As for caprine, oocytes were retrieved from LOPU procedure whereas for bovine, oocytes were obtained from the local abattoir.

3.3.5.2 (a) *Caprine oocytes retrieval through laparoscopic oocytes pick up (LOPU)*

There were several important steps carried out in order to retrieve caprine oocytes through LOPU procedure including oestrus synchronisation, superovulation, anaesthesia and sedation, disinfection of surgical instruments and surgical area of does as well as the preparation pre-LOPU, during LOPU and post-LOPU.

3.3.5.2 (a) (i) *Oestrus synchronisation and superovulation of caprine donor*

Before retrieving the oocytes through laparoscopic oocytes pick-up procedure, the experimental does underwent oestrus synchronisation to manipulate the oestrus cycle of the subjected does in order to manage time of oocytes retrieval during LOPU. In order to synchronise the oestrous cycle, a Controlled Intravaginal Drug Release device (CIDR®, 0.3 g progesterone; EAZI BREED™, CIDR®, Pharmacia and Upjohn, New Zealand) was inserted into the vagina of the caprine donor with the help of a clean and sterile CIDR® applicator (Pharmacia and Upjohn, New Zealand) with the application of water-based lubricant (K-Y Jelly, Pharmedica Lab, South Africa).

The controlled intravaginal drug release device remained in the vagina for 14 days (at 0900 hours on day 0 of CIDR insertion) before being removed. The CIDR device is made of an inert silicone elastomer that is non-porous and does not readily absorb bodily fluids; once properly inserted deep into the donor‟s vagina, the controlled intravaginal drug release device unfolds into a „T‟ like formation that aids in retention. Daily monitoring of the device was performed to confirm that it had not been inadvertently removed. At approximately 36 hours prior to controlled intravaginal drug release device removal, a luteolytic treatment (125 µg cloprostenol; Estrumate®; Schering-plough, Australia) was

injected intramuscularly (at 0900 hours on day 12 of CIDR insertion) to regress the corpus luteum that facilitates initiation of pro-oestrus and eventually resulted in oestrogen surge for the onset of oestrus (heat).

On the next day (at 1600 hours on day 13 of CIDR imsertion), follicle stimulating hormone (1500 IU; Folligon®, Intervet International, Holland) was injected intramuscularly. Upon removal of controlled intravaginal drug release device (at 2100 hours on day 13 of CIDR insertion), a gonadotrophin treatment of (250 IU; Ovidrel™, Merck Serono, Switzerland) was injected intramuscularly. Two days after the controlled intravaginal drug release device removal, the female caprine was prepared for laparoscopic oocytes pick-up. The oestrus was detected by the twigling of its tail and by using a teaser buck. Surgery was done 60 hours post-ovidrel (at 0900 hours on day 16) to stimulate multiple follicular developments (Figure 3.2).

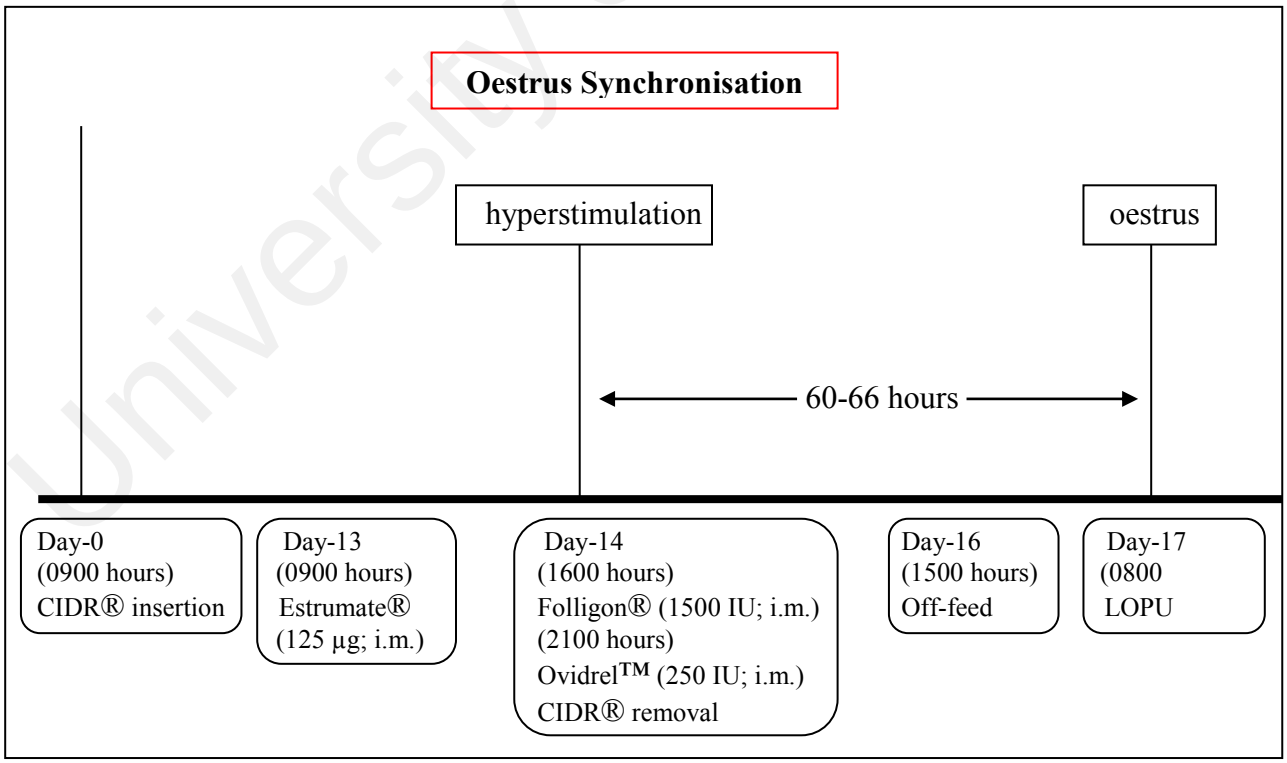

Figure 3.2: A schematic diagram of the treatments performed on a donor caprine to synchronise oestrus cycle for LOPU.

Figure 3.3: (a) Controlled intravaginal drug releasing (CIDR) device, CIDR applicator, gloves and K-Y Jelly. (b) Insertion of CIDR. (c) Removal of CIDR.

3.3.5.2 (a) (ii) *Pre-LOPU preparation and anaesthetisation of caprine donor*

Identification tag of the caprine was determined and the caprine donors were off-feed and water (at 1500 hours on day 15 of CIDR insertion) for 18 to 24 hours before to oocyte retrieval surgery (at 0900 hours on day16 of CIDR insertion). The caprine donors were put in a separated area specifically in off-feed pen and were brought to NaTuRe laboratory in the morning day of surgery. Anaesthesia was administrated to a caprine with mixture of xylazine hydrochloride (0.22 mg/kg body weight; Ilium Xylazine-20; Troy Laboratorries, Australia) and ketamine hydrochloride (11 mg/kg body weight; Ketamil; Troy

Laboratorries, Australia) via intramascular (i.m.) injection. The caprine donor was maintained under anaesthesia with Ketamine hydrochloride (0.1 mg/kg bwt) injected intramuscularly as maintenance doses every 20 to 30 minutes or as required.

3.3.5.1 (a) (iii) *Preparation and setting up of surgical instruments, aspiration-flushing system, CO_2 insufflator, light system and imaging device for LOPU*

Prior to surgery, non-autoclavable surgical instruments (trocar and cannula, atraumatic grasper, fibre optic cable, light probe for endoscope, flushing system silicone tubing and CO_2 silicone tubing) were exposed under UV light radiation for 20-30 minutes as a procedure for disinfection. For the autoclavable surgical instruments, it was ready to be used as long as the autoclave package was not torn and dry. The outer wrapping of the sterile surgical package (surgical set, catgut suture, scalpel blade, round-bottom test tubes, aspiration needle, sterile gauze, sterile surgical gloves, sterile towels, sterile drapes and sterile table cloth) was opened and unfolded carefully without touching the sterilized instruments inside. All the sterile surgical instruments were assembled and arranged properly on a sterile table-cum-trolley which was covered with a sterile table cloth.

The laparoscopic system and equipment used were purchased from Aesculap A.G. and Company, Germany consist of a paediatric Storz laparoscope (7.0 mm; PE 688A), an Aesculap endoscopic camera system (PV431), a charge couple device (CCD) camera (David3; PV430), a light probe with fibre optic cable (OP913), a light system (Light source 300 W; OP927), an electronic CO_2 gas insufflators unit (PG001), a Verrus needle (PG3) attached to a silicone tubing, a trocar and canula (7.0 mm) for the endoscope (EJ457), an antraumatic grasping forceps or paediatric grasper (PO951R) and a small trocar with cannula (5.5 mm; EJ456) for passing the grasper. The light probe was

connected to a light system via fibre optic cable. The light system was also connected to a CCD camera and computer monitor. The lens of the light probe and the entire length of the fibre optic cable were disinfected by wiping with 70% ethanol and white balance was adjusted using sterile gauze as a reference.

The flushing and aspiration system was purchased from Cook, Australia composed of a pedal operated aspiration pump (Aspiration system, K-MAR-5100; Cook, Australia) for providing a constant vacuum for LOPU, a flushing pump (a microprocessor controlled pump for automated follicle flushing) (Flushing system; K-MAR-4000; Cook, Australia), a test tube heater (K-FTH-1012; Cook, Australia) for maintaining a constant temperature (38.5°C) during LOPU, a silicone connecting tubing that connects an aspiration needle to a vacuum pump and a series of collection tubes. A pair of flushing and aspiration pedal (Herga Electric Limited, UK), connected, respectively, to the flushing and aspiration system through cable.

A follicle puncture or aspiration needle (FAS Set C2) was purchased from Gynetics Medical Product, Belgium. The collection medium (flushing medium) for oocytes collection was pre-warmed (38.5°C) and filled in a sterile luer slip syringe (50 ml; Terumo, USA) and placed horizontally in the electronic vacuum pump (aspiration system). The other sterile luer slip syringes filled with flushing medium were kept in a polysterene box with a glass bottle with luke-warm water in it to keep the temperature of the flushing medium consistent during LOPU commencement. The collection tubes used were disposable round-bottom test tubes (14 ml; Falcon™; Becton Dickinson Labware, USA) with inlet and outlet ports in the stopper were placed in a test tube heater to maintain constant temperature (38.5°C) during LOPU. The inlet and outlet ports were connected to the aspiration needle and to a vacuum pump respectively, with silicone tubing. The

vacuum pressure was regulated with a flow valve (-100 mm Hg) and measured as drops of collection medium per minute entering the collection tube. Usually, it was adjusted to 50 to 70 drops per minute. The complete aspiration set was gas sterilised and rinsed with collection medium prior to use (Figure 3.4).

(continued)

(continued)

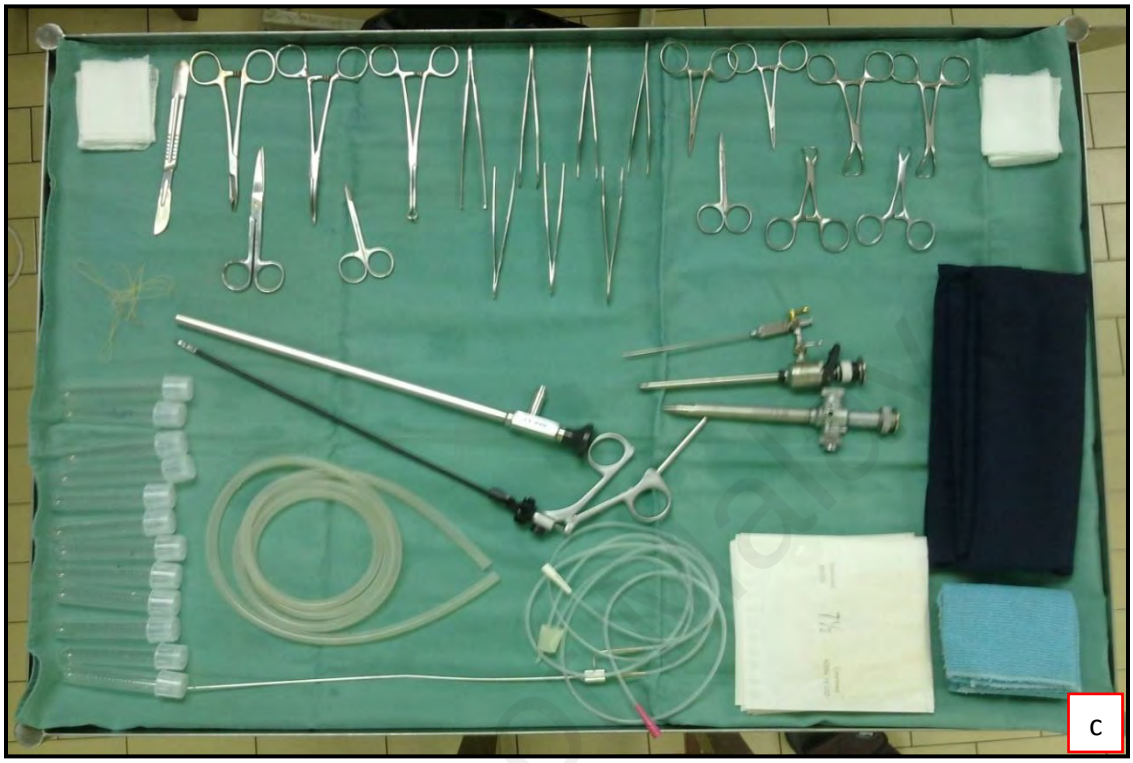

Figure 3.4: (a) Flushing and aspiration system. (b) Light system and imaging device. (c) Surgical instrument that used for laparoscopic oocyte pick-up (LOPU).

3.3.5.2 (a) (iv) *Disinfection surgical skin area of donor caprine*

The anaesthetised caprine donor was placed on a clean small ruminant restraining cradle. The restraining cradle was set at a 45º angle where the head of the animal lowered to facilitate the laparoscopy procedure. By using clean gauzes, the abdominal area of the caprine donor was disinfected with diluted Hibiscrub (10%; International Plc, UK) and the hair was shaved. After shaving, the bare skin was disinfected again with absolute Hibiscrub (100%) and subsequently with iodine solution (10%; Weak iodine; ICN Biomedicals, USA). The caprine donor was then covered with a sterile drape with an opening to reveal the disinfected bare skin and was ready for oocyte retrieval surgery. The

drape used was isolated the surgical site and minimise wound contamination. The drape was positioned without the fabric dragging across a non-sterile surface and secured in place with towel clamps at the 4 corners of the surgical site.

3.3.5.2 (a) (v) *Procedure during LOPU commencement*

LOPU is a microsurgical procedure to retrieve oocytes from matured follicles which can be visualised as „pimple-like" protrusions on the surface of the ovary via four minor incisions on the abdomen of donor caprine. Oocytes were retrieved by aspiration of follicular contents under laparoscopic observation. The anaesthetised caprine was restrained in a cradle in the standard position for laparoscopic surgery, a small incision was made on the disinfected abdominal area and a trocar connected to a CO_2 gas insufflator was inserted into the incision to insufflate the abdominal cavity with CO_2. The peritoneum cavity was filled with filtered CO_2 in order to facilitate visualisation of the reproductive tract.

Another three small incision (3 to 5 mm) were made once the peritoneum cavity was expended enough with CO_2. One incision was made near the umbilicus to facilitate the insertion of trocar for passing the light probe, one on the right side of lower-ventral abdomen to insert the trocar for passing the grasping forceps and the other one on the left side of lower-ventral abdomen for passing aspiration needle. The uterine horns were gently manipulated by the grasper to allow visualisation of each stimulated ovary. Once an ovary was identified, the ovarian ligament was grasped using a forceps without damaging the oviduct, to effectively stabilise the ovary for follicular puncture. The ovarian characteristics (size of ovary, estimated number of follicles and the stimulation response) were observed and recorded for each experimental donor before follicular puncture. When

flushing the follicles, extra care was taken to avoid leakage of follicular fluid and the inadvertent oocytes loss from the follicle. During the surgery, the ovaries were exposed by pulling the fimbria in different directions by manipulation the grasping forceps to ensure that all the follicles content were collected. The collection tube containing aspirated fluid (3-5 ml) was dispensed into a sterile Petri dish (60 mm) for oocytes searching and identification by embryologist under a stage warmer stereomicroscope (38.5ºC). Oocytes were then washed in flushing medium three times followed by IVM medium and cultured in CO_2 incubator (5%; 38.5ºC) according to oocyte grades for the subsequent post-IVM experiments.

After aspiration, the ovary was rinsed repeatedly with warm heparinised physiological saline (38.5ºC) using a syringe (50 ml) introduced through one of the trocars to aid in reducing adhesion following oocytes aspiration. The ovary was then released and the contra-lateral ovary was similarly aspirated. The incisions were closed with suture and the incision area was sprayed with antisecptic and insecticide containing cyphenothrin i.e. iodine solution (10%; Weak iodine; ICN Biomedicals, USA) and wound spray (Wound Sarex, Canaan Alpha Sdn. Bhd., Malaysia). The donor caprine was carefully lifted and removed from the cradle to be sent back to the farm. The donor caprine was administrated with oxytetracycline (20 mg/kg bwt) via i.m injection once in 4 days for 2 weeks period to avoid the possible post-surgical infection.

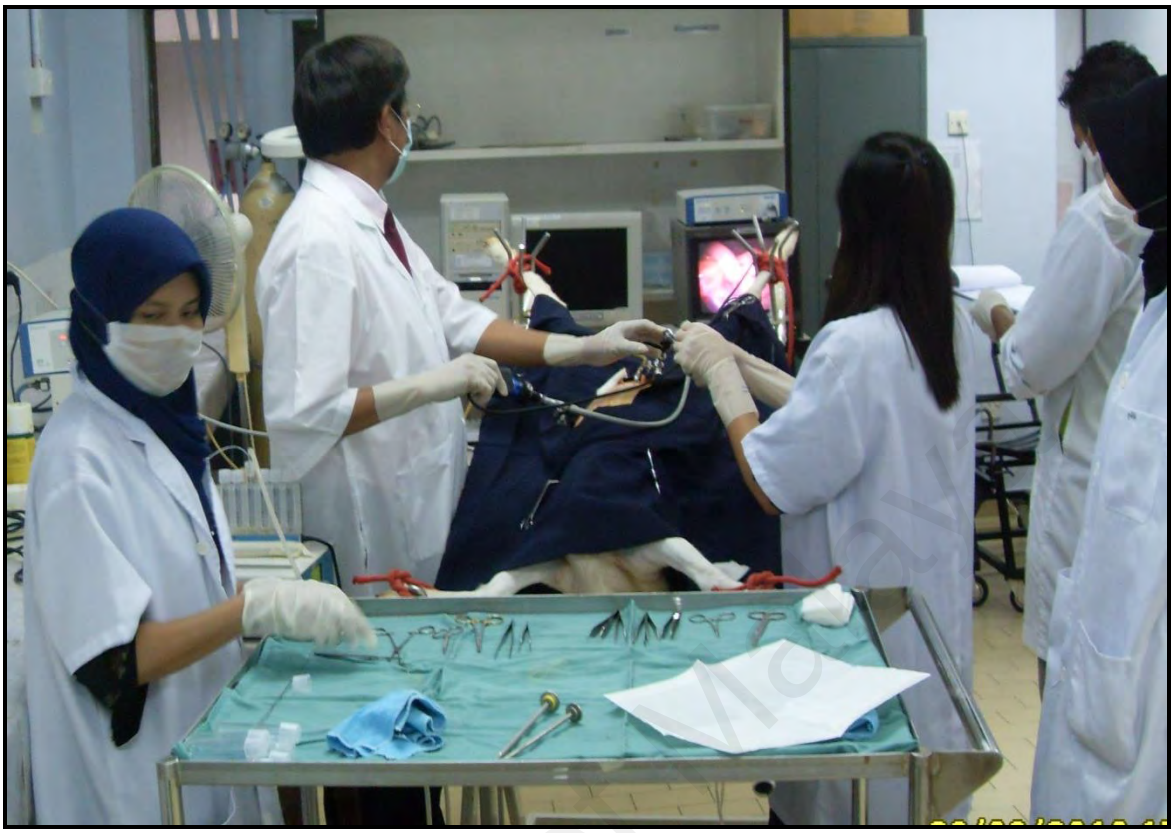

Figure 3.5: LOPU commencement.

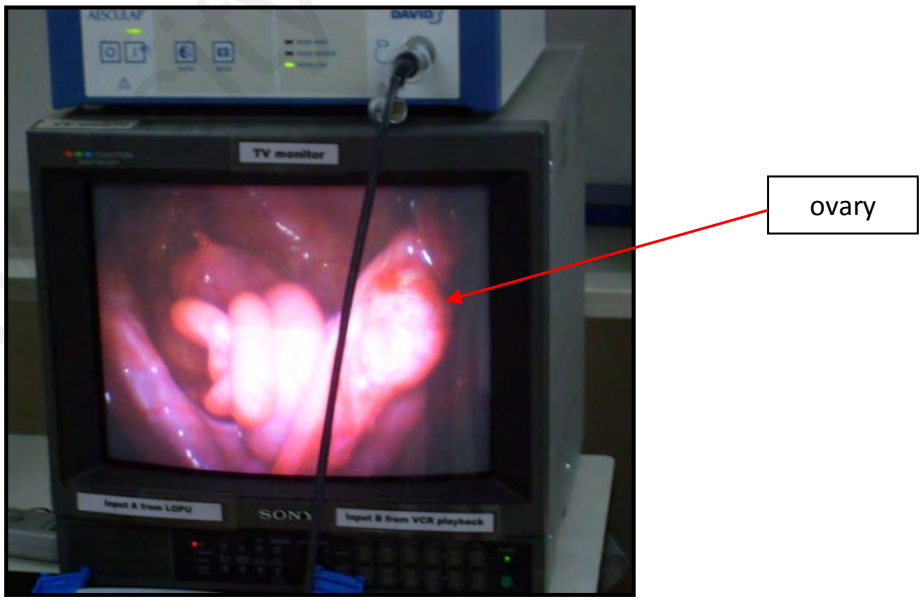

Figure 3.6: Caprine ovary observation through endoscopic camera system during LOPU.

3.3.5.2 (a) (vi) *Post-LOPU management*

As the surgery was completely finished, all the surgical instruments were washed and cleaned using a smooth sponge with a diluted 7X®-PF solution and immediately rinsed thoroughly running tap water and immersed completely in Gigasept® and subsequently rinsed with sterile autoclaved distilled water. The instruments then drain dried on a sterile paper towel before separated according to the autoclavable and non-autoclavable instruments into autoclave bag and sealed. Non-autoclavable instruments were straight away dried in oven while autoclavable instruments were autoclaved fisrt before dried in oven (56°C). for the aspiration needle, each opening ends were flushed with ethanol (70%) three times followed by sterile autoclaved distilled water three times before dried in oven for the next surgery procedure. The cradle and the table-cum-trolley was sterilised and wiped by ethanol (70%) while the floor was sweep and mopped with diluted Dettol antiseptic solution.

3.3.5.2 (b) *Bovine oocytes from abattoir-derived ovaries*

The bovine ovaries were obtained from the Department of Veterinary Services and Abattoir Complex, Shah Alam (Selangor) and Senawang (Negeri Sembilan). Two thermoses (one for rinsing the ovaries and another one for collecting the ovaries) were filled with warmed (38.5ºC) saline supplemented with penicillin G and streptomycin sulphate. A pair of surgical scissors was brought over and used to cut the ovaries from the slaughtered caprine. The excess tissues of the ovaries were trimmed and some blood was rinsed with the warmed saline before put into the thermos.

The ovaries that were collected from slaughterhouse were transported to the laboratory within 1 to 2 hours in warm saline medium which were maintained between 30°C to 38°C. After arriving at the laboratory, the ovaries were rinsed thoroughly with warm saline medium (38.5°C) and placed in a beaker (100 ml) containing warm saline medium (38.5°C). The bovine ovaries were placed in a Petri dish (60 mm) containing TL-Hepes working solution on a stage warmer (38.5ºC). The ovary was sliced individually and the COCs were searched and later cultured in IVM medium.

3.3.5.2 (b) (i) ***Ovary slicing***

Each collected ovary was freed from the surrounding tissues and overlaying bursa using surgical scissors and forceps under the laminar flow and rinsed three times using flushing medium. The ovary was placed in a Petri dish (60 mm) containing warm (38.5ºC) TL-Hepes working solution on a stage warmer at 38.5ºC. The ovary was held with the help of forceps and each ovary was sliced individually by checker-board incision technique on the whole ovarian surface. Systematic slices were given along the whole ovarian surface using a sharp razor blade held with sterile haemostat. While retrieving the oocytes by slicing method, the ovary was kept completely dipped in the TL-Hepes working solution. The Petri dish was examined under warmer stage stereomicroscope (38.5ºC) (magnification 20x or 40x). Oocytes were then washed in TL-Hepes working solution followed by IVM medium and subsequently cultured in CO_2 incubator (5%; 38.5ºC), according to oocyte grades for subsequently post-IVM experiments.

3.3.5.2 (c) *Caprine/bovine oocytes grading*

The caprine/bovine oocytes were identified and graded base on the criteria stated below:

Table 3.21: Oocyte grading based on cumulus cell layers and cytoplasm uniformity

Oocyte grade	Cumulus cell layers and cytoplasm uniformity
Grade A	COC with ≥5 multi-layered compact cumulus cells with evenly granulated cytoplasm
Grade B	COC with partially 5 layers or complete 3 to 4 layers of cumulus cells with evenly granulated cytoplasm
Grade C	COC with partially 3 to 4 layer s or complete 1 to 2 layers of cumulus cells with evenly granulated cytoplasm
Grade D	COC with partially 1 to 2 layers of cumulus cells with evenly granulated cytoplasm
Grade E	Naked oocytes with pale-coloured or heterogeneous cytoplasm*

*Heterogeneous cytoplasm: non-homogenous cytoplasm, in which the oocyte cytoplasm showed pigmentation in some areas.
(Adapted from Rahman, 2008)

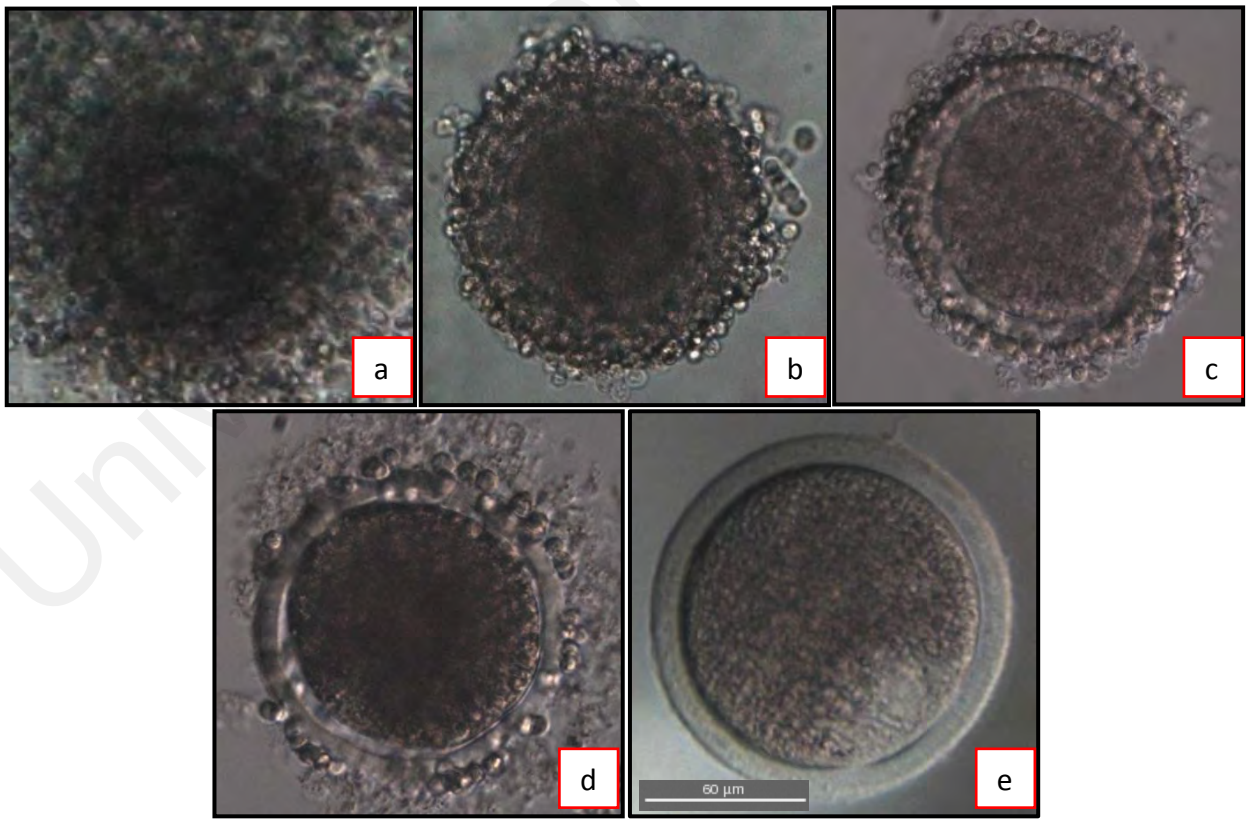

Figure 3.7: Morphology of different grades of caprine oocytes based on the cumulus cells evaluation: (a) Grade A, (b) Grade B, (c) Grade C, (d) Grade D and (e) Grade E.

3.3.5.2 (d) *In vitro maturation (IVM) procedure in caprine/bovine oocytes*

IVM medium was prepared in a small polystyrene culture dish (35 mm) in the size of microdroplets (80 µl each droplet; 7 microdroplets per culture dish) and overlaid with equilibrated mineral oil. The prepared dish was placed in the CO_2 incubator (5%; 38.5ºC) and equilibrated overnight. Basically, after finished washing and grading, the caprine oocytes were separately placed in IVM microdroplets (10 to 20 oocytes/80 µl) according to the oocytes grading. The COCs were cultured in CO_2 incubator (5%; 38.5ºC) for 18 to 25 hours (LOPU-derived oocytes). As for bovine, maturation was done for 24 hours duration.

3.3.5.2 (e) *Cumulus oocyte complexes COCs denuding*

After the respective IVM duration, the COCs were transferred into hyaluronidase droplet (0.1%; 100 µl). Using the disposable yellow tips (100 µl), attached with micropipettor (10 to 100 µl), the hyaluronidase medium contains COCs were suck in and out a few times to make the cumulus cells dispersed and totally removed from oocytes. If the cumulus cells still surround the oocytes after denuding, the oocyte were denuded again by gentle pipetting in and out using a narrow-bored Pasture pipette (80 to 100 µm) in hyaluronidase (0.1%) covered with mineral oil (<5 minutes) and washed 5 times in TL-Hepes working solution (100 µl/microdroplet). Only the matured oocytes (MII) with first extruding polar body were chosen for the subsequent experiments. Oocytes without first polar bodies were considered immature and discarded.

3.3.5.2 (f) *Activation of caprine/bovine oocytes*

Mature oocytes subjected to activation were divided into several different groups based on the type of the activation chemical used, duration of the treatment and the concentration of the chemicals.

3.3.5.2 (f) (i) *Activation of caprine oocytes by A23187or Iono*

For single activation, one culture dishes were made for activation medium, either for A23187 or for Iono. Each culture dish (35 mm) contains 7 microdroplets (100 µl/microdroplet) of activation medium overlaid with mineral oil and equilibrated in CO_2 incubator (5%; 38.5ºC) overnight. The naked oocytes were treated in A23187 or with Iono microdroplet respectively for 5 minutes in CO_2 incubator (5%; 38.5ºC) and rinsed in TL-Hepes three times followed by equilibrated KSOM three times to wash off the A23187 or Iono residue before transferred directly into IVC medium (KSOM) and culture inside CO_2 incubator (5%; 38.5ºC).

3.3.5.2 (f) (ii) *Activation of caprine/bovine oocytes by A23187 and 6-DMAP*

Basically, two culture dishes were made for activation medium, one for A23187 and the other one for 6-DMAP. Each culture dish (35 mm) contains 7 microdroplets (100 µl/microdroplet) of activation medium overlaid with mineral oil and equilibrated in CO_2 incubator (5%; 38.5ºC) overnight. The naked oocytes were first treated in A23187 microdroplet for 5 minutes in CO_2 incubator (5%; 38.5ºC) and rinsed in TL-Hepes three times followed by equilibrated KSOM three times to wash off the A23187 residue before

transferred into 6-DMAP microdroplet. The culture dish were culture inside CO_2 incubator (5%; 38.5ºC) for 3 to 6 hours accordingly.

3.3.5.2 (f) (iii) ***Activation of caprine/bovine oocytes by Iono and 6-DMAP***

Basically, two culture dishes were made for activation medium, one for Iono and the other one for 6-DMAP. Each culture dish (35 mm) contains 7 microdroplets (100 µl/microdroplet) of activation medium overlaid with mineral oil and equilibrated in CO_2 incubator (5%; 38.5ºC) overnight. The naked oocytes were first treated in Iono microdroplet for 5 minutes in CO_2 incubator (5%; 38.5ºC) and rinsed in TL-Hepes three times followed by equilibrated KSOM three times to wash off the Iono residue before transferred into 6-DMAP microdroplet. The culture dish were culture inside CO_2 incubator (5%; 38.5ºC) for 3 to 6 hours accordingly.

3.3.5.2 (f) (iv) ***Activation of caprine/bovine oocytes by Iono and CHX***

Basically, two culture dishes were made for activation medium, one for Iono and the other one for CHX. Each culture dish (35 mm) contains 7 microdroplets (100 µl/microdroplet) of activation medium overlaid with mineral oil and equilibrated in CO_2 incubator (5%; 38.5ºC) overnight. The naked oocytes were first treated in Iono microdroplet for 5 minutes in CO_2 incubator (5%; 38.5ºC) and rinsed in TL-Hepes three times followed by equilibrated KSOM three times to wash off the Iono residue before transferred into CHX microdroplet. The culture dish were culture inside CO_2 incubator (5%; 38.5ºC) for 3 to 6 hours accordingly.

3.3.5.2 (f) (v) ***Activation of caprine oocytes by Iono and CB***

Basically, two culture dishes were made for activation medium, one for Iono and the other one for CB. Each culture dish (35 mm) contains 7 microdroplets (100 µl/microdroplet) of activation medium overlaid with mineral oil and equilibrated in CO_2 incubator (5%; 38.5ºC) overnight. The naked oocytes were first treated in Iono microdroplet for 5 minutes in CO_2 incubator (5%; 38.5ºC) and rinsed in TL-Hepes three times followed by equilibrated KSOM three times to wash off the Iono residue before transferred into CB microdroplet. The culture dish were culture inside CO_2 incubator (5%; 38.5ºC) for 3 to 6 hours accordingly.

3.3.5.2 (f) (vi) ***Activation of bovine oocytes by EtOH and 6-DMAP***

Basically, two culture dishes were made for activation medium, one for EtOH and the other one for 6-DMAP. Each culture dish (35 mm) contains 7 microdroplets (100 µl/microdroplet) of activation medium overlaid with mineral oil and equilibrated in CO_2 incubator (5%; 38.5ºC) overnight. The naked oocytes were first treated in EtOH microdroplet for 5 minutes in CO_2 incubator (5%; 38.5ºC) and rinsed in TL-Hepes three times followed by equilibrated KSOM three times to wash off the EtOH residue before transferred into 6-DMAP microdroplet. The culture dish were culture inside CO_2 incubator (5%; 38.5ºC) for 4 hours.

3.3.5.2 (g) *In vitro fertilisation (IVF) of caprine/bovine oocytes*

A few hours after IVM treatment, cumulus cells of COCs were partially removed by gentle repeated pipetting to leave few layers of corona cells surrounding the oocytes in order to make oocytes ready for IVF procedure.

3.3.5.2 (g) (i) *Sperm capacitation*

Equilibrated Sp-TALP medium was divided into 2 parts (5 ml) in each centrifuge tube (15 ml). Meanwhile, three straws of frozen semen was removed aseptically from the liquid nitrogen (LN_2) tank using a pair of long forceps and pre-thawed at room temperature (25°C; 1 minute) followed by thawing in a water bath (37°C; 3 minutes). The straws were removed from the water bath and the outer surface was disinfected with ethanol (70%) and allowed to dry. Using a pair of sterile scissors, the straws were cut at both ends (first was the end with cotton plug, then the sealed end) and the semen was slowly released into the one of the centrifuge tube. The tube was closed tightly and put inside the centrifuge machine (200 x *g*; 5 minutes).

After that, the supernatant of the semen was carefully sucked out using micropipettor (1000 µl) to leave the sediment and a small amount of the supernatant. The sediment was mixed well with the supernatant residue and sucked out and dispensed at the bottom of the other centrifuge contain equilibrated Sp-TALP. The tube was closely capped and centrifuged again (200 x *g*; 5 minutes). The supernatant was carefully discarded without agitating the sperm pallet and the bottom layer of the supernatant was left (800 µl). The bottommost part of supernatant (upper layer of sediment) was carefully sucked out and dispensed at the bottom part of a microcentrifuge tube (1.5 ml) contain 1 ml of equilibrated

Sp-TALP. The tube was cultured inside CO_2 incubator (5%; 38.5ºC) for 45 minutes to 1 hour to allow the sperm swim-up.

3.3.5.2 (g) (ii) ***Preparation of oocytes for IVF***

Oocytes were transferred from IVM medium into hyaluronidase solution (0.1%; 100 µl) and partially denuded by sucking it in and out of the pipette. Oocytes with 1 to 2 layers of cumulus cells left were washed thrice in a microdroplet of equilibrated IVF-TALP before being transferred into IVF-TALP culture dish (80 µl/microdroplet; 5-10 oocytes/microdroplet) overlaid with mineral oil. The culture dish was cultured inside CO_2 incubator (5%; 38.5ºC) to allow oocytes ready for insemination later.

3.3.5.2 (g) (iii) ***Insemination process of caprine/bovine***

After 1 hour of sperm capacitation and oocytes were ready for insemination process, 10 µl of sperm suspension from Sp-TALP microcentrifuge tube incubated earlier was took out and dispensed on the glass slide and observed under inverted microscope to examine the sperm motility and availability. When the sperm confirmed present (alive; concentration $1.0x10^6$ sperm/ml), 20 µl of sperm suspension from Sp-TALP microcentrifuge tube was dispensed into each IVF-TALP microdroplet (20 µl/5-10 oocytes) and allowed to inseminated in CO_2 incubator (5%; 38.5ºC) for 18 to 24 hours.

3.3.5.2 (h) *In vitro culture (IVC) of activated and in vitro fertilised caprine/bovine embryos*

Culture dish (35 mm) contains 7 microdroplets (80 µl/microdroplet) of KSOM working solution was overlaid with mineral oil and equilibrated in CO_2 incubator (5%; 38.5ºC) overnight. All the activated and fertilised embryos were washed thrice respectively in TL-Hepes followed by equilibrated KSOM after the few hours of the respective treatments in order to wash off the residue of activation medium (PA); sperm and cumulus cells (IVF). Activated oocytes and fertilised embryos were then transferred into KSOM microdroplet (10-15 oocytes/microdroplet) respectively, and subsequently cultured in CO_2 incubator (5%; 38.5ºC) for later embryos development. The IVC medium was changed at day-3 and -5 with KSOM working solution supplemented with glucose (0.004g/10 ml; 2.85 mM). The embryos were checked at Day-3, -5 and -7 accordingly and their development was recorded.

3.3.5.3 Hoechst Staining of Parthenotes

In order to ensure the specific embryonic developmental stage, the embryos were stained with Hoechst at the particular embryonic development stage. The number of nuclei in the blastomeres of the particular embryos was determined and counted using epifluorescence microscope. Basically, a five microdroplets of PBS (-) (100 µl) and fixative solution (100 µl) were made and overlaid with mineral oil on the Petri dish (60 mm). The cleaved embryos were washed in PBS (-) five times followed by in fixative solution thrice on stage warmer stereomicroscope (38.5°C). Then, the embryos were placed to the last droplet of fixative solution for 5 minutes at 25°C. Four small drops of Vaseline-wax mixture were made on the centre of the glass slide to make a border. After 5 minutes, the embryos were transferred on centre of the slide and the excessive fixative solution was slowly aspirated

out from the embryos before mounted with a coverslip. Hoechst stain solution (10 µl) was sipped through the coverslip and leave for a while to let the embryos absorb the stain. The coverslip was sealed with a layer of nail polish, labeled, wrapped in aluminium foil and kept in refrigerator (4°C) before being examined under epifluorescence microscope.

3.4 EXPERIMENTAL DESIGN

The present study was carried out to investigate and evaluate the ability of the certain chemicals to activate murine, caprine and bovine oocytes artificially as well as to compare the development competency of the respective embryos activated by different activation chemicals and with control (*in vitro* fertilised embryo). Other factors that affect the development of parthenotes such as hormone administration on LOPU caprine donor, IVM duration of caprine oocytes and post-hCG duration on murine oocytes were also evaluated in this study.

3.4.1 Effect of Different Combinations of Activation Chemical on the Production of Parthenogenetic Murine Embryos as Model Animals (Experiment 1)

The objectives of this experiment were (i) to develop a protocol of parthenogenetic activation in murine as model animals, (ii) to compare effect of the different activation chemicals in term of combination, duration of treatment and chemical concentration on the murine embryonic development and to (iii) to determine the best activation chemical in order to activate murine oocytes in the optimal way. The naked murine oocytes with the first polar body were treated in several different combinations of activation chemicals (i) $SrCl_2$ (concentration: 2, 4, 6, 8 and 10 mM; incubation duration: 1, 2, 3, 4 and 5 hours) + CB (5 µg/ml) in CZB Ca^{2+} free medium, (ii) CaI (5 µM; 5 minutes) + 6-DMAP (2 mM; 4

hours), (iii) CaI (5 µM; 5 minutes) + CHX (10 µg/ml; 4 hours), (iv) CaI (5 µM; 5 minutes) + CB (5 µg/ml; 4 hours), and (v) EtOH (7, 8, and 9%; 5 minutes) + 6-DMAP (2 mM; 4 hours). At the end of this experiment, the development of the parthenotes activated by different combination treatments was compared. The parameters such as oocytes maturation rate, embryo cleavage rate and embryo developmental rate between different chemical treatments were also evaluated.

3.4.2 Effect of Post-hCG Duration on the Production of Murine Oocytes and its Subsequent Parthenote Development (Experiment 2)

The objective of this experiment was to compare the effect post-hCG duration on the maturation rate, cleavage rate and embryonic developmental rate of murine oocytes. There were two groups of post-hCG duration investigated (Group 1: 13-15 hours and Group 2: 16-18 hours). The parameters such as oocytes maturation rate and embryonic developmental rate between two different groups of post-hCG duration were determined.

3.4.3 Effect of Different Combinations of Activation Chemical on the Production of Parthenogenetic Bovine Embryos as a Model Species of Livestock Animals (Experiment 3)

The objectives of this experiment were (i) to develop a protocol of parthenogenetic activation in bovine as a model species of livestock animals, (ii) to compare effect of the different activation chemicals in term of combination, duration of treatment and chemical concentration on the bovine embryonic development and to (iii) to determine the best activation chemical in order to activate bovine oocytes in the optimal way. Naked bovine oocytes with the first polar body were treated in several different combinations of activation chemicals (i) CaI (5 µM; 5 minutes) + 6-DMAP (2 mM; 4 hours), (ii) Iono (10 µM; 5 minutes) + 6-DMAP (2 mM; 4 hours), (iii) EtOH (8%; 5 minutes) + 6-DMAP (2

mM; 4 hours) and (iv) Iono (10 µM; 5 minutes) + CHX (10 µg/ml; 4 hours). The embryonic development of the parthenotes activated by different combination treatments was compared in order to find the optimal treatment that induced bovine parthenogenesis *in vitro*. At the end of this experiment, the development of the parthenotes was also compared with the *in vitro* fertilised embryos as a control group. The effect of oocytes maturation rate and the embryonic developmental rate on the different oocytes grade were also compared. The parameters such as oocytes maturation rate, embryo cleavage rate and embryo developmental rate between different chemical treatments were evaluated.

3.4.4 Effect of Different Combinations of Activation Chemical on the Production of Parthenogenetic Caprine Embryos (Experiment 4)

In this experiment, single and combination treatment of chemicals were used in order to activate caprine oocytes artificially. The objectives of this experiment were (i) to develop a protocol of parthenogenetic activation in caprine, (ii) to compare effect of the different activation chemicals in term of single or combination treatments, duration of treatment and chemical concentration on the caprine embryonic development and (iii) to determine the best activation chemical in order to activate caprine oocytes in the optimal way. Naked caprine oocytes with the first polar body were treated in a single treatment: (i) Iono (10 µM; 5 minutes) and (ii) CaI (5 µM; 5 minutes) as well as in different combination treatments: (i) CaI (5 µM; 5 minutes) + 6-DMAP (2 mM; 3, 4, 5 and 6 hours), (ii) Iono (10 µM; 5 minutes) + 6-DMAP (2 mM; 3, 4, 5 and 6 hours), (iii) Iono (10 µM; 5 minutes) + CHX (10 µg/ml; 3, 4, 5 and 6 hours) and (iv) Iono (10 µM; 5 minutes) + CB (5 µg/ml; 3, 4, 5 and 6 hours). The embryonic development of the parthenotes in each group was compared and the later comparison was with the best combination in each group in order to find the optimal treatment that induced caprine parthenogenesis *in vitro*. At the end of this

experiment, the development of the parthenotes was also compared with the *in vitro* fertilised embryos as a control group. The effect of oocytes maturation rate and the embryonic developmental rate on the different grade of oocytes were also compared. The parameters such as oocytes maturation rate, embryo cleavage rate and embryo developmental rate between different chemical treatments were evaluated.

3.4.5 Effect of *In Vitro* Maturation (IVM) Duration on the Production of Caprine Oocytes and its Subsequent Parthenote Development (Experiment 5)

The objective of this experiment was to compare the effect IVM duration on the oocyte grades, maturation rate and embryonic developmental rate of caprine oocytes from LOPU. There were two groups of IVM duration evaluated (18-21 and 22-25 hours). The parameters such as number of oocytes retrieved, oocytes maturation rate, embryo cleavage rate and embryo developmental rate between different groups and oocyte grades were evaluated.

3.5 STATISTICAL ANALYSIS

The data obtained were expressed as mean and Standard Error of Mean (mean±SEM) and a probability of $P<0.05$ were considered as significant for all statistical tests (Steel and Torrie, 1981). All data were analysed using one-way Analysis of Variance (ANOVA). Difference between developmental rate in each treatment and embryo stage were further analysed using the Duncan Multiple Range Test (DMRT). The software used was SPSS (Statistics Package for Social Science) for Windows, (Version 17.0; SPSS Inc., Chicago, IL, USA).

For Experiment 1, effect of the factors (type of chemical used as activation medium, combination agent, duration of the activation treatment and concentration of the activation chemical of murine oocytes) on the parameters (cleavage rate at each particular embryonic developmental stage) were determined. For Experiment 2, effect of the factor (duration of post-hCG treatment in murine oocytes) on the parameters (maturation rate, cleavage rate at each particular embryonic developmental stage) were determined. For Experiment 3, effect of the factors (type of chemical used as activation medium, combination agent, duration of the activation treatment and concentration of the activation chemical of caprine oocytes) on the parameters (maturation rate, cleavage rate at each particular embryonic developmental stage as well as on the quality of oocytes) were determined. For Experiment 4, effect of the factor (duration of *in vitro* maturation duration of caprine oocytes) on the parameters (maturation rate, cleavage rate at each particular embryonic developmental stage as well as on the quality of oocytes) were determined. For Experiment 5, effect of the factors (type of chemical used as activation medium, combination agent, duration of the activation treatment and concentration of the activation chemical of bovine oocytes) on the parameters (maturation rate, cleavage rate at each particular embryonic developmental stage as well as on the quality of oocytes) were determined.

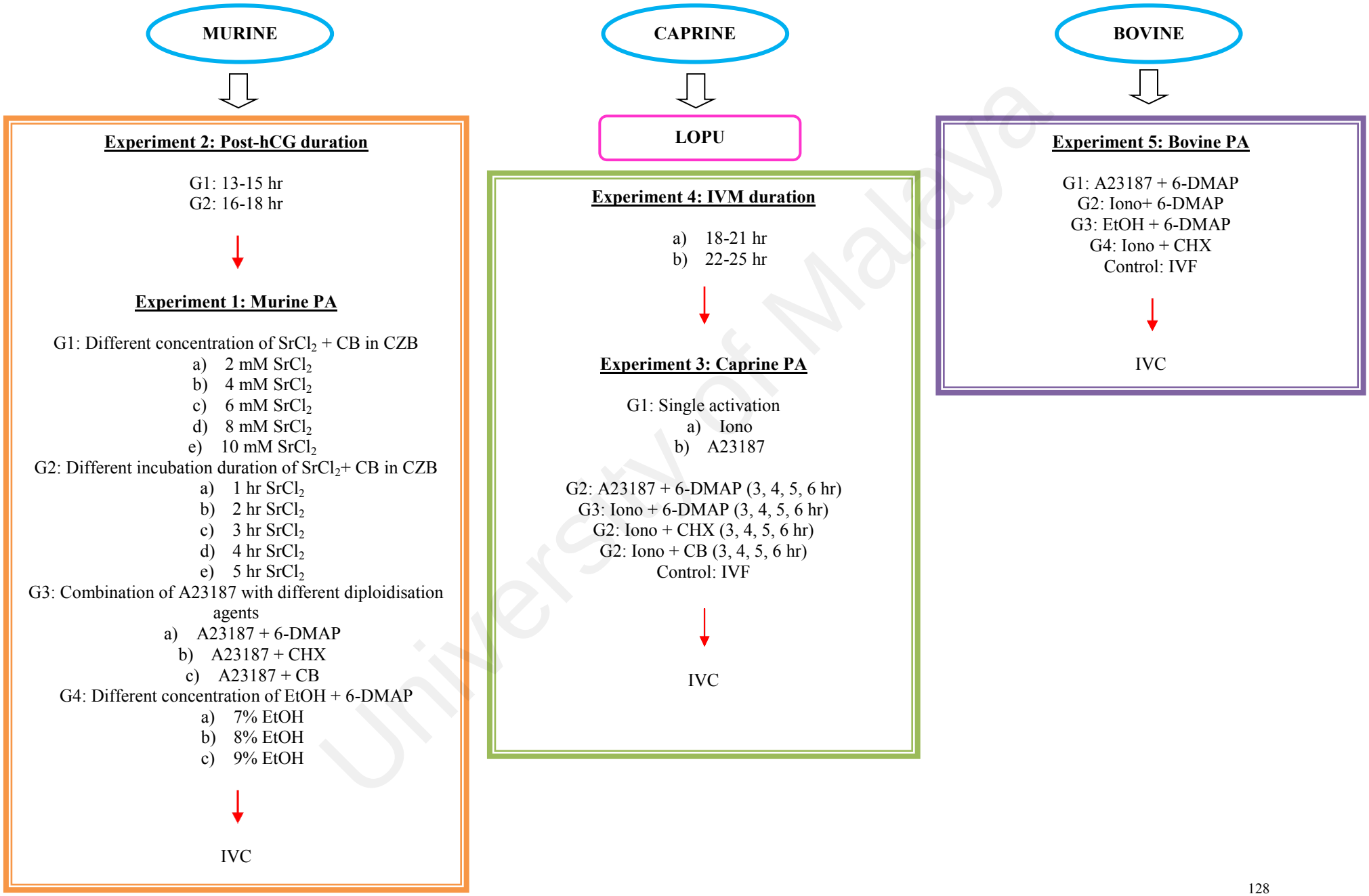

Figure 3.8: Flow chart of experimental design.

Chapter 4

4.0 RESULTS

Chapter 4

4.0 RESULTS

4.1 EFFECT OF DIFFERENT COMBINATIONS OF ACTIVATION CHEMICAL ON THE PRODUCTION OF PARTHENOGENETIC MURINE EMBRYOS AS MODEL ANIMALS (EXPERIMENT 1)

A total of 5479 oocytes were recovered from 381 donor murine to collect oocytes as shown in Table 4.1. Out of this, 73.26% of oocytes were at MII stage (presence of polar body) and the rest 26.74% were absent of polar body (Figure 4.1).

Table 4.2 shows the comparison of murine embryonic development after activation of matured oocytes by using five different concentrations of strontium chloride ($SrCl_2$). There were no significant differences ($P>0.05$) among all five concentrations of $SrCl_2$ at every embryonic developmental stages with the values of 80.96-89.06% (2-cell); 67.84-80.00% (4-cell); 55.29-69.79% (8-cell); 44.44-59.36% (morula) and 31.02-46.83% (blastocyst), respectively, when oocytes were treated with five different concentrations of $SrCl_2$ + 5 µg/ml CB in CZB Ca^{2+}-free medium for 5 hours. However, treatment with 10 mM $SrCl_2$ showed the highest cleavage (89.06±2.76%) and blastocyst (46.83±5.37%) rates.

Table 4.3 shows the comparison of murine embryonic development after activation of matured oocytes by using five different durations of 10 mM $SrCl_2$ + 5 µg/ml CB in CZB Ca^{2+}-free medium. Even though there were insignificant differences ($P>0.05$), generally oocytes treated with 10 mM $SrCl_2$ + 5 µg/ml CB in CZB Ca^{2+}-free medium for 3 hours incubation duration showed the highest cleavage rates 93.83±1.82% (2-cell); 84.30±3.06% (4-cell); 79.24±3.74% (8-cell); 68.78±4.56% (morula) and 60.42±4.92% (blastocyst), respectively, followed by 4, 5, 2 and 1 hours. Figure 4.2 shows the morphological changes

of cleaved parthenogenetic murine embryos activated by 10 mM $SrCl_2$ + 5 µg/ml CB in CZB Ca^{2+}-free medium for 3 hours.

In evaluating optimal combination treatment after treated with calcium ionophore (A23187) (Table 4.4), the combination of A23187 + 6-DMAP shows the significantly ($P<0.05$) highest cleavage rates at all embryonic stages compared to the combination of A23187 + CB (2-cell: 79.48±5.63% vs. 61.88±5.07%; 4-cell: 59.83±6.29% vs. 43.53±5.08%; 8-cell: 50.58±35.15% vs. 29.66±5.08%; morula: 30.29±5.51% vs. 16.34±3.98% and blastocyst: 23.28±4.62% vs. 9.76±2.74%, respectively). However, treatment with combination of A23187 + CHX only shows significant different ($P<0.05$) with treatment with combination of A23187 + 6-DMAP at 8-cell stage (50.58±35.15% vs. 31.14±4.79%, respectively) until blastocyst (23.28±4.62% vs. 9.24±3.02%, respectively).

In evaluating optimal concentration of ethanol (EtOH) as combination with 2 mM 6-DMAP for 4 hours incubation duration (Table 4.5), treatment with 8% EtOH exhibited the highest cleavage rates at all embryonic stages and only significantly ($P<0.05$) higher from the other two combinations at 8-cell stage (39.70±4.73%), morula (21.50±4.14%) and blastocyst stage (13.86±3.64%).

When comparing the optimal methods of activation protocols from each group of combination treatment (Table 4.6), combination of 10 mM $SrCl_2$ + 5 µg/ml CB gave significantly the highest ($P<0.05$) cleavage rates at all embryonic stages compared to the other two treatments. The cleavage rates were decreased in all cases from early to the later stages of embryonic development significantly ($P<0.05$).

Table 4.1: Number and percentages of oocytes obtained through oviduct oocytes retrieval

Total no. of mice	Total no. of ovaries*	Total no. of oocytes	Total no. of oocytes/ovary	Percent of oocytes with polar body	Percent of oocytes without polar body
381	733	5479	7.47	73.26 (n=4014)	26.74 (n=1465)

* Total number of ovaries was based on ovaries that contain oocytes.

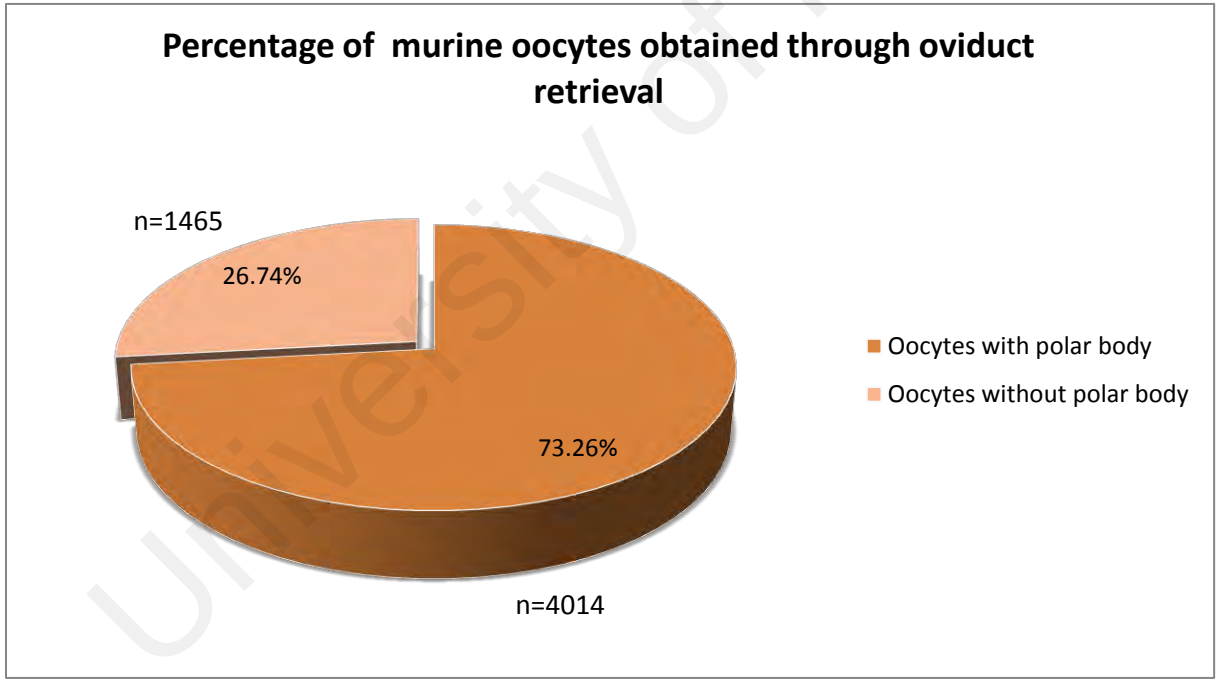

Figure 4.1: Pie chart of percentage of murine oocytes obtained through oviduct retrieval.

Table 4.2: Comparison of *in vitro* embryonic development of murine parthenotes after activation by using five different concentrations of $SrCl_2$

$SrCl_2$ concentration (mM)	No. of oocyte retrieved	Percent of oocyte with polar body (%)	Embryos cleavage rate (%)*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
2	347	75.79 (n=263)	86.82±3.19^{az} (n=223)	71.16±4.20^{az} (n=191)	56.02±5.50^{ay} (n=158)	44.44±5.52^{axy} (n=152)	33.42±5.11^{ax} (n=96)
4	347	74.93 (n=260)	85.11±2.95^{az} (n=215)	67.84±4.64^{ay} (n=171)	55.29±5.97^{axy} (n=137)	45.14±6.42^{awx} (n=108)	31.02±5.97^{aw} (n=74)
6	348	75.29 (n=262)	88.80±2.70^{az} (n=224)	77.66±5.13^{ayz} (n=193)	66.20±5.44^{axy} (n=162)	53.04±5.65^{awx} (n=133)	39.26±6.11^{aw} (n=102)
8	348	75.00 (n=261)	80.96±3.99^{az} (n=214)	71.00±4.46^{ayz} (n=187)	59.00±4.40^{axy} (n=153)	50.80±4.48^{awx} (n=131)	40.70±5.37^{aw} (n=102)
10	348	75.86 (n=264)	89.06±2.76^{az} (n=231)	80.00±3.21^{ayx} (n=200)	69.79±3.75^{axy} (n=174)	59.36±4.48^{ax} (n=143)	46.83±5.37^{aw} (n=111)

n: no. of oocytes/embryos

* Cleavage rate was based on percent of oocyte with polar body

[a] Means with similar superscripts in a column were insignificantly different (P>0.05)

[wxyz] Means with different superscripts in a row were significantly different (P<0.05)

Table 4.3: Comparison of *in vitro* embryonic development of murine parthenotes after activation by using five different incubation durations of $SrCl_2$

$SrCl_2$ duration (hours)	No. of oocyte retrieved	Percent of oocyte with polar body (%)	Embryos cleavage rate (%)*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
1	345	76.52 (n=264)	74.05 ± 3.37^{az} (n=188)	62.34 ± 3.39^{ay} (n=264)	48.46 ± 3.48^{ax} (n=128)	38.17 ± 3.75^{aw} (n=102)	29.62 ± 3.73^{aw} (n=79)
2	348	75.28 (n=262)	82.51 ± 2.92^{abz} (n=215)	67.80 ± 2.85^{aby} (n=176)	57.35 ± 3.48^{abx} (n=150)	45.95 ± 3.79^{abw} (n=116)	33.39 ± 4.48^{abv} (n=82)
3	348	75.28 (n=262)	93.83 ± 1.82^{cz} (n=242)	84.30 ± 3.06^{cyz} (n=211)	79.24 ± 3.74^{cxy} (n=193)	68.78 ± 4.56^{cwx} (n=164)	60.42 ± 4.92^{cw} (n=138)
4	346	74.57 (n=258)	88.93 ± 3.21^{bcz} (n=217)	78.70 ± 4.59^{bcyz} (n=187)	66.29 ± 5.67^{bxy} (n=156)	58.21 ± 6.15^{bcwx} (n=130)	48.00 ± 7.18^{bcw} (n=101)
5	346	75.43 (n=261)	81.13 ± 3.71^{abz} (n=204)	73.88 ± 4.37^{bcyz} (n=179)	61.82 ± 5.00^{bxy} (n=150)	51.32 ± 5.81^{abwx} (n=124)	40.19 ± 5.83^{abw} (n=91)

n: no. of oocytes/embryos

* Cleavage rate was based on percent of oocyte with polar body

^{abc} Means with different superscripts in a column were significantly different ($P<0.05$)

^{vwxyz} Means with different superscripts in a row were significantly different ($P<0.05$)

Table 4.4: Comparison of *in vitro* embryonic development of murine parthenotes after activation by using calcium ionophore (A23187), followed by different combination (6-DMAP, CHX or CB)

Combination	No. of oocyte retrieved	Percent of oocyte with polar body (%)	Embryos cleavage rate (%)*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
6-DMAP	370	68.38 (n=253)	79.48±5.63^{bz} (n=216)	59.83±6.29^{by} (n=174)	50.58±35.15^{by} (n=147)	30.29±5.51^{bx} (n=97)	23.28±4.62^{bx} (n=52)
CHX	371	68.46 (n=254)	72.62±5.17^{abz} (n=194)	45.26±4.10^{aby} (n=134)	31.14±4.79^{ax} (n=97)	15.01±3.69^{aw} (n=52)	9.24±3.02^{aw} (n=19)
CB	369	69.11 (n=255)	61.88±5.07^{az} (n=172)	43.53±5.08^{ay} (n=125)	29.66±5.08^{ax} (n=95)	16.34±3.98^{aw} (n=54)	9.76±2.74^{aw} (n=15)

n: no. of oocytes/embryos

***** Cleavage rate was based on the percent of oocyte with polar body

^{ab} Means with different superscripts in a column were significantly different (P<0.05)

^{wxyz} Means with different superscripts in a row were significantly different (P<0.05)

Table 4.5: Comparison of *in vitro* embryonic development of murine parthenotes after activation by using three different concentrations of ethanol followed by 6-DMAP

EtOH concentration (%)	No. of oocyte retrieved	Percent of oocyte with polar body (%)	Embryos cleavage rate (%)*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
7	299	70.90 (n=212)	54.33±6.36^{az} (n=123)	35.29±6.29^{ay} (n=83)	23.20±4.93^{ay} (n=57)	9.28±2.65^{ax} (n=23)	4.10±1.62^{ax} (n=10)
8	300	70.33 (n=211)	77.07±4.21^{bz} (n=161)	53.96±4.89^{by} (n=109)	39.70±4.73^{bx} (n=90)	21.50±4.14^{bw} (n=51)	13.86±3.64^{bw} (n=36)
9	299	70.90 (n=212)	64.37±4.52^{abz} (n=141)	39.56±5.41^{aby} (n=90)	24.87±4.28^{ax} (n=60)	10.31±2.78^{aw} (n=30)	5.12±1.82^{aw} (n=16)

n: no. of oocytes/embryos

***** Cleavage rate was based on percent of oocyte with polar body

^{ab} Means with different superscripts in a column were significantly different ($P<0.05$)

^{wxyz} Means with different superscripts in a row were significantly different ($P<0.05$)

Table 4.6: Comparison of *in vitro* embryonic development of murine parthenotes between three best results of oocytes activation using $SrCl_2$+CB, A23187+DMAP and EtOH+DMAP

Combination	No. of oocyte retrieved	Percent of oocyte with polar body (%)	Embryos cleavage rate (%)*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
$SrCl_2$ + CB	348	75.28 (n=262)	93.83 ± 1.82^{bz} (n=242)	84.30 ± 3.06^{byz} (n=211)	79.24 ± 3.74^{bxy} (n=193)	68.78 ± 4.56^{bwx} (n=164)	60.42 ± 4.92^{bw} (n=138)
CaI + DMAP	370	68.38 (n=253)	79.48 ± 5.63^{az} (n=216)	59.83 ± 6.29^{ay} (n=174)	50.58 ± 35.15^{ay} (n=147)	30.29 ± 5.51^{ax} (n=97)	23.28 ± 4.62^{ax} (n=52)
EtOH + DMAP	300	70.33 (n=211)	77.07 ± 4.21^{az} (n=161)	53.96 ± 4.89^{ay} (n=109)	39.70 ± 4.73^{ax} (n=90)	21.50 ± 4.14^{aw} (n=51)	13.86 ± 3.64^{aw} (n=36)

n: no. of oocytes/embryos

***** Cleavage rate was based on the percent of oocyte with polar body

^{ab} Means with different superscripts in a column were significantly different ($P<0.05$)

^{wxyz} Means with different superscripts in a row were significantly different ($P<0.05$)

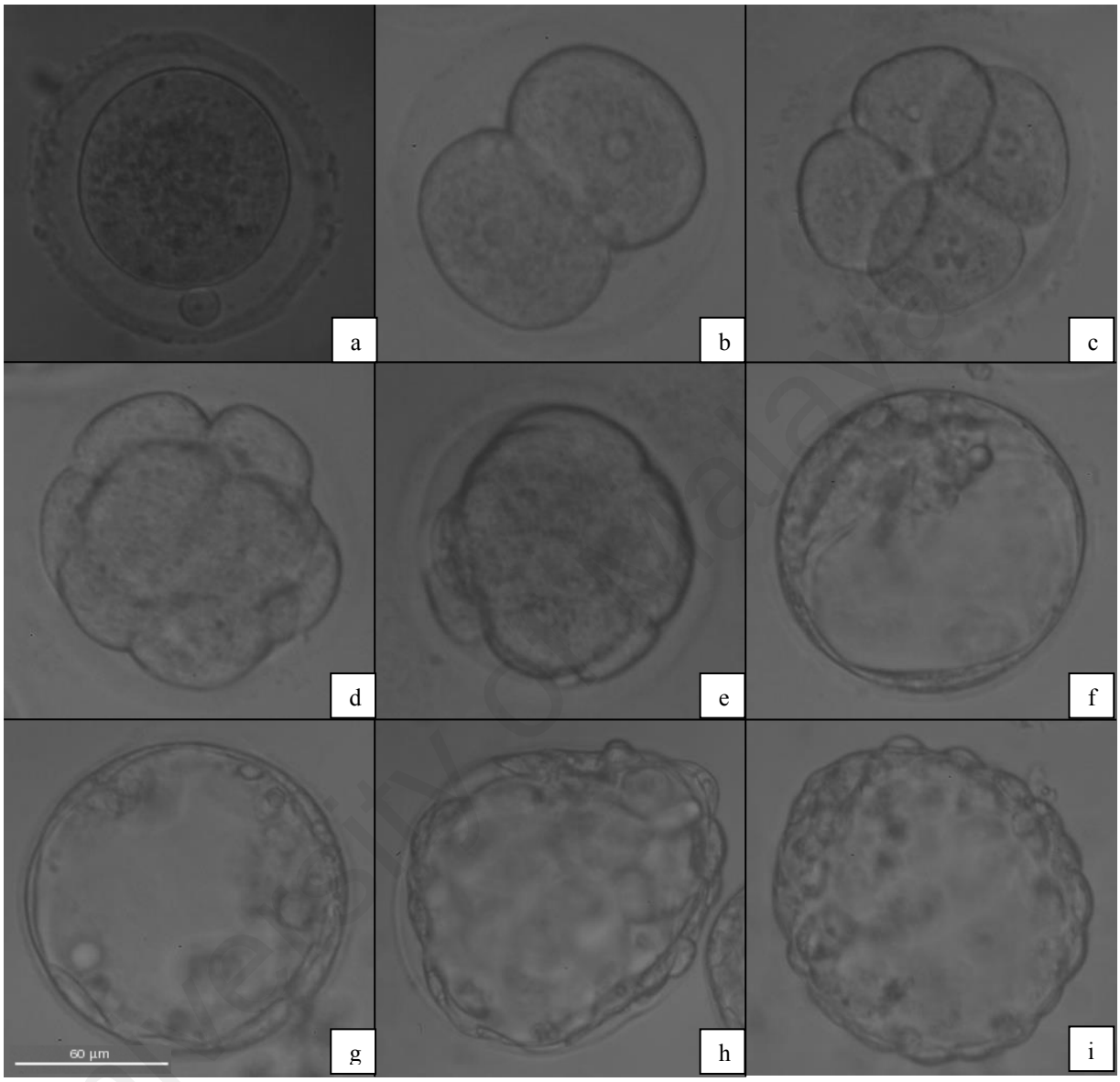

Figure 4.2: Morphological changes of parthenogenetic murine embryos activated by 10 mM $SrCl_2$ + 5 µg/ml CB in CZB Ca^{2+}-free medium for 3 h at different cell stages at (a) 1-cell, (b) 2-cell, (c) 4-cell, (d) 8-cell, (e) morula, (f) expanding blastocyst, (g) fully expanded blastocyst, (h) hatching blastocyst and (i) hatched blastocyst stage.

4.2 EFFECT OF POST-hCG DURATION ON THE PRODUCTION OF MURINE OOCYTES AND ITS SUBSEQUENT PARTHENOTES DEVELOPMENT (EXPERIMENT 2)

Two groups (13-15 hours and 16-18 hours) of post-hCG duration were evaluated in this experiment to determine the effect of post-hCG administration durations on the oocyte retrieval. Subsequent maturation rate and embryonic development were also evaluated (Table 4.7). A total of 5479 oocytes were obtained from 381 donor murine which were further divided into two groups consisting of 2673 and 2806 oocytes for 13-15 hours and 16-18 hours durations, respectively.

Even though insignificance ($P>0.05$) in the percent of oocyte with polar body between these two groups of post-hCG duration (70.69±1.04% vs. 70.08±1.05%), group of 13-15 hours post-hCG duration were significantly ($P<0.05$) higher than group of 16-18 hours in all embryonic developmental stages (2-cell: 81.90±1.53% vs. 76.41±1.64%; 4-cell: 67.87±2.02% vs. 60.20±1.92%; 8-cell: 55.50±2.25% vs. 48.37±1.96%; morula: 43.68±2.42% vs. 35.19±2.01% and blastocyst: 34.36±2.34% vs. 26.04±1.88%, respectively). The cleavage rates were decreased in all cases from early to the later stages of embryonic development significantly ($P<0.05$).

Table 4.7: Comparison of *in vitro* embryonic development of murine parthenotes between two different post-hCG durations

Post-hCG duration (hour)	No. of oocytes retrieved	Percent of oocyte with polar body (%)	Embryo cleavage rate (%)*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
13-15	2673	70.69±1.04^{a} (n=1979)	81.90±1.53^{bz} (n=1566)	67.87±2.02^{by} (n=1318)	55.50±2.25^{bx} (n=1045)	43.68±2.42^{bw} (n=856)	34.36±2.34^{bv} (n=581)
16-18	2806	70.08±1.05^{a} (n=2035)	76.41±1.64^{az} (n=1607)	60.20±1.92^{ay} (n=1251)	48.37±1.96^{ax} (n=1021)	35.19±2.01^{aw} (n=737)	26.04±1.88^{av} (n=533)

n: no. of oocytes/embryos

* Cleavage rate was based on the maturation rate

^{ab} Means with different superscripts in a column were significantly different (P<0.05)

^{vwxyz} Means with different superscripts in a row were significantly different (P<0.05)

4.3 EFFECT OF DIFFERENT COMBINATIONS OF ACTIVATION CHEMICAL ON THE PRODUCTION OF PARTHENOGENETIC BOVINE EMBRYOS AS A MODEL SPECIES TO LIVESTOCK ANIMALS (EXPERIMENT 3)

A total 918 oocytes were retrieved from 77 ovaries using ovary slicing procedure as shown in Table 4.8. As shown in Figure 4.3, 61.22% oocytes were matured (presence of polar body) and the rest 38.78% were not matured (absence of polar body).

Table 4.9 shows comparison of embryonic development after activation of matured oocytes by using combination of different types of chemical. Treatment with combination of 10 μM Iono + 2 mM 6-DMAP indicated the highest embryonic cleavage rates with the values of 60.96±4.34% (4-cell), 42.38±4.90% (8-cell), 24.32±3.97% (morula) and 7.10±1.43% (blastocyst), compared to the other combination treatments. Treatment with Iono + CHX was significantly lower ($P<0.05$) than other combination treatments at 8-cell stage. There were significant differences ($P<0.05$) between combination of Iono + 6-DMAP with combination of Iono + CHX and IVF control at morula (24.32±3.97% vs. 8.80±2.89% and 24.32±3.97% vs. 3.40±1.97%, respectively) and blastocyst (7.10±1.43% vs. 2.16±1.56% and 7.10±1.43% vs. 0.00±0.00%, respectively) stages, respectively. Figure 4.4 shows the image of activated bovine oocyte with polar body, whereas Figure 4.5 shows the morphological changes of cleaved parthenogenetic bovine embryos activated by various combinations of chemical treatment.

Table 4.10 shows the development of bovine parthenotes according to the oocyte grades from every activation protocols. Grade B oocytes from A23187 + 6-DMAP treated group shows significant different ($P<0.05$) in maturation rates than Grades A, B and C oocytes those treated with Iono + CHX. Grade C oocytes from EtOH + 6-DMAP indicated significantly higher ($P<0.05$) cleavage rate (2-cell) than Grade B those treated with Iono + CHX. However, no differences ($P>0.05$) were observed in blastocyst rates for each oocyte

grade from every activation protocols and IVF control. Generally, Grade A oocytes from each activation protocol shows highest percentage in both maturation and cleavage rates (2-cell to blastocyst). The cleavage rates were decreased in all cases from early to the later stages of embryonic development significantly ($P<0.05$).

Table 4.8: Number and percentage of oocytes obtained from ovary slicing

Total no. of ovaries*	Total no. of oocytes	Total no. of oocytes/ovary	Percent of oocytes with polar body (%)	Percent of oocytes without polar body (%)
77	918	11.92	61.22 (n=562)	38.78 (n=356)

* Total number of ovaries was based on ovaries that contain oocytes.

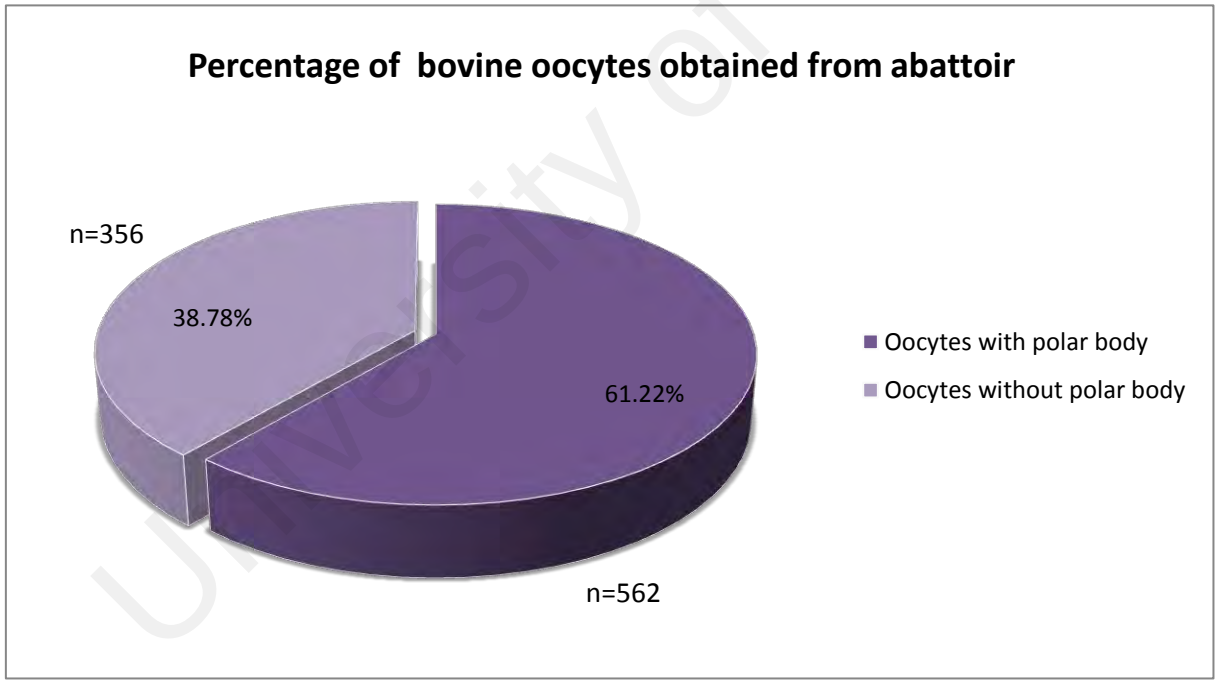

Figure 4.3: Pie chart of percentage of bovine oocytes obtained from abattoir.

Table 4.9: Comparison of *in vitro* embryonic development of bovine parthenotes after activation by various combination treatments

Treatment	No. of oocytes retrieved	Maturation rate (%)	Embryo cleavage rate (%)*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
CaI + DMAP	209	52.15 (n=109)	74.86±5.64^{az} (n=83)	57.01±3.66^{ay} (n=62)	41.79±4.33^{bx} (n=44)	17.90±3.60^{bcw} (n=20)	4.24±2.52^{abv} (n=5)
Iono + DMAP	223	65.92 (n=147)	73.60±4.68^{az} (n=109)	60.96±4.34^{ay} (n=89)	42.38±4.90^{bx} (n=63)	24.32±3.97^{cw} (n=35)	7.10±1.43^{bv} (n=13)
EtOH + DMAP	169	53.85 (n=91)	74.20±2.84^{az} (n=64)	55.97±3.46^{ay} (n=51)	37.53±3.71^{bx} (n=36)	18.55±4.49^{bcw} (n=18)	0.00±0.00^{av} (n=0)
Iono + CHX	188	72.34 (n=136)	63.48±9.12^{az} (n=87)	46.28±7.09^{ay} (n=63)	19.66±4.44^{ax} (n=27)	8.80±2.89^{abx} (n=12)	2.16±1.56^{abx} (n=3)
IVF**	129	61.24 (n=79)	73.00±7.22^{az} (n=53)	62.90±9.30^{az} (n=45)	37.63±8.71^{by} (n=29)	3.40±1.97^{ax} (n=4)	0.00±0.00^{ax} (n=0)

n: no. of oocytes/embryos
* Cleavage rate was based on the maturation rate
** IVF was used as a control
^{abc} Means with different superscripts in a column were significantly different (P<0.05)
^{vwxyz} Means with different superscripts in a row were significantly different (P<0.05)

Table 4.10: Comparison of *in vitro* embryonic development of bovine parthenotes according to oocyte grades from every activation protocols

Treatment	Oocyte grade	Total oocyte retrieved	Maturation rate (%)	Embryos cleavage rate (%)*				
				2-cell	4-cell	8-cell	Morula	Blastocyst
A23187 + DMAP (4 hr)	A	88	60.74±4.09^{abc} (n=53)	80.09±8.03^{abz} (n=45)	64.04±5.76^{ayz} (n=36)	49.45±8.86^{bcy} (n=27)	20.75±4.33^{abcdex} (n=12)	7.95±4.95^{ax} (n=4)
	B	31	50.27±6.54^{a} (n=16)	86.12±9.04^{abz} (n=14)	58.33±9.38^{ay} (n=8)	52.77±10.02^{cy} (n=7)	25.00±9.38^{bcdex} (n=4)	0.00±0.00^{av} (n=0)
	C	34	60.96±6.97^{abc} (n=20)	84.53±7.80^{abz} (n=16)	57.14±7.14^{ay} (n=11)	29.76±8.50^{abcy} (n=6)	16.67±8.91^{abcdevx} (n=3)	2.39±2.39^{av} (n=1)
Iono + DMAP (4 hr)	A	62	74.74±8.15^{bcd} (n=49)	85.92±8.45^{abz} (n=40)	69.23±9.93^{ayz} (n=31)	50.24±6.64^{bcy} (n=22)	28.70±1.63^{cdex} (n=14)	8.90±2.40^{av} (n=5)
	B	69	64.70±3.04^{abcd} (n=45)	72.78±4.92^{abz} (n=35)	63.40±4.19^{az} (n=29)	39.38±7.03^{abcy} (n=20)	25.78±8.13^{bcdey} (n=13)	8.32±3.64^{ax} (n=6)
	C	57	72.76±8.95^{abcd} (n=39)	75.56±7.73^{abz} (n=26)	66.68±10.28^{ayz} (n=22)	50.66±16.16^{bcyz} (n=16)	34.66±16.44^{exy} (n=8)	3.56±2.29^{ax} (n=2)
EtOH + DMAP (4 hr)	A	78	62.37 ±6.99^{abc} (n=52)	77.22±6.96^{abz} (n=40)	65.30±7.92^{ayz} (n=32)	47.82±5.85^{bcxy} (n=24)	33.10±7.46^{dex} (n=15)	0.00±0.00^{av} (n=0)
	B	24	61.12±8.44^{abc} (n=13)	81.95±8.72^{abz} (n=10)	63.88±11.72^{ayz} (n=7)	51.38±16.73^{bcy} (n=5)	0.00±0.00^{ax} (n=0)	0.00±0.00^{ax} (n=0)
	C	22	56.25±4.00^{abc} (n=13)	92.85±7.15^{bz} (n=11)	73.23±15.53^{az} (n=7)	13.40±7.77^{ay} (n=3)	9.83±6.08^{abcdy} (n=2)	0.00±0.00^{ay} (n=0)

(continued)

(continued)

Iono + CHX (4 hr)	A	55	85.16±6.49^{d} (n=44)	68.40±13.20^{abz} (n=27)	49.52±10.17^{ayz} (n=20)	19.08±4.09^{abxy} (n=9)	8.02±3.50^{abcx} (n=4)	1.43±1.43^{ax} (n=1)
	B	43	77.32±1.77^{cd} (n=33)	60.42±11.65^{az} (n=21)	41.50±8.97^{az} (n=14)	20.92±6.91^{abcz} (n=7)	12.22±5.60^{abcdez} (n=4)	2.86±2.86^{ay} (n=1)
	C	58	78.94±2.78^{cd} (n=46)	69.40±7.88^{abz} (n=34)	51.90±5.02^{ay} (n=25)	20.76±5.27^{abcx} (n=10)	8.04±3.76^{abcvx} (n=4)	2.00±2.00^{av} (n=1)
IVF**	A	44	54.60±8.22^{ab} (n=27)	79.05±13.03^{abz} (n=17)	74.63±15.29^{az} (n=15)	42.70±4.61^{abcy} (n=10)	4.43±2.60^{abcx} (n=2)	0.00±0.00^{ax} (n=0)
	B	41	58.55±9.32^{abc} (n=25)	75.00±10.21^{abz} (n=17)	62.50±16.14^{acz} (n=16)	39.38±17.63^{abcz} (n=10)	3.13±3.13^{aby} (n=1)	0.00±0.00^{ay} (n=0)
	C	41	64.25±6.93^{abcd} (n=27)	71.88±3.73^{abz} (n=19)	55.63±7.93^{acy} (n=14)	33.75±6.88^{abcx} (n=9)	3.13±3.13^{abv} (n=1)	0.00±0.00^{av} (n=0)

n: no. of oocytes/embryos

* Cleavage rate was based on the maturation rate

^{abcde} Means with different superscripts in a column were significantly different (P<0.05)

^{vwxyz} Means with different superscripts in a row were significantly different (P<0.05)

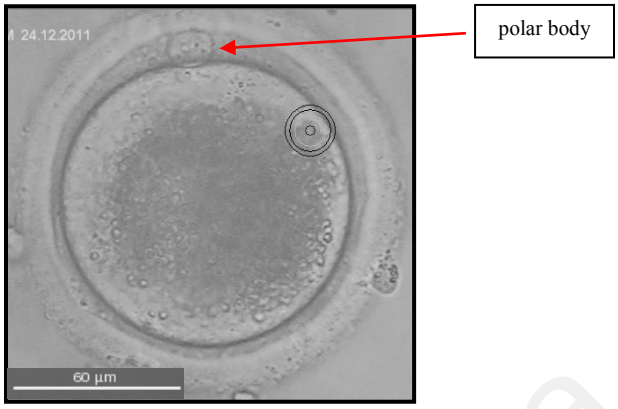

Figure 4.4: Activated bovine oocytes with polar body.

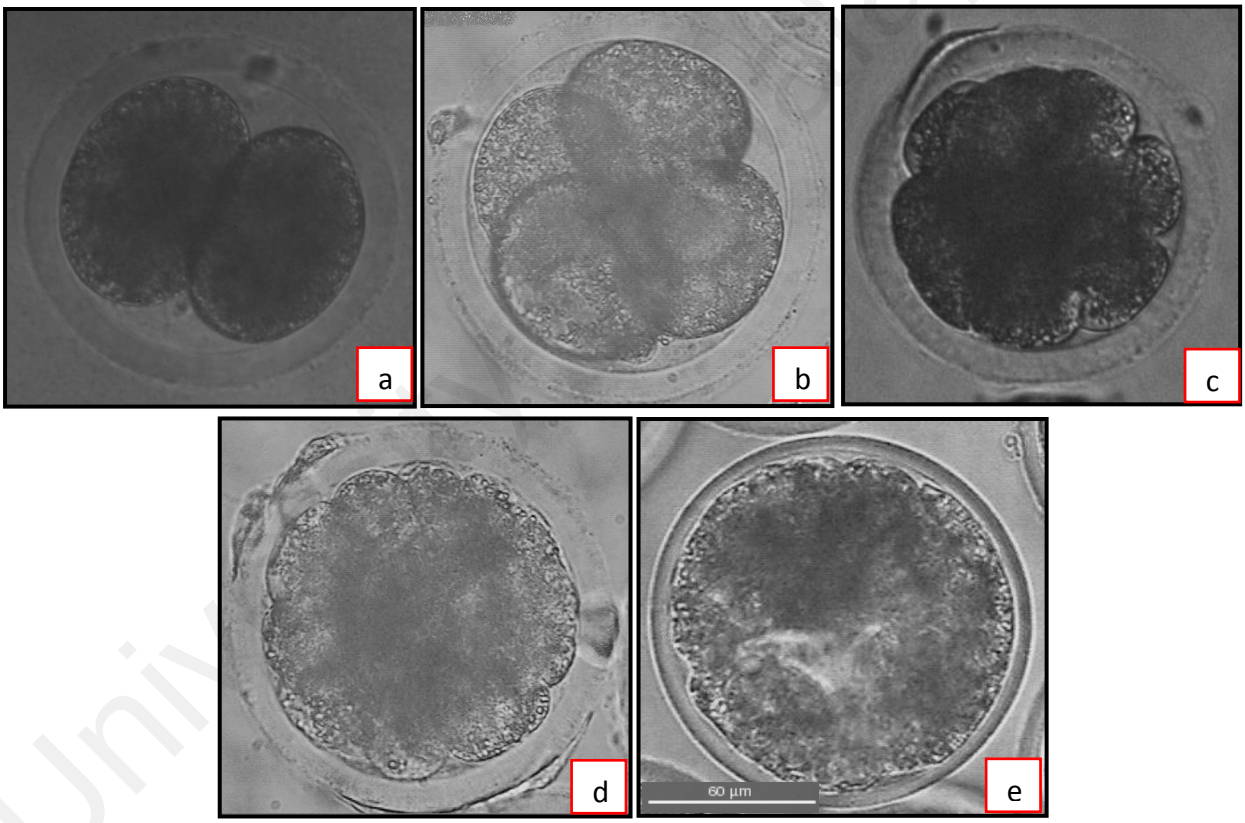

Figure 4.5: Parthenogenetic bovine embryos at: (a) 2-cell, (b) 4-cell, (c) 8-cell, (d) compacting morula and (e) early blastocyst.

4.4 EFFECT OF DIFFERENT COMBINATIONS OF ACTIVATION CHEMICAL ON THE PRODUCTION OF PARTHENOGENETIC CAPRINE EMBRYOS (EXPERIMENT 4)

A total of 64 donor caprine were used in LOPU procedure in order to collect the oocytes. Some donors were used two to three times to carry out the LOPU procedure. Out of 136 ovaries collected, 837 oocytes were obtained (Table 4.11) in which 73.48% of oocytes were matured (presence of polar body) and the rest 26.52% were not matured (absence of polar body) (Figure 4.6).

Table 4.12 shows comparison of caprine embryonic development after activation of matured oocytes by using a single chemical. There were no significant differences ($P>0.05$) between these two chemicals (Iono vs. A23187) at each embryonic developmental stage in maturation rate (61.54% vs. 59.46%) and cleavage rates (2-cell: 95.00±5.00% vs. 83.33±10.91%; 4-cell: 95.00±5.00% vs. 83.33±10.91%; 8-cell: 75.66±8.19% vs. 72.61±13.21%; morula: 53.34±16.16% vs. 45.24±14.53% and blastocyst: 20.34±12.46% vs. 9.53±7.14%, respectively).

Table 4.13 shows comparison of embryonic development after activation of matured oocytes by using combination of 5 μM A23187 + 2 mM 6-DMAP with different incubation durations in 6-DMAP (3, 4, 5 and 6 hours). Similarly, there were no significant differences ($P>0.05$) among these combination treatments at every embryonic developmental stage with treatment of A23187 + 6-DMAP for 3 hours incubation duration gave embryonic cleavage rates of 95.25±4.75% (2-cell), 92.85±7.15% (4-cell), 90.07±7.14% (8-cell), 47.22±14.27% (morula) and 10.72±5.49% (blastocyst), compared to the other incubation durations.

As shown in Table 4.14 in comparison of combination treatment by using 10 μM Iono + 2 mM 6-DMAP with different incubation durations in 6-DMAP (3, 4, 5 and 6 hours), treatment with the combination of Iono + 6-DMAP for 6 hours incubation duration, cleavage rates were naturally decreasing at each embryonic developmental stage as follows: 96.15±3.85% (2-cell), 84.28±10.89% (4-cell), 79.05±13.95% (8-cell), 56.35±12.15% (morula) and 35.00±13.72% (blastocyst). Blastocyst rate of 6 (35.00±13.72%) and 3 hours (24.66±10.40%) incubation durations in 6-DMAP was significantly higher ($P<0.05$) than those of 4 (0.00±0.00%) and 5 hours (0.00±0.00%) incubation durations.

In comparison of combination treatment by using 10 μM Iono + 10 μg/ml CHX with different incubation durations in CHX (3, 4, 5 and 6 hours) as shown in Table 4.15, treatment with Iono + CHX for 4 hours incubation duration apparently gave higher cleavage rates than the other incubation durations with the values of 91.67±4.82% (2-cell), 76.87±6.48% (4-cell), 68.50±10.20% (8-cell), 36.13±15.47% (morula) and 11.10±11.10% (blastocyst). As for morula stage, the incubation duration for 6 hours in CHX gave significantly higher ($P<0.05$) cleavage rate than 5 hours (14.93±9.16% vs. 49.35±3.57%, respectively).

In evaluating combination treatment by using 10 μM Iono + 5 μg/ml CB with different incubation duration in CB (3, 4, 5 and 6 hours) as shown in Table 4.16, treatment with Iono + CB for 5 hours incubation duration shows the highest embryonic cleavage rates with the values of 92.17±7.83% (2-cell), 88.23±11.77% (4-cell), 87.87±12.13% (8-cell) and 55.80±22.00% (morula). However, there was no embryo develop beyond morula stage at each incubation duration. The cleavage rates at 8-cell stage of 5 hours incubation duration were significantly different ($P<0.05$) from other incubation durations.

Table 4.17 shows that combination of 10 μM Iono + 2 mM 6-DMAP for 6 hours generally gave higher cleavage rates in most cases followed by 5 μM A23187 + 2 mM 6-DMAP for 3 hours, 10 μM Iono + 10 μg/ml CHX for 4 hours and 10 μM Iono + 5 μg/ml CB or 5 hours. In all cases, the cleavage rates were higher than those of IVF. Figure 4.7 shows image of activated oocyte with polar body whereas Figure 4.8 shows the morphological changes of cleaved parthenogenetic caprine embryos activated by various chemical combination treatments.

Table 4.18 shows the embryonic development of parthenotes according to the oocytes grades regardless the activation protocol used to activate oocytes. Grade C oocytes showed the highest number of total oocytes retrieved (40.30±2.29%) whereas Grade B oocyte showed the highest maturation rate (91.09±2.17%). The maturation rate of Grade B oocyte was significant ($P<0.05$) with Grades D and E oocytes. Cleavage rate (95.81±1.51%) of Grade B oocyte was the highest among the other grades. However, Grade A (12.35±3.29%) oocytes showed the highest blastocyst rate followed by Grades B, C, D and E. The cleavage rates were decreased in all cases from early to the later stages of embryonic development significantly ($P<0.05$).

Table 4.19 shows the development of caprine parthenotes according to the oocyte grades from the optimal activation protocol in each group. There were no significant differences ($P>0.05$) were observed in the maturation rate for each oocyte grade from every activation protocols. Grade A oocytes IVF control group was significantly lower ($P<0.05$) in cleavage rate (2-cell) from the other oocyte grades. However, no differences ($P>0.05$) were observed in morula and blastocyst rates for each oocyte grade from every activation protocols and IVF control. No differences ($P>0.05$) were indicated in maturation, cleavage (2-cell) and blastocyst rates for each oocyte grades from A23187 + 6-DMAP, Iono + 6-DMAP, Iono + CHX and Iono + CB treated groups. In contrast, Grade A

oocytes shows significant difference ($P<0.05$) in cleavage (2-cell) rate from other oocyte grades in IVF group. Generally, each grades of oocyte from every activation protocols and IVF control shows high percent in maturation, cleavage and blastocyst rates. The cleavage rates were decreased in all cases from early to the later stages of embryonic development significantly ($P<0.05$).

Table 4.11: Number and percentages of oocytes obtained from LOPU

Total no. of goats*	Total no. of ovaries**	Total no. of oocytes	Total no. of oocytes/ovary	Percent of oocytes with polar body (%)	Percent of oocytes without polar body (%)
64	136	837	6.15	73.48 (n=615)	26.52 (n=222)

* Some goats were used two to three times for LOPU.
** Total number of ovaries was based on ovaries that contain oocytes.

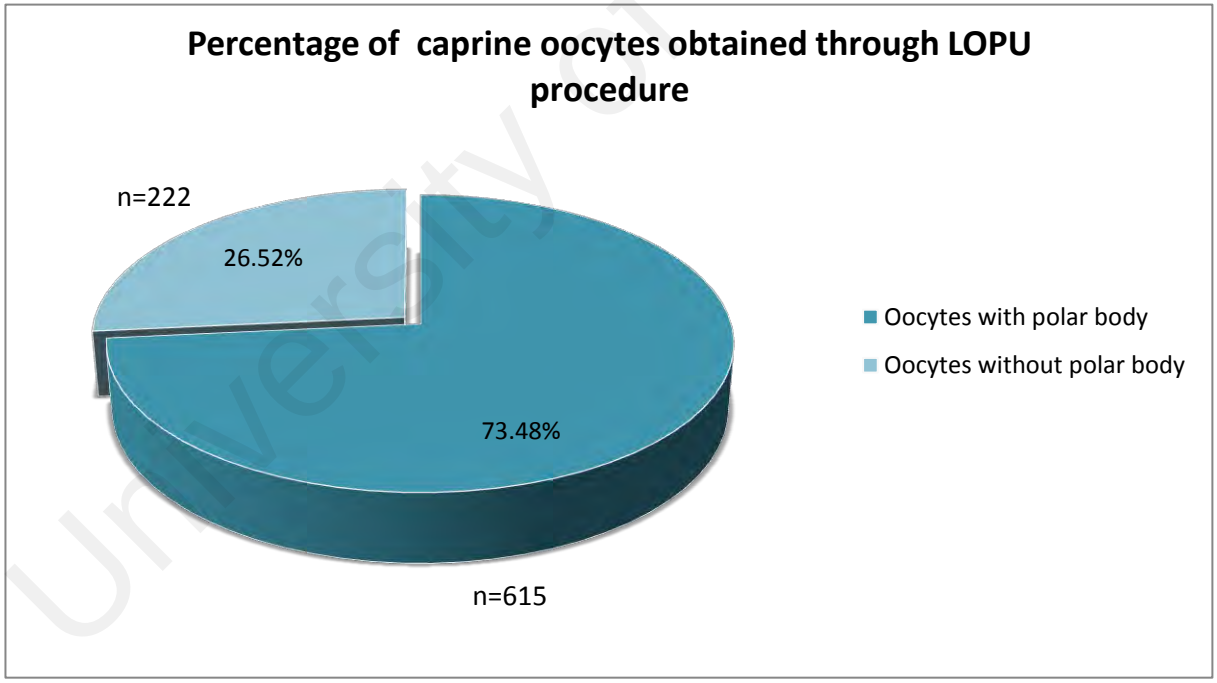

Figure 4.6: Pie chart of percentage of caprine oocytes obtained from LOPU procedure.

Table 4.12: Comparison of *in vitro* embryonic development of caprine parthenotes after activation by a single chemical

Treatment	No. of oocytes retrieved	Maturation rate (%)	Embryo cleavage rate (%)*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
Iono	39	61.54 (n=24)	95.00±5.00^{az} (n=23)	95.00±5.00^{az} (n=23)	75.66±8.19^{ayz} (n=18)	53.34±16.16^{ay} (n=15)	20.34±12.46^{ax} (n=6)
A23187	37	59.46 (n=22)	83.33±10.91^{az} (n=18)	83.33±10.91^{az} (n=18)	72.61±13.21^{ayz} (n=16)	45.24±14.53^{ay} (n=11)	9.53±7.14^{ax} (n=2)

n: no. of oocytes/embryos

* Cleavage rate was based on the maturation rate

[a] Means with similar superscripts in a column were insignificantly different (P>0.05)

[xyz] Means with different superscripts in a row were significantly different (P<0.05)

Table 4.13: Comparison of *in vitro* embryonic development of caprine parthenotes after activation by combination treatment using 5 µM A23187 (5 min) + 2 mM 6-DMAP (3, 4, 5 and 6 hours)

Treatment	No. of oocytes retrieved	Maturation rate (%)	Embryo cleavage rate (%)*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
A23187 + DMAP (3 hr)	49	77.55 (n=38)	95.25±4.75^{az} (n=36)	92.85±7.15^{az} (n=35)	90.07±7.14^{az} (n=34)	47.22±14.27^{ay} (n=13)	10.72±5.49^{ax} (n=4)
A23187 + DMAP (4 hr)	67	58.21 (n=39)	93.33±6.68^{az} (n=31)	91.68±8.33^{az} (n=30)	65.00±12.58^{ayz} (n=24)	53.33±17.00^{ay} (n=21)	0.00±0.00^{ax} (n=0)
A23187 + DMAP (5 hr)	41	82.93 (n=34)	92.37±3.58^{az} (n=30)	79.17±6.93^{ayz} (n=25)	60.28±10.10^{axy} (n=16)	40.82±12.05^{ax} (n=8)	5.55±3.62^{aw} (n=4)
A23187 + DMAP (6 hr)	51	64.71 (n=33)	85.11±6.91^{az} (n=30)	83.93±4.85^{az} (n=27)	63.70±9.48^{ay} (n=20)	38.09±6.46^{ax} (n=9)	1.79±1.79^{aw} (n=1)

n: no. of oocytes/embryos

***** Cleavage rate was based on the maturation rate

[a] Means with similar superscripts in a column were insignificantly different (P>0.05)

[wxyz] Means with different superscripts in a row were significantly different (P<0.05)

Table 4.14: Comparison of *in vitro* embryonic development of caprine parthenotes after activation by combination treatment using 10 µM Iono (5 min) + 2 mM 6-DMAP (3, 4, 5 and 6 hours)

Treatment	No. of oocytes retrieved	Maturation rate (%)	Embryo cleavage rate (%)*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
Iono + DMAP (3 hr)	58	89.66 (n=52)	80.00±14.23^{az} (n=47)	80.0±14.23^{az} (n=47)	65.88±14.03^{ayz} (n=43)	45.12±14.43^{ayz} (n=28)	24.66±10.40^{by} (n=18)
Iono + DMAP (4 hr)	56	78.57 (n=44)	93.52±4.34^{az} (n=41)	90.7±5.82^{az} (n=39)	79.04±8.35^{ayz} (n=34)	66.43±10.56^{ay} (n=27)	0.00±0.00^{ax} (n=0)
Iono + DMAP (5 hr)	48	75.00 (n=36)	80.68±6.46^{az} (n=31)	78.40±7.69^{az} (n=30)	78.40±7.69^{az} (n=30)	49.33±16.15^{ay} (n=18)	0.00±0.00^{ax} (n=0)
Iono + DMAP (6 hr)	51	82.35 (n=42)	96.15±3.85^{az} (n=40)	84.28±10.89^{ayz} (n=35)	79.05±13.95^{ayz} (n=32)	56.35±12.15^{axy} (n=21)	35.00±13.72^{bx} (n=12)

n: no. of oocytes/embryos

***** Cleavage rate was based on the maturation rate

^{ab} Means with different superscripts in a column were significantly different ($P<0.05$)

^{xyz} Means with different superscripts in a row were significantly different ($P<0.05$)

Table 4.15: Comparison of *in vitro* embryonic development of caprine parthenotes after activation by combination treatment using 10 µM Iono (5 min) + 10 µg/ml CHX (3, 4, 5 and 6 hours)

Treatment	No. of oocytes retrieved	Maturation rate (%)	Embryo cleavage rate (%)*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
Iono + CHX (3 hr)	42	64.29 (n=27)	80.70±6.63^{az} (n=21)	80.70±6.63^{az} (n=21)	61.68±10.74^{az} (n=15)	20.83±7.21^{aby} (n=5)	11.80±6.84^{ay} (n=3)
Iono + CHX (4 hr)	42	78.57 (n=33)	91.67±4.82^{az} (n=30)	76.87±6.48^{az} (n=25)	68.50±10.20^{ayz} (n=22)	36.13±15.47^{abxy} (n=11)	11.10±11.10^{ax} (n=3)
Iono + CHX (5 hr)	37	72.97 (n=27)	90.88±5.51^{az} (n=24)	84.63±5.60^{az} (n=22)	47.10±8.62^{ay} (n=12)	14.93±9.16^{ax} (n=5)	3.13±3.13^{ax} (n=1)
Iono + CHX (6 hr)	36	55.56 (n=20)	82.85±6.07^{az} (n=17)	77.85±8.44^{az} (n=16)	71.60±4.25^{az} (n=15)	49.35±3.57^{by} (n=10)	6.25±6.25^{ax} (n=1)

n: no. of oocytes/embryos

***** Cleavage rate was based on the maturation rate

^{ab} Means with different superscripts in a column were significantly different ($P<0.05$)

^{xyz} Means with different superscripts in a row were significantly different ($P<0.05$)

Table 4.16: Comparison of *in vitro* embryonic development of caprine parthenotes after activation by combination treatment using 10 µM Iono (5 min) + 5 µg/ml CB (3, 4, 5 and 6 hours)

Treatment	No. of oocytes retrieved	Maturation rate (%)	Embryo cleavage rate (%)*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
Iono + CB (3 hr)	38	76.32 (n=29)	71.57±3.43^{az} (n=20)	65.70±9.30^{az} (n=17)	49.50±14.87^{ayz} (n=11)	16.67±16.67^{abxy} (n=2)	0.00±0.00^{ax} (n=0)
Iono + CB (4 hr)	43	79.07 (n=34)	81.77±9.74^{az} (n=26)	63.80±12.16^{ayz} (n=20)	36.33±13.48^{axy} (n=10)	6.67±6.67^{awx} (n=1)	0.00±0.00^{aw} (n=0)
Iono + CB (5 hr)	38	92.11 (n=35)	92.17±7.83^{az} (n=31)	88.23±11.77^{az} (n=29)	87.87±12.13^{bz} (n=25)	55.80±22.00^{bz} (n=16)	0.00±0.00^{ay} (n=0)
Iono + CB (6 hr)	35	62.86 (n=22)	71.60±12.30^{az} (n=15)	64.48±14.25^{ayz} (n=13)	42.15±5.09^{ay} (n=9)	0.00±0.00^{ax} (n=0)	0.00±0.00^{ax} (n=0)

n: no. of oocytes/embryos

***** Cleavage rate was based on the maturation rate

^{ab} Means with different superscripts in a column were significantly different (P<0.05)

^{wxyz} Means with different superscripts in a row were significantly different (P<0.05)

Table 4.17: Comparison of *in vitro* embryonic development of caprine parthenotes after activation by various optimal combination treatments and IVF control

Treatment	No. of oocytes retrieved	Maturation rate (%)	Embryo cleavage rate (%)*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
A23187 + DMAP (3 hr)	49	77.55 (n=38)	95.25±4.75^{az} (n=36)	92.85±7.15^{az} (n=35)	90.07±7.14^{bz} (n=34)	47.22±14.27^{aby} (n=13)	10.72±5.49^{abx} (n=4)
Iono + DMAP (6 hr)	51	82.35 (n=42)	96.15±3.85^{az} (n=40)	84.28±10.89^{ayz} (n=35)	79.05±13.95^{byz} (n=32)	56.35±12.15^{bxy} (n=21)	35.00±13.72^{bx} (n=12)
Iono + CHX (4 hr)	42	78.57 (n=33)	91.67±4.82^{az} (n=30)	76.87±6.48^{az} (n=25)	68.50±10.20^{byz} (n=22)	36.13±15.47^{abxy} (n=11)	11.10±11.10^{abx} (n=3)
Iono + CB (5 hr)	38	92.11 (n=35)	92.17±7.83^{az} (n=31)	88.23±11.77^{az} (n=29)	87.87±12.13^{bz} (n=25)	55.80±22.00^{bz} (n=16)	0.00±0.00^{ay} (n=0)
IVF**	29	82.76 (n=24)	71.00±10.90^{az} (n=16)	67.00±10.81^{az} (n=15)	30.66±6.60^{ay} (n=7)	7.50±5.00^{ax} (n=3)	0.00±0.00^{ax} (n=0)

n: no. of oocytes/embryos
* Cleavage rate was based on the maturation rate
** IVF was used as a control
^{ab} Means with different superscripts in a column were significantly different ($P<0.05$)
^{xyz} Means with different superscripts in a row were significantly different ($P<0.05$)

Table 4.18: Comparison of *in vitro* embryonic development of caprine parthenotes according to the oocyte grades regardless activation protocols

Oocyte Grade	Total oocytes retrieved	Maturation rate	Embryos cleavage rate*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
A	35.95±2.35^{bc} (n=217)	90.08±2.04^{c} (n=197)	92.09±2.13^{abz} (n=172)	87.75±2.75^{bz} (n=162)	71.16±3.97^{by} (n=129)	37.04±4.86^{ax} (n=70)	12.35±3.29^{aw} (n=20)
B	28.28±2.34^{ab} (n=150)	91.09±2.17^{c} (n=128)	95.81±1.51^{bz} (n=119)	92.70±2.01^{bz} (n=113)	76.07±4.65^{by} (n=94)	45.90±5.62^{ax} (n=54)	8.47±2.59^{aw} (n=13)
C	40.30±2.29^{c} (n=267)	81.07±2.67^{bc} (n=210)	91.17±2.17^{abz} (n=191)	85.70±2.93^{bz} (n=170)	72.79±4.42^{by} (n=144)	47.04±4.87^{ax} (n=90)	6.46±2.16^{aw} (n=21)
D	19.45±2.11^{a} (n=48)	77.17±4.55^{b} (n=42)	91.36±3.60^{abz} (n=34)	86.42±4.54^{bz} (n=31)	64.20±8.74^{aby} (n=22)	25.92±8.17^{ax} (n=8)	3.70±2.57^{aw} (n=2)
E	53.10±7.13^{d} (n=46)	50.09±10.21^{a} (n=17)	83.32±10.91^{az} (n=12)	71.41±13.48^{ayz} (n=9)	47.61±14.74^{ayz} (n=6)	40.47±17.00^{ay} (n=4)	0.00±0.00^{ax} (n=0)

n: no. of oocytes/embryos

***** Cleavage rate was based on the maturation rate

^{abc} Means with different superscripts in a column were significantly different (P<0.05)

^{wxyz} Means with different superscripts in a row were significantly different (P<0.05)

Table 4.19: Comparison of *in vitro* embryonic development of caprine parthenotes according to oocyte grades from the optimal activation protocol in each group

Treatment	Oocyte grade	Total oocyte retrieved	Maturation rate (%)	Embryos cleavage rate (%)*				
				2-cell	4-cell	8-cell	Morula	Blastocyst
A23187 + DMAP (3 hr)	A	12	93.34±6.66^{a} (n=11)	96.00±4.00^{bz} (n=10)	92.00±8.00^{bcz} (n=9)	82.00±11.14^{cz} (n=8)	24.00±19.39^{ay} (n=2)	24.00±19.39^{ay} (n=2)
	B	13	96.00±4.00^{a} (n=11)	100.00±0.00^{bz} (n=11)	100.00±0.00^{cz} (n=11)	100.00±0.00^{cz} (n=11)	60.00±24.49^{ay} (n=7)	10.00±10.00^{ax} (n=1)
	C	19	81.12±8.50^{a} (n=14)	100.00±0.00^{bz} (n=14)	100.00±0.00^{cz} (n=14)	100.00±0.00^{cz} (n=14)	47.23±16.34^{ay} (n=4)	16.67±10.54^{ax} (n=2)
Iono + DMAP (6 hr)	A	13	88.75±6.57^{a} (n=11)	100.00±0.00^{bz} (n=11)	87.50±12.50^{abcyz} (n=9)	87.50±12.50^{cyz} (n=9)	60.43±15.73^{ay} (n=6)	20.83±12.50^{ax} (n=2)
	B	9	93.33±6.67^{a} (n=8)	100.00±0.00^{bz} (n=8)	91.67±8.3^{bcz} (n=7)	91.67±8.33^{cz} (n=7)	72.23±14.70^{az} (n=5)	19.43±10.01^{ay} (n=2)
	C	20	95.23±4.77^{a} (n=19)	94.43±5.57^{bz} (n=18)	83.33±16.67^{abcyz} (n=16)	74.73±20.88^{cyz} (n=14)	40.40±11.92^{axy} (n=9)	12.13±12.13^{ax} (n=5)
Iono + CHX (4 hr)	A	10	91.67±8.33^{a} (n=9)	100.00±0.00^{bz} (n=9)	77.80±11.10^{abcyz} (n=7)	66.67±19.25^{cyz} (n=5)	22.23±22.23^{ay} (n=2)	22.23±22.23^{ay} (n=2)
	B	17	100.00±0.00^{a} (n=17)	85.7±8.26^{bz} (n=13)	65.07±4.21^{abyz} (n=11)	60.30±3.20^{bcyz} (n=10)	31.77±17.47^{axy} (n=4)	11.10±11.10^{ay} (n=1)
	C	4	100.00±0.00^{a} (n=4)	100.00±0.00^{bz} (n=4)	100.00±0.00^{cz} (n=4)	100.00±0.00^{cz} (n=4)	66.67±33.33^{az} (n=3)	0.00±0.00^{ay} (n=0)

(continued)

(continued)

Iono + CB (5 hr)	A	18	100.00±0.00^{a} (n=18)	95.83±4.17^{bz} (n=17)	95.83±4.17^{bcz} (n=17)	80.97±19.03^{cz} (n=13)	67.63±12.37^{az} (n=10)	0.00±0.00^{ay} (n=0)
	B	4	100.00±0.00^{a} (n=4)	100.00±0.00^{bz} (n=4)	83.33±16.67^{abcz} (n=3)	83.33±16.67^{cz} (n=3)	66.67±33.33^{az} (n=2)	0.00±0.00^{ay} (n=0)
	C	14	83.33±8.33^{a} (n=12)	88.90±11.10^{bz} (n=10)	83.33±16.67^{abcz} (n=9)	83.33±16.67^{cz} (n=9)	44.47±22.23^{az} (n=4)	0.00±0.00^{ay} (n=0)
IVF**	A	15	95.23±4.77^{a} (n=14)	57.80±8.90^{az} (n=8)	57.80±8.90^{az} (n=8)	28.87±4.43^{aby} (n=4)	12.23±6.19^{axy} (n=2)	0.00±0.00^{ax} (n=0)
	B	4	100.00±0.00^{a} (n=4)	83.33±16.67^{bz} (n=3)	83.33±16.67^{abcz} (n=3)	0.00±0.00^{ay} (n=09)	0.00±0.00^{ay} (n=0)	0.00±0.00^{ay} (n=0)
	C	6	83.33±16.67^{a} (n=5)	100.00±0.00^{bz} (n=5)	88.90±11.10^{abcyz} (n=4)	77.77±22.23^{cyz} (n=3)	33.33±33.33^{axy} (n=1)	0.00±0.00^{ax} (n=0)

n: no. of oocytes/embryos

* Cleavage rate was based on the maturation rate

^{abc} Means with different superscripts in a column were significantly different (P<0.05)

^{xyz} Means with different superscripts in a row were significantly different (P<0.05)

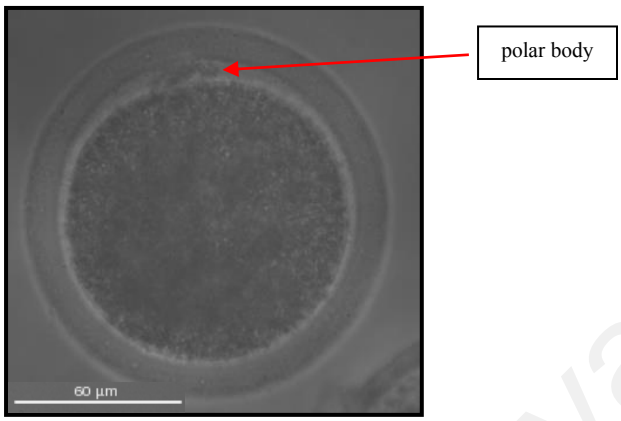

Figure 4.7: Activated caprine oocytes with polar body.

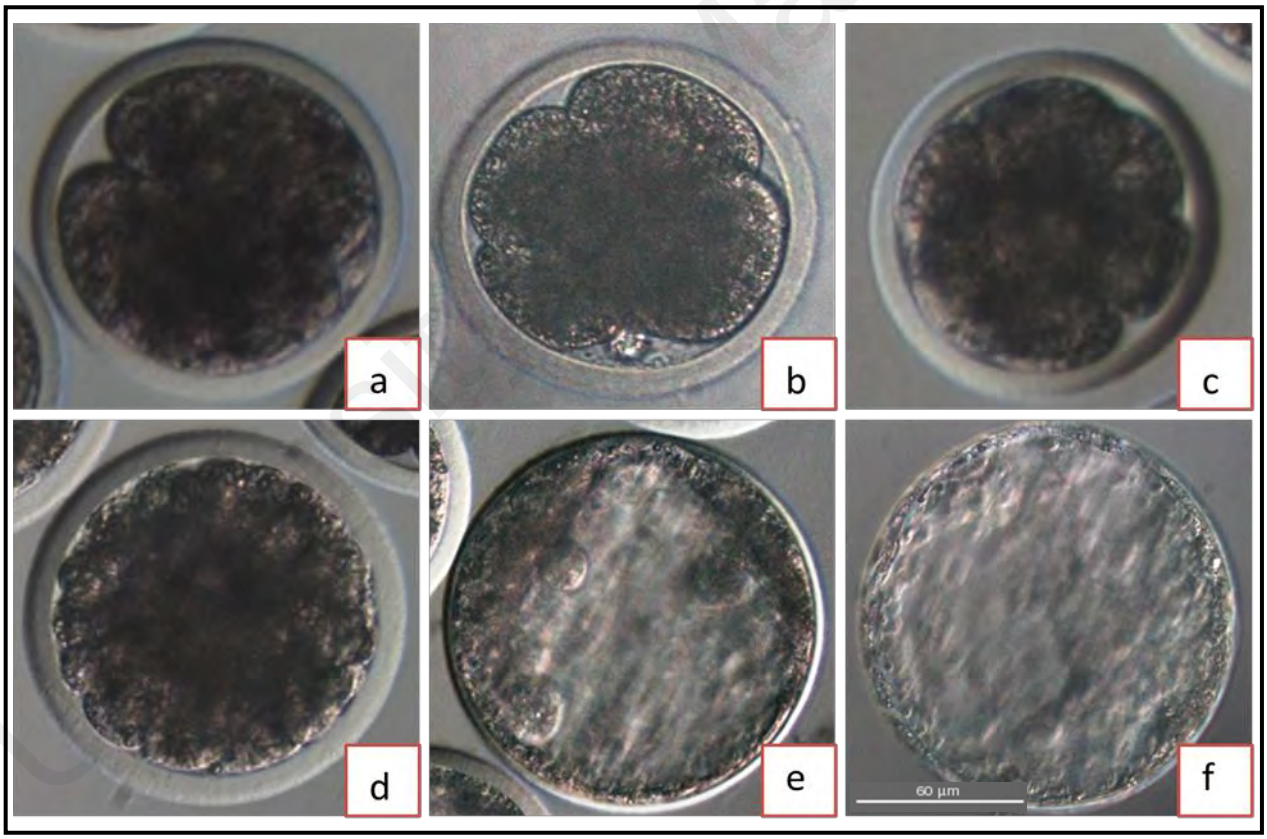

Figure 4.8: Parthenogenetic caprine embryos at: (a) 2-cell, (b) 4-cell, (c) 8-cell, (d) compacting morula, (e) fully expended blastocyst and (f) hatched blastocyst.

4.5 EFFECT OF *IN VITRO* MATURATION (IVM) DURATION ON THE PRODUCTION OF CAPRINE OOCYTES AND ITS SUBSEQUENT PARTHENOTE DEVELOPMENT (EXPERIMENT 5)

Table 4.20 shows the comparison of *in vitro* development of caprine parthenote at different IVM durations according to the grade of oocytes derived from LOPU procedure. Only four grades of oocyte that gave the highest cleavage rates were selected for this evaluation which is Grades A, B, C and D whereas Grade E was not included as it shows low number of total oocytes as well as matured oocytes. There were two groups of IVM duration evaluated in this experiment (18-21 hours and 22-25 hours) before the activation procedure.

At 18-21 hours IVM duration, Grade D oocytes showed significantly lower ($P>0.05$) in maturation rate compared to Grades A and B oocytes. Similarly, at 22-25 hours IVM duration, the maturation rates of Grades A and B oocytes were significantly higher ($P<0.05$) than Grade D oocytes. In addition, maturation rate of Grades A and B oocytes from group of 18-21 hours IVM was significantly higher ($P<0.05$) than Grade D oocytes from 22-25 hours IVM group. No differences ($P>0.05$) were observed in cleavage and blastocyst rates for all oocyte grades from group of 18-21 hours IVM duration. In contrast, at 22-25 hours IVM duration, cleavage rate (2-cell) of Grade C oocytes was significantly lower ($P<0.05$) from Grade D oocytes (84.75±4.73% vs. 96.15±3.85%, respectively), whereas blastocyst rate of Grade A oocytes was significantly higher ($P<0.05$) from Grade D oocytes (23.22±6.36% vs. 7.69±5.21%, respectively).

In addition, blastocyst rates for all grades from group 18-21 hours IVM duration were significantly different ($P<0.05$) from Grades A, B and C from 22-25 hours IVM group. At 18-21 hours IVM duration, Grade B oocytes gave the highest cleavage rates at all stages of embryonic development, followed by Grades C, A and D. Similarly, Grade B

oocytes from the 22-25 hours IVM group gave the highest cleavage rates at all stages of embryonic development, followed by Grades A, C and D.

Table 4.20: Comparison of *in vitro* caprine parthenotes development at different IVM duration according to the grade of oocytes derived from LOPU

IVM duration (hours)	Oocyte grade	No.of oocytes	Maturation rate (%)	Embryos cleavage rate (%)*				
				2-cell	4-cell	8-cell	Morula	Blastocyst
18-21	A	102	91.61±2.70^{c} (n=94)	89.86±3.94^{abz} (n=83)	84.17±4.74^{ayz} (n=76)	71.64±6.07^{ay} (n=61)	32.52±7.08^{abx} (n=37)	3.43±2.05^{aw} (n=3)
	B	83	93.33±2.83^{c} (n=73)	96.87±1.70^{bz} (n=68)	94.62±2.41^{az} (n=64)	78.98±5.77^{ay} (n=51)	47.97±7.98^{bx} (n=27)	3.81±2.98^{aw} (n=2)
	C	149	80.47±3.58^{abc} (n=119)	94.21±2.17^{abz} (n=109)	88.79±3.52^{az} (n=100)	73.54±5.71^{ay} (n=79)	48.38±6.30^{bx} (n=51)	0.82±0.58^{aw} (n=2)
	D	32	77.39±6.68^{ab} (n=23)	86.91±5.84^{abz} (n=18)	80.95±7.17^{az} (n=16)	68.45±11.98^{ayz} (n=11)	42.86±13.73^{by} (n=6)	0.00±0.00^{ax} (n=0)
Total		366	86.80±1.84 (n=309)	92.93±1.55 (n=278)	88.25±2.06 (n=256)	73.96±3.26 (n=202)	43.24±3.94 (n=121)	2.28±1.02 (n=7)
22-25	A	105	88.17±3.29^{bc} (n=83)	94.95±2.59^{abz} (n=76)	91.85±3.47^{ayz} (n=72)	78.99±5.31^{ay} (n=57)	43.35±6.98^{bx} (n=30)	23.22±6.36^{cw} (n=17)
	B	64	86.29±3.65^{bc} (n=52)	95.84±2.31^{abz} (n=49)	90.91±3.37^{az} (n=46)	81.82±6.34^{az} (n=41)	48.87±7.99^{by} (n=26)	17.04±4.79^{bcx} (n=11)
	C	92	82.87±4.21^{abc} (n=77)	84.75±4.73^{az} (n=66)	79.89±5.56^{az} (n=61)	70.85±7.65^{az} (n=52)	46.40±8.05^{by} (n=33)	17.14±5.56^{bcx} (n=16)
	D	26	73.08±6.41^{a} (n=18)	96.15±3.85^{bz} (n=16)	92.31±5.21^{az} (n=15)	67.31±12.46^{ay} (n=11)	15.38±8.74^{ax} (n=3)	7.69±5.21^{abx} (n=1)
Total		287	84.11±2.08 (n=230)	92.62±1.78 (n=207)	88.50±2.24 (n=194)	75.82±3.60 (n=161)	41.47±4.08 (n=92)	17.85±2.99 (n=45)

n: no. of oocytes/embryos

* Cleavage rate was based on the maturation rate

^{abc} Means with different superscripts in a column were significantly different (P<0.05)

^{wxyz} Means with different superscripts in a row were significantly different (P<0.05)

Chapter 5

5.0 DISCUSSION

Chapter 5

5.0 DISCUSSION

5.1 EFFECT OF DIFFERENT COMBINATIONS OF ACTIVATION CHEMICAL ON THE PRODUCTION OF PARTHENOGENETIC MURINE EMBRYOS AS MODEL ANIMALS (EXPERIMENT 1)

When denuded oocytes were stimulated with 10 mM $SrCl_2$ + 5 µg/ml CB in CZB Ca^{2+}-free medium for 6 hours as suggested for the mice (Kishikawa *et al.,* 1999), most oocytes in this study were observed to undergo shrinking of cytoplasm, which was similar to that reported by O'Neill *et al.* (1991) with the lysis of oocytes. When cumulus-free oocytes were cultured in 1.6 mM $SrCl_2$ for more than 20 minutes, their results showed that the lysed oocytes were reduced to 6%, similar to those co-cultured with high density of cumulus cells or cumulus-intact oocytes. Although the concentration and duration of $SrCl_2$ treatments greatly differ among the studies (Marcus, 1990; O'Neill *et al.,* 1991; Bos-Mikich *et al*., 1995a; Wakayama *et al*., 1998; Otaegui *et al.,* 1999; Ono *et al.,* 2001; Kono *et al.,* 2004), Ma *et al.* (2005) reported that these factors had marked effect on the parthenotes development.

Ma *et al.* (2005) found that the best concentration for strontium chloride ($SrCl_2$) treatment was when mouse oocytes were treated with 10 mM $SrCl_2$ (cleavage rate: 95.2% and blastocyst rate: 56.1%) for 2.5 hours incubation duration. Krivokharchenko *et al.* (2003) showed that treatment of cumulus-free rat oocytes by 2 mM Sr^{2+} for 15 minutes was sufficient for oocytes activation (3.4%), and increase in duration for 2 hours in 2 mM Sr^{2+} resulted in a significant increase in oocytes activation (21.2%). In this experiment, concentration of 10 mM $SrCl_2$ for 3 hours incubation duration was found to be optimal for

mouse oocytes activation without cytoplasmic shrinkage as well as high cleavage (93.83±1.82%) and blastocyst (60.42±4.92%) rates.

Strontium is a very effective agent and very popular used for parthenogenetic activation in murine oocytes (Krivokharchenko *et al.,* 2003). A short exposure of murine oocytes to medium contained 1.6 mM $SrCl_2$ for 2-10 minutes can induced a high incidence of parthenogenesis. Even so, when oocytes were incubated in $SrCl_2$ for 20-60 minutes, the rate of oocytes activation was lower and a significant degree of oocyte degeneration was observed (O"Neill *et al.,* 1991). Still, many researchers have published great variances of strontium activation procedures for murine oocytes: 4.6 or 9.2 mM Sr^{2+} for 30-40 minutes (Kline and Kline, 1992), 1.71 mM Sr^{2+} for 1 hours (Bos-Mikich *et al.,* 1995b) , 10 mM Sr^{2+} for 1.5 hours (Kono *et al.,* 1996), 10 mM Sr^{2+} for up to 24 hours (Bos-Mikich *et al.,* 1997), 10 mM Sr^{2+} for 6 hours (Kishikawa *et al.,* 1999), and 25 mM Sr^{2+} for 1 hours (Otaegui *et al.,* 1999) and 10 mM Sr^{2+} for 2.5 hours (Ma *et al.,* 2005).

As fertilisation involves an increase in intracellular calcium concentration [$(Ca^{2+})_i$] and the activation of a calcium-dependent protein kinase, therefore it is important to induce the metaphase II (MII) arrested oocytes with the chemicals which can triggered these two kinds of intracellular mechanism. Calcium ionophore (A23187) was commonly used in oocytes activation in order to trigger the repetitive [$(Ca^{2+})_i$] to allowed oocytes exit MII and enter anapahase II (AII) subsequently resume meiosis. Oocytes later on will restart meiosis, undergo emission of second polar body, formation and migration of pronuclei, initiation of maternal mRNA translation and finally synthesis new DNA (Nuccitelli, 1991).

Ethanol (EtOH) has been used as activation agent in mammalian oocytes. However, ethanol alone is not sufficient to induce parthenogenesis in murine oocytes. The activation rate (38.91%) was the highest when activated by 9% EtOH compared to 7 and 8% EtOH. However, the embryo development was stopped at 8-cell stage at all three concentrations

in the study conducted by Ata‟allah (2012). Even so, when 7, 8 and 9% EtOH was combined with 2 mM 6-DMAP, the combination of 9% EtOH with 6-DMAP gave the highest activation rate (73.76%). The embryos still could not reach the blastocyst stage as it stopped at morula stage (5.39%). In contrast, in the present study, when murine oocytes were activated by three different concentrations of ethanol (7, 8 and 9%) combined with 2 mM 6-DMAP for 4 hours, embryos could reach up until blastocyst stage for each treatment. The optimal cleavage (77.07±4.21%) and blastocyst rates (13.86±3.64%) were achieved from activation by 8% EtOH + 6-DMAP, even though Uranga *et al.* (1996) described that the activation of murine oocytes can be achieved by 7% EtOH alone for 5 minutes with 74.3% of oocytes cleaved and the cleaved embryos were able to achieved blastocyst stage (23.7%). Still, activation of murine oocytes by 5 µM A23187 for 5 minutes indicated higher cleavage (76.2%) and blastocyst rates (32.3%) when compared to activation by ethanol (Uranga *et al.*, 1996).

Inconsistent results were obtained in regard to activation of rat oocytes by ethanol. Zernicka-Goetz (1991) and Jiang *et al.* (2002) reported that ethanol alone could activate rat oocytes but the later showed that parthenotes could not develop beyond 2-cell stage when diploidisation was omitted. Parthenogenetic activation was obtained only after combined treatment between ethanol with cycloheximide (CHX) (Hayes *et al.,* 2001), and the blastocyst formation was only observed when the rat oocytes were treated with ethanol and 6-dimethylaminopurine (6-DMAP) (Jiang *et al.,* 2002). In their study to compare the activation of rat oocytes by Iono + 6-DMAP and electrical stimulation + 6-DMAP, Mizutani *et al.,* (2004) described that both pronuclear formation (96.7% vs. 96.7%) and blastocyst rate (42.7% vs. 42.3%) did not differ between two activation procedures. However, activation by Iono + 6-DMAP exhibited higher both cleavage and blastocyst rates.

6-DMAP is a specific protein phosphorylation induced diploidisation in rat, whereas treatment of 6-DMAP with bovine oocytes after electroactivation suppressed the second reduction division. Therefore, it was suggested that karyokinesis did not occur and oocytes entered interphase of the first mitotic cycle as uniform diploid parthenotes (Susko-Parish *et al.,* 1994). It was also found that 6-DMAP inhibited meiosis reinitiation in murine, bovine and porcine oocytes by preventing chromatin condensation and germinal vesicle breakdown (GVBD) (Liu *et al.*, 1997). 6-DMAP itself does not induce the inactivation of histone H1 kinase in MII-arrested oocytes. Post-fertilisation events such as the formation of the interphase network of microtubules, the remodeling of sperm chromatin and pronucleus formation were controlled by 6-DMAP-sensitive kinases (Szöllösi *et al.,* 1993). According to Liu *et al.* (1997), more than 70% activation rate could be obtained when murine oocytes were treated with 2 mM 6-DMAP for 2 to 4 hours. However, the highest blastocyst rate (20.3%) was obtained when murine oocytes were treated in 2 mM 6-DMAP for 2 hours. The cleavage rate of activated oocytes also declined gradually as the exposure time increased from 0.5 to 3 hours (Liu *et al.*, 1997). In the present study, the combination of A23187 + 6-DMAP apparently ($P>0.05$) gave higher activation and blastocyst rates when compared to the combination of EtOH + 6-DMAP (79.48±5.63% vs. 77.07±4.21%) and (23.28±4.62% vs. 13.86±3.64%), respectively. Previously, Mizutani *et al.* (2004) also found that although there were no significant differences between these two treatments, the cleavage and blastocyst rates tended to be higher when rat oocytes were activated by Iono + 6-DMAP rather than by electrical stimulation + 6-DMAP (77.3% vs. 66.3%) and (42.7% vs. 42.3%).

In a study conducted on parthenogenetic porcine oocytes by Jilek *et al.* (2000), the results indicated that the activation of porcine oocytes using calcium ionphore alone did not sufficiently suppress synthesis of the proteins that were responsible for blocking

meiosis at the MII stage, proving that activation with calcium ionphore did not provide a full activation stimulus to the oocytes. The highest activation rate was obtained when pig oocytes were exposed to a combined treatment of calcium ionphore (25 or 50 μM for 7 min) + CHX (10 μg/ml for 6 hours) (Jilek *et al.,* 2000). Even so, when reconstructed bovine oocytes were activated with the combination of A23187 + 6-DMAP, the cleavage and blastocyst rates were significantly higher than those treated in the combination of A23187 + CHX + CB (98.9% vs. 79.6%) and (51.6% vs. 14.3%) (Shen *et al.,* 2008). In the present study, the results showed that activation of murine oocytes with A23187 + DMAP exhibited better results than activation with A23187 + CHX in term of cleavage rate (79.48±5.63% vs. 72.62±5.17%) and significantly higher ($P<0.05$) at blastocyst formation (23.28±4.62% vs. 9.24±3.02%). Treatment of mouse oocytes with CHX resulted in oocytes activation, as assessed by emission of the second polar body and formation of a pronucleus (Siracusa *et al.,* 1978; Clarke and Masui, 1983; Fulka *et al.,* 1994). Oocytes activation induced by CHX presumably occurs by permitting the turnover of cyclin B that resulted in a concomitant decrease in cdc2/cyclin B kinase activity (Moos *et al.,* 1996).

According to the study conducted by Ma *et al.,* (2005), even though the activation rates were no differences with the presence or absence of cytochalasin B (CB) (98.9% vs. 96.2%), there were more oocytes developed into blastocyst when CB was present during oocyte activation (56.3% vs. 15.6%). The reason why embryos more proned to develop until blastocyst with the presence of CB, it could be due to the diploidisation of the parthenotes, in which oocytes were activated in CB resulted in formation of two pronuclei, whereas most of those activated in the absence of CB contained only one pronucleus. Therefore, it was obvious that the CB in activation media induced a diploid parthenotes. It is well known that diploid parthenotes are less apoptotic and have greater developmental capacity compared to haploid parthenotes (Kaufman and Sachs, 1976; Cha *et al.,* 1997;

Latham *et al.,* 2002; Liu *et al.,* 2002b). Neuber *et al.* (2002) and Hao *et al.* (2004) mentioned that there was a higher percentage of apoptosis in parthenogenetic embryos, relative to IVF embryos. Nevertheless, apoptosis was not caused by parthenogenetic activation and parthenogenesis themselves. It was probably due to haploidy formation that increased the incidence of apoptosis in preimplantation embryos (Liu *et al*., 2002a,b). In the present research, it was vividly shown that combination of $SrCl_2$ + CB gave higher cleavage (93.83±1.82% vs. 61.88±5.07%) and blastocyst (60.42±4.92% vs. 9.76±2.74%) rates rather as opposed to combination of A23187 + CB. This phenomenon could be due to the chemicals compatibility and integration among the chemicals in the medium.

It is well known that during fertilisation of the oocytes, an increase in $[(Ca^{2+})_i]$ is a universal response elicited by the sperm (Whitaker and Patel, 1990). Based on this important theory of fertilisation, most of the currently used oocytes activation procedures depend on methods to induce an intracellular calcium; $(Ca^{2+})_i$ increase. The capability of Ca^{2+} rose to release oocytes from the meiotic arrest was related to the ability to trigger persistent inactivation of maturation promoting factor (MPF) and cytostatic factor (CSF), which was the result of c-mos and mitogen activated protein kinase (MAPK) activity (Collas *et al.,* 1993a; Lorca *et al.,* 1993). Collas *et al.* (1993a) and Lorca *et al.* (1993) mentioned that a single Ca^{2+} rise, which was evoked by most of the currently used activation procedures, caused only a transient decline of MPF and CSF activity, which was not adequate for full oocyte activation. Moreover, Vitullo and Ozil, (1992) previously demonstrated that the most effective stimuli were those which promoted multiple intracellular Ca^{2+} peaks. Based on these findings, alternative methods of activation have been developed that combine a transient inactivation of MPF obtainable with a single $[(Ca^{2+})_i]$ rise, with a persistent inhibition of MPF, induced by addition of either non-specific kinase inhibitors (e.g. 6-DMAP; Liu *et al.,* 1998a) or protein synthesis inhibitors

(e.g. CHX; Bos-Mikich *et al.,* 1995a). Non-specific protein kinase inhibitors inhibit kinase activity of MPF by inactivating MAPK (Motlik *et al.,* 1998; Gordo *et al.,* 2000) while protein synthesis inhibitors usually restrict the synthesis or re-accumulation of cyclin B, thus, blocking the re-synthesis of MPF activity (Presicce and Yang, 1994a,b). Combination treatments with different chemicals had been broadly used for reconstructed oocytes for parthenogenetic activation (ovine: Schnieke *et al.,* 1997; bovine: Cibelli *et al.,* 1998; caprine: Keefer *et al.,* 2001, 2002) to increase [$(Ca^{2+})_i$] (e.g. electric pulse, ethanol, calcium ionophore, or ionomycin) and inhibited protein synthesis (e.g. CHX) or MPF activity (e.g. 6-DMAP) (Shen *et al.,* 2008).

5.2 EFFECT OF POST-hCG DURATION ON THE PRODUCTION OF MURINE OOCYTES AND ITS SUBSEQUENT PARTHENOTE DEVELOPMENT (EXPERIMENT 2)

There were two groups (13-15 hours and 16-18 hours) of post-hCG duration evaluated in this experiment. Previous studies showed that most of the researchers using the range of 13-15 hours duration between post-hCG administration and ovulated-oocyte retrieval, specifically 13 hours (Hiller *et al.,* 1985), 14 hours (Martin-Coello *et al.,* 2008) and 14.5 hours (Vergara *et al.,* 1997). These durations were chosen because murine ovulation starts after 11 to 12 hours post-hCG administration (Hogan *et al.,* 1986). It was reported that the quality of oocytes was affected by several factors such as dose and timing of gonadotrophins (Edgar *et al.,* 1987a,b; Vergara *et al.,* 1997), strain of murine (Ainul Bahiyah, 2010), age of murine (Hogan *et al.,* 1986; Ozgunen *et al.,* 2001) and weight of murine (Hogan *et al.,* 1986).

Even though there was insignificant difference ($P>0.05$) in the percent of oocytes with polar body between two respective groups of post-hCG administration duration (70.69±1.04% vs. 70.08±1.05%) whereby 13-15 hours group showed higher percent of oocytes with polar body compared to 16-18 hours group. Ata"allah (2012) showed that percent of oocytes with polar body of 13-15 hours post-hCG duration was found to be significantly higher ($P<0.05$) than those undergo 16-18 hours and 19-21 hours post-hCG administration duration (78.6% vs. 61.4% and 36.1%%, respectively). The ovulation (MII stage; extrusion of first polar body) and fertilisation occurred optimally within 11-14 hours range of post-hCG treatment. It was suggested that subsequent extrusion of second polar body occurred at 17-23 hours of post-hCG administration (Hogan *et al.,* 1986). Therefore, matured oocytes (MII stage) should be fertilised within the time range of optimal fertilisation, which was shown to be variable depending on species, such as mice (8-12

hours), rats (12-14 hours), rhesus monkeys and humans (<24 hours) (Austin, 1974). Moreover, Miao *et al.* (2009) stated that if the oocytes are not fertilised within the fertilisation window, it will undergo a time-dependent deterioration in quality or commonly known as oocyte aging.

Ata‟allah (2012) also showed that the cleavage rate of murine parthenote development from 13-15 hours post-hCG duration (52.78%) was significantly higher ($P<0.05$) than parthenotes from the other post-hCG groups of 16-18 hours (14.49%) and 19-21 hours (8.33%). In the present experiment, the cleavage rates of parthenotes from 13-15 hours were found to be significantly higher ($P<0.05$) than group of 16-18 hours duration at all embryonic stages (2-cell: 81.90±1.53% vs. 76.41±1.64%; 4-cell: 67.87±2.02% vs. 60.20±1.92%; 8-cell: 55.50±2.25% vs. 48.37±1.96%; morula: 43.68±2.42% vs. 35.19±2.01% and blastocyst: 34.36±2.34% vs. 26.04±1.88%, respectively). These results clearly showed that the post-hCG administration duration at 13-15 hours was the optimal for oocyte retrieval and the subsequent parthenote development as oocyte aging might have occurred at 16-18 hours post-hCG duration. However, it has been demonstrated in the previous study on murine (Otaegui *et al.,* 1999), bovine (Susko-Parrish *et al.,* 1994; Presicce and Yang, 1994a,b) and rabbit (Collas and Robl, 1990) that the ability of oocytes to be parthenogenetically activated by chemicals with regard to post-hCG administration duration depended upon age of oocytes, and aged oocytes can be more efficiently activated. The differences between the post-hCG duration results of the current experiment and the previous ones may due to the difference in strains and activation protocols used.

In other study by Bos-Mikich *et al.* (1995a), 96% of hybrid (B6CB F1) murine oocytes were activated by treatment with 10 mM $SrCl_2$ for 2 hours recovered after 15 hours post-hCG. Marcus (1990) reported that 86.3% of TRf mouse oocytes were activated when treated with 1.6 mM $SrCl_2$ for 1 hour collected after 20-22 hours post-hCG, however,

only 42.4% ICR oocytes were activated under the same treatment. In the later study by Otaegui *et al.* (1999) on murine oocytes collected from C57BL/6 x CBA/Ca strain 18 hours post-hCG, 89% of oocytes were activated by treatment with 5 mM $SrCl_2$ for 1 hour. Ma *et al.* (2005) reported that when the murine oocytes treated with 10 mM $SrCl_2$ (2.5 hours) + 2 mM 6-DMAP (3.5 hours) after 13 hours post-hCG injection, 94.1% were activated, with 60.1% of them developing into blastocysts. However, when oocytes were cultured in the same treatment after 18 hours post-hCG injection, the blastocyst rate of decreased significantly (28.3%), although the activation rate (92.7%) remained high. Activation of murine oocytes by $SrCl_2$ was dependent on the post-hCG duration, the concentration of $SrCl_2$, and the incubation duration of $SrCl_2$ (Otaegui *et al.,* 1999). Ma *et al.* (2005) suggested that stronger stimuli might be needed to activate recently ovulated oocytes (young oocytes) than those oocytes present in the oviduct for at least a few hours (aged oocytes).

In the study of effect of post-hCG duration on parthenote-rat oocytes by Krivokharchenko *et al.* (2003), treatment with EtOH for parthenogenetic activation was highly effective only for aged oocytes (22-24 hours post-hCG) than young oocytes (12-14 hours post-hCG). The incidence of pronuclear formation and cleavage rate was significantly highest (73.5% and 94.7%, respectively). Electrical stimulation also was found to be less effective ($P<0.05$) in young (12-14 hours post-hCG) compared to aged rat oocytes (20-22 hours post-hCG), respectively, in parameters such as pronuclear formation (44.7 vs. 77.8%) and cleavage rate (38.2% vs. 66.4%). However, in a treatment with $SrCl_2$ for 15 minutes, the efficiencies of pronuclear formation and the cleavage rate was equal for all ages of oocytes. Similarly, treatment with $SrCl_2$ for 2 hours resulted in a significantly increase in proportion of active animals with increase in duration after hCG injection. The efficiencies of activation and cleavage were similarly high for oocytes of all ages, but the

cleavage rate was significantly ($P<0.05$) the highest (12-14 hours: 93.7% vs. 16-18 hours: 89.6% and 20-22 hours: 87.8%) for young oocytes (12-14 hours post-hCG) (Krivokharchenko *et al.*, 2003). Krivokharchenko *et al.* (2003) also found that without any treatment, rat oocytes could form pronuclear and subsequently cleaved. However, the ability to form a pronucleus and activated increased proportionally with the age of oocytes (>22 hours post-hCG).

Wilcox *et al.* (1998) stated that oocyte aging before fertilisation may result in low embryonic development as well as failure in early pregnancy in several mammalian species. Thus, it is advisable to avoid using aged oocyte during IVP procedure in order to achieve successful embryonic development and pregnancy.

5.3 EFFECT OF DIFFERENT COMBINATIONS OF ACTIVATION CHEMICAL ON THE PRODUCTION OF PARTHENOGENETIC BOVINE EMBRYOS AS A REFERENCE OF LIVESTOCK ANIMALS (EXPERIMENT 3)

In the study conducted by Abdalla *et al.* (2009), when bovine oocytes were treated in the combination of 5 µM Iono (5 minutes) + 1.9 mM 6-DMAP (3 hours), the cleavage rate (20% vs. 18%) obtained was higher than those treated with the combination of 5 µM Iono (5 minutes) + 10 µg/ml CHX (5 hours) whereas only few cleaved oocytes developed to blastocyst (2% vs. 1%). In the present study, the cleavage (73.60±4.68% vs. 63.48±9.12%, respectively) and blastocyst (7.10±1.43% vs. 2.16±1.56%, respectively) rates obtained are comparatively higher compared with those reported by Abdalla *et al.* (2009) when bovine oocytes were treated with the combination of 10 µM Iono (5 minutes) + 2 mM 6-DMAP (4 hours) and 10 µM Iono (5 minutes) + 10 µg/ml CHX (5 hours), respectively. Results in the present study showed that the combination treatment of Iono + 6-DMAP gave higher cleavage and blastocyst rates compared to those treated in the combination of Iono + CHX support the results obtained by Abdalla *et al.* (2009).

Previous study by Bhak *et al.* (2006) on activation of bovine oocytes showed that the cleavage (52% vs. 38%) and blastocyst (12% vs. 5%) rates of oocytes activated by combination of 5 µM Iono (5 minutes) + 1.9 mM 6-DMAP (3 hours) was significantly higher than those treated by the combination of 5 µM Iono (5 minutes) + 10 µg/mL CHX (3 hours). This finding is in agreement with the current research where bovine oocytes treated with combination of Iono + 6-DMAP showed the better embryonic development. In a separate study, when bovine oocytes were treated with the combination of 5 µM Iono (5 minutes) + 2 mM 6-DMAP (3 hours), the pronuclear formation, cleavage and blastocyst

rates were significantly higher (P<0.05) (67.1%, 69.2% and 28.0%, respectively) compared to other treatments (Wang *et al.,* 2008). In contrast, in the current study, the cleavage rate is higher (73.6% vs. 67.1%) when bovine oocytes treated with Iono + 6-DMAP than those by Wang *et al.* (2008), however lower in blastocyst rate (7.1% vs. 28.0%).

When bovine oocytes were treated with 5 µM Iono (5 minutes) + 7% EtOH (5 or 10 minutes) or 5 µM Iono (5 minutes) + 10 µg/mL CHX (5 hours) post-ICSI, the cleavage rates of ICSI oocytes were higher than that of non-treated oocytes (69-78% vs. 39%, respectively) (Abdalla *et al.,* 2009). Moreover, the results showed that Iono + EtOH treatment significantly improved the proportions of ICSI oocytes developing into blastocysts until Day-8 (30%) compared with that of nontreated oocytes (12%). However, treating oocytes with EtOH alone, Iono alone, or Iono + 6-DMAP did not improve the cleavage rate (Abdalla *et al.,* 2009). Bhak *et al.* (2006) demonstrated that the cleavage rate (53% vs. 32%) of bovine oocytes activated by combination of 5 µM Iono (5 minutes) + 1.9 mM 6-DMAP (3 hours) post-SCNT was significantly higher than those treated by the combination of 5 µM Iono (5 minutes) + 10 µg/mL CHX (3 hours) with blastocyst rate (16 vs. 13%). This finding is contradictory to the report by Abdalla *et al.* (2009) where the treatment with combination of Iono + CHX gave better cleavage and blastocyst rates compared to the treatment with Iono + 6-DMAP.

Previous study also reported that the treatment of bovine oocytes post-ICSI with combination of Iono + 6-DMAP effectively improved blastocyst yield (Rho *et al.,* 1998; Ock *et al.,* 2003; Oikawa *et al.,* 2005). Also, Galli *et al.* (2003) suggested that using CHX instead of 6-DMAP improved the cleavage rate but not the blastocyst yield. Moreover, both activation treatments (Iono + CHX or Iono + 6-DMAP) resulted in obviously low blastocyst rate (2-7%). Therefore, the significantly lower blastocyst yields might be due

the adverse effects of the inhibitors (CHX and 6-DMAP) on the activity of other kinases or other proteins synthesis involved in cell functions (Soloy *et al.,* 1997). Perecin *et al.* (2007) suggested that the side effects of CHX or 6-DMAP can be avoided by using more specific kinase inhibitors for cell cycle regulation.

Presicce and Yang (1994a,b) and Winger *et al.* (1997) reported that ethanol, ionomycin or calcium ionophore with or without combination of CHX were not sufficient to induce parthenogenetic development in bovine oocytes. Thus, induction of diploidisation by combining the calcium oscillation-inducing activators with cytochalasin or 6-DMAP was necessary to improve the parthenogenetic development of bovine oocytes (Liu *et al.,* 1998b; Meo *et al.,* 2004). The rate of blastocyst development in oocytes activated with Iono + DMAP was greater ($P<0.05$) than those in other treated groups. These findings are in agreement with previous studies, in that 6-DMAP treatment of artificially activated oocytes increased the developmental potential of the parthenotes in bovine, murine and porcines (Susko-Parrish *et al.,* 1994; Leal and Liu, 1998; Liu *et al.,* 1998a). Similarly, previous studies have shown that combining Iono + 6-DMAP was particularly effective in inducing bovine oocyte activation and has been used in nuclear transfer studies (Wells *et al.,* 1999; Bhak *et al.,* 2006).

Wang *et al.* (2008) indicated that bovine oocyte activation is more desirable for combination of Iono + 6-DMAP or EtOH + CHX than for other combination or single treatments. Presicce and Yang (1994a) reported that activation rates achieved with the combined EtOH + CHX treatment were high for both young and aging oocytes. The activation with ionomycin alone and in combination of CHX caused an increase in haploid parthenotes, whereas the 6-DMAP treatment resulted in diploid parthenotes (Szollosi *et al.,*

1993) is the reason why oocytes treated in 6-DMAP is more competent than CHX-treated oocytes.

5.4 EFFECT OF DIFFERENT COMBINATIONS OF ACTIVATION CHEMICAL ON THE PRODUCTION OF PARTHENOGENETIC CAPRINE EMBRYOS (EXPERIMENT 4)

Ethanol (Nagai, 1987), ionomycin and calcium ionophore (Jones *et al.,* 1995a; Tesarik and Sousa, 1995) are chemical agents usually used to activate mammalian oocytes. These chemicals elevate [$(Ca^{2+})_i$], similarly as observed in normal fertilisation after sperm penetration (Yoshida and Plant, 1992). Ionophores such as calcium ionophore and ionomycin induces a great single $(Ca^{2+})_i$ rise in metaphase II (MII) oocytes from the internal deposits (Hoth and Penner, 1992) and resulted in activation of several calcium-dependent proteolytic pathways which later lead to the destruction of cyclin B, reduction of MPF activity and resumption of meiosis (Rinaudo *et al.,* 1997; Tomashov-Matar *et al.,* 2005; Jellerette *et al.,* 2006). In the present experiment, two chemicals that help to elevate [$(Ca^{2+})_i$] which are calcium ionophore and ionomycin were compared in order to find out which ionophores gave the highest cleavage rates on the caprine parthenotes. Even though there were no significant differences ($P>0.05$) between these two chemicals at every embryonic developmental stages, treatment with ionomycin showed the highest embryonic cleavage rates (2-cell: 95.00±5.00% vs. 83.33±10.91%; 4-cell: 95.00±5.00% vs. 83.33±10.91%; 8-cell: 75.66±8.19% vs. 72.61±13.21%; morula: 53.34±16.16% vs. 45.24±14.53% and blastocyst: 20.34±12.46% vs. 9.53±7.14%, respectively), compared to treatment with calcium ionophore. Calcium ionophore increased $(Ca^{2+})_i$ which in turn destroyed the existing Ca-sensitive CSF and resulted in the reduction of MPF activity (Swann and Ozil, 1994).

In a study conducted by Uranga *et al.* (1996) to compare the effect of two ionophores (calcium ionophore and ionomycin) to induce an increase in [$(Ca^{2+})_i$] in murine oocytes, calcium ionophore induced only slight activation at 1 μM concentration; however,

incubation with 5 μM for 5 minutes resulted in 56% of oocytes reached 2-cell stage and 40% when the treatment was lengthened to 10 minutes. However, when oocytes were treated with ionomycin either with 1 μM or 5 μM for 5 or 10 minutes, both indicated oocyte sensitivity to ionomycin treatment with 50-60% embryonic mortality. Their results also indicated that the cellular fragmentations and the death rate of embryos at 24 hours after exposure to the activating agents were higher with ionomycin than with calcium ionophore. According to Colonna *et al.* (1989), murine oocyte exposure to 1 μM ionomycin yielded only 1 μM increase in $[(Ca^{2+})_i]$. This increase is five times smaller than the one obtained when oocytes were activated with ethanol (Cuthbertson *et al.,* 1981). On the other hand, calcium ionophore induced significant parthenogenetic activation yielding high rates of development to blastocyst (Uranga *et al.,* 1996).

Navara *et al.* (1994) and Susko-Parrish *et al.* (1994) stated that treatment with inhibitors of protein synthesis, protein phosphorylation, or histone kinase improved the efficiency of oocyte activation. Histone kinase inhibitor prevents re-accumulation of maturation promoting factor (Ongeri *et al.,* 2001). 6-DMAP, the histone kinase inhibitor, has been reported to accelerate and enhance the formation of pronuclei in non-aged MII oocytes and parthenogenetic development in murine and bovine oocytes by inhibiting protein kinase functions and promoting mitosis (Leal and Liu, 1998).

Results from the current caprine experiment showed that when oocytes were treated in the combination of 5 μM A23187 + 2 mM 6-DMAP with different incubation durations in 6-DMAP (3, 4, 5 and 6 hours), there were no significant differences ($P>0.05$) among these combination treatments at every embryonic-developmental stages. However, treatment with A23187 + 6-DMAP for 3 hours incubation duration showed the highest cleavage rates with the values of 95.25±4.75% (2-cell); 92.85±7.15% (4-cell); 90.07±7.14% (8-cell); 47.22±14.27% (morula) and 10.72±5.49% (blastocyst), respectively,

compared to the other incubation durations. In contrast, treatment with the combination of Iono + 6-DMAP for 6 hours incubation duration showed the highest cleavage rates with the values of 96.15±3.85% (2-cell); 84.28±10.89% (4-cell); 79.05±13.95% (8-cell); 56.35±12.15% (morula) and 35.00±13.72% (blastocyst), respectively, compared to the other incubation durations. Blastocyst rate of 6 hours incubation duration in 6-DMAP was significantly higher ($P<0.05$) with 4 and 5 hours incubation durations (35.00±13.72% vs. 0% and 0%, respectively).

The present caprine results were in agreement with the study by Lan *et al.* (2005), whereby the best protocol for caprine oocyte activation (pronuclear formation: 87-95%) was obtained when oocytes were treated with 2.5 μM ionomycin (1 minute) followed by 2 mM 6-DMAP (6 hours). Lan *et al.* (2005) found that the duration of 6-DMAP treatment could be reduced to 1 hour from 2 to 4 hours after ionomycin treatment to produce higher activation rates (>85%). Moreover, activated caprine oocytes began pronuclear formation at 3 hours and completed it by 5 hours post-ionomycin treatment. In addition, an extended incubation in 6-DMAP shows: (i) improvement in the development of caprine parthenotes; (ii) quickened both the release from metaphase arrest and the pronuclear formation; and (iii) inhibit the chromosome movement at anaphase II (AII) and telophase II (TII), leading to the formation of one pronucleus without extrusion of second polar body (PBII) (Lan *et al.,* 2005).

Previous study by Ongeri *et al.* (2001) showed that there were no significant differences ($P>0.05$) in cleavage (57.8% vs. 58.0%) and blastocyst (28.5% vs. 27.4%) rates when caprine oocytes were treated in the combination of Iono + 6-DMAP and EtOH + 6-DMAP, respectively. Therefore, Ongeri *et al.* (2001) suggested that these two combination treatments induced development rates for caprine oocytes efficiently. These two combination activation protocols also have been shown to be the most effective method for

inducing parthenogenesis of bovine oocytes, as both protocols mimic natural fertilisation process (Jones *et al.,* 1995b; Kim *et al.,* 1997; Rho *et al.,* 1998). However, Baguisi *et al.* (1999) stated that in caprine, oocytes that were activated by ethanol did not develop to term pregnancy despite good cleavage rates after SCNT. The reason for this phenomenon was not explained by the researchers. In the recent study by De *et al.* (2012), there were no significant differences found between the activation of caprine oocytes by 5 µM A23187 (5 minutes) + 2 mM 6-DMAP (3 hours) and 2.31 kV/cm EP (15 µs) + 2 mM 6-DMAP (3 hours) in the cleavage (70.44% vs. 72.10%) and blastocyst (21.09% vs. 23.11%) rates. Bhak *et al.* (2006) also demonstrated that parthenotes and SCNT embryos activated with Iono + 6 DMAP showed abnormal time-dependent patterns to cleavage stages and more rapid than other treatments. Particularly, approximately 30% of parthenotes and SCNT embryos treated with Iono + 6-DMAP cleaved at 19 hours post-activation, whereas few or none had cleaved in other chemical activation treatments (Bhak *et al.,* 2006).

Also, the incubation duration and type of parthenogenetic activation chemicals or agents were shown to influence chromosomal complement and subsequent development of caprine parthenotes (Van De Velde *et al.,* 1999). Although Van De Velde *et al.* (1999) found that there was no significant difference in the cleavage rate of oocytes activated by sequential treatment with A23187 + 6-DMAP for 3.5 hours versus the IVF controls, prolonged treatment with 6-DMAP for 6.5 hours resulted in a significantly lower proportion of diploid embryos complement with higher proportion of abnormal chromosomal composition compared to the IVF or short term 6-DMAP treatment. This finding suggested that 6-DMAP treatment induced diploid activation by preventing chromosomal separation and extrusion of the second polar body, while longer treatment in 6-DMAP might cause irreversible injury to normal microtubule spindle formation, which later resulting in abnormal division and abnormal ploidy (Liu *et al.,* 1998a). The authors

showed that the decrease in histone kinase activity, a prerequisite for oocyte activation, was more rapid in oocytes treated with a calcium ionophore followed by 6-DMAP, whereas the decrease was slower in oocytes treated with a calcium ionophore alone. Moreover, MAPK activity remained high in the calcium ionophore-treated oocytes and was comparable to MII control oocytes. Liu *et al.,* (1998a) also suggested that combination treatment with calcium ionophore and 6-DMAP resulted in one diploid pronucleus without second polar body extrusion. In addition, combination treatment of Iono + 6-DMAP also improved pronuclear formation after ICSI procedure in bovine oocytes (Rho *et al.,* 1998).

The combination of A23187 + 6-DMAP was found to induce high rates of oocyte activation, pronucleus formation and development to blastocyst in bovine (Liu *et al.,* 1998a) and ovine (Loi *et al.,* 1998). These findings are in agreement with previous studies, in that combination treatment with 6-DMAP successfully activated oocytes artificially as well as increased the developmental potential of the parthenotes in bovine, murine and porcine (Susko-Parrish *et al.,* 1994; Leal and Liu, 1998; Liu *et al.,* 1998a; Bhak *et al.,* 2006). Even though the reason of the abnormal time dependent rate of oocytes activated with Iono + 6-DMAP remains unclear, Lonergan *et al.* (2000) suggested that it may be related to association of gene expression or re-entering of some nuclei to S-phase of the cell cycle without having passed through metaphase (De La Fuente and King, 1998). However, De La Fuente and King (1998) stated that there is one disadvantage when activating oocytes with Iono + 6-DMAP as it caused DNA injury which could be observed in abnormal pattern of karyokinesis at the first cell cycle.

Treatment of murine oocytes with CHX resulted in oocytes activation, as assessed by emission of the second polar body and formation of a pronucleus (Siracusa *et al.,* 1978; Clarke and Masui, 1983; Fulka *et al.,* 1994). Oocytes activation induced by CHX

presumably occurs by permitting the turnover of cyclin B that resulted in a concomitant decrease in cdc2/cyclin B kinase activity (Moos *et al.,* 1996).

Results in the current experiment indicated that treatment with 10 μM Iono + 10 μg/ml CHX for 4 hour incubation duration showed the highest cleavage rates (2-cell: 91.67±4.82%; 4-cell: 76.87±6.48%; 8-cell: 68.50±10.20%; morula: 36.13±15.47% and blastocyst: 11.10±11.10%, respectively), compared to the other incubation durations. The significance was only shown at morula stage where incubation duration in CHX for 5 hours was significant ($P<0.05$) with 6 hours (14.93±9.16% vs. 49.35±3.57%, respectively).

In the assessment of activation protocols on bovine oocytes by Bhak *et al.* (2006), treatment with 5 μM Iono (5 minutes) + 1.9 mM 6-DMAP (3 hours) showed significantly greater ($P<0.05$) cleavage and blastocyst rates from the treatment with 5 μM Iono (5 minutes) and 5 μM Iono (5 minutes) + 10 μg/ml CHX (cleavage: 85.5 vs. 30.3 and 57.9%; blastocyst: 12.3 vs. 0.7 and 5.3%, respectively). Results from the present study was in agreement with the finding by Bhak *et al.* (2006), whereby apparently the highest cleavage (96.15±3.85%) and blastocyst (35.00±13.72%) rates obtained from the oocytes treated with 10 μM Iono + 2 mM 6-DMAP for 6 hours. Similarly, previous study by Alexander *et al.* (2006) showed that the blastocyst rate of parthenogenetic ovine embryos was significantly higher ($P<0.05$) in combination with 6-DMAP activated oocytes compared to those activated with combination of CHX (21.0% vs. 14.9%, respectively). However, no difference ($P>0.05$) was observed in blastocyst rate between the two activation treatment groups for SCNT embryos. Further chromosomal analysis demonstrated that all the parthenogenetic embryos derived from 6-DMAP treatment were chromosomally and significantly higher ($P<0.05$) than those treated in CHX (100.0 vs. 93.6%, respectively) (Alexander *et al.*, 2006).

The activation with ionomycin alone and in combination of CHX caused an increase in haploid parthenotes, whereas the 6-DMAP treatment resulted in diploid parthenotes (Szollosi *et al.,* 1993) which was the reason why oocytes treated in 6-DMAP was more competent than CHX-treated oocytes. This theory could be observed in the results of the present study, whereby when comparing various optimal combination treatments and IVF control, oocytes from the treatment with combination of 6-DMAP and CB achieved more than 50% cleaved embryos at morula stage in contrast with oocytes from the treatment with combination of CHX (morula: 36%). However, all embryos from CB-treated oocytes were unable to develop beyond morula stage. The reason for this happening could not be explained in the present experiment.

The current results indicate that treatment with 10 μM Iono + 5 μg/ml CB for 5 hours incubation duration showed the highest embryonic cleavage rates with the values of 92.17±7.83% (2-cell); 88.23±11.77% (4-cell); 87.87±12.13% (8-cell) and 55.80±22.00% (morula), respectively, when comparing different incubation duration in CB (3, 4, 5 and 6 hours). However, there was no embryo develop beyond morula stage at each incubation duration. The cleavage rates at 8-cell stage of 5 hours incubation duration were significantly different ($P<0.05$) from other incubation durations.

Similarly, in the study of comparison of different activation protocols on reconstructed bovine oocytes, there were no significant differences ($P>0.05$) were found in cleavage (84.4% vs. 87.1%) and blastocyst rates (36.7% vs. 33.3%) in reconstructed bovine oocytes activated by A23187 + 6-DMAP and Iono + 6-DMAP (Shen *et al.,* 2008). However, cleavage (98.9% vs. 79.6%) and blastocyst (51.6% vs. 14.3%) rates were found to be significantly higher ($P<0.01$) in oocytes activated by A23187 + 6-DMAP and A23187 + CHX + CB (Shen *et al.,* 2008). In the previous research to evaluate the effect of different protein synthesis inhibitors on the parthenogenetic development of porcine

oocytes (Yi and Park, 2005), cleavage (66.4%) and blastocyst (25.0%) yields were improved when oocytes were treated with combination of EtOH + CHX + CB + 6-DMAP than in other treatments (cleavage: 24.9-57.6%; blastocyst: 0.0-19.3%).

These results indicate that full activation of caprine oocytes requires inactivation of both MPF and MAPK activity. Oocytes only achieved partial activation (exit from MI1 arrest but no pronuclear formation) when MPF was inactivated but MAPK activity remained high (Liu *et al.,* 1998a). Therefore, exposure of oocytes to protein synthesis inhibitors such as 6-DMAP or CHX functions to inactivate MAPK activity. Furthermore, treatment of oocytes with 6-DMAP resulted in instant destruction of the spindle suggesting that MAPK is responsible for spindle assembly (Liu *et al.,* 1998a).

6-DMAP, a non-specific protein kinase inhibitor, inhibited kinase activity of MPF by inactivating MAPK (Motlik *et al.,* 1998; Gordo *et al.,* 2000). Activation treatment with 6-DMAP caused extrusion of the polar body, thus generating a diploid zygote (Susko-Parrish *et al.,* 1994). Moreover, 6-DMAP could restrain the activity of MPF and CSF by hampering protein phosphorylation, which was required in spindle assembly; consequently, extrusion of the second polar body would be disrupted (Presicce and Yang, 1994a). It was reported that 6-DMAP could enhance the speed of pronuclear formation compared with CHX in bovine and ovine oocytes (De La Fuente *et al.,* 1998; Alexander *et al.,* 2006). On the other hand, CHX, a protein synthesis inhibitor, restricted the synthesis or re-accumulation of cyclin B and subsequently blocked the re-synthesis of MPF activity, as well as prohibited the synthesis of proteins such as CSF and MPF, resulting in low CSF concentration and decreased MPF activity (or inactivation) which later led to the resumption of second meiotic division and the activation of oocytes (Presicce and Yang, 1994b). In contrast, CB was reported to inhibit extrusion of the second polar body after activation of the oocytes as well as led to the occurrence of chromosomes segregation, thus

further inhibited the cytokinesis which resulted in diploid zygotes with two pronuclei (Fukui *et al.,* 1992; Presicce and Yang, 1994a,b; Liu *et al.,* 1998a). Also, CB would help prevent fragmentation of embryos (Collas and Robl, 1990; Yang *et al.,* 1992).

When comparing the embryonic development of the parthenotes from the optimal treatment in each group of combination with IVF as a control, every activation treatment showed a significant difference ($P<0.05$) with IVF control at 8-cell stage. Treatment with 10 μM Iono + 2 mM 6-DMAP for 6 hours exhibited the highest cleavage (96.15±3.85%) and blastocyst rates (35.00±13.72%) compared to other combination treatments and significantly higher ($P<0.05$) with IVF control from 8-cell stage to blastocyst. Results from the present experiment were in agreement with the earlier study by Ongeri *et al.* (2001), in which the cleavage (57.8% and 58.0% vs. 47.2%, respectively) and blastocyst (28.5% and 27.4% vs. 10.2%, respectively) rates of caprine oocytes activated by the combination of Iono + 6-DMAP and EtOH + 6-DMAP were significantly higher ($P<0.05$) than the IVF control. Van De Velde *et al.* (1999) showed that bovine oocytes activated by combination of A23187 + 6-DMAP exhibited no difference from IVF in the cleavage rate. In a study by Boediono *et al.* (1995), bovine oocytes activated with ethanol alone or in combination with CB as well as with IVF oocytes, did not differ in cleavage and blastocyst rates. No embryos were observed to develop beyond morula stage from the IVF control in the present study that could be due to the numerous reasons including unavailable efficient IVF protocol as well as incompetence of the personnel carrying out the caprine IVF. Previous studies by Brackett and Oliphant (1975) and De Smedt *et al.* (1992) had reported that 8 to 20% polyspermy normally occurred in caprine IVF. In addition, Ongeri *et al*. (2001) suggested that penetration of multiple sperm into an oocyte could lead to fragmentation after a few cell divisions; and this phenomenon could explain why the

earlier stage cleavage rates were relatively much higher than the blastocyst rates. Similar patterns were observed in the present study.

5.5 EFFECT OF *IN VITRO* MATURATION (IVM) DURATION THE PRODUCTION OF CAPRINE OOCYTES AND ITS SUBSEQUENT PARTHENOTES DEVELOPMENT (EXPERIMENT 5)

Two groups of IVM durations were designed in this experiment (18-21 hours and 22-25 hours) before activation procedures were carried out. Grade D oocytes showed the lowest maturation rate for both IVM groups, whereas Grades B and A oocytes showed the highest maturation rates for 18-21 and 22-25 hours IVM, respectively. The range of IVM duration at 18-21 hours was chosen because at this range, the oocyte maturation rate was high. From the previous protocol of our laboratory, it was hypothesised that the oocyte donor caprine treated hormonally (60 hours of post-PMSG and 66 hours post-hCG) from LOPU procedure could give good quality oocytes which were developmentally competence. The present results clearly demonstrated that the duration of IVM as well as the quality of oocytes affected maturation rate, oocyte activation and subsequent embryonic development.

Cognie *et al.* (2003) showed that the maturation rate of caprine oocytes is 21 to 72% when *in vitro* matured at 16-24 hours. Phua (2006) obtained 31 to 56% maturation rate at 25-28 hours IVM duration with LOPU-derived caprine oocytes, whereas Chan (2008) reported that the maturation rate of caprine oocytes that were *in vitro* matured at 21-24 hours was significantly higher ($P<0.05$) from those matured at 25-28 hours (71.23% vs. 56.75%, respectively). This finding is in agreement with study by Kong (2010), where IVM duration of caprine oocytes at 22-25 hours showed better maturation rate compared to those *in vitro* matured at 26-29 hours (71.60% vs. 38.70%, respectively). However, in the present study, caprine oocytes *in vitro* matured at 18-21 hours apparently gave the higher maturation rate from those matured at 22-25 hours (86.80±1.84% vs. 84.11±2.08%). It is clearly shown that the maturation rate from the current study was improved than the

previous study by Chan (2008) and Kong (2010) (84.11% vs. 71.23% and 71.60%, respectively). As for slaughterhouse oocytes, the IVM duration suggested to be 24-27 hours (Kong, 2010) which was higher than LOPU oocytes (21-24 hours). The reasons for higher maturity in the current study using 18-21 hours IVM duration could not be explained, but it could be due to oocytes were already undergoing process of meiosis at the time of LOPU oocyte retrieval, resulting in at shortening of IVM duration. Besides this, it could be due to factors specific to the laboratories such as the method of IVM preparation, different IVM ingredients, duration of IVM as well as time interval between PMSG-hCG and LOPU commencement. From the results of present study, the duration of IVM at 18-21 hours from the present study is suggested to obtain for the LOPU oocytes before subsequent embryo procedures to be carried out in the caprine species.

When using the same range of IVM duration (21-25 hours) of LOPU materials in this study, the results of cleavage (92.62%) and morula (41.47%) rates of oocytes were found to be higher than that of Chan (2008) who produced *in vitro* fertilised caprine embryo at rates of 64.46 and 14.38%, respectively. By contrast, Lan *et al.* (2005) demonstrated that the best caprine oocyte activation from slaughterhouse (pronuclear formation: 87-95%), was obtained when oocytes matured *in vitro* for 27 hours were treated with 2.5 μM ionomycin (1 minute) followed by 2 mM 6-DMAP (6 hours). In addition, activation rates of oocytes *in vitro* matured for 27, 30, and 33 hours were higher ($P<0.05$) than those matured 24 hours when treated with Iono + 6-DMAP (6 hours). However, activation of oocytes with Iono + 6-DMAP (4 hours) enhanced the activation rate of oocytes matured by 24 hours (Lan *et al.,* 2005).

Chan (2008) reported that the beneficial results obtained from ICSI as compared to IVF were due to the additional effect of the chemical activation of oocyte cleavage. The possible reasons related to the higher percentage of embryonic cleavage obtained by

parthenogenetic activation could be due to the additional effect of chemical activation treatment. This explanation might be applied to the current experiment as the present results showed higher cleavage and morula rates compared to those obtained by Chan (2008). It is well known that calcium ionophore, ionomycin and ethanol were able to mimic fertilisation very closely by elevating [$(Ca^{2+})_i$] levels in oocytes (Jones *et al.,* 1995b). Moreover, protein kinase inhibitor such as 6-DMAP helped in preventing re-accumulation of MPF, thus resulted in improvement the efficiency of oocyte activation (Susko-Parrish *et al.,* 1994). In addition, cysteamine used in IVM ingredients had been reported to improve oocyte maturation and subsequent embryonic development (Cognie *et al.,* 2002).

Regardless of IVM duration, oocytes from Grades A, B and C were apparently more competent to develop until morula stage compared to Grade D oocytes. These observations brought out the important role of cumulus cells in influencing maturation and the subsequent development of caprine embryo (Chan, 2008). The cumulus cells surrounding the immature oocytes played an important role in nuclear and embryonic development (Hashimoto *et al.,* 1998; Taghe *et al.,* 2002). Therefore, the integrity of (cumulus oocyte complexes) COCs during maturation was necessary for embryonic development (Shioya *et al.,* 1988; Vanderhyden and Armstrong, 1989). Maturation rates of Grades A and B oocytes were significantly higher ($P<0.05$) than Grade D oocytes at both IVM durations (18-21 hours: 91.61±2.70% and 93.33±2.83% vs. 77.39±6.68%, respectively; 22-25 hours: 88.17±3.29% and 86.29±3.65% vs. 73.08±6.41%, respectively). Moreover, it is believed that oocyte quality would influence the *in vitro* production of caprine embryos (Katska-Ksiazkiewicz *et al.,* 2007).

The present results showed that good quality oocytes which were surrounded by multi-layers of compact investment with homogenous ooplasm (Grades A, B and C) had

higher embryonic development rates up to blastocyst compared to those of poor quality oocytes (Grade D). This finding suggested the essential role of cumulus cells in promoting normal cytoplasmic maturation of oocytes necessary for activation, cleavage and the subsequent parthenote development. Eppig (1991) reported that cumulus cells were a sub-population of granulosa cells surrounding the oocyte, providing nutrients and signals that regulated its growth and maturation. In addition, the presence of cumulus cells around the oocyte was essential to initiate the transport of nutrients and signals into and out of the oocytes (Moor and Seamark, 1986). Maedomari *et al.* (2007) also suggested that the presence of cumulus cells around the oocytes during IVM had been reported to be important to the blastocyst stage after IVC, where these abilities were regulated by intracytoplasmic glutathione which was synthesised by cumulus cells and accumulated in the oocytes during the later stage of maturation. Moreover, oocyte quality was determined by diverse scale such as the degree of expansion, texture of ooplasm and number of cumulus cells layers, where these criteria were found to have significant effects on the *in vitro* maturation, fertilisation and development (Tanghe *et al.,* 2002). This theory was consistent with the current experiments whereby oocytes with 3 to 5 layers of cumulus cells and homogenous ooplasm (Grades A, B and C) indicated higher cleavage rates up to blastocyst compared to oocytes originating from <3 layers of cumulus cells (Grade D) though not significantly different. Besides, it has been demonstrated in some reports that removal of cumulus cells had adverse effect on oocyte maturation, fertilisation and embryonic development (Cecconi *et al.,* 1996; Goud *et al.,* 1998).

Regardless of oocyte grades, there were no significant differences observed in maturation and cleavage rates for all embryonic developmental stages between 18-21 and 22-25 hours IVM durations. These findings suggested that *in vitro* matured oocytes at 18-21 and 22-25 hours had same fertilisation and embryonic developmental ability. Although

there were no significant differences at all embryonic developmental stages in both IVM durations, 18-21 hours IVM duration gave higher results at all embryonic development stages (except morula stage) than 22-25 hours. It is well known that the degree of cytoplasm maturation will determine the developmental competence of IVM oocyte to undergo further embryonic development (Combelles *et al.,* 2002; Inoue *et al.,* 2008).

Apoptosis was also observed in some embryos in the present experiment, particularly in morula stage whereby these embryos were barely able to develop beyond the morula stage either the embryos were stopped at this stage or undergo fragmentation. Chan (2008) suggested that the reason could probably due to the sub-optimal culture medium. However, Jimenez-Macedo *et al.* (2007) suggested that one of the functions of apoptosis during development was to remove cells that are no longer required.

5.6 GENERAL DISCUSSION

The results of the current research showed the feasibility of obtaining parthenogenetic embryos by using various types of chemical activation in murine, bovine and caprine species. Generally, there are many factors that affect production of parthenogenetic embryos such as post-hCG duration (in mice), source of oocytes (in caprine), IVM duration (in caprine and bovine) hormonal administration, type of chemical used, duration of incubation in activation chemical, concentration of activation chemical and type of chemical used as combination treatment.

Although low percentages of blastocyst were obtained from the various chemical treatments, the performance of further studies could be improved if proper precautions and organisation are carried out, such as proper hormonal administration used, selection of high quality oocytes, proper handling of oocytes including aseptic techniques, meiotic stages of oocytes retrieved as well as choice of chemicals used for oocyte activation. The

following section will provide a brief discussion on other specific factors affecting parthenote developmental competence not covered in this study.

5.6.1 Summary of Significant Findings

In summary, all the objectives of this project were achieved. Parthenogenesis protocol in murine, bovine and caprine species was developed in ABEL laboratory that could be used integratively with other related embryo technologies in our laboratory. For murine study, combination of strontium chloride and cytochalasin B is the optimal way to produce parthenogenetic murine embryos, even though other chemicals have the same potential to produce parthenogenetic embryos which develop until blastocyst stage. Duration of 13-15 hours post-hCG administration was found to be a better choice to give higher percentage of oocytes with polar body and subsequent parthenote development in murine species. As for bovine study, treatment with combination of ionomycin and 6-DMAP was the optimal method to produce parthenogenetic bovine embryos although other chemicals could also produce bovine parthenotes. As for caprine species, combination of ionomycin and 6-DMAP was the optimal protocol to produce parthenogenetic caprine embryos even though other chemicals had the same potential to produce parthenogenetic embryos and develop until blastocyst stage. Duration of 18-21 hours IVM was a better choice to give higher percentage in maturation rate and subsequent parthenote development in caprine species. Grades A, B and C oocytes were found to be more competence than Grades D and E oocytes in both caprine and bovine species.

5.6.2 Oestrus Synchronisation and Superovulation

In order to produce large amount of oocytes as a source for production of parthenogenetic embryos, oestrus synchronisation and superovulation of the female oocyte donor are

conventionally necessary. The efficiency of superovulation in female oocyte donors depends on several factors. The factors include the strains of donor, their physical condition such as age, weight and sexual maturity, the type of hormonal treatment, dose of gonadotrophins administered and timing of gonadotrophins administration (Hogan *et al.*, 1994) as well as external factors such as the presence of pheromones produced by male (Zarrow *et al.*, 1971). It was suggested that successful superovulation in donor animal requires the synergistic effects of both pregnant mare‟s serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) injection. PMSG mimics the effects of follicle stimulating hormone (FSH) produced which induce follicular growth and subsequently prepares them for ovulation whereas hCG mimics the luteinising hormone (LH) to stimulate the ovulation of mature ova (Christenson and Eleftheriou, 1972).

Oestrus synchronisation was successfully performed in caprine whereas superovulation was successfully performed in both caprine and murine in the present study as favourable number of total oocytes recovered was obtained. Nevertheless, some of the donors indicate poor response to the hormonal treatment. As for caprines that were used for LOPU, some of the donors underwent several repeated surgeries which mean repeated oestrus synchronisation and superovulation had been carried out to the same donor. Therefore, it is suggested that antibody formation to chorionic gonadotrophin might the possibility of the poor response to the hormonal treatment (Baril *et al.,* 1996; Herve *et al.,* 2004). Moreover, selection of the healthy donor, proper preparation and timing of the hormonal treatment, interval between the current and next LOPU cycles and post-LOPU treatment should be optimised in order to achieve good stimulation response, hence obtaining good quality and high yield of oocytes.

5.6.3 Oocyte Quality

The quality of oocytes which was assessed by cumulus cells expansion and ooplasm homogeneity seemed to be adequate enough to differentiate between competent (Grades A, B and C) and incompetent oocytes (Grades D and E). Nevertheless, it should be noted that the morphology of cumulus cells and ooplasm might not be a precise indicator of oocyte developmental competence due to the several aspects that might be affect cumulus investment during oocytes retrieval such as vacuum pressure, type and diameter of needle used as well as the aspiration technique. It has been reported that COCs retrieved from LOPU might have few layers less cumulus cells than the actual layers due to the mechanical factors during LOPU procedure (Chan, 2008). Therefore, the reduction in the vacuum pressure during aspiration technique could improve the quality of oocytes.

In the current study, not all competence oocytes (Grades A, B and C) developed to the blastocyst stage. This phenomenon would suggest that developmental potential of the individual oocyte might not possibly be predicted based on the visual appearance of the oocytes (Blondin and Sirard, 1995; De Wit and Kruip; 2001). Other aspects had been reported to affect competency of oocytes such as diameter of the oocytes as well as morphology and size of both follicle and ovary (Jewgenow *et al..,* 1999; Hendriksen *et al.,* 2000).

5.6.4 *In Vitro* Culture (IVC) System

In the present study, oocytes from all three species (murine, bovine and caprine) were able to develop until blastocyst stage although low number of blastocyst was obtained. Despite the activation treatment used to activate oocytes, it could be suggested that the culture condition also contribute the developmental competence of parthenogenetic embryos. An optimal culture system was an essential requirement on the developmental potential of *in*

vitro produced embryos since the embryos underwent according critical events which were maturation, chemical activation, fertilisation, cleavage, embryonic genome activation, compaction of morula as well as blastocyst formation (Chan, 2008). Thus, improvement in IVC systems would likely produce better quality of embryos (Galli *et al,* 2001).

Moreover, it has been reported that the number of embryos in a culture droplet (Khurana and Niemann, 2000) as well as droplet size also influenced the developmental potential of embryos. In the present experiment, embryos were cultured in a small group according to the oocyte grades (bovine and caprine) for further identification throughout the experiments. Glucose was used as an energy substrate at the earlier stage of embryonic development in a culture media. However, earlier stages of embryo have a limited capacity to utilise glucose (Thompson *et al.,* 1996). Kim *et al.* (1993) reported that glucose in the culture media has an adverse effect on embryonic development and it is not required until Day-3 or -4 of development. In contrast, Thompson *et al.* (1996) stated that high glucose concentration is also shows detrimental effect to embryonic development. Kwong *et al.* (2012) suggested that increasing glucose concentration (2.78 mM) in KSOM medium at later stage of embryonic development (8-cell onwards) could supported the hatching of caprine cloned blastocyst. The blastocyst rates of SCNT embryos were significantly higher ($P<0.05$) when glucose was added into KSOM compared to those non-added glucose (Kwong *et al.,* 2012).

Several studies have been carried out in order to optimise culture condition and many chemically-defined media had been proposed to support caprine embryonic development *in vitro*. Even so, knowledge about the exact requirements of culturing caprine embryos *in vitro* was not well recorded, hence the success rate of *in vitro* produced caprine embryos still varies widely. One of the main limitations *in vitro* produced embryos

is the slow growth rate and low percentage of developing caprine embryos reaching blastocyst stage as compared to *in vivo* embryonic development.

5.6.5 Constraints of the Studies

During the research duration, numerous difficulties were encountered including:

5.6.5.1 Skill attainment

Learning curve was done in murine model to practice in proper handling of oocytes/embryos, preparation of microtools and media which took approximately 5 months due to lack of model animals, facilities, chemicals, reagents and tools as well as lack in skills and expertise in the laboratory. Moreover, purchasing of hormones, chemicals and reagents also took a long period (approximately 3 months) as many bureaucratic processing procedures had to be gone through before approval of the purchase.

5.6.5.2 Facilities

As our laboratory comprised many post-graduate students, the facilities, equipment and reagents available were relatively inadequate to enable the current research to be carried out in a more comprehensive manner. The situation worsened due to sharing of equipment and facilities with other researchers that resulted in compromising the outcome of the experiments. For example, the author was needed to carry out LOPU procedures at different laboratories and therefore had to transport the oocytes from LOPU laboratory to the other embryo manipulation laboratory in order to carry out the subsequent experiments. The development of oocytes might be disrupted during the journey as well as in the transferring process. Electricity breakdown was common at the laboratory also could affect

the embryonic development as there was no back-up generator provided by the management.

5.6.5.3 Source of oocytes

The consistency in the number of oocytes yield from every LOPU procedure was varied due to several factors such as age, breed, health and physiological status of caprine oocyte donors which could not be standardised throughout the research duration. Moreover, repeated LOPU procedures were done on the same animal due to difficulty in finding sufficient number of caprine donors might contribute to the adhesion problem of reproductive tract. Low productivity in caprine oocyte donor which later produced low number and quality in oocyte yielded was routinely experienced in this study. Since our laboratory comprises many post-graduate students, we have to share caprine oocytes from the same sources. Therefore, the number of oocytes obtained for experiments was relatively low per replicate.

5.6.5.4 Embryo developmental arrest

One of the major problems faced by almost researchers in this laboratory was to obtain blastocyst in their research. The embryo development was unable to develop beyond the morula stage when using mSOFaa as IVC medium previously. This unfavourable scenario was solved by using KSOMaa as a basal medium after a series of several inventions by using substitution of BSA with glucose and FBS (Kwong *et al.,* 2012).

5.6.6 Future Directions

Although parthenogenetic embryos have much beneficial effect, however, it cannot be used to treat infertility alone, as parthenogenetic embryos would be non-viable at early

post-implantation stage. The possible reason might be due to the absences of paternally imprinted which was a requirement in the developmental stage, especially for the formation of extra-embryonic tissues. Another deficiency of parthenogenetically activated oocytes that was oocytes tend to rely on the maturation time or oocytes age. As suggested in the literature, parthenogenetic activation was easier to be achieved in the aged oocytes. However, aging oocytes managed to alter oocyte cytoskeleton components, impairs enucleation, high frequency in producing fragmented oocytes, besides being responsible for causing cell apoptosis (Meo *et al.,* 2004).

Since the technique of nuclear transfer and cloning could greatly increase the efficiency of producing transgenic caprine in industry sector, therefore, it is needed to develop an effective protocol for oocyte activation that can be used during nuclear transfer in caprine. However, limited information is available in the literature on the efficient parthenogenesis and developmental competence of caprine parthenotes. In the present study, it is demonstrated that murine, bovine and caprine embryos could be generated through parthenogenesis approach using various types of chemical and further develop until blastocyst stage. However, numerous factors influencing the efficiency of caprine parthenotes development need to be identified and studied in details in order to be used in cloning, nuclear transfer and stem cell procedures for the production of better results for biological, medical research and industry application. Since it is difficult to obtain high percentage of caprine blastocyst in normal IVF, ICSI and SCNT techniques, thus, the optimised protocol of caprine parthenogenesis hopefully could be enhanced and improved in cloning, nuclear transfer and stem cell procedures. In addition, parthenotes have been used in co-transfer experiments for the establishment of pregnancies in studies of SCNT (De Sousa *et al.*, 2002).

Chapter 6

6.0 CONCLUSIONS

Chapter 6

6.0 CONCLUSIONS

This study was carried out to produce embryos through parthenogenesis approach as well as to investigate the effect of different activation protocols using various single and combination treatments on the production and parthenotes developmental performance in murine, caprine and bovine species. Specific conclusions can be made based on the findings from this research as follows:

a) This is the first comprehensive report of production of murine, caprine and bovine embryos through parthenogenesis approach using various combinations of activation chemicals in the local setting at Animal Biotechnology-Embryo Laboratory, University of Malaya, Malaysia.

b) The optimal protocol for production of murine parthenotes is by using combination of 10 mM $SrCl_2$ + 5 μg/ml CB in CZB Ca^{2+}-free medium (3 hours) though other activation protocols in this study are able to activate oocytes and subsequently develop throughout the pre-implantation stage.

c) Higher percent of oocytes with polar body was obtained from murine parthenotes derived from 13-15 hours post-hCG administration duration and significantly higher ($P<0.05$) in developmental competence compared to those derived from 16-18 hours post-hCG administration duration.

d) The optimal protocol for production of bovine parthenotes is by using combination of 10 μM Iono (5 minutes) + 2 mM 6-DMAP (4 hours) though other activation protocols in this study are able to activate oocytes and subsequently develop throughout pre-implantation stage.

e) Generally, maturation rates of Grades A, B and C bovine oocytes from abattoir sample were more than 50%. Bovine parthenotes derived from Grades A, B and C oocytes treated with various combination treatments showed high developmental capacity and able to develop untill blastocyst stage.

f) Caprine parthenotes can be produced successfully until blastocyst using both single and combined activation chemical treatments.

g) The optimal protocol for production of caprine parthenotes is by using combination of 10 μM Iono (5 minutes) + 2 mM 6-DMAP (6 hours) though other activation protocols in this study are able to activate oocytes and subsequently develop throughout pre-implantation stage.

h) Maturation rates of Grades A and B caprine oocytes from LOPU procedure were significantly higher ($P<0.05$) than Grades D and E oocytes. Caprine parthenotes derived from Grades A, B and C oocytes showed higher developmental competence than those derived from Grades D and E.

i) Higher maturation rate was obtained from caprine parthenotes derived from 18-21 hours IVM duration than those derived from 22-25 hours of IVM duration. However, both IVM durations had the same potential in the development of caprine parthenotes.

j) In a nutshell, murine, caprine and bovine parthenotes were successfully *in vitro* produced until blastocyst stage using various chemical activation treatments.

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