USING MOUSE EMBRYONIC FIBROBLAST (MEF) AS FEEDER CELLS FOR PRODUCTION OF EMBRYONIC STEM CELL (ESC) LINE IN THE MURINE AND CAPRINE

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ABSTRACT

Embryonic stem cells (ESC) have unlimited potential in the field of biological sciences and regenerative medicine due to their pluripotency and ability to self-renew indefinitely. With the goal to establish, isolate and culture murine (mESC) as well as caprine (gESC) embryonic stem cells, *in vivo*- and *in vitro*-derived blastocysts were used as a source in producing mESC and gESC lines. Both mESC and gESC were cultured *in vitro* using mouse embryonic fibroblast (MEF) as feeder cell layer in this study. The aims of this study were: a) to compare the effects of murine strain, blastocyst stage and inner cell mass (ICM) isolation techniques on the efficiency of deriving murine embryonic stem cell (mESC) lines in murine species and b) to compare the effects of *in vivo*- and *in vitro*-derived blastocyst sources as well as to establish effective technologies to isolate and culture ESC in caprine species. Mouse embryonic fibroblasts (MEF) were derived from murine foetuses (13.5 to 14.0 d.p.c.), cultured up to Passage 2 (P2), cryopreserved and thawed at each passage to be used as feeder cell layer for mESC and gESC cultures. In order to obtain the blastocyst sources for production of mESC and gESC lines, somatic cell nuclear transfer (*in vitro*) and *in vivo* flushing were carried out in this study. For isolation of the inner cell mass (ICM) from blastocyst, whole blastocyst culture, manual cut and laser dissection were compared among respective treatment groups to derive mESC and gESC lines. In murine, a total of 71 (ICR), 38 (CBA/ca), 22 (C57BL/6J) mESC lines were produced from 971, 758 and 709 murine blastocysts, respectively. Five blastocyst stages were cultured on the MEF with 3 ICM isolation techniques. ICM outgrowths were disaggregated by trypsin/EDTA (0.05%) and manual dissociation, cultured on new inactivated MEF in CO_2 (5%) incubator at 37℃. The attachment, primary ICM outgrowth and successful consecutive passages rates up to P3 were compared among the murine strains, blastocyst stages and

ICM isolation techniques. There were significant differences ($P<0.05$) in successful passage rate at P3 between CBA/ca with ICR and C57BL/6J (19.81% vs. 9.00% and 8.50%), respectively, also mESC at Passage 1 (P1) for mid-, expanded- and hatching blastocyst stages versus early- and hatched blastocyst (45.35%, 52.79% and 43.01% vs. 27.88% and 24.53%), respectively. Manual cut ICM isolation technique consistently gave the highest attachment, primary ICM outgrowth and successful mESC P2 and P3 rates compared with whole blastocyst culture and laser dissection techniques (78.03% vs. 66.52% and 71.06%; 78.35% vs. 75.32% and 75.67%; 52.06% vs. 41.62% and 45.06%; 36.52% vs. 25.77% and 30.49%), respectively. In summary, the CBA/ca strain, expanded blastocyst stage and manual cut for ICM isolation techniques showed the optimal outcomes obtained in production of mESC lines. A total of 156 and 13 caprine blastocysts were obtained from *in vitro*- and *in vivo*-derived blastocyst, respectively. The *in vivo*-derived blastocsyts gave significant difference in production gESC lines at P3 compared with *in vitro*-derived blastocysts (91.67% vs. 20.83%). The caprine ICM outgrowths for gESC production were then disaggregated by trypsin/EDTA (0.05%) and manual dissociation and cultured on new inactivated MEF feeder cell layer in CO_2 (5%) incubator at 37℃. Manual cut for ICM isolation technique consistently gave the highest successful rates of gESC in P1 and P3 compared with whole blastocyst culture and laser dissection technique (71.28% vs. 39.58% and 43.89%; 35.04% vs. 12.50% and 23.33%), respectively. The mESC and gESC were stained to evaluate the expression of alkaline phosphates (AP) and positive results confirming the pluripotency of mESC and gESC were obtained. The ICM outgrowths for mESC were also characterised for Oct 4 and SSEA 1, whereas ICM outgrowths for gESC were characterised using Oct 4 and SSEA 3 and positive results were detected. It is concluded that ICM cells could be isolated from *in vivo*- and *in vitro*-derived murine and caprine blastocysts using whole blastocysts culture, manual cut and laser dissection

techniques and subsequently cultured to produce mESC and gESC lines as confirmed by positive expression of AP, Oct 4, SSEA 1 and SSEA 3. It is hoped that the findings obtained from this research will provide the fundamental information for future studies regarding establishment of ESC and MEF cell lines that can be potentially applied to overcome issues in livestock production, wildlife conservation and human regenerative medicine.

ABSTRAK

Sel batang embrionik (ESC) mempunyai potensi yang tidak terbatas dalam bidang sains biologi dan perubatan regeneratif oleh kerana pluripotensi dan keupayaannya untuk memperbaharui selnya sendiri tanpa batasan. Dengan sasaran untuk membangun, mengasing dan mengkultur sel batang embrionik mencit (mESC) dan kambing (gESC), blastosis yang diperolehi secara *in vivo* dan *in vitro* digunakan sebagai suatu sumber dalam menghasilkan titisan-titisan mESC dan gESC. Kedua-dua mESC dan gESC dikultur *in vitro* dengan menggunakan fibroblas embrionik mencit (MEF) sebagai lapisan sel pembantu dalam kajian ini. Tujuan kajian ini adalah: a) untuk membanding kesan strain mencit, peringkat blastosis dan teknik mengasingkan jisim sel dalaman (ICM) terhadap kecekapan dalam memperolehi titisan sel batang embrionik mencit (mESC) dalam spesies mencit dan b) untuk membanding kesan sumber blastosis diperolehi secara *in vivo* dan *in vitro* serta membangunkan teknologi yang berkesan untuk mengasing dan mengkultur ESC dalam spesis kambing. Fibroblast embronik mencit (MEF) telah diperolehi daripada janin mencit (13.5 hingga 14.0 d.p.c.), dikultur sehingga Pasaj 2 (P2), dikrioawet dan dinyahsejukbeku pada setiap pasaj untuk digunakan sebagai lapisan sel pembantu bagi pengkulturan mESC dan gESC. Untuk mendapatkan sumber blastosis bagi menghasilkan titisan-titisan mESC dan gESC, pemindahan nuklear sel somatik (*in vitro*) dan pengepaman keluar *in vivo* telah dijalankan dalam kajian ini. Untuk mengasingkan jisim sel dalaman (ICM) daripada blastosis, kultur seluruh blastosis, pemotongan secara manual dan pembedahan laser telah dibandingkan antara kumpulan perlakuan masing-masing untuk memperolehi titisan-titisan mESC dan gESC. Dalam mencit, sejumlah 71 (ICR), 38 (CBA/ca), 22 (C57BL/6J) titisan-titisan mESC telah dihasilkan daripada 971, 758 dan 709 blastosis mencit, masing-masing. Lima peringkat blastosis telah dikultur atas MEF dengan 3

teknik pengasingan ICM. Pertumbuhan ICM telah dipisahkan dengan cara trypsin/EDTA (0.05%) dan penceraian secara manual, dikultur di atas MEF baru yang telah teraktif di dalam inkubator CO_2 (5%) pada 37℃. Pelekapan, pertumbuhan ICM primer dan kadar pasaj turutan yang berjaya sehingga ke P3 telah dibanding antara strain mencit, peringkat blastosis dan teknik pengasingan ICM. Terdapat perbezaan signifikan ($P<0.05$) dalam kadar pasaj yang berjaya pada P3 antara CBA/ca dengan ICR dan C57BL/6J (19.81% vs. 9.00% dan 8.50%), masing-masing juga mESC pada P1 bagi peringkat blastosis pertengahan-, pengembangan dan penetasan berlawan dengan peringkat blastosis awal- dan menetas (45.35%, 52.79% dan 43.01% vs. 27.88% dan 24.53%), masing-masing. Teknik penceraian ICM secara pemotongan manual dengan konsisten memberi kadar paling tinggi pelekapan, pertumbuhan ICM primer serta P2 dan P3 bagi mESC yang berjaya berbanding dengan kultur seluruh blastosis dan teknik pembedahan laser (78.03% vs. 66.52% dan 71.06%; 78.35% vs. 75.32% dan 75.67%; 52.06% vs. 41.62% dan 45.06%; 36.52% vs. 25.77% dan 30.49%), masing-masing. Secara ringkasnya, strain CBA/ca, peringkat pengembagan blastosis dan pemotongan secara manual bagi teknik pengasingan ICM menunjukkan hasilan optimal diperolehi dalam menghasilkan titisan-titisan mESC. Sejumlah 156 dan 13 blastosis kambing telah diperolehi daripada blastosis berasal *in vitro* dan *in vivo*, masing-masing. Blastosis berasal *in vivo* telah memberi perbezaan yang signifikan dalam menghasilkan titisan gESC pada P3 berbanding dengan blastosis berasal *in vitro* (91.67% vs. 20.83%). Pertumbuhan ICM kambing untuk penghasilan gESC kemudian dipisahkan dengan trypsin/EDTA (0.05%) dan penceraian secara manual serta dikultur di atas lapisan sel pembantu MEF yang telah teraktif baru dalam inkubator CO_2 (5%) pada 37℃. Pemotongan secara manual bagi teknik pengasingan ICM secara konsisten memberi kadar kejayaan yang tertinggi bagi gESC pada P1 dan P3 berbanding dengan kultur seluruh blastosis dan teknik pembedahan laser (71.28% vs. 39.58% dan 43.89%;

35.04% vs. 12.50% dan 23.33%), masing-masing. mESC dan gESC telah diwarnakan untuk menilai ekspresi fosfat alkali (AP) dan keputusan positif diperolehi yang mengesahkan pluripotensi mESC dan ESC. Pertumbuhan ICM bagi mESC juga telah dicirikan dengan menguna Oct 4 dan SSEA 1, manakala pertumbuhan ICM bagi gESC telah dicirikan denagan mengguna Oct 4 dan SSEA 3 dan keputusan positif telah dikesan. Dirumuskan bahawa sel ICM boleh diasingkan daripada blastosis mencit dan kambing berasal dari *in vivo* dan *in vitro* dengan menggunakan kultur seluruh blastosis, teknik-teknik pemotongan secara manual dan pembedahan laser dan seterusnya dikulturkan bagi menghasilkan titisan-titisan mESC dan gESC sebagaimana disahkan oleh ekspresi positif AP, Oct 4, SSEA 1 dan SSEA 3. Adalah diharapkan semoga penemuan yang diperolehi daripada kajian ini akan menyediakan maklumat asas bagi kajian-kajian masa hadapan yang berhubung kait dengan pembangunan titisan-titsan sel ESC dan MEF yang mempunyai potensi untuk diaplikasi bagi menyelesaikan isu-isu dalam produksi haiwan ternakan, konservasi hidupan liar dan perubatan regeneratif manusia.

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Sincerely,

Goh Siew Ying

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
℃	degree Celcius
µl	microlitre
µm	micrometer
µs	microsecond
cm	centimeter
CO_2	carbon dioxide
Hg	hydrargyros
Kg	kilogram
L	litre
M	molar
MΩ	milliohm
mg	milligram
min	minute
ml	millilitre
mm	millimeter
mM	millimole
mOsm	milliOsmole
N_2	nitrogen
O_2	oxygen
pH	hydrogen potential
rpm	revolutions per minute
β	beta
s	second
ABEL	Animal Biotechnology-Embryo Laboratory

AC	adenylyl cyclase
ANOVA	analysis of variance
AP	alkaline phosphatase
ART	assisted reproduction technology
bFGF	basic fibroblast growth factors
bHLH	basic helix-loop-helix
BIO	6-bromoindirubin-3’-oxime
BME	basal medium Eagle
BMP	bone morphogenetic protein
BMPR	bone morphogenetic protein receptor
BSA	bovine serum albumin
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CB	cytochalasin B
CF	cystic fibrosis
CHX	cycloheximide
CIDR	Controlled Intravaginal Drug Release device
CNS	central nervous system
COC	cumulus oocyte complex
CR1	Charles Rosenkrans medium
DIA	differentiation inhibitory factor
DMAP	dimethylaminopurine
DMEM	Dulbecco’s modified Eagle’s medium
αMEM	alpha minimum essential medium Eagle
DMRT	Duncan’s Multiple Range Test
DMSO	dimethyl sulfoxide

d.p.c.	days post-coitus
Dsh	dishevelled
e.g.	for example
EC	embryonal carcinoma
eCG	equine chorionic gonadotrophin
EDI	electrodeionisation
EDTA	ethylenediaminetetraacetic acid
EGC	embryonic germ cells
EGF	epidermal growth factor
EMiL	Embryo Micromanipulation Laboratory
ERK	extracellular regulated kinase
ESC	embryonic stem cell
et al.	et alii (and others)
FBS	foetal bovine serum
FSH	follicle stimulating hormone
G	gauge
gESC	caprine embryonic stem cell
gp130	glycoprotein 130
hCG	human chorionic gonadotrophin
HCL	hydrogen chloride
HFL	hydrogen fluoride
HMG	high mobility group
HWM	Hepes Whitten's medium
ICI	intracytoplasmic injection
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection

Id	inhibitor of differentiation
ID	inner diameter
Igf2	insulin-like growth factor 2
Igf2r	insulin-like growth factor 2 receptor
i.m.	intramascular
i.p.	intraperitoneal
IPS	Institute of Postgraduate Studies
ISB	Institute of Biological Sciences
IU	international unit
IVC	*in vitro* culture
IVF	*in vitro* fertilisation
IVM	*in vitro* maturation
IVMFC	*in vitro* maturation, fertilisation and culture
IVP	*in vitro* production
KCL	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
Klf4	kruppel-lile factor 4
KSOM	k simplex optimisation medium
LED	lymphoid enhancer factor
LH	luteinising hormone
LIF	leukaemia inhibitory factor
LIFRh	leukaemia inhibitory factor-specific receptor subunit
LN_2	liquid nitrogen
LOPU	laparoscopic ovum pick-up
MAPC	multipotent adult progenitor cell
MAPK	mitogen-activated protein kinases
mDPBS	modified Dulbecco’s phosphate buffered saline

MEF	mouse embryonic fibroblast
MEK	methyl ethyl ketone
mESC	murine embryonic stem cell
Mg^{2+}	magnesium
MII	metaphase II
MPF	maturation promoting factor
mRNA	messenger ribonucleic acid
MTC	mitomycin C
MZT	maternal zygotic transcription
NaCL	sodium chloride
$NaHCO_3$	sodium bicarbonate
Na_2HPO_4	sodium pyrophosphate
NaTuRe	Nuclear Transfer and Reprogramming Laboratory
NEBD	nuclear envelope break down
ntES	nuclear transfer of embryonic stem cells
OCS	oestrus cow serum
Oct 3	Octamer 3
Oct 4	Octamer 4
OD	outer diameter
P0, P1, P2, P3	Passage 0, Passage 1, Passage 2, Passage 3
PA	parthenogenetic activation
PBS	phosphate buffered saline
PGC	primary germ cells
PMSG	pregnant mare’s serum gonadotrophin
POMC	proopiomelanocortin
PS	penicillin-streptomycin
PVA	polyvinyl alcohol
PVP	polyvinylpyrrolidone

RO	reverse osmosis
SCF	stem cell factor
SCID	severe compromised immune deficient
SCNT	somatic cell nuclear transfer
SEM	standard error of means
siRNA	small inhibitory ribonucleic acid
SOF	synthetic oviductal fluid
SSEA	stage specific embryonic antigen
SR	serum replacement
Sr^{2+}	strontium
TE	trophectoderm
UK	United Kingdom
USA	United States of America
UV	ultra-violet
vs.	versus
WCICI	whole cell intracytoplasmic injection
WM	Whitten’s medium

Chapter 1

1.0 INTRODUCTION

Chapter 1

1.0 INTRODUCTION

At the turn of the twentieth century, development of stem cells from a variety of sources has led to the rapid worldwide progression in stem cell research for basic biological information and therapeutic applications. Stem cells are cells that consist of prolonged or unlimited capacity for self-renewal in an undifferentiated state, which have the capacity to develop into at least one type of highly differentiated descendent. They are pluripotent and capable to differentiate to specialised cell types when induced by appropriate stimulants under suitable conditions (Bongso and Lee, 2005). *In vitro* studies have repeatedly shown that cells grown under the control of specific growth factors can differentiate into cardiomyocytes, epithelial cells, and neurons (Shamblott *et al*., 2001). According to Thomson *et al*. (1998), stem cells can be classified into 2 groups, namely adult stem cells and embryonic stem cells (ESC). Conventionally, adult stem cells are isolated from various tissues and organs, such as the blood (heamatopoietic stem cell), cord blood (cord blood stem cell), bone marrow (mesenchymal stem cell), skin and hair (epidermal stem cell) and amniotic stem cell (Coppi *et al*., 2007). However, adult stem cells (multipotent) have limited differentiation capability compared to embryonic stem cells (pluripotent) where the deriving cell types of its own origins (Kondo and Raft, 2000). The former may develop into many different cells, but not all cell types. It has become increasingly clear that embryonic stem cells have distinct advantages over adult-derived stem cells. Embryonic stem cells are more potent than adult stem cell because it typically derived from the inner cell mass (ICM) of blastocyst stage embryos (Thomson *et al*., 1998). Similar to cells of the inner cell mass, embryonic stem cells are pluripotent diploid cells and capable to differentiate into all cell types of the 3 embryonic germ layers, namely

ectoderm, mesoderm and endoderm, including germ cells both *in vivo* and *in vitro* (Smith, 2001; Hoffman and Carpenter, 2005; Soto-Gutierrez *et al*., 2006). Embryonic stem cells are remarkable because they can be cultured and manipulated relatively easily *in vitro* without losing their developmental potential and behave like normal embryonic cells when they are injected into host blastocysts (Robertson, 1987) or 8-cell stage embryos (Tokunaga and Tsunoda, 1992).

Sources of blastocyst are an important factor to obtain inner cell mass in producing the embryonic stem cells. There are two ways to obtain inner cell mass, namely *in vivo* flushing and somatic cell nuclear transfer (SCNT). Blastocysts also can be obtained by other methods such as *in vitro* fertilisation (IVF), intracytoplasmic sperm injection (ICSI) and parthenogenesis (pathenogenetic activation; PA). However, the focus of this project was *in vivo* flushing (*in vivo* fertilisation), cloning (SCNT) and parthenogenetic activation because it is routine in our laboratory to produce blastocyst through *in vivo* flushing, cloning (SCNT) and pathenogenetic activation (PA), although other researchers obtain blastocyst sources from *in vitro* fertilisation and intracytoplasmic sperm injection procedures.

Primary mouse embryonic fibroblast (MEF) is used as feeder cell layer to prevent embryonic stem cell differentiation. Mouse embryonic fibroblast continues to be the most commonly used feeder cell type for the culture and maintenance of murine and human derived embryonic stem cell lines. Mouse embryonic fibroblast provides a complex, but unknown mixture of nutrients and substrates for the long term growth and proliferation of undifferentiated pluripotent embryonic stem cells. Mouse embryonic fibroblast has been used as feeder cell layer for the culture of embryonic stem cells since the first murine embryonic stem cells were derived in 1981 (Evan and Kaufman, 1981). Mouse embryonic fibroblast is currently giving encouraging results as feeder cell layer for culturing embryonic stem cells. The advantages of mouse embryonic fibroblast

are easier to prepare, cheaper comparing to STO cells, and it is available in our laboratory. However, the researchers in ABEL laboratory mostly used murine species to produce murine embryonic stem cells (mESC). Some results with other species such as ovine (Meinecke-Tillmann *et al*., 2011) and bovine (Roach *et al*., 2006) are just encouraging as well. In this study, the mouse embryonic fibroblast as feeder cell layer for caprine species to obtain caprine embryonic stem cell (gESC) line was tested. In relation to this, the effect of culture medium and protein source on the formation of pluripotent primary outgrowths from *in vitro* (SCNT) produced and *in vivo* (flushing) derived murine and caprine embryos as the first step towards the isolation of embryonic stem cells was examined. Dulbecco's Modified Eagle Medium (DMEM), which has been widely used for embryonic stem cells isolation was also compared with Alpha Modified Eagle Medium (αMEM), both supplemented with foetal bovine serum (FBS), which has shown promising results in previous studies by Ivan *et al*. (1995).

1.1 BACKGROUND

Since the past 2 decades, the concept of using embryonic stem cells as a source of multiple cell types for use in tissue repair has existed. Keller and Snodgrass (1999) reported that because many degenerative and chronic disease states involve the dysfunction or destruction of a single tissue, the derivation of embryonic stem cells, whose differentiation *in vitro* might be guided towards the affected cell types, raises the attractive prospect of cell replacement therapy. Although the concept of exploiting the pluripotency of embryonic stem cells to aid tissue repair was first conceived over 20 years ago, it is only recently that various technological advances have begun to realise this vision, heralding the emerging field of regenerative medicine. Approximately 3000 people estimated die every day in the United States from diseases that could have been treated with stem cell–derived tissues (Lanza *et al.*, 2001). It is not surprising that

embryonic stem cell research has been rapidly expanding where it provide the therapeutic potential and growing public awareness of stem cells to treat degenerative diseases since mouse embryonic stem cells were first isolated in 1981 (Evans and Kaufman, 1981; Martin, 1981) followed by the isolation of human embryonic stem cells (hESC) in 1998 (Shamblott *et al.*, 1998; Thomson *et al.*, 1998) from the inner cell mass of human blastocysts. Although adult stem cells such as bone marrow transplantation since the 1960s have been used clinically and hold great therapeutic promise, embryonic stem cells represent an alternative source of cells, with benefits and advantages including ease of isolation, ability to propagate rapidly without differentiation, and potential to form all cell types in the body. Besides murine embryonic stem cells , embryonic stem cells had also been derived in different animal species such as rabbit (Graves and Moreadith, 1993), mink (Polejaeva *et al*., 1997), equine (Saito *et al*., 1992), porcine (Chen *et al*., 1999; Li *et al*., 2003a, 2004), bovine (Wang *et al*., 2005), primate (Thomson *et al*., 1995; Suemori *et al*., 2001; Vrana *et al*., 2003; Shoukhart, 2006), and human (Thomson *et al*., 1998; Cowan *et al*., 2004; Heins *et al*., 2004; Lee *et al*., 2005; Ludwig *et al*., 2006; Baharvand *et al*., 2006).

Establishment of embryonic stem cells from domestic animals would be one of the most important milestones in the history of farm animal breeding. Recent breakthroughs in somatic nuclear transfer and human embryonic stem cells derivations have produced new and exciting avenues of research basic scientific information and applications in this area. For example, establishment of embryonic stem cell lines that are customised or tailored and genetically identical as well as immunologically compatible to the individual patients would be new innovative procedures to treat degenerative diseases that could not be treated before. Embryonic stem cells are promising sources of cells for regenerative therapy, hence an enhanced understanding of the molecular mechanisms that regulate their propagation and pluripotency will allow us to better utilise them for clinical

treatments. Preceding to this technology, the advent of somatic stem cell nuclear transfer has allowed us and others to produce cloned blastocsyt as a source for production of embryonic stem cells (Wilmut *et al*., 1997). However, somatic cell nuclear transfer suffers from the limitation that somatic cells have a limited lifespan in culture which makes it impossible to perform multiple rounds of gene targeting, which is required for producing expressing multiple transgenes for xenotransplantation research (Nottle *et al*., 2001), highlighting the need for embryonic stem cells for this as well as other applications. Hundreds of cloned animals exist today, but the number of different species is limited. Somatic cell nuclear transfer technique of Dolly the sheep in 1997 can be used to produce an embryo from which cells called embryonic stem cell could be extracted to use in research into potential therapies for a wide variety of diseases such as Alzhemier, Leukaemia, Parkinson, Stroke and Huntington diseases. Some researchers compared the efficiency with which primary cell lines could be established from *in vitro* versus *in vivo* derived whole blastocyst cultured. The present study aimed to develop conditions for the isolation of homogenous pluripotent outgrowths from whole embryos, as the majority of methods that have been used so far result in heterogenous outgrowths which may inhibit isolation (Vackova *et al*., 2007). Separation of blastomeres into the trophoblast and the inner cell mass is the first visible stage of embryo differentiation. To prevent further differentiation, the cells must be disaggregated and seeded onto a feeder cell layer. The cells containing the smallest amount of cytoplasm and highest rate of proliferation should be the embryonic stem cells. This technique was introduced by Evans and Kaufman (1981) and Martin (Matsui *et al*., 1992) in order to isolate murine embryonic stem cells. Later, this method was used to isolate embryonic stem cells from other species: Syrian golden hamster (Doetchman *et al*., 1988), porcine (Notarianni *et al*., 1983) rabbit (Graves *et al*., 1993), bovine (Strelchenko *et al*,. 1991), mink (Sukoyan *et al*,. 1993), rodent (Iannaccone *et al*., 1994) and primate (Thomson, *et al*., 1995). However, there is no

report on caprine embryonic stem cells was found in the literature. The goal of this project was an attempt to establish caprine embryonic stem cell lines through *in vitro* propagation and expansion of inner cell mass obtained from blastocysts using mouse embryonic fibroblast as feeder cell layer.

1.2 STATEMENT OF PROBLEMS

Below are some of the pertinent issues regarding the performance of production of embryonic stem cells culture in caprine and murine species:

a) How can caprine and murine embryonic stem cells be maintained through many passages in a truly stable state?
b) Which passages of caprine and murine embryonic stem cells will obtain pure embryonic stem cells line?
c) Can inner cell mass produce the embryonic stem cell lines in caprine species?
d) Is there any difference between *in vivo* or *in vitro* caprine and murine produced embryos for blastocyst production as a source to produce optimum quality of embryonic stem cell lines?
e) Which inner cell mass isolation techniques are more efficient in producing caprine and murine embryonic stem cell lines?
f) Is it possible to produce totipotent tissues or organs from single embryonic stem cell through nuclear transfer of embryonic stem cells (ntES)?
g) Which strains of murine would be suitable to produce mouse embryonic fibroblast as a feeder cell layer for caprine and murine embryonic stem cells culture?
h) Which stage(s) of blastocyst would give optimum embryonic stem cell lines production?

i) Why is it difficult to derive and maintain embryonic stem cells from being differentiated?

j) How many inner cell mass cells are needed to produce embryonic stem cell lines in caprine and murine species?

k) Which developmental stages of embryos are suitable to do chimaera in caprine and murine species?

l) Can fresh cumulus cell produce cloned caprine and murine embryos for blastocyst production through whole cell intracytoplasmic injection (WCICI) technique?

m) Can caprine ear fibroblast cells produced cloned caprine embryos for blastocyst production through WCICI technique?

n) How many passages of caprine ear fibroblast cells to stabilise cell lines for caprine cloning?

o) Is there any difference in caprine breed to obtain suitable embryonic stem cell lines?

p) How could the culture conditions be improved for sustaining embryonic stem cells development?

q) How the embryonic stem cell lines could be expressed to their optimal pluripotency traits?

r) How the embryonic stem cell lines can be applied in human degenerative diseases?

s) Are embryonic stem cells true pluripotent stem cells?

t) What are the similarities and differences between murine and caprine embryonic stem cells culture?

u) How can embryonic stem cells be directed to differentiate reproducibly into given cell types?

v) How can we ensure that embryonic stem cells will not be tumorigenic *in vivo*?

1.3 JUSTIFICATION

The main purpose of this study was to produce caprine embryonic stem cells after culturing inner cell mass using mouse embryonic fibroblast as feeder cell. The inner cell mass cells were obtained from blastocysts using cloning (SCNT) and *in vivo* fertilised techniques. Currently, Malaysian government gives a high priority to agriculture including caprine commercialisation as third engine of economic growth. Caprine species has the comparative advantages to other livestock animals due to less fat content in the carcass, easy to manage, more economical in production and could be consumed by all the ethnic groups of Malaysia. In addition, caprine is a excellent animal model for the study and applications of embryos cloning and stem cell research in human therapeutic purposes. However, embryonic stem cells and embryo culture in caprine as any other mammalian species is dependent on breed, age and reproductive status of the animals as well as culture requirements and conditions. Several embryo biotechnology techniques already developed in caprine and other species could be utilised and complementary to caprine cloning and stem cells research. By producing the large number of embryonic stem cells that lead to cloning of embryonic stem cells (ntES), it will accelerate the rate of propagation of commercially desirable traits, overcome infertility problems and upgrading the quality of economically valuable caprine breed. The embryonic stem cells in caprine that are used in cloning could be applied not only to reproductive cloning but also to therapeutic cloning to produce pharmaceutical drugs, tissues for repair and organ for xenotransplant. However, we are constantly lacking of caprine samples in carrying out the experiments. We were using murine and bovine model as learning curve as well as subsequently applied in caprine species since they are more easily available in our laboratory. Murine was chosen as our animal model due

to easier to manage, handle and prolificacy. Also, a short gestation length, short oestrous cycle (5 days) and large litters make murine an ideal candidate. Also, murine can be backcrossed to achieve animal sizes that are more easily managed in animal care facilities and maintained under laboratory condition. Murine are the species of choice because of the wealth of biological information known about the murine genome, and a multiple homologous recombination strategy for disrupting genes has already been demonstrated in murine. Murine is inexpensive and non-seasonal breeders which can be carried out the experiment at anytime. Murine embryonic stem cells have been routinely used and preferred by many researchers to produce cloned murine including nuclear transfer of embryonic stem cells (ntES) technique. However, Stice *et al*. (1996) and Cibelli *et al*. (1998) have extensive experience in bovine and porcine embryonic stem-like cells in both gene modification and cloning, and after extensive nuclear transfer studies, they were never able to produce a cloned offspring derived from their embryonic stem-like cell lines (Stice *et al*., 1996). In the present study, caprine species was chosen as a model species in an attempt to produce embryonic stem cell lines towards applications in human therapeutic cloning. Related reproductive techniques in the caprine have already developed and therefore, they provide convenient and complementary way of obtaining a large number of oocytes and embryos for the stem cells research in caprine species.

1.4 APPLICATIONS

Embryonic stem cells provide with great characteristics potential for use in cell-based drug discovery and regenerative medicine (Fan *et al*., 2010). It is a need for derivation new embryonic stem cell lines to meet emerging requirements for their use in cell replacement therapies, disease modeling and basic research (Fan *et al*., 2010). Embryonic stem cells are isolated more easily in single cell type populations, multiply

more readily in the laboratory, and are more proficient in producing much specialised cell types including pancreatic islet and neurons cells. The current and potential applications of embryonic stem cells for use in biomedical and biotechnological applications are advancing rapidly. Yong and Yuqiang (1998) reported that the application of nuclear transfer technology using blastomeres of early caprine embryos. Six generations of cloned caprine embryos were produced by using serial nuclear transfer. With the embryonic stem cells in caprine, it increase production of caprine, improve the quality of caprine for commercialisation at the very rapid rate as well as its human applications to help cure diabetes, to cure burn patients, may provide a cure for Parkinson's disease, help many other diseases, help cloned organs using therapeutic stem cells and create organs for xenotransplantation in human. There were more than 62,000 people in United States of America are on waiting lists to receive donated organs, to mass-produce pharmaceuticals where a gene can be inserted into an animal to make that animal produce a particular protein in its milk, for example, insulin, to save endangered species, to cloned pets or animals, to repair damage tissues and to do organ for xenotransplant. Parkinson's patients were treated by surgical implantation of foetal cells into their brain even though not completely effective, perhaps owing to lack of sufficient numbers of dopamine secreting cells, similar experiments using appropriately differentiated stem cells should overcome those obstacles. More complex experiments have already been successfully conducted in rodent models of Parkinson's. Similar approaches could be developed to replace the dead or dysfunctional cells in cortical and hippocampal brain regions that are affected in patients with Alzheimer's. Transplants of haematopoietic stem cells (the cells which eventually produce blood) following treatments for cancer, for example, have been done for years now in human beings. Further, somewhat cruder experiments (for example, the transplantation of foetal tissue into the brains of Parkinson's patients) indicate that the expectation that stem cell

therapies could provide robust treatments for many human diseases is a reasonable one. The interplay between developmental biologists and stem cell biologists will be key to a fundamental understanding of stem cell development and its translation into therapeutic outcomes. Pluripotent stem cells, instructed to differentiate into a particular pancreatic cell called a beta cell (β-cell), could overcome the shortage of therapeutically effective material to transplant. Pluripotent stem cells could be used to create an unlimited supply of cells, tissues, or organs that could be used to restore function without the requirement for toxic immunosuppression and without regard to tissue matching compatibility. In gene therapy, genetic material that provides a missing or necessary protein, or causes a clinically-relevant biochemical process, is introduced into an organ for a therapeutic effect. For gene-based therapies (specifically, those using DNA sequences), it is critical that the desired gene be introduced into organ stem cells in order to achieve long-term expression and therapeutic effect. Although techniques for delivering the therapeutic DNA have been greatly improved since the first gene therapy protocol almost 10 years ago, there are as yet no *bona fide* successes. The science of stem cells dates to the mid-1960s, and many papers have been published on the isolation and laboratory manipulation of stem cells from animal models. While these models are imperfect, they are accepted in the scientific community as good initial predictors of what occurs in human beings. Despite these and other advances in the prevention and treatment of human diseases, devastating illnesses such as heart disease, diabetes, cancer, and diseases of the nervous system such as Alzheimer's disease present continuing challenges to the health and well-being of people everywhere. All this research on embryonic stem cells, both murine and human as well as caprine, will provide new insights into embryonic development and new clues as to how to isolate and characterise new stem cells from different embryonic or adult tissues. Much work remains to be done, to gain more insight into the basic mechanisms regulating

proliferation, differentiation and reprogramming of embryonic and somatic stem cells. We are looking forward to future developments in this field.

1.5 OBJECTIVES

a) To develop a protocol for production of murine embryonic stem cell (mESC) lines in murine.
b) An attempt to develop a protocol for production of caprine embryonic stem cell (gESC) lines in caprine.
c) To produce cleaved murine embryos through *in vivo* fertilisation and somatic cell nuclear transfer (SCNT) for blastocyst formation to be used in embryonic stem cell lines development.
d) To produce cleaved bovine embryos through somatic cell nuclear transfer (SCNT) as a model to develop somatic cell nuclear transfer (SCNT) protocol in caprine.
e) To produce caprine cleaved embryos through somatic cell nuclear transfer (SCNT), *in vivo* fertilisation and parthenogenesis as a source of blastocyst for embryonic stem cell lines production.

Chapter 2

2.0 REVIEW OF LITERATURE

Chapter 2

2.0 REVIEW OF LITERATURE

2.1 STEM CELL BACKGROUND

Several types of stem cell have been discovered from embryo, germ cells, foetus and adult. Each of them promised to revolutionise the future of regenerative medicine through the provision of cell replacement therapies to treat a variety of debilitating and degenerative diseases. Stem cell research is raises many ethical and religious debates and generates a great deal of public interest. A capability of embryonic stem cells (ESC) versus the adult stem cell (somatic stem cells) has ignited debates as to the choice of one cell type over another for future application. However, the biology of these mysterious cells have yet to be more understood and a lot more basic research is needed to carry out before applied new therapies using stem cell differentiated derivatives. We always read and listen to news reports everyday about how stem cells change our lives with remedy for every imaginable disease, for example rhetoric that stem cell therapy will some day delay the process of aging. We hope someday many degenerative human diseases will be treated with stem cell therapy examples of whole organ and limb regeneration in animals, as well as the historical success of bone marrow transplants (xenotransplantation), which have improved the lives of many patients suffering from leukaemia, immunological and other blood disorders. However, there will be quite long road towards the development of an effective cell based therapy for widespread use and involves overcoming many technical, ethical and safety issues. According to Alison *et al*. (2002), stem cell is a cell with the ability to divide indefinitely *in vitro* culture and with the potential to give rise to mature specialised cell types.

Stem cells can be isolated from many adult tissue types (Vogel, 2001), foetal and embryo sources. Usually, isolation of stem cell has been most successful in cells

from tissues that undergo rapid senescence (aging), cell turnover, and replacement such as germ cells, epithelia, and hematopoietic system as well as in some tissues that have a more limited cellular regeneration, for example, hepatocytes (Vogel, 2001). Adult stem cells are mostly considered as multipotent, meaning that they may develop into many different cell types, but not all cell types whereas embryonic stem cells are pluripotent where it capable to develop into all cell type, except extraembryonic. By working with animal models has shown that transplantation of pluripotent stem cells can be successfully treat a variety of disorders such as Duchenne muscular dystrophy, spinal cord injuries, and heart failure and are recently being applied to clinical models (Van and Weiss, 2000; Watt and Hogan, 2000; Odorico *et al*., 2001). Both embryonic and adult stem cells have enormous potential to further our understanding of basic developmental processes. It is very important to identify the nature of the pluripotent state in embryonic stem cells. How it is acquired, maintained and propagated and which genes confer pluripotency. By understanding the fundamental mechanisms, cell fate is determined during embryonic development will prove informative for the *in vitro* manipulation of stem cells.

An early stem cell research goal is to find out how to isolate and store stem cells from different types of tissues. Theoretically, stem cells could be collected, grown and stored to provide a plentiful supply of healthy replacement tissue for transplantation into anybody site using much less invasive surgery than conventional transplants. Scientists have already reported success with human brain tissue transplants to treat Parkinson's disease and with stem cell-derived murine heart-muscle cell grafts. Stem cell has symmetrical dividing property that could generate 2 daughter cells which absolutely identical to mother cell. Besides that, stem cell also has capacity to self-renewal (Figure 2.1).

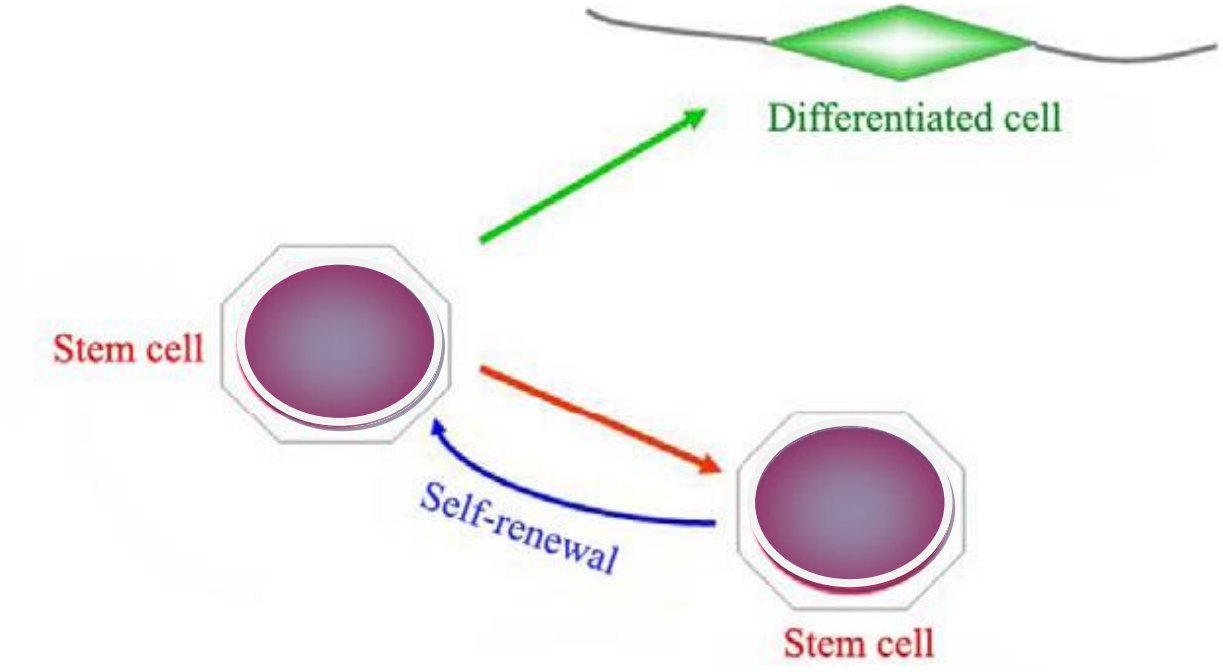

Figure 2.1: Stem cell has capacity to self-renew and asymmetric division. At each cell division, stem cells have to choose between self-renewal (stem cells) and differentiation (somatic or mature cells) (Cai *et al*., 1997; Lu *et al*., 2000).

2.1.1 Totipotency, Pluripotency and Multipotency

Stem cells can be classified as totipotent, pluripotent and multipotent. Totipotency stem cell have unlimited capability where they can basically form the whole organisms, including the entire foetus and placenta (for example, early mammalian preimplantation embryos are clusters of totipotent cells). Pluripotency has the ability to form several cell types of all 3 germ layers (ectoderm, mesoderm and endoderm) but not the whole organism. Theoretically, pluripotent stem cells have the ability to form all the 200 or so cell types in the body. There are divided into 4 classes of pluripotent stem cells, namely embryonic stem cells (ESC), embryonic germ cells (EGC), embryonic carcinoma cells (EC) and recently the discovery of a fourth class of pluripotent stem cell, the multipotent adult progenitor cell (MAPC) from bone marrow (Smith, 2001) (Figure 2.2). It is generally assumed that the potential fates for embryonic germ cells will be relatively limited compared to embryonic stem cells because embryonic germ cells are

much further along in the schema of embryonic development. Embryonal carcinoma cell lines are derived from tumours of germ cell origin and have long served as the human counterpart of murine embryonic carcinoma cells for studying human development and differentiation *in vitro* (Andrews, 2000). Not all embryonic carcinoma cell lines are pluripotent and some feeder-independent embryonic carcinoma lines have been reported to be nullipotent. Jiang *et al*. (2002) isolated murine multipotent adult progenitor cells from murine bone marrow and demonstrated that these cells express telomerase and that a single multipotent adult progenitor cell could be expanded clonally into a large number of daughter cells. Additionally, under appropriate conditions, multipotent adult progenitor cell differentiate into ectoderm, endoderm and mesoderm and are capable of generating chimaeric murine when injected into murine blastocysts.

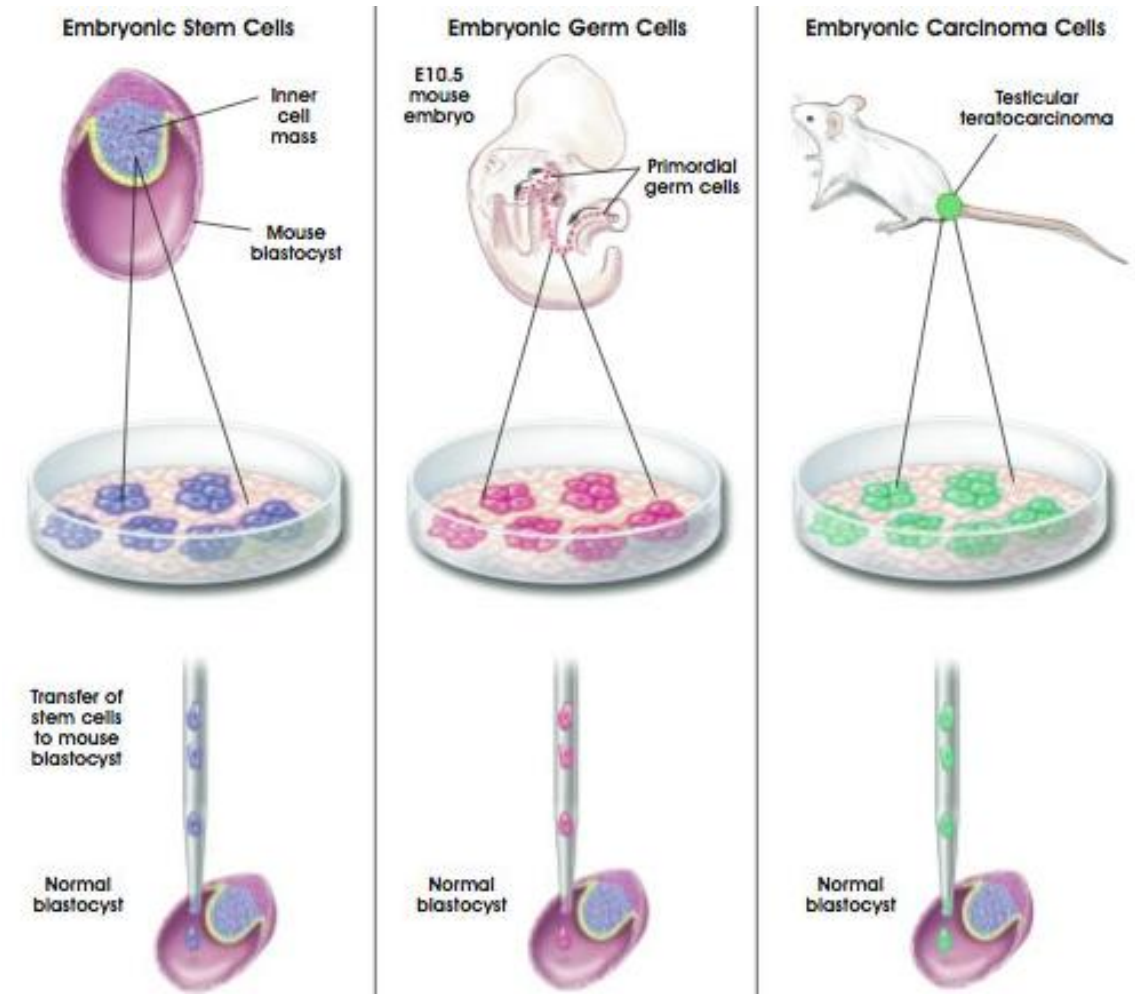

Figure 2.2: Origins of pluripotent stem cell (Brook and Garder, 1997).

Multipotency stem cell has the ability of giving rise to a limited range of cells and tissues appropriate to their location, for example, blood stem cells give rise to red blood cells, white blood cells and platelets, whereas skin stem cells give rise to the various types of skin cells. Some current reports suggested that adult stem cells, such as haemopoietic stem cells, neuronal stem cells and mesenchymal stem cells, could cross boundaries and differentiate into cells of a different tissue (Bjornson *et al*., 1999; Jackson *et al*., 1999; Clarke *et al*., 2000; Krause *et al*., 2001).

2. 2 BACKGROUND HISTORY OF CLONING

The idea of cloning came about in the early 1900s where a German scientist named Hans Spemann first thought of the cloning methods around 1938. Although nuclear transfer in cloning mammals began more than 20 years ago, the basic biology underlying this process still remains unclear. After the birth of Dolly the sheep (Wilmut *et al*., 1997), the first cloned mammal using somatic cell nucmear transfer, success in cloning using adult somatic cells has been reported in other mammalian species including murine (Wakayama and Yanagimachi 1999; Wakayama *et al*., 1998, 2000), bovine (Kato *et al*., 1998; Galli *et al*., 1999; Renard *et al.,* 1999; Wells *et al*., 1999; Zakhartchenko *et al*., 1999; Hill *et al*., 2000; Kubota, 2000), caprine (Baguisi *et al*., 1999), porcine (Polejaeva *et al*., 2000), and *Bos gaurus* (gaur) (Lanza *et al*., 2000). The efficiency of cloning has increased from around 1 out of 300 to 1 out of 10 to 30 reconstructed embryos transferred depending on the study (Kato *et al*., 1998; Wells *et al*., 1999; Kubota, 2000). Smith (1989) reported that a clone can be defined as a set of genetically identical animals and very useful in animal selection for the determination of the genetic component of zootechnical traits or in research to reduce the number of experimental animals (Lamberson, 1994). Cloning technology has made several steps forward in recent years, most notably in somatic cell nuclear transfer (SCNT) and in combining cloning with transgenic technologies. The advantages of somatic cell nuclear transfer are cloning valuable research animals may alleviate a limited availability of highly informative genotypes while to obtain optimum results by reducing the number of animals used. Cloning and transgenics are now available for producing phenotypes in large animals to study devastating disease. Today, cloning plays a relatively small role in research and commercial endeavors. Cloning technology is used to produce therapeutics in milk and for cell replacement or cell therapy. In these areas, along with xenotransplantation and animal agriculture transgenics require approval in the form of

clinical trials or safety studies. Obviously, cloned animals can replace more genetically dissimilar animals in studies when the trait being investigated is highly heritable.

The first attempt on cloning was by a scientist named Adolph Eduard D. Genzyme began a program to produce therapeutic proteins in the milk of transgenic goats in the early 1990s (Genzyme, 1990). They inserted a genetic construct into the nucleus of a one-cell embryo by using microinjection technique. Professor Zhang Yong from the Northwest University of Science and Technology for Agriculture and Forestry from China has successfully produced the first cloned caprine from ear fibroblast cells (Yong *et al*., 2002). Besides that, the first success in cloning Asian Yellow Goat by nuclear transfer had recently been achieved in east China's Shandong Province (Chen *et al*., 2007). Asian Yellow Goat (*Capra hircus*) also named Nanjiang yellow goat which is a new breed developed after 40 years by efforts from Chinese scientists. The improvement of the cloning technique may provide new approaches for conserving and saving endangered and valuable animal species. Yong *et al*. (1991) reported that successfu1 nuclear transfer has also been reported in caprine; a number of attempts has been made to try intergeneric (interspecies) nuclear transfer, for example, bovine embryonic nuclei into bison, caprine or hamster oocyte recipient cytoplasms, or caprine nuclei into ovine or bovine oocytes, but very limited development to the blastocyst stage was obtained (l to 2%) (Wolfe and Kraemer,1992). Cloning by nuclear transfer has great potential application in pharmaceutical protein production, xenotransplanation, and most excitingly, therapeutic cloning. In therapeutic cloning a patient's own skin cells can be used to generate cloned embryos from which embryonic stem cells are isolated. Through targeted differentiation, embryonic stem cells can be directed to develop into the desired tissues/organs for replacement. According to Wakayama *et al*. (1998), a piezo-electric nuclear transfer method has been used to generate cloned murine from cumulus cells and tail-tip cells of adult animals as well as

embryonic stem cells. Cloning live murine using Sertoli cells by the same piezo-electric injection method (Ogura *et al*., 2000a). Ogura *et al*. (2000b) reported that cell fusion or injections without a piezo-electric device were used successfully to produce live cloned murine. However the percentage of live offspring obtained in murine cloning is less than 3%. The success rate of cloning so low may be due to a few percent of any cell population are 'cloning competent' and technical problems. Many technical factors such as cell cycle, oocyte activation and chemical agents affect development of cloned embryos as well as reprogramming of donor nucleus and recipient cytoplast.

The cloned ovine (Dolly) in 1997 began a biological revolution which showed us the way to physiologically differentiate already committed somatic cells, opening the gate to a whole new world of possibilities in the study of basic biological mechanisms that touch the origins of life as its core (Cibelli *et al*., 1998). After Dolly, nuclear transfer enabled the cloning of bovine, caprine and porcine. The era of livestock cloning had arrived. The debate about therapeutic cloning has led to a consensus in which embryonic stem cells are adjudged to offer the best short term prospects for tissue repair, while adult stem cells seem destined to predominate in the longer term (Robert *et al*., 1998). There were early reports of nuclear transfer in the rabbit during the late 1970s where McGrath and Solter (1983) demonstrated that by using microsurgery, pronuclei could be exchanged between murine zygotes and development still be obtained. Subsequently, Willadsen (1986) produced live lambs after transferring nuclei from 8- to 16-cell ovine embryos into enucleated MII oocytes. Although a lot of attention has been paid on the possibility, cloning can be used as a great biochemical tool in providing invaluable advances in medicine and pharmacology. Cloning is in transition from a distinguished past to a new future. Robert *et al*. (1998) reported that if used in an imaginative manner, this move will ensure that nuclear transplantation

continues to serve as a beacon of scientific excellent and as an important contributor to human health.

2.3 TIMELINE OF MURINE, BOVINE AND CAPRINE SOMATIC CELL NUCLEAR TRANSFER (SCNT)

Table 2.1 shows the timeline of significant findings in somatic cell nuclear transfer (SCNT) in murine, bovine, gaur and caprine

Table 2.1: Timeline of significant findings in somatic cell nuclear transfer (SCNT) in murine, bovine, gaur and caprine

Year	Author	Species	Significant finding
1997	Wilmut *et al.*	Ovine	Successful somatic cell nuclear transfer (SCNT) responsible for cloning of the first mammalian species (Dolly).
1998	Cibelli *et al.*	Bovine	Live cloned bovine offspring produced through somatic cell nuclear transfer (SCNT).
1998	Kato *et al.*	Bovine	Produced 8 cloned calves (4 calves die after birth).
1998	Wakayama *et al.*	Murine	Produce cloned murine using cumulus cells as donor karyoplasts through whole cell intracytoplasmic injection (WCICI) technique.
1998	Zhang and Li	Caprine	Production of caprine by serially cloning embryos.
1999	Baguisi *et al.*	Caprine	Caprine offspring produce through somatic cell nuclear transfer (SCNT) by using cumulus cells.
2000	Lanza *et al.*	Gaur	Cloned gaur produce through somatic cell nuclear transfer (SCNT) cloning.

(Continued)

(Continued)

2000	Wang *et al.*	Caprine	Production of cloned caprine (*Gapra hircus*) from foetal fibroblast cell lines.
2001	Reggio *et al.*	Caprine	Production of transgenic caprine offspring from oocytes derived from follicle stimulating hormone stimulated and nonstimulated abattoir derived ovaries.
2001	Ono *et al.*	Murine	Cloned murine from foetal fibroblast cells arrested at metaphase by a serial nuclear transfer.
2002	Baldassare *et al.*	Caprine	Production of transgenic caprine using laparoscopic ovum pick-up and in embryo production technology.
2002	Hitoshi *et al.*	Bovine	86.00% of cleaved bovine embryos obtained by using cumulus cells as donor cell.
2002	Keefer *et al.*	Caprine	Production of cloned caprine after nuclear transfer using caprine granulose cells and foetus fibroblast cells.
2003	Gasparrini *et al.*	Murine	Cloned murine derived from embryonic stem cell karyoplasts and activated cytoplasts prepared by induced enucleation.
2007	Chen *et al.*	Caprine	First success in cloning Asian Yellow Goat by whole cell intracytoplasmic injection (WCICI) technique.
2009	Tao *et al.*	Caprine	Production of Boer goat (*Gapra hircus*) by nuclear transfer of cultured and cryopreerved ear fibroblast cells into slaughterhouse derived oocytes.
2011	Tang *et al.*	Caprine	Production of 5 cloned caprine using foetus fibroblast cell lines as donor cell through somatic cell nuclear transfer (SCNT) technique.

2.4 SOURCES OF OOCYTES

Success of any *in vitro* produced system including somatic cell nuclear transfer is mostly dependant on the good quality oocytes supply. Caprine oocytes can be collected from either both live and dead or killed does. For live does, oocyte recovery may be obtained via laparoscopic ovum pick-up (LOPU), ovariectomy and laparotomy techniques. On the other hand, oocytes also can derived from dead does from abattoir (slaughterhouse) or during post-mortem. Since collecting ovaries from dead does in largely depended on the availability and uncertain, access to abattoir derived ovaries is easier, cheap and provides large sample source of oocytes.

2.4.1 Recovery of Oocytes from Laparoscopic Ovum Pick-up (LOPU) Technique

Although abattoir ovary source is easier and cheaper compared to laparoscopic ovum pick-up technique, ovary source from abattoir is very limited in some countries such as Malaysia. Therefore, laparoscopic ovum pick-up technique could be an alternative method for caprine oocyte recovery to carry out somatic cell nuclear transfer in present study. Snyder and Dukelow (1974) first described the recovery of 6 oocytes from an ovine by follicular aspiration of 21 follicles under laparoscopic observation. However, the technique was not fully developed until recent years, in conjunction with the development of *in vitro* embryo production technologies (Baldassarre *et al*., 1994, 1996, 2001; Graff *et al*., 1995, 1999; Tervit 1995). Laparoscopic ovum pick-up is a convenient methodology by which oocytes can be recovered and used either for *in vitro* production of zygotes or as a source of cytoplasts in nuclear transfer procedures (Balsassare *et al*., 2002). The laparoscopic ovum pick-up is one of the best techniques for oocyte recovery from live does. The laparoscopic ovum pick-up is advantageous than standard laparotomy-based method which allows repetition of the laparoscopic procedure more frequently and more times during the reproductive life of a valuable

female (Baldassarre *et al*., 2007). The main advantages of the laparoscopic ovum pick-up methodology over the utilisation of *in vivo* produced zygotes or *in vivo* matured oocytes are improved control over the stage of maturation/development of zygotes and oocytes and the less invasive means of recovery, thereby allowing for repeating the laparoscopic ovum pick-up procedure to the donor animals several times. The laparoscopic ovum pick-up can be repeated several times without ovarian damage or decrease in the donors' fertility (McKelvey *et al*., 1986; Stangl *et al*., 1999; Alberio *et al*., 2002; Pierson *et al*., 2004). Additionally, compared with oocytes sourced from abattoir ovaries, laparoscopic ovum pick-up allows one to source the oocytes from live animals of known health status, which is very important for the production of therapeutic proteins for pharmaceutical applications. Laparoscopic ovum pick-up technique already allows field application in commercial programmes (Hasler *et al*., 1995). In addition, this method can be applied to prepubertal animals and thereby could contribute to a considerable shortening of the generation interval. Usually, oocytes used for cloning were obtained from the ovaries of slaughtered caprine so that genetic background of the oocytes was unknown. Such an approach would be unsuitable when oocytes with defined genomes or defined sources are required. A good method for obtaining oocytes from a defined source is to use the laparoscopic ovum pick-up technology which allows harvesting of oocytes from defined individual caprine (De Roover *et al*., 2008).

2.4.2 Oocyte Recovery from Abattoir

According to Rajikin (1995) and Amir (2007), abattoir source is easier and cheaper way of obtaining oocytes for various reproductive technologies. For example, in Malaysia, this source of oocyte is extremely limited due to less slaughtering activities as shortage of breeding stock, normally older or culled does are chosen. Generally, the quality of

the oocytes recovered from abattoir is quite low compared to laparoscopic ovum pick-up technique. Caprine oocytes source from slaughterhouse are obtained via slicing or follicle aspiration technique. Simple aspiration of follicular contents performed by using a syringe and a needle 18 to 22 G (Keskintepe and Brachett, 1996; Keskintepe *et al.*, 1998 Yadav *et al*., 1998). Ovaries have been subjected to dissection and isolation of individual follicles followed by rupture of the latter (Le Gal, 1996; Crozet *et al*., 2000) or slicing of the ovaries followed by simple rinsing of the slices (Pawshe *et al*., 1994b, 1996; Ongeri *et al*., 2001; Rho *et al*., 2001). Ovary slicing was a simpler and more efficient technique than follicle aspiration in that an average of 6 COC per ovary were obtained by slicing as opposed to between 1.5 to 2.0 oocytes per ovary by aspiration (Martino *et al*., 1994; Pawshe *et al*., 1994a).

2.5 FACTORS AFFECTING DEVELOPMENT OF NUCLEAR TRANSFER RECONSTRUCTED EMBRYOS

Since the birth of Dolly in 1997 (Wilmut *et al*., 1997), this success revealed the extraordinary capacity of the oocyte to erase the process of genome cell differentiation and to reprogramme the genetic information to produce a new individual (Campbell *et al*., 2002). Li *et al*. (2004) reported that this process is attainable by nuclear transfer, which consists of the replacement of the maternal genetic material of the oocyte by the genetic information of donor cells after nuclear transfer. Unfortunately, although cloning is possible now, nuclear transfer is a very complex, poorly understood, time consuming and inefficient process. In fact, the efficiency of nuclear transfer has been estimated to be between 1 and 2% of all oocytes used (Polejaeva *et al*., 2000). Several reasons for this low efficiency can be attributed to the source and quality of oocytes, the preparation of the recipient cytoplasm, the donor cell type, the synchronisation of the cell cycle of both, recipient cytoplasm and donor cells, the failure to reprogramme the

transplanted nucleus and finally the failure of artificial activation methods. Embryo manipulations such as nuclear transfer or unusual embryo culture environments may also result in inappropriate epigenetic modification of imprinted genes during early embryogenesis.

2.5.1 *In Vitro* Maturation (IVM) of Caprine Oocytes

In vitro maturation of oocyte is a reproductive technology which enables the production of mature oocytes by culturing them in standard cell culture conditions until they reach metaphase II stage (Gilchrist and Thompson, 2007). Before oocyte able to mature *in vitro*, it must be visualised as being normal. Usually, normal oocyte should have cumulus cells surrounding the zona pellucida, absence of cracked zona pellucida and vesicles in the ooplasm. The more the cumulus cell layers presence is consider better. Rajikin *et al*. (1994) described good quality of caprine oocyte will appear golden, golden yellow or brownish in colour and have granulated appearance in the ooplasm. Size of an oocyte is also very important for the attainment of maturation. There are 86% of caprine oocytes from follicles 2 to 6 mm in diameter progressed to metaphase II, whereas only 24% of oocytes from follicles 1 to 1.8 mm reached metaphase II stage (Smedt *et al*., 1992). Maturation of oocytes was assessed by cumulus expansion and the extrusion of the first polar body. Cumulus cells were removed through use of finely drawn glass pipettes with 0.2% hyaluronidase (Sigma). Only oocytes that had extruded the first polar body with a complete perivitelline space separating the zona pellucida and cell membrane and with evenly granulated cytoplasm were used for enucleation experiments (Bavister *et al*., 1989).

2.5.2 Oocyte Enucleation

Quality of the recipient oocyte is important factor in successful nuclear transfer. All critical maternal factors (such as proteins and messenger RNA required for synthesis of new proteins) required for early embryo development are stored and synthetised in the oocyte cytoplasm during oocyte maturation. Therefore, it would be logical to assume that the amount of these factors in cytoplasm would also play a role in successful cloning. One of the major steps involved in the nuclear transfer procedure is the removal of important cytoplasmic components (genetic material) from the recipient oocyte (enucleation), which may reduce cytoplast viability. Usually, one third or more of the ooplasm is removed that would result in a corresponding decrease in the total cell number of cloned blastocysts. This procedure and technique requires time and limits the number of oocytes available for cloning. Therefore, the oocyte enucleation procedure is crucially important to cloning efficiency by eliminating any genetic contribution of the recipient cytoplasm, and for excluding the possibility of parthenogenesis (Dominko *et al*., 2000). According to Wilmut *et al*. (1997), mammalian oocyte cytoplasts are prepared by physically removing nuclear chromatin by micromanipulation techniques in preparation to receive the donor genome. Enucleated oocytes arrested at MII are subsequently ‘‘reconstructed’’ by the combination of the donor karyoplast, typically using either electrofusion (Wilmut *et al*., 1997) or microinjection techniques (Wakayama *et al*., 1998). Generally, enucleation is preferably guided by the polar body which may be used to identify the position of the metaphase plate. Enucleated oocytes were stained with Hoechst 33342 and exposure to ultra-violet may cause damages of cytoplasmic organelles and mitochondrial DNA and these results in low cloning efficiency. In bovine and caprine, the position of the polar body can be used to identify the position of the metaphase plate because visual observation of chromatin in oocytes is impossible due to dark, opaque cytoplasm. Besides that, chemically assisted

enucleation is probably a better procedure for animal cloning. Recently, with colcemid assisted enucleation, cloned rabbit foetus (Yin *et al*., 2002a), porcine (Yin *et al*., 2002b), and murine (Gasparrini *et al*., 2003) were produced. Most studies on nuclear transfer have relied on enucleation by aspiration where it depending on the skill of the micromanipulator and between 5 to 50% of the cytoplasm is removed with a metaphase plate (Westhusin *et al*. 1992; Westhusin and Azambujam, 1996). Another method of enucleation is to bisect oocytes and discard the chromatin containing half, resulting in loss of 50% of the volume. Enucleation rate is very high (96%), which insures that the maternal genetic material is removed, and eliminates the possibility of the involvement of maternal genetic residual in nuclear transfer embryos. In addition, any oocyte derived chromatin left in to the reconstituted embryo could cause disturbances in the process of normal chromosome and cell division even without participating directly to embryonic development. Therefore, it prevents proper development of nuclear transfer embryos.

2.5.2.1 Enucleation techniques

Several enucleation methods have been published (Willadsen, 1986) and most rely on the removal of a karyoplast via micromanipulation in combination with a DNA fluorescent stain to confirm positive enucleation (Westhusin *et al*., 1992). This technique has improved enucleation and development as compared with enucleation by blind oocyte biopsy (Willadsen, 1986), but it has limitations associated with the time required for the production of large numbers of nuclear transfer embryos through micromanipulation. In the murine, the spindle is identifiable as a translucent region. The karyoplasts removed by micromanipulation were individually stained with Hoechst 33342 and then checked for the presence of metaphase II chromosomes by epifluorescent microscopy to confirm whether the corresponding oocyte had been successfully enucleated (Brendan *et al*., 1995). According to Wakayama *et al*. (1998),

enucleation of oocytes was performed by removing the metaphase II spindle with a small amount of ooplasm, and the first polar body. However, enucleation is a major limiting step in the nuclear transfer procedure. The successful and efficient removal or the destruction of genetic material in the recipient oocyte is dependent upon the technique used. Staining by places the oocyte under stresses associated with exposure to Hoechst 33342 and ultra-violet irradiation (Smith, 1993), which may also affect development. In earlier reports, germinal vesicle enucleation was performed rather 'indirectly' by increasing the pressure inside a holding pipette to expel a germinal vesicle karyoplast (nucleus) through a slit made in the zona (Sun *et al*., 1991). The zona pellucida was penetrated by pressing a glass microneedle tangentially into the perivitelline space against the holding pipette. The germinal vesicle nucleus surrounded by a small amount of cytoplasm (germinal vesicle karyoplast) was removed by a micropipette with a 20 μm inner diameter.

2.5.3 Donor Cell Preparation

Cloning of mammals by nuclear transfer of differentiated cells has become a tool to propagate valuable animals (Wilmut *et al*., 1997) and can be used as an avenue to produce genetically modified animals (Willadsen *et al*., 1991). The stage of donor cell cycle is a major factor in the success of nuclear transfer in mammalian cloning (Campbell *et al*., 1996). Quiescent donor cells arrested in G_0/G_1 phases of the cell cycle have been used to produce cloned murine (Wakayama *et al.*, 1998), ovine (Well *et al*., 1997), caprine (Baguisi *et al*., 1999), porcine (Polejaeva *et al*., 2000), rabbits (Chesne *et a*l., 2002) and bovine (Kato *et al*.,1998). Quiescent cells arrested in the G_0/G_1 phases of the cell cycle by either serum starvation or growth arrest when cultured cells reach confluence have been used as donors to produce cloned animals. Recently, Sezen *et al.* (1997) developed a novel and effective method using roscovitine to synchronise adult

bovine granulose cells in the G_0/G_1 cell cycle stage. The sizes of donor cells varied with different confluence degrees even though in the same culture dish. According to the experimental results of Tao *et al*. (1999), it was shown that donor cell size could affect the reprogramming of nuclear transfer embryos. The percentage of chromosome condensation and nuclear formation were higher in reconstructed embryos derived from small donor cells (15 μm) compared to large donor cells (20 μm), respectively. Variety of donor cell types have been used to generate viable cloned offspring in ovine (Schnieke *et al*., 1997; Wilmut *et al*., 1997), bovine (Cibelli *et al*., 1998; Kato *et al*., 1998; Wells *et al*., 1999), murine (Wakayama *et al*., 1998), caprine (Baguisi *et al*., 1999), porcine (Onishi *et al*., 2000; Polejaeva *et al*., 2000 ; Lee *et al*., 2007), rabbits (Chesne *et al*., 2002), and feline (Shin *et al*., 2002). This study was to investigate the effects of donor cell passage, size and type on the development of nuclear transfer embryos.

2.5.4 Whole Cell Intracytoplasmic Injection (WCICI) Technique

In 2007, used simplified manipulation procedure to reconstruct Asian Yellow Goat cloning embryos by Professor D.Y. Chen and colleagues known as direct whole cell intracytoplasmic injection into *in vitro* matured oocytes enucleated at telophase II stage (Chen *et al*., 2007). High pregnancy rate (24%), high birth rate (20%), high natural delivery rate (100%), and high survival rate (83%) were achieved by this method. Jang *et al*. (2004) reported that the first time that directly injecting a whole cell into the cytoplasm of an enucleated porcine oocytes (WCICI, whole cell intracytoplasmic injection) is feasible for the production of cloned embryos and piglet. Whole cell injection for nuclear transfer had not been attempted previously because of concerns that the plasma membrane of the donor cell may persist in the oocytes resulting in failure to release the nucleus. Majority, the membrane of the donor cell dissolved

relatively rapidly, within an hour of injection. However, the exact mechanism for the donor cell's plasma membrane dissolution in the oocyte is still unclear. There are 2 possible mechanisms can potentially explain the dissolution process. First, the oocyte might have actively recognised the donor cell's plasma membrane or its surface proteins as belonging to a cell surface, not to the cytoplasm of the oocyte. By this recognition, it would lead the oocyte to actively degrade the donor cell's plasma membrane or transport it to the oocyte's cell surface. This active dissolution would then release the nucleus for reprogramming (Lee *et al*., 2003). Thus, it actively dissolved the membrane of the whole cell and recycled it to the oocyte's surface membrane. The second possible mechanism for donor cell membrane dissolution may be that the donor cell membrane was damaged during whole cell injection and the leaky plasma membrane then released the nucleus, which resulted in its reprogramming. In previous report, they found that different donor cell types require different amounts of conditioning time in the oocytes before activation. Fibroblast cells required longer exposure to the methaphase II (MII) cytoplasm than granulosa cells when whole cells were used for injection. These results are consistent with those in murine and porcine showing that delayed activation for fusion as well as nucleus injection methods was beneficial to embryo development (De *et al*., 2002). I*n vitro* blastocyst development rate obtained as high as 37% by using *in vitro* matured porcine oocytes and the simple whole cell intracytoplasmic injection technique and conventional micromanipulation equipment. This attribute the high efficiency of our new cloning procedure to the following: 1) technique for the injection of whole cells reduced the manipulation time of donor cells and recipient oocytes as compared with the other 2 nuclear transfer methods and, therefore, is beneficial to embryo development; 2) the injection of a whole cell assured delivery of all cellular components to the enucleated oocytes; these components of the donor cells might be important for later development in caprine; the microtubule organising centre is needed

during natural fertilisation in most mammals except for the murine (Schatten *et al*., 2005); and 3) whole cell injection assured delivery of DNA into each injected oocyte and thus avoided fusion failure and potential damage to the nucleus during isolation. Whole cell intracytoplasmic injection technique yet helped improve our nuclear transfer efficiency may be that we avoided ultra-violet exposure and the use of Hoechst staining during enucleation, which was a common practice for cloning (Betthauser *et al*., 2000) and was found to cause abnormal meiosis and poor development (Tsunoda *et al*., 1988).

2.5.5 Tools and Skills for Micromanipulation

Anyone who researched embryo micromanipulation in the 1970s and 1980s would remember the necessary emphasis on producing the appropriate micropipettes and micromanipulators, and learning how to handle embryos gently and deftly. One of the pioneers in this field, Lin (1971) described equipment used to inject murine oocyte and for intracytoplasmic sperm injection (ICSI) of murine sperm. Later, Yang *et al.* (1990) expanded this space in rabbit oocytes by using hypertonic media to facilitate nuclear transfer without mechanically disturbing the cytoplast. Gardner (1988) used microinjection techniques extensively to produce chimaeras. He emphasised the need for patience as much as manual dexterity and need to have spare needles because as he stated "trying to continue with broken needles or partly occluded pipettes leads to a vicious circle of abortive operations and increasing exasperation. Micromanipulation is comparatively a recent term of biological microdissection that known as "micrurgy" in the old term. The term "micrurgy" was first coined by Peterfi (1923) for biological micro-dissection which also known as "micrurgery" (cited by Timson and McDermott, 1994). The first micromanipulatior with a joystick was designed that could directly transmit the movement required by the operator (El Badry, 1963). Nuclear transfer involves a manual manipulation process *in vitro* which requires exposure of oocytes and

cells to fluctuations in light, temperature, atmospheric conditions, and different media. Different systems have been used for enucleation (micromanipulation, chemical and zona free), transfer of the donor nucleus (electrofusion and direct injection) and activation stimulus (electrical, chemical and biological extracts). A minor alteration at any one step in the process can have significant effects on the success rates. Particularly, species specific requirements and sensitivities need to be identified to achieve successful nuclear transfer that adjustments in media components, temperatures or decreased exposure to detrimental conditions (Ribas *et al*., 2006; Hinrichs, 2010).

2.5.6 Activation Oocytes

After nuclear transfer, the recipient oocyte must be stimulated to initiate development. In normal fetilisation, this stimulation is achieved by inducing changes in the oocyte cytoplasm that normally are triggered by the sperm. Changes will be occurred where a transient increase in the intracellular free calcium concentration induced by an electrical pulse or by chemical agents. Ca^{2+} as the trigger of oocyte activation. Ca^{2+} transient is registered in echinoderm, fish, and frog oocytes (Jaffe, 1983). Repetitive calcium oscillations that last several hours are observed in mammals (Miyazaki *et al*., 1993; Sun *et al*., 1994; Nakada and Mizuno, 1998). This has also been shown in murine, hamster, rat, rabbit, porcine, bovine, and human oocytes (reviewed by Jones, 1998). The first calcium wave originates from the penetration site (Miyazaki *et al*., 1986), whereas subsequent oscillations arise in the cortical region of the vegetal hemisphere with a non-wave type uniform calcium rise in murine oocytes (Deguchi *et al*., 2000). Usually, calcium oscillations are of low frequency and appear at intervals of 6 to 31 minutes in murine (Deguchi *et al*., 2000) and 8 to 25 minutes in bovine oocytes (Fissore *et al*., 1992). The interval of Ca^{2+} transients is prolonged with time and last for several hours, until pronuclear formation in murine oocytes (Kline and Kline, 1992; Deguchi *et al*.,

2000) whereas these Ca^{2+} oscillations last for 22 hours in bovine with a decline in the amplitude by 12 to15 hours (Nakada *et al*., 1995). Completion of meiosis is observed when a single Ca^{2+} pulse is applied in a murine oocyte, but no pronuclear formation takes place. Kubiak *et al*. (1993) reported that a metaphase plate (or MII) forms after extrusion of the second polar body instead of a pronucleus. A donor nucleus is introduced into the cytoplasm of an "enucleated," unfertilised oocyte during nuclear transfer. Thus, in the absence of fertilisation, the oocyte must be exposed to artificial stimuli that are expected (Verma *et al*., 1994)**.** Susko-Parrish *et al*. (1994) described an activation procedure where the nuclear transfer oocytes were activated between 24 and 28 hours after the start of maturation (6 to10 hours post-nuclear transfer). The nuclear transfer oocytes were washed through Ca^{2+} and Mg^{2+} free TALP-Hepes and then exposed to 5 mM ionomycin for 4 minutes, washed in bovine serum albumin in TALP-Hepes (30 mg/ml), and incubated in 1.9 mM 6-dimethylaminopurine (6-DMAP) (Susko-Parrish *et al*., 1994) in CR1aa (Rosenkrans *et al*., 1993) for 4 hours at 39.8˚C and CO_2 (5%) in air stained with aceto-orcein. According to Wakayama *et al*. (1998, 2000), this activation protocol was used for the production of first cloned murine 'Cumulina' and her clones. When the oocytes were activated in cytochalasin B, the chromosomes were transformed into 2 or more pseudo-pronuclei. The reconstructed embryos were activated with ionomycin (5 μM) in phosphate buffer saline for 5 minutes, followed by exposure to bovine serum albumin (30 mg/ml) for 4 minutes in order to stop the activation. In order to maintain the low level of maturation or mitosis promoting factor (MPF), the reconstructed oocytes were further incubated in *in vitro* culture medium containing DMAP (2 μM) for 4 hours. Then, the reconstructed embryos were cultured in SOFaa (0.5 ml), supplemented with foetal bovine serum (5%) in 4-well dishes overlaid with mineral oil at 38.5 ℃ in a humidified atmosphere with CO_2 (5%). Cleaved embryos were further cultured in SOFaa medium supplemented with

bovine serum albumin (2 mg/ml) for 7 days (Xiang *et al*., 2007). For a frog oocyte initiates pronuclear formation within minutes after sperm penetration. In mammals, this is observed after 2 to 6 hours depending on the species (murine: 3 to 4 hours, Krishna and Generoso, 1977; bovine: 4 to 6 hours, Liu and Yang, 1999). A single Kubiak *et al*. (1993) reported that Ca^{2+} transient is not sufficient to induce definitive maturation or mitosis promoting factor degradation in young matured murine oocytes.

2.5.7 Efficiency of *In Vitro* Culture Methods

Except rabbit, most embryos of domestic mammals especially ruminants often exhibit an *in vitro* arrest of development that coincides with the period of maternal zygotic transition (MZT). Co-culture system using different supporting cells (such as oviduct epithelial cells and granulosa cells) have been used for developing *in vitro* produced embryos to overcome this *in vitro* block stage (Goto *et al*., 1992; Rehman *et al*., 1994). Besides co-culture with somatic cells, proteins and other undefined substances contained in *in vitro* culture medium (Chung *et al*., 1998; Joo *et al*., 1998; Uhm *et al*., 1998). It is also to achieve in *vitro* produced embryos as well as co-culture with somatic cells (Vansteenbrugge *et al*., 1994; Palma *et al*., 1997). Eckert and Niemann (1996) studied the importance of the supplementation of media with proteins with the final goal to develop a fully defined culture medium. The protein supplements are play important roles in culture medium which it reducing embryo toxicity of medium supplements or products from embryonic metabolism. Second, the proteins supplement serving as a source for basic nutritive requirements of early embryos and as a source of growth or proliferation. However, further studies are needed on the road towards a completely defined culture medium for the efficient *in vitro* production of caprine embryos (Keskintepe and Brachett, 1996). Embryos from farm animals can be routinely cultured up to the blastocyst stage with increased suitability of culture medium and culture

related techniques. According to Takano *et al*. (1997), the proportions of oocytes receiving somatic cells that developed into blastocysts were higher than those obtained in multiple-nuclear transfer of embryonic nuclei (27% to 37%). As compared with *in vivo* derived embryos, culture reduced total cell number and inner cell mass: trophectoderm cell ratio. *In vitro* culture system is one of the most useful strategies for understanding the mechanisms of early development and establishing optimum culture conditions. Usually, a medium including fractionated bovine serum albumin is defined as a chemically defined medium. In order to establish a simple and optimum culture system for the *in vitro* development of porcine embryos, parthenogenetic diploid embryos (Van Thuan *et al*., 2002) were used instead of fertilised embryos and their ability to develop to the blastocyst stage was examined under various culture conditions. With the presence of non-polar essential amino acids especially valine, leucine, isoleucine and methionine during the first 48 hours of culture after electrical stimulation causing severe delay of the first division of the parthenogenetic embryos (Van Thuan *et al*., 2002), hence, inhibiting their further development beyond the 4-cell stage. The presence of non-essential amino acids (NEAA) in the medium for embryos beyond the 4-cell stage produced favorable conditions for blastocyst expansion. Osmolarity changing in culture media after the 4-cell stage increased the rate of expanded blastocyst formation in porcine parthenogenetic diploids. By comparing with *in vivo* produced control embryos, level of messenger RNA (mRNA) expression in *in vitro* produced embryos can differ with stage of embryo development as well as with the base medium and supplements used for embryo culture (Wrenzycki *et al*., 2004). Several culture medium have been used for the culture of caprine embryos derived somatic cell nuclear transfer, including TCM 199 (Baguisi *et al*., 1999), Charles Rosenkrans medium (CR1) (Guo *et al*., 2002; Lan *et al*., 2006; Chen *et al*., 2007) and synthetic oviductal fluid (SOF) (Melican *et al*., 2005). Generally, *in vitro* culture media

generally require supplementation with additional compounds which can aid *in vitro* development but in the long term, can alter embryo quality. Serum is known to be detrimental to embryonic and foetal development. The effects of serum compared to bovine serum albumin supplementation during *in vitro* murine development results in lower preimplantation development and neonatal offspring, which demonstrated behavioral abnormalities in anxiety and deficiencies in implicit memories (Fernandez-Gonzalez *et al*., 2004). The addition of foetal bovine serum or oestrus cow serum (OCS) to TCM 199 or synthetic oviductal fluid (SOF) media enhanced blastocyst yield compared to TCM 199 containing polyvinyl alcohol (PVA) (Wreanzycki *et al*., 1999, 2000). Generally, a higher developmental rate to the blastocyts stage was obtained from media supplemented with serum (Pinyopummintr and Bavister, 1994; Van Langendonckt *et al*., 1997). Bovine embryos generated in the synthetic oviductal fluid system were suggested to be more similar to their *in vivo* counterparts with regard to gene expression patterns (Niemann and Wrenzycki, 2000; Sagirkaya *et al*., 2006), although Bhuiyan *et al*. (2004) observed greater development from (KSOM). Watson *et al*. (1994) reported that the use of low oxygen systems in the absence of co-culture has also been reported to improve development.

2.5.8 Reprogramming

Reprogramming usually occurs aberrantly in most cloned embryos. Incomplete reprogramming may contribute to the low efficiency of cloning. Global epigenetic reprogramming of somatic nuclei is mainly in most preimplantation cloned embryos (Dean *et al*., 2001). Therefore, correct epigenetic reprogramming is necessary for successful and normal developmental of clones (Dean *et al*., 2001). All animals derived from nuclear transfer, whether from somatic or embryonic stem cells, have some abnormal gene expression, after analysis of more than 10,000 genes. The low success

rate could be caused by an incomplete reprogramming of the epigenetic state of nuclei from differentiated donor cells. It is easy to understand that genes permanently switched off in the course of cell differentiation may be hard to reactivate efficiently. An example of such a gene is Oct 4, whose product is required for embryonic development and the maintenance of stem cell status (Nichols *et al*., 1998). The incomplete reactivation of Oct 4 may account for some failures of nuclear transfer embryo development (Boiani *et al*., 2002; Bortvin *et al*., 2003).

2.5.8.1 Pre-zygotic reprogramming

Most of cloning used an unfertilised and mature oocyte as recipient, reprogramming occurred within the brief interval between the transfer of the donor nucleus into the enucleated oocyte and here the start of zygotic transcription. Thus, pre-zygotic modifications (any modifications that have occurred before the mature oocyte stage) are expected to be less efficiently reprogrammed than post-zygotic modifications. The fact is that imprints in donor nuclei are usually not corrected in the clones. Epigenetic modification of DNA resulting in the mono-allelic and parent-of-origin–specific expression of certain genes are named as genomic imprinting. Young *et al*. (1998) reported that the dysregulation of imprinted genes is particularly pronounced in cloned animal derived from embryonic stem cells, because cultured embryonic stem cells are epigenetically very unstable and frequently gain or lose genomic imprints. According to Humpherys *et al*. (2002), cloned animal derived from uncultured cumulus cells with normal imprints also have aberrant expression of imprinted genes, proving that the dysregulation of imprinted genes is influenced by both the epigenetic state of the donor cell and the nuclear transfer procedure. Since imprinted genes are important for foetal growth and placental function, aberrant expression of these genes might account for the severely abnormal foetal and placental phenotypes in many clones. A sub-group of

these genes was found to be misexpressed exclusively in clones derived from cumulus cells, whereas another sub-group was aberrantly expressed only in clones derived from embryonic stem cells showed a result consistent with the finding that clones derived from different types of donor cells can have different abnormalities (Hochedlinger and Jaenisch, 2002; Ogonuki *et al*., 2002; Tamashiro *et al*., 2002). Therefore, pre-zygotic reprogramming, which affects the expression of imprinted and most non-imprinted genes, appears to be faulty in clones.

2.5.8.2 Post-zygotic reprogramming

Most cloned animals, the lengths of telomeres are normal or even longer than normal (Lanza *et al*., 2000; Tian *et al*., 2000; Wakayama *et al*., 2000; Betts *et al*., 2001). In normal fertilisation, telomeres undergo progressively shorten with each cell division, and this shortening has been correlated with organismal and cellular aging. The function of telomeres is structures that protect the ends of chromosomes. Pre-zygotic modifications that usually occur during gametogenesis are not corrected in the clones. Inactivation of one X chromosome in female cells is a mechanism that to ensure equal dosage of X linked genes in the 2 sexes. In early embryogenesis, it is accomplished by the random and stable silencing of one of the 2 X chromosomes. Inactive X was found to be reactivated properly, resulting in random X inactivation in cloned murine embryos from female somatic cells (Eggan *et al*., 2000). Thus, all available evidence indicates that reproductive cloning, in contrast to normal development or *in vitro* fertilisation, is limited by the fundamental biologic problem of epigenetic reprogramming of the donor nucleus. This incomplete reprogramming may result in abnormal phenotypes, aberrant gene expression, and the death of most clones.

2.5.8.3 Maternal zygotic transcription (MZT)

The onset of maternal zygotic transition is species dependent such as murine 1 to 2-cell stage (Bolton *et al*., 1984); bovine, caprine and ovine 8- to 16-cell stage (Calarco and McClaren, 1976; Camous *et al*., 1986) and Xenopus approximately 4000 (Newport and Kirschner, 1982). With improvements in nuclear transfer techniques, development from later developmental stages has been achieved and live offspring have been reported following direct transfer of karyoplasts to enucleated metaphase II oocytes in the murine from morula stage embryos and in ovine and bovine from inner cell mass cells (Smith and Wilmut, 1989; Collas and Barnes, 1994). In murine (Howlett *et al*., 1987) and rabbit (Kanka *et al*., 1996), inhibition of transcription and establishment of zygotic gene expression profiles have been reported when transferring transcriptionally active blastomere nuclei, suggesting 'reprogramming'.

2.5.9 Effects of Cytoplast Cell Cycle Stage

When using different donor cell cycle phases a direct comparison of all cytoplast recipients is not possible. Consider the fate of the chromatin from a diploid donor nucleus on transfer to an metaphase II or a pre-activated cytoplast recipient is very important. The chromatin transferred to the methaphase II cytoplast will undergo an additional round of chromosome condensation in comparison to the pre-activated oocyte. The period of exposure of the chromatin to the cytoplasm of the oocyte can be increased by utilising an unactivated metaphase II oocyte as cytoplast (Campbell, 1999). Nuclear structure and function are intimately related. In addition, precise nuclear structure is required for some of the physical events required for embryo development. Alternatively nuclear remodeling may occur not as a single event but during successive mitosis and therefore, the effects of an extra round of nuclear envelope break down (NEBD) is additive. The ideas suggest that nuclear remodeling is essential for some

processes during early development. However, whether nuclear envelope break down is necessary is presently unclear and what changes are essential in different species are also unclear.

2.5.10 Cell Cycle Stage of Donor Nucleus

The donor cells in late G_2, M or early G_1 phases of the cell cycle are more able to promote development (Kwon and Kono, 1996). Recently, live offspring have been reported from embryonic foetal and adult derived cell populations that were in the G_0 phase of the cell cycle at the time of transfer (Campbell *et al*., 1996; Wilmut *et al*., 1997; Wells *et al*., 1997; Schnieke *et al*., 1997; Wakayama *et al*., 1998). During the late G_2 phase of the cell cycle, nelcaer envelope break down and chromosome condensation occur as the cell enters mitosis. During mitosis, the chromosomes remain condensed and are segregated to the 2 new daughter cells. Then, during the G_1 period, chromosome decondensation occurs and the nuclear envelope reforms. Martinez-Balbas *et al*. (1995) described during mitosis transcription is dramatically reduced and transcription factors become displaced from the chromatin. Cell cycle coordination between donor cells and recipient oocytes has been stressed importantly (Campbell *et al*., 1996, 1999). Although, it was once thought that donor cells must be at the G_0 stage of a cell cycle during nuclear transfer for successful cloning (Wilmut *et al*., 1997), subsequent studies revealed that this is not necessarily the case. However, the donor cells at the G_1 or G_2 stage and even those at the M stage can be used as long as the cloned embryos are diploid (Campbell *et al*., 1996; Tani *et al*., 2001; Ono *et al*., 2001). When we injected cumulus cell nuclei (which were mostly at the G_0 and G_1 stage) into murine oocytes and then activated it, part of cumulus chromosomes (2n) were shed into pseudo-second polar bodies which resulting in the production of hypoploidy embryos which were doomed to die. To avoid this problem, culturing nucleus-injected oocytes in a medium

which activated oocytes and inhibited their cytokinesis simultaneously should be carried out. The medium used was a Ca^{2+} free medium containing strontium (Sr^{2+}) and cytochalasin B (CB). Strontium activated the oocytes and cytochalasin B inhibited cytokinesis of the activated oocytes (Wakayama *et al*., 1998). The first production of live murine was obtained by transferring nuclei from 2-cell embryos at the G_2 phase to ooplasts (Kono *et al.*, 1991, 1992).

2.5.11 Genomic Imprinting

Genomic imprinting is an epigenetic chromosomal modification in the germ line that leads to preferential expression of one of the 2 parental alleles in a parent-of origin-specific manner. The study of imprinting is provides new insights into epigenetic gene modification during development. Genomic imprinting in mammals was discovered in the early 1980s. In early 1990s, the discovery of the first imprinted genes, which were expressed differently on maternal and paternal chromosomes (Barlow *et al*., 1991; Bartolomei *et al*., 1991; DeChiara *et al*., 1991). According to Barlow (1995), imprinting genes are particularly implicated in the regulation of foetal growth, development, and function of placenta in mammals. DNA methylation by the enzyme *Dnmt1* plays a central role in genomic imprinting, and may be the main epigenetic mechanism. The most common phenotypes observed in animals cloned from either somatic cell nuclei or embryonic stem cell nuclei are foetal growth abnormalities for example increased placental and birth weight. Jaenish (1997) reported that similar phenotypes have been observed in human patients and in murine as a consequence of both naturally occurring and targeted mutagenesis of imprinted genes, these apparent similarities suggested that aberrant expression of imprinted genes might cause some of the abnormalities in clones. Humpherys *et al*. (2001) have examined imprinted gene expression in both murine cloned by nuclear transfer and embryonic stem cell donor populations from which they

were derived in an effort to correlate gene expression with foetal and survival overgrowth. Epigenetic state of the embryonic stem cell genome was found to be extremely unstable. Imprinting is an important genetic mechanism in mammals, and is thought to influence the transfer of nutrients to the foetus and the newborn from the mother. DNA methylation was a key molecular mechanism of imprinting, the methylation marks the imprinted genes differently in egg and sperm, and inheritance of these epigenetic marks leads to differential gene expression (Reik *et al*., 1987; Sapienze *et al*., 1987; Swain *et al*., 1987; Chaillet *et al*., 1991; Sasaki *et al*., 1991). The evolutionary understanding of imprinting and its likely biological purposes is increasing (Moor and Haig, 1991; Hurst and Mc Vean, 1997) and the study of imprinting is providing general insights into the importance of epigenetic mechanisms in development. Genomic imprinting, the preferential expression of one parental allele, is regulated primarily by DNA methylation of CpG islands. Wilkins and Haig (2003) reported that the majority of imprinted genes have roles in foetal growth and development, and both the maternal and paternal genomes are required for normal development. The phenomenon of genomic imprinting is mainly observed in eutherian mammals, suggesting that it evolved when placental mammals developed direct maternal-foetal interactions throughout pregnancy (Wilkins and Haig, 2003; Wagschai and Feil, 2006). The time course of imprinting has been best studied in the murine where distinct allelic expression patterns of imprinted genes can be established as early as the 2-cell stage, and by the blastocyst stage (Latham, 1999; Monk and Salpekar, 2001). Monoallelic expression of many imprinted genes is observed, which closely follows the reestablishment of genome-wide DNA methylation in early development (Latham, 1999; Monk and Salpekar, 2001).

2.5.12 DNA Methylation

DNA methylation is a major epigenetic modification of the genome that regulates crucial aspects of its function. In mammals there are at least 2 developmental periods: in germ cells and in preimplantation embryos, in which methylation patterns are reprogrammed genome wide, generating cells with a broad developmental potential (Reik *et al*., 2001). Therefore, during cell division, the methylation pattern should also pass over to the daughter cell. Genomic methylation patterns in somatic differentiated cells are generally stable and heritable. According to Bird and Wolffe (1999), methylation of cytosine residues is implicated in several genomic functions, such as imprinting, X chromosome inactivation, genomic stability, immobilisation of mammalian transposons, and tissue-specific gene expression. The somatic donor nuclei that have been used include foetal and adult fibroblasts, embryonic stem cells, and other somatic cells that were presumably differentiated. All these cells have a highly methylated genome, which is the characteristic of somatic cells (Kang *et al*., 2001). Aberrant DNA methylation patterns have been reported in genomic repetitive elements in cloned bovine blastocysts (Bourc'hi *et al*., 2001; Dean *et al*., 2001; Kang *et at*., 2001). In cloned bovine morulae and blastocysts, the methylation levels of several repeat and unique sequences were found by bisulfite analysis to be much higher than that in normal embryos, and thus resembled methylation levels in the donor cell genome (Kang *et al*., 2001). Dean *et al*. (2001) reported that reorganisation of the nuclear pattern of methylation to resemble that of differentiated nuclei occurred prematurely in cloned embryos. Chung *et al*. (2003) found cloned preimplantation murine embryos aberrantly expressing the somatic form of the *Dnmt1* DNA (cytosine-5) methyltransferas, this confirmed the front speculation. Kang *et al*. (2003) studied a unique DNA methylation change occurring at bovine neuropeptide galanin gene sequence in cloned preimplantation bovine embryos. Recently, epigenetic alterations in

DNA methylation and imprinted gene expression arising from derivation or culture murine embryonic stem cells do not become corrected during post-implantation development and are associsted with aberrant gene expression in the embryonic stem cell derived foetus (Dean *et al.*, 1998). In all embryonic stem cell lines studied, the maternal Igf2 allele was unusually methylated, in addition to the paternal allele. In several foetuses this resulted in expression of Igf2 from both alleles, but particularly from the maternal allele. Finally the Igf2 allele gene was maternally methylated in all embryonic stem cell lines and in most embryonic stem cell foetuses. In all embryonic stem cell lines the pattern of Igf2r expression was biallelic but in the majority of embryonic stem cell foetuses was only from the maternal allele. This is consistent with the patterns of Igf2r methylation and expression found in normal murine blastocyst stage embryos (from which embryonic stem cells are derived) and foetuses. As in the age effect suggested for donor somatic nuclei from older animals (Jaenisch, 1997), imprinting errors may also be expected to increase with age and passage number in embryonic stem cells. There have been recent reports of the isolation of both bovine and human (Thomson *et al*., 1998) embryonic stem-like cells from blastocyst inner cell mass cells. During normal early embryonic development in mammals, the global pattern of genomic DNA methylation undergoes marked changes. The level of methylation is high in male and female gametes. *De novo* methylation in murine occurs at the blastocyst stage, preferentially in the inner cell mass. *De novo* methylation in bovine embryos happens at the 8- to 16-cell stage, concurrent with zygotic genome activation. It has been proposed that chromatin configuration has a significant role in establishing the ‘histone code’, a cellular memory that is responsible for maintaining the identity of differentiated cells (Bird, 2002). Histones are the proteins that make up chromatin, and their N-terminal tails can be modified in several ways, including methylation, acetylation, phosphorylation and ubiquitinylation.

2.6 HISTORICAL BACKGROUND OF EMBRYONIC STEM CELL

In 1998, researchers at the University of Wisconsin building on earlier stem cell research, isolated cells from the inner cell mass (ICM) of the early human embryo, called the blastocyst. This is the first human embryonic stem cell lines development (Thomsom *et al*., 1998). During that exact same year, Gearhart, from Johns Hopkins University, derived germ cells from cells in foetal gonad tissue; pluripotent stem cell lines were developed from both sources. Since then, it has made a lot of progress, with 2,000 research papers on embryonic and adult stem cells being published in reputed scientific journals every year. Now, adult stem cells are being used to treat over 100 conditions, including leukaemia, Hunter's syndrome and heart disease. However, embryonic stem cell research is still in its infancy, especially when it is yet to yield any clinical trials. In 1988, embryonic stem cell lines were created from a hamster. The first development of 1990s, in context of stem cell research, comprised of the derivation of the first embryonic stem cell line from a primate, in 1995. Two years later, the world witnessed its first case of cloning, where cloned lamb were created from stem cells. The same year, haematopoietic stem cell was found as the origin of leukaemia, indicating possible proof of cancer stem cells. Today, it is believed that embryonic stem cells can become almost any of the specialised cells in the body and thus, have the ability to generate replacement cells for a wide range of tissues and organs in the body, like heart, liver, pancreas and nervous system. Smith and Wilmut (1989) reported that nuclei isolated from the inner cel1 mass of ovine blastocysts are totipotent as one full-term lamb was obtained after fusing such cells to enucleated recipient oocytes. According to Campbell *et al*. (1995), more recent experiments in the same laboratory demonstrated that even after several passages in culture such cells derived from ovine inner cell mass can be transferred and result in the birth of lambs. High developmental rate to the morula stage, up to 70% can be obtained in sheep using 16-cel1 stage donor morula and

in vivo enucleated and preactivated oocytes (Loi *et al*., 1995). Embryonic stem cell lines have been established from murine blastocysts generated by nuclear transfer of murine somatic cell nuclei. The somatic cell types were ovarian cumulus cells (Munsie *et al*., 2000; Wakayama *et al*., 2001), tail-tip fibroblasts (Wakayama *et al*., 2001), and fetal neuronal cells (Kawase *et al*., 2000a). The 3 primary embryonic lineages were all represented in teratomas (Kawase *et al*., 2000b; Munsie *et al*., 2000) and normal chimaeric progeny were obtained when the embryonic stem cells were injected into host blastocysts (Munsie, 2000; Wakayama *et al*., 2001). The embryonic stem cells contributed to all tissues of the chimaeric murine, including germ line transmission. The primary difficulty for nuclear transfer in murine is the efficiency of insertion of somatic cell nuclei into oocytes (Munsie, 2000). It is difficult to electrofuse cells that are very different sizes, and attempts to vary the techniques of electrofusion, reduced size differences. Therefore, other parameters such as nuclear injection has remained the method of choice (Trounson *et al*., 1998). The use of piezo-electronic impact micromanipulation (Prime Tech Ltd or Burleigh Instruments) enables very improved efficiencies of nuclear transfer to produce viable embryos (Wakayama *et al*., 1998). This method was used to produce nuclear transfer embryos for embryonic stem cells production in all the studies reported to date. There were no obvious differences between strains of murine as donor cells and between cumulus cells or fibroblasts, in the development to blastocysts. Wakayama *et al*. (2001) have been established embryonic stem cell lines and number of chimaeric offspring produced by combining nuclear transfer of embryonic stem cells (ntES) with wild-type blastocysts. He suggested that the donor cell origin or tissue type has no effect on the ability to generate embryonic stem cells, and this would be an attractive strategy to explore for generation of embryonic stem cells for human cell therapies. Embryonic stem cells therapies have been proposed for regenerative medicine and tissue replacement after injury or disease

because of their plasticity and potentially unlimited capacity for self-renewal. Embryonic stem cells also known as “master cells” which able to develop into almost any cell in the human body. Most of research has focused on the potential that embryonic stem cells where they can offer to treat diseases and conditions as well as to generate replacement tissues for disfunctioning cells, tissues or organs (Judith *et al*., 2001). Research efforts also have focused on spinal cord injury, multiple sclerosis, Parkinson’s disease, Alzheimer’s disease, diabetes, and other degenerative diseases. The embryo is destroyed in the removal process in order to extract the stem cells found within the embryo. In 1981, embryonic stem cells were first derived from murine embryos by Martin Evans and Matthew Kaufman at the Department of Genetics, University of Cambridge. Martin is credited with coining the term "Embryonic Stem Cell" (Martin, 1981). A breakthrough occurred when researchers, led by James Thomson at the University of Wisconsin-Madison, first developed a technique to isolate and grow in cell culture human embryonic stem cells *(*Thomson *et al*., 1998). It was discovered that there was a way to extract embryonic stem cells without destroying the actual embryo *(*Klimanskaya *et al.*, 2005). This technical achievement enable scientists to work with new lines of embryonic stem cells derived using public funding in the United State, where federal funding was at the time limited to research using embryonic stem cell lines derived prior to August 2001. Zangani *et al*. (2007) reported that Professor Shinya Yamanaka had a recent breakthrough in which the skin cells of laboratory murine were genetically manipulated back to their embryonic state. This was confirmed by 2 other groups, demonstrating that the addition of just 4 genes (Oct 3/4, Sox 2, Klf4, and c-Myc) could reprogram murine skin cells into embryonic stem like cells. This finding is important in science and stem cell field where after Hwang Woo-Suk from Korea fabricated data, claiming to have generated human embryonic stem cells from cloned embryos. Yamanaka as well as the other laboratories demonstrated all

the hallmarks of embryonic stem cells including the ability to form chimaeric murine and contribute to the germ line. In 2008, these embryos can be harvested for patient matching embryonic stem cells. First isolated in 1997 by researchers at the Johns Hopkins Medical Institutions the embryonic stem cell is a fairly recent addition to the list stem cell types.

Over past 15 years, many reports have been published on bovine, porcine, caprine, ovine and equine embryonic stem cell lines are often called as "embryonic stem-like" cell lines. This is similar to a few embryonic stem-like cell lines from rodents other than the murine (hamster, Doetschman *et al*., 1988; rat, Iannaccone *et al*., 1994; Vassilieva *et al*., 2000) and from rabbit (Schoonjans *et al*., 1996) have also reported. None of these cell lines has been definitively proven to be embryonic stem cells. With our knowledge, none has been successfully used as a biological reagent in a manner similar to the use of human, monkey or murine embryo such as directed pluripotent *in vitro* differentiation (Perrier *et al*., 2004; Takagi *et al*., 2005) or embryonic chimaera formation as a means of genetically engineering (Bradley, 1987). Embryonic stem cell lines were first isolated from explant cultures of *in vivo* derived murine blastocyst stage embryos (Evans and Kaufman, 1981; Martin, 1981; Robertson, 1987). Embryonic stem cell lines have also been derived from 8-cell and morula stage embryos prior to the first embryonic differentiation events, such as formation of the inner cell mass and trophectoderm (TE) of the blastocyst (Eistetter, 1989; Delhaise *et al*., 1996; Strelchenko *et al*., 2004). In general, primate embryonic stem cell lines have been established from *in vivo* derived blastocysts of monkeys (Thomson *et al*., 1995) and *in vitro* fertilised (IVF) as well as *in vitro* cultured (IVC) blastocysts of humans (Thomson *et al*., 1998; Reubinoff *et al*., 2000; Lee *et al*., 2005).

An attempt to create embryonic stem cell lines of the porcine, caprine, ovine and equine have most often used *in vivo* blastocysts acquired from the reproductive tract at

various stages, but generally from the early blastocyst stage to the later elongated or filamentous stage. Efficient and cost effective *in vitro* produced (IVP) embryo culture systems are commonly used for bovine which early blastocyst stage embryos are the most frequent starting material for attempts at making embryonic stem cell lines. Production of these *in vitro* produced embryos usually involves *in vitro* maturation (IVM), *in vitro* fertilisation and culture to morula or blastocyst stages. *In vitro* produced blastocysts may be altered in terms of cell metabolism, epigenetic status and constituent cell numbers, but it is probably that some will prove competent for the establishment of bovine embryonic stem cell lines. This seems reasonable since human embryonic stem cell lines have been derived from *in vitro* fertilisation or *in vitro* culture embryos, and because culture of *in vitro* produced derived bovine epiblast tissue displays normal differentiation and pluripotency *in vitro* (Talbot *et al*., 1995). Besides that, peer-reviewed reports of porcine embryonic stem cells, embryonic stem-like or inner cell mass cell lines have been published by at least 6 groups, all of which used *in vivo* derived blastocysts as their primary culture material (Notarianni *et al*., 1990, 1991; Piedrahita *et al*., 1990; Hochereau-de Reviers and Perreau, 1993; Wheeler, 1994; Chen *et al*., 1999; Li *et al*., 2003a, 2004a, 2004b). Li *et al*. (2004b) reported that putative porcine embryonic stem cell lines also isolated from *in vitro* produced porcine embryos, as were several bovine embryonic stem cells or embryonic stem-like cell lines from *in vitro* produced early blastocyst stage embryos (Evans *et al*., 1990; Cibelli *et al*., 1998; Iwasaki *et al*., 2000; Mitalipova *et al*., 2001; Saito *et al*., 2002, 2003; Wang *et al*., 2005). Production of ovine, equine and caprine embryonic stem-like cell lines has also been reported (Notarianni *et al*., 1991; Keefer *et al*., 1996; Saito *et al*., 2002). There are also several reports of similar embryonic stem-like cell lines of the porcine, caprine and bovine that was derived from primordial germ cells found in the early genital ridge tissue, sometimes referred to as embryonic germ cell lines (Piedrahita *et al*., 1997; Shim

et al., 1997; Mueller *et al*., 1999; Tsung *et al*., 2003; Rui, 2004). Both murine and human embryonic germ cell lines have been proven or are assumed to be functionally equivalent to embryonic stem cell lines (Matsui *et al*., 1992; Resnick *et al*., 1992; Shamblott *et al*., 1998). Through the above mentioned studies have provided us valuable information on ungulate embryonic cell culture, future studies need to focus more intensely on the critical characteristics to define true embryonic stem cell lines.

Embryonic stem cells can be derived from numerous techniques through *in vitro* produced embryos [such as *in vitro* fertilisation (IVF), intracytoplasmic sperm injection (ICSI), parthenogenesis (PA) and somatic cell nuclear transfer] and *in vivo* produced embryos (oviductal and uterine flushing). Based on above sources, only parthenogenesis, somatic cell nuclear transfer and flushing techniques have been utilised to obtain embryonic stem cells in caprine in our study. Since inner cell mass could develop to be a foetus, so it is a valuable source for embryonic stem cells establishment. There are several techniques to isolate a clump of inner cell mass cells such as blastocyst outgrowth (Tabar, 2005; Genbacev *et al*., 2005), mechanical dissection (Wang *et al*., 2005), laser assisted blastocyst dissection (Tanaka *et al*., 2006) and immunosurgery (Solter and Knowles, 1975; Reubinoff *et al*., 2000; Mateizel *et al*., 2006; Tanaka *et al*., 2006). Immunosurgery technique is one of the most efficient techniques used for isolating inner cell mass cells. Once isolated and cultured *in vitro* under appropriate conditions, the inner cell mass cells could propagated as an embryonic stem cell line.

2.7 EMBRYONIC STEM CELLS PROPERTIES

The embryonic stem cells are pluripotent where they can differentiate into almost all tissues of the 3 embryonic germ layers (ectoderm, mesoderm and endoderm) after *in vitro* induced differentiation. Besides that, embryonic stem cells are capable to form

teratocarcinomas after subcutaneous injection into severe compromised immune deficient (SCID) murine, except embryonic stem cells could not differentiated to any cell types of the trophoblast lineage (Cavaleri and Scholer, 2004). The general characteristics of embryonic stem cells were: a) embryonic stem cells derived from preimplantation embryo, b) pluripotent (capable of differentiating into specialised cells from all three embryonic germ layers), c) immortal (the cell cultures can be maintained indefinitely by long term cultures or continuous passages (Suda *et al*., 1987; Amit *et al*., 2000), d) maintaining normal karyotype after prolonged culture, e) ability to contribute to all embryonic germ layers including the germ line after injection into blastocyst, f) expressing unique pattern of embryonic stem cells markers such as Oct 4, Sox 2, Nanog and cell surface markers such as stage specific embryonic antigen SSEA 1, SSEA 2, SSEA 3, SSEA 4, tumor resistant antigen TRA-1-60 and TRA-1-81 and g) clonogenic (each individual cell processing the above characteristics).

The term "cell line" indicated the maintenance of the cell culture's original phenotype during continuous culture or passage, such as hundreds of population doublings and also implies homogeneity of phenotype within the population of cells. In 1981, the derivations of pluripotent cell lines from murine blastocyst by 2 different groups (Evan and Kaufman, 1981; Martin, 1981). Therefore, murine embryonic stem cells establishment has been used for establish embryonic stem cell lines from different species including rabbit, hamster, pig, monkey and human (Gardner, 2004). Althoguh embryonic stem cell lines were considered as pluripotent, they exhibit different properties from murine embryonic stem cells such as morphology of undifferentiated colonies and the expression profile of stem cell markers. According to Thomson *et al*., (1995), the morphologically appearance of embryonic stem cell colonies vary in different animal species such as murine embryonic stem cells undifferentiated colonies which showed colonies with dome shape, clear edge and high nuclear-cytoplasmic ratio

while the undifferentiated primate embryonic stem cells as appeared as flat colonies with high nuclear–cytoplasmic ratio. The primate embryonic stem cells are differed from the murine embryonic stem cells in the expression of stem cell markers pattern and cytokine factors that are important for maintaining at undifferentiated stage.

Stem cell markers can be proved where samples tested should be free of feeder cells. This is because feeder cells may contain a mixture of cell types or have undefined gene expression profiles which can complicate interpretation of results (Talbot *et al*., 2004). Moreover, embryoid body formation demonstrations by ungulate embryonic stem-like cells must differentiate embryoid-like bodies from similar multicellular vesicle-like bodies that are commonly produced by the anchorage-independent growth of ungulate trophectoderm and visceral endoderm cells or by other polarised, dome-forming epithelial cell lines (Robertson, 1987) for a clear description of murine embryonic stem cells embryoid bodies. By proving the pluripotency of primate and murine embryonic stem cells is that of teratoma formation in immune-compromised murine. In recent research, embryonic stem cells have been successfully established from early embryos, 2-cells to morula stage, instead of using the inner cell mass of a blastocyst (Bradley *et al*., 1984; Eistetter, 1989; Strelchenko *et al*., 2004; Tesar, 2005). Furthermore, embryonic stem cells have been successfully established from a single biopsied blastomere of early preimplantation stage murine and human embryo (Chung *et al*., 2006; Klimanskaya *et al*., 2006, Wakayama *et al*., 2007). These breakthroughs have demonstrated the feasibility of deriving personal embryonic stem cells. Some embryonic stem-like cell lines were used as nuclear donor cells to create cloned animals by nuclear transfer (Chen *et al*., 1999; Saito *et al*., 2003). The use of the cells in nuclear cloning in no way proves their embryonic stem cells character since many types of fully differentiated somatic cell nuclei have proven competent for the creation of cloned animals (Kato *et al*., 2000; Wakayama and Yanagimachi, 2001) and nuclear cloned

animals have also been created from trophectoderm cells (Tsunoda and Kato, 1998). The latter demonstration is very significant because trophectoderm cells are easily confused with epiblast cells and a common cell contaminant in attempts to establish embryonic stem cell lines.

Embryonic stem cell colonies usually grow quickly to contain hundreds if not thousands of cells per colony. Thus, the colonies will fuse with one another to form monolayers if there are sufficient colonies in close proximity. Murine embryonic stem cells will begin to spontaneously differentiate at the periphery of the colony with the formation of flatter, larger and irregularly cuboidal visceral endoderm cells. On the same time, other somatic cell types may appear in or around the differentiating colony. Primate embryonic stem cells colony morphology is different from murine embryonic stem cells in that the monkey or human embryonic stem cells are generally flatter in appearance and spontaneous differentiation tends to begin in the center of colonies if they are left undisturbed for a week or more without passage (Thomson *et al*., 1995, 1998; Thomson and Marshall, 1998). Some researchers reported that cell morphology of embryonic stem cells and primary cultures of epiblast cells are very similar across species (Robertson, 1987; Talbot *et al*., 1993; Thomson *et al.,* 1995, 1998; Thomson and Marshall, 1998). The general size of embryonic stem cells is 10 to 15 μm in diameter with a round to oval shape. Their most distinctive morphological feature when viewed by phase contrast microscopy is their large nucleus surrounded by a narrow band of non-granular cytoplasm. Most nuclei are observed to contain 1 or 2 very large and distinct nucleoli. An ultrastructural study of the inner cell mass of *in vivo* porcine blastocysts and of primary cultures of porcine epiblast cells showed contrast to primate and murine embryonic stem cells where the porcine epiblast cells develop relatively robust complex junctions or tight junctions shortly after blastocyst formation (Talbot and Garrett, 2001). Another important property of embryonic stem cells is that of

asymmetric division, yielding one differentiated progeny and one stem cell daughter (Cai *et al*., 1997; Lu *et al*., 2000). Table 2.2 shows the comparision between murine and human embryonic stem cells.

Table 2.2: Comparision between murine and human embryonic stem cells

Pluripotent markers	Murine embryonic stem cells	Human embryonic stem cells
AP	+	+
SSEA-1	+	-
SSEA-3	-	+
SSEA-4	-	+
TRA-1-60	-	+
TRA-1-81	-	+
Oct-4	+	+
Feeder cell	Need	Need
Cytokine factor control self-renew	LIF and some growth factors that work through GP130 receptor	Feeder cell and bFGF
Morphology of undifferentiated colony	Multi layers clump, dome shape, clear edge	Mono layer clump, loose, flat colony
Embryoid body formation	Yes	Yes
Teratoma formation	Yes	Yes
Chimaera formation	Yes	Yes

2.8 MAINTAINING OF EMBRYONIC STEM CELLS IN THEIR UNDIFFERENTIATED STATE

Embryonic stem cells are derived from totipotent cells of the early mammalian embryo and are capable of unlimited, undifferentiated proliferation *in vitro* (Evans and Kaufman 1981; Martin, 1981; Morrison *et al*., 1981). Undifferentiated murine embryonic stem cells can be maintained for extended periods in media containing the cytokine, leukaemia inhibitory factor or Millipore's proprietary embryonic stem cell culture reagent ESGRO (Smith *et al*., 1988; Williams, 1988). Upon removal of leukaemia inhibitory factor from the culture medium, *in vitro*, the murine embryonic stem cells

differentiate into cells derived from all 3 germ layers. In contrast, human embryonic stem cells cultures required mouse fibroblasts as feeder cells and cannot be maintained with leukaemia inhibitory factor for self-renewal (Shamblott *et al*., 1998; Thomson and Marshall, 1998).

Embryonic stem cells can be maintained in a proliferative, undifferentiated state *in vitro* by growing them on feeder layers of mouse embryonic fibroblast cells. Smith (2001) and Williams *et al*. (1988) have been reported that an alternative to culture on feeder layers is the addition of leukaemia inhibitory factor to the culture medium. Leukaemia inhibitory factor is produced by feeder cells and it allows embryonic stem cells *in vitro* to continue proliferating without differentiating (Rathjen *et al.*, 1990).

2.8.1 Leukaemia Inhibitory Factor (LIF)

Gearing *et al.* (1987) had identified the role of leukaemia inhibitory factor in maintaining stem cell functions. This approach was applied by Williams *et al.* (1988) who used it to maintain stem cell properties in cultures of murine embryos. The leukaemia inhibitory factor receptor consists of the luekaemia inhibitory factor-specific receptor subunit (LIFRh) and the common signal transducer gp130 (Davis *et al*., 1993). Added leukaemia inhibitory factor enhances embryonic stem cell self-renewal suggesting a combinational effect of these pathways on self-renewal (Chambers *et al*., 2003; Mitsui *et al*., 2003). Yoshida *et al*. (1994) reported that the effect of leukaemia inhibitory factor on self-renewal is not exclusive but is also seen by a small group of related cytokines which act via the common gp130 receptor.

2.8.2 Cytokine Control Pluripotent State

Cytokine is a polypeptide protein that could maintain self-renewal of embryonic stem cell and control the undifferentiated state of embryonic stem cells. The important

cytokine for embryonic stem cell maintaining self-renewal state is leukaemia inhibitory factor which secreted from feeder cell and added into culture medium. In the past, culture conditions for the establishment of embryonic stem cells and embryonic stem-like cells in murine, ungulates and other animals have relied on a system of co-culture with mouse embryonic fibroblasts (primary mouse embryonic fibroblasts or transformed STO cells) and supplementation of medium with foetal bovine serum (FBS), glutamine and beta-mercaptoethanol. Both foetal bovine serum and the medium conditioned by feeder cells comprise a poorly defined system of growth factors and cytokines. In murine embryonic stem cells, supplementation with leukaemia inhibitory factor obviates the need for any other additive in feeder and serum free conditions. Therefore, supplement the culture medium with a combination of growth factors and cytokines proven beneficial for murine embryonic stem cells. The most common supplements used to establish and maintain embryonic stem-like cells include leukaemia inhibitory factor (Strelchenko, 1996; Iwasaki *et al*., 2000; Saito *et al*., 2003; Li *et al*., 2004c; Yadav *et al*., 2005), basic fibroblast growth factors (bFGF) (Strelchenko, 1996; Li *et al*., 2004c; Yadav *et al*., 2005), stem cell factor (SCF) and epidermal growth factor (EGF) (Saito *et al*., 2003). It has been known for some time that leukaemia inhibitory factor and its related family members namely oncostatin M, interleukin-6 and ciliary neurotrophic factor are key cytokines for the maintenance of murine embryonic stem cell lines (Piquet-Pellorce *et al*., 1994; Yoshida *et al*., 1994), whereas they do not appear to function in human embryonic stem cell lines (Thomson and Marshall, 1998). Thus, some of the challenges in establishing embryonic stem cell lines may be reflected in the differences identified in cytokine requirements for the maintenance of undifferentiated murine and human embryonic stem cells. In previous report, they found that the bovine inner cell mass and its primary outgrowths do have the leukaemia inhibitory factor receptor and gp130 signal transducer (Pant and Keefer,

2006). The maintenance of pluripotency is probably the most critical problem to be solved in establishing embryonic stem cell lines and should be the major focus of current and future research. Identification of functional pathways in ruminant embryonic cells will help determine what cytokines are beneficial and which may be inhibiting the establishment of true embryonic stem cells.

2.8.3 Mouse Embryonic Fibroblast (MEF)

Mouse embryonic fibroblast have been used reliably as feeder cell layer for murine embryonic stem cells since the early 1980s when the first murine embryonic stem cell lines were being derived and cultivated (Puck *et al.*, 1956). Feeder cell layer are usually used in culturing embryonic stem cells to maintain their undifferentiated and pluripotent status. Mouse embryonic fibroblast serve as feeder cell layer in embryonic stem cell culture (Evans and Kaufman, 1981; Thomson *et al*., 1998; Smith, 2001; Turksen, 2002). Williams *et al*. (1988) reported that one of the substances released by mouse embryonic fibroblast that inhibit the differentiation of embryonic stem cells is leukaemia inhibitory factor. The first published derivation of human embryonic stem cells used mouse embryonic fibroblast as feeder cell layer, and many laboratories continue to use them routinely for long-term human embryonic stem cell culture. Mouse embryonic fibroblast are primary cells derived from days 12.5–14.5 foetuses. It is primary cells that do not continue to proliferate indefinitely. Once the cells begin to senesce they seem to lose their capacity to support undifferentiated growth and proliferation of human embryonic stem cells. So, usually mouse embryonic fibroblast is used optimally between Passages 1 to 3. Process of preparation mouse embryonic fibroblast to be repeated only occasionally which usually large batches are made, tested, and cryopreserved. It is important that mouse embryonic fibroblast are mitotically inactivated before being co-cultured with murine or human embryonic stem cells, or the

mouse embryonic fibroblast will become a growing contaminant cell type that is difficult to remove. There are 2 common ways of inactivating mouse embryonic fibroblast: irradiation and mitomycin C (MTC) treatment. The cells can be cryopreserved either before or after mitotic inactivation. Embryonic stem cells are usually grown on a layer of mitotically inactivated primary mouse embryonic fibroblast to promote growth and prevent differentiation. These cells stop dividing after a couple of passages, so embryonic fibroblast need to be isolated freshly from time to time. To avoid isolation at time to time we need to be preserve them safely. Mouse embryonic fibroblast cells are isolated from murine embryos at day 14 of gestation as described by Robertson (1987). The specific role of mouse embryonic fibroblast in prolonging embryonic stem cell culture is less clear. It is generally known that mouse embryonic fibroblast provides a befitting environment for the interplay of signaling networks that regulate the fate of embryonic stem cells (Xu *et al*., 2001; Lim and Bodnar, 2002). However, the feeder cell layer of xenogeneic or allogeneic origin make it possible to introduce inter-and intra-species transfer of pathogens (Odorico *et al*., 2001; Richards *et al*., 2002). In addition, the use of mouse embryonic fibroblast as feeders has other disadvantages, including a limited performance window that is optimal only between the 4th and 6th passages, so it is necessary to repeatedly sacrifice murine foetuses (Odorico *et al*., 2001; Richards *et al*., 2002; Stojkovic *et al*., 2005). To avoid these issues, recently some investigators have successfully established novel feeder systems that were derived from human embryonic stem cells themselves (Stojkovic *et al*., 2005; Wang *et al*., 2005; Yoo *et al*., 2005). However, a correlative study about deriving feeders from murine embryonic stem cells has not been reported.

Historically, embryonic stem cells have been cultured under a variety of conditions to prevent differentiation (Wurst and Joyner, 1993). The most common method utilises feeder layers prepared from mouse embryo fibroblast or from SIM

mouse embryo fibroblast (STO cells) resistant to thioguanine and oubain (Martin and Evans, 1975; Hogan *et al*., 1994). The advantage of mouse embryonic fibroblast is that they provide a more consistent source of feeder cells. Early passage cells with reproducible characteristics must be used because they rapidly lose their ability to divide. Long-term propagation of STO cells can lead to changes that result in characteristics that are less favorable for embryonic stem cell growth. Because of these potential problems, researchers who are new to embryonic stem cell culture and who wish to generate embryonic stem cell clones capable of germline transmission will probably have more success with primary mouse embryonic fibroblasts. Mouse embryonic fibroblast cells form intercellular junctions such as gap junctions, adherens junctions and tight junctions with the embryonic stem cell culture (Ehmann *et al.*, 1998). Therefore, Ehmann *et al.* (1998) reported that the mouse embryonic fibroblasts providing physical support to assist in the attachment of the embryonic stem cells. Furthermore, mouse embryonic fibroblasts also secrete factors such as fibroblast growth factors (FGF) (Shimoaka *et al.*, 2002) and anti-apoptotic factors (Tseng *et al.*, 1996) that support the clonal growth and expansion of the embryonic stem cell culture. Mouse emberyonic fibrolbast cells will provide certain nutrients which not found in the medium (Fisher and Puck, 1956). The mouse embryonic fibroblast as feeder cell layer as well as providing growth factors to support the embryonic stem cell culture. Mouse embryonic fibroblast cells also function in the removal of toxicants from the culture medium (Rajabalian *et al.*, 2003). In 1972, Evans described the use of chick embryonic fibroblast as feeder cell layer for culture of embryonic carcinoma cells. Martin and Evans (1975) used a type of mouse embryonic fibroblast called STO cell lines to replace the use of chick fibroblast as feeder cell layer. Later, they were also used for murine embryonic stem cells culture (Evans and Kaufman, 1981; Martin, 1981).

2.8.4 Signaling Pathway in Embryonic Stem Cells

There are numerous defined and undefined signaling pathways involved in proliferation and self-renewal embryonic stem cells such as leukaemia inhibitory factor receptor–STAT3 **(**LIF-STAT), Wnt and bone morphogenetic proteins (BMP) signaling pathway. It is even more difficult to understand the cascade of control since the signal transductions are able to cross talk among other pathways. Therefore, the Wnt and bone morphogenetic proteins pathway is an ongoing effort. Only the leukaemia inhibitory factor receptor–STAT3 pathway has been defined in detail.

2.8.4.1 Leukaemia inhibitory factor (LIF) receptor (LIFR) – STAT3 pathway

Leukaemia inhibitory factor is also known as differentiation inhibitory factor (DIA). It secreted cytokine to inhibit the spontaneous differentiation of embryonic stem cells (Smith *et al*., 1988; Willium *et al*., 1988). Activation of the STAT3 pathway on engagement of the leukaemia inhibitory factor receptor is essential for embryonic stem cell self-renewal, and indeed under certain experimental systems STAT3 signalling seems to be sufficient for self-renewal (Matsuda *et al*., 1999). Recruitment and activation of STAT3 on engagement of the leukaemia inhibitory factor receptor is necessary for self-renewal of embryonic stem cells as interference in this process inhibits embryonic stem cell self-renewal (Niwa *et al*., 1998; Ernst *et al*., 1999). Leukaemia inhibitory factor bind with 2 part receptor complex, namely leukaemia inhibitory factor receptor and the glycoprotein-130 receptor. With this binding of leukaemia inhibitory factor will triggers the activation of the latent transcription factor STAT3 where in a necessary the continued proliferation of embryonic stem cells *in vitro* (Niwa *et al*., 1998; Burdon *et al*., 1999; Matsuda *et al*., 1999). The function of leukaemia inhibitory factor by binding to leukaemia inhibitory factor receptor at the cell surface, which causes it to heterodimerise with another transmembrane protein called

glycoprotein-130 (gp130). The dimerisation of STAT3 proteins will move into nucleus and activated the DNA for self-renewal. Besides that, the self-renewal of embryonic stem cells also influenced by SHP-2 and extracellular regulated kinase (ERK) activity. SHP-2 is a tyrosine phosphatase, an enzyme that removes phosphate groups to the tyrosine residues of various proteins and it interacts with the intracellular (amino terminus) domain of the gp130 receptor. The protein tyrosine phosphatase SHP-2 is recruited to the receptor where it associates with Gab1. This promotes the activation of Ras which initiates a cascade of transphosphorylations involving Raf and MAPK kinase (MEK) kinases resulting in the activation of extracellular regulated kinase (Takahashi-Tezula *et al*., 1998; Kolch, 2000). SHP-2 modulates embryonic stem cell self-renewal and differentiation via bi-directional regulation of the extracellular regulated kinase and STAT3 pathways. Extracellular regulated kinase is one kind of enzymes that become activated when the glycoprotein-130 receptor and other cell-surface receptors are stimulated. Extracellular regulated kinase can phosphorylate cytoplasmic proteins and be translocated to the nucleus where they can modulate the activities of transcriptional regulators. In embryonic stem cells, activation of this pathway on stimulation of the leukaemia inhibitory factor receptor is a pro-differentiation signal rather than contributing to self-renewal (Cheng *et al*., 1998; Burdon *et al*., 1999). Therefore, the addition of MEK inhibitors to growth medium can promote self-renewal by preventing the activation of this pathway leading to enhanced STAT3 signaling. Both extracellular regulated kinase and SHP-2 are components of a signal-transduction pathway that counteracts the proliferative effects of STAT3 activation (Figure 2.3). Therefore, if extracellular regulated kinase and SHP-2 are active, they inhibit embryonic stem cell self-renewal (Burdon *et al*., 1999). The STAT3 pathway is active in many cell types other than embryonic stem cells; embryos deficient in the LIF/STAT3 pathway develop normal inner cell mass; some murine embryonic stem cell lines do not require

leukaemia inhibitory factor, yet can still self-renew (Berger and Strum, 1997; Dani *et al*., 1998). This suggests that there is an underlying feature of embryonic stem cells that determines the embryonic stem cell phenotype that is independent of the STAT3 pathway (Figures 2.3 and 2.4).

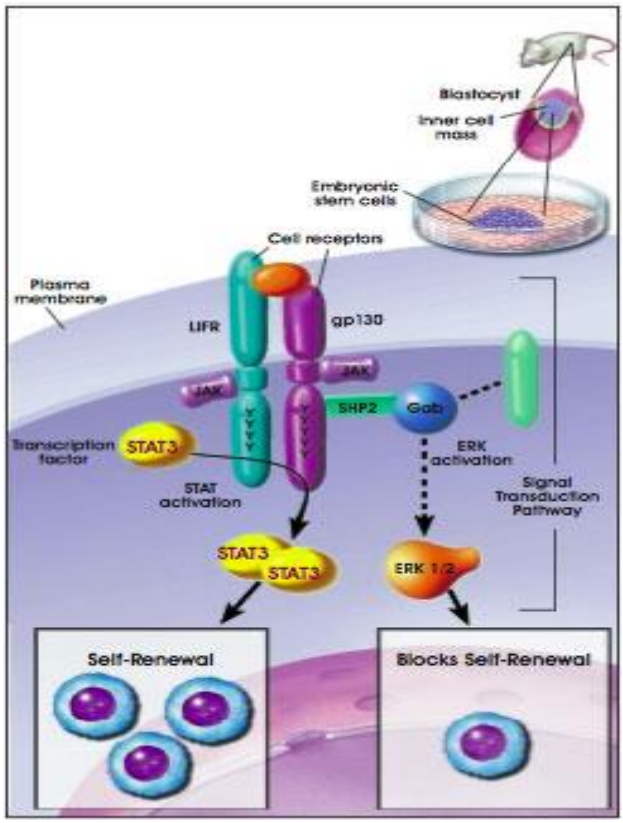

Figure 2.3: LIF–STAT3 signaling pathway control embryonic stem cells self renewal as well as in the mean time, it could inhibit extracellular regulated kinase (ERK) signaling which usually induce the differentiation of cell (Burdon *et al*., 1999).

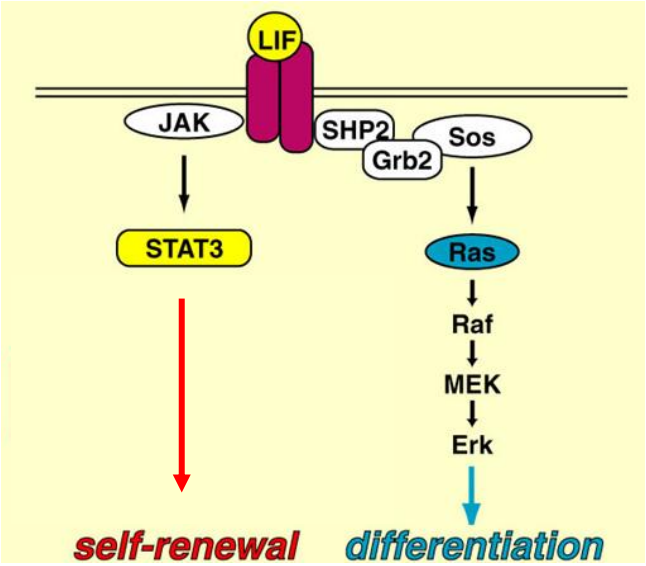

Figure 2.4: LIF-STAT3 signaling pathway control embryonic stem cells self renewal whereas extracellular regulated kinase (ERK) signaling which induce the differentiation of cell (Burdon *et al*., 1999).

2.8.4.2 Wnt signaling in embryonic stem cells

Activation of Wnt signal transduction pathways upon ligand binding can regulate diverse processes including cell proliferation, migration, polarity, differentiation and axon outgrowth. According to Cadigan and Nusse (1997), Wnt proteins are very important regulators of cell proliferation and differentiation. Wnt molecules will secrete lipid modified signaling proteins that bind to Frizzed receptors in the cell surface (Figure 2.4). Wnt signaling pathways involves proteins that directly participate in both cell adhesion and gene transcription. Several cytoplasmic components transduce the signal to beta-catenin, which enter the nucleus and forms a complex with a high mobility group (HMG) box containing DNA binding protein such as TCF (T cell factor) and lymphoid enhancer factor (LEF). The beta-catenin is a central player of the canonical Wnt signaling pathway, which is degraded in the absence of Wnt in cytoplasm.

In the presence of Wnt signaling, Dishevelled (Dsh) becomes activated which leads to the uncoupling of beta-catenin from the degradation pathway by inhibition of

GSK-3beta activity. This caused the accumulation of beta-catenin, which enters the nucleus and interacts with partners such as TCF or LEF. Therefore, stabilisation of beta-catenin and its accumulation in the cytoplasm is a crucial step in canonical Wnt dependent target gene expression (Eisenmann, 2005; Paratore and Sommer, 2006). Currently, Miyabayashi *et al*. (2007) reported that Wnt/beta-catenin has been considered to maintain long-term pluripotency state of embryonic stem cells. The large-scale gene expression profiling studies of undifferentiated murine and human embryonic stem cells found that the main components of the canonical Wnt signaling pathways are expressed (Aubert *et al*., 2002; Sato *et al*., 2003) and the activation of Wnt signaling pathway could inhibit embryonic stem cells from being differentiated. Recent studies have been showed that activation of Wnt signaling pathway using either a specific pharmacological inhibitor of glycogen synthase kinase 3 (GSK03), 6-bromoindirubin-3-oxime (BIO), (Sato *et al*., 2005) or a small molecule, IQ 1, (Miyabayashi *et al*., 2007) or a purine derivative, QS11, (Zhang *et al*., 2007) is sufficient to maintain embryonic stem cell at undifferentiated state of by working through different down stream targets of Wnt signaling pathway (Figure 2.5).

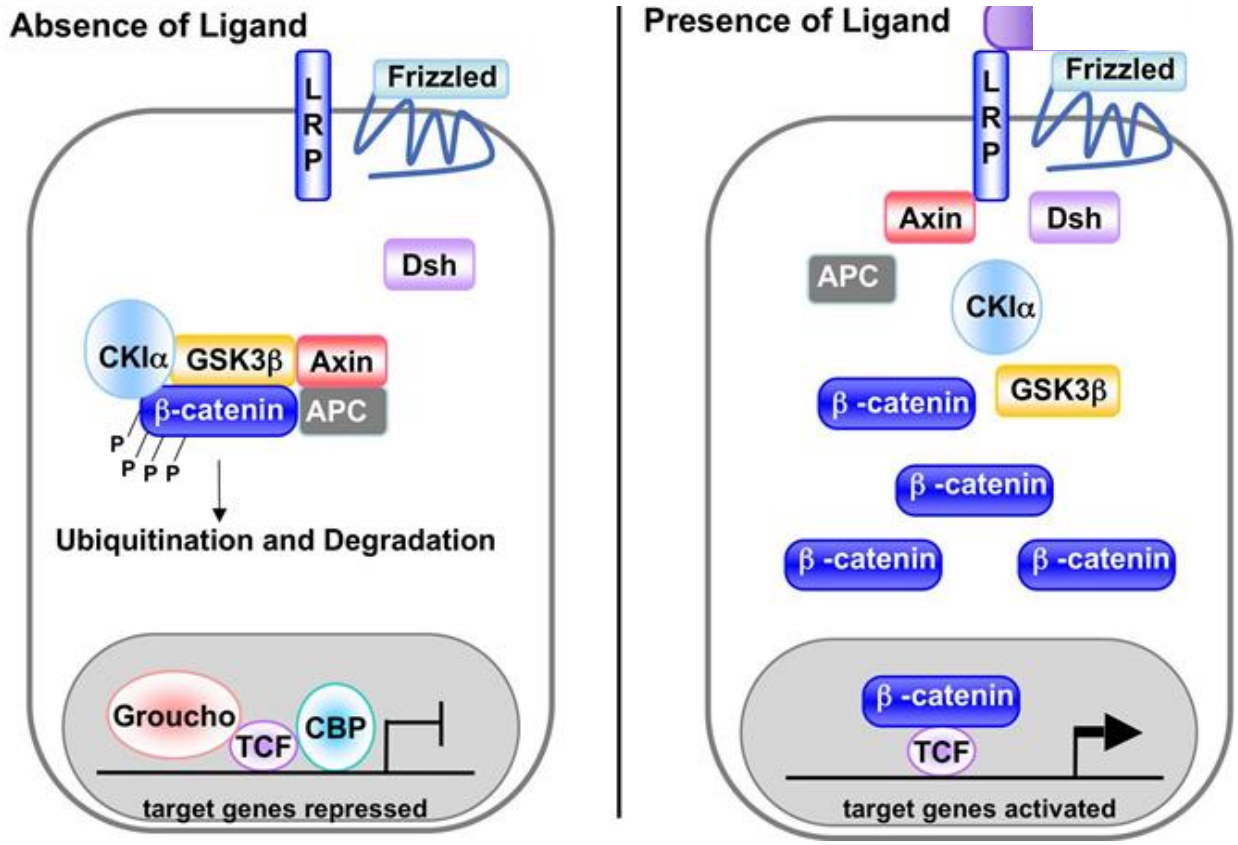

Figure 2.5: A canonical Wnt signaling pathway. Eisenmann (2005) described the Wnt mechanism where without a signal, action of the destruction complex (CKIα, GSK3β, APC, Axin) creates a hyperphosphorylated β-catenin, which is a target for ubiqitination and degradation by the proteosome. Wnt ligand bind to a Frizzled receptor complex leads to stabilisation of hypophosphorylated β-catenin, which interacts with TCF or LEF proteins in the nucleus to activate transcription.

2.8.4.3 Bone morphogenetic proteins (BMP) signaling in embryonic stem cells

Leukaemia inhibitory factor alone is insufficient to prevent murine embryonic stem cells from differentiating into other cell types and also not sufficient to support self-renewal of human embryonic stem cell. Bone morphogenetic proteins might support embryonic stem cell self-renewal especially in serum-free culture environment. Ying and colleagues (2003) and Qi and colleagues (2004) reported that the combination of bone morphogenetic proteins (BMP) and leukaemia inhibitory factor is sufficient to support the self-renewal of murine embryonic stem cells. The effects of bone morphogenetic proteins on embryonic stem cells involve induction of inhibitor of differentiation (Id) proteins, and inhibition of extracellular receptor kinase and p38

mitogen-activated protein kinases (MAPK). Bone morphogenetic proteins initiate signalling from the cell surface by interacting with heterodimers of type I and type II serine-threonine receptors (Canalis *et al*., 2003). In embryonic stem cells the 2 signaling pathways initiated by leukaemia inhibitory factor and bone morphogenetic proteins act in combination and are highly controlled in order to sustain self-renewal. Cytoplasmic proteins called Smads are activated by phosphorylation, form heterodimers with Smad 4 and are translocated to the nucleus where they inhibit or activate target genes. According to Ying *et al*. (2003), bone morphogenetic proteins 4 has no direct effect on STAT3 signalling and it inhibit the extracellular regulated kinase pathway. The bone morphogenetic proteins signaling is parallel pathway to LIF/STAT3. According to Ying *et al*. (2003), bone morphogenetic proteins 4, transcripts for types I and II serine–threonine and GDF6 are found are found in undifferentiated embryonic stem cells. Inhibitor of differentiation (Id) genes encode negative bHLH (basic helix–loop–helix) factors and these proteins bind to ubiquitous helix–loop–helix (HLH) factors, the E proteins (Norton, 2000). The inhibitor differentiation proteins were shown to be the factors that directly involved in self-renewal as expression of Id1, Id2 or Id3 in the absence of bone morphogenetic proteins caused the cells to grow as well as when cultured with leukaemia inhibitory factor and bone morphogenetic proteins. It was also seen that serum induces inhibitor differentiation gene expression in embryonic stem cells (Ying *et al*., 2003). Therefore, the inhibitor differentiation proteins may act in an antidifferentiation manner by sequestering E proteins which normally partner pro-neural basic helix–loop–helix factors, such as Mash-1 which is expressed in embryonic stem cells (Jen *et al*., 1992; Ying *et al*., 2003). Bone morphogenetic proteins were chosen as it is an anti-neural factor in embryos. As embryonic stem cells grown without serum develop spontaneously into neural phenotypes (Ying *et al*., 2003) it was thought that an anti-neurogenic factor may play a role in maintaining the self-renewal phenotype. When

embryonic stem cells are cultured in bone morphogenetic proteins containing media in the absence of leukaemia inhibitory factor, the embryonic stem cells differentiate into mesoderm and hematopoietic cells (Johansson and Wiles, 1995; Ying *et al*., 2003). However, in conjunction with leukaemia inhibitory factor, bone morphogenetic proteins 4 or GDF6 acts to maintain pluripotency. The balance of those pathways to remain in a self-renewal state is needed since there are several signaling pathways which involved for maintaining embryonic stem cell in pluripotency state. Embryonic stem cells might begin to differentiate if the balance shifts (Figure 2.6).

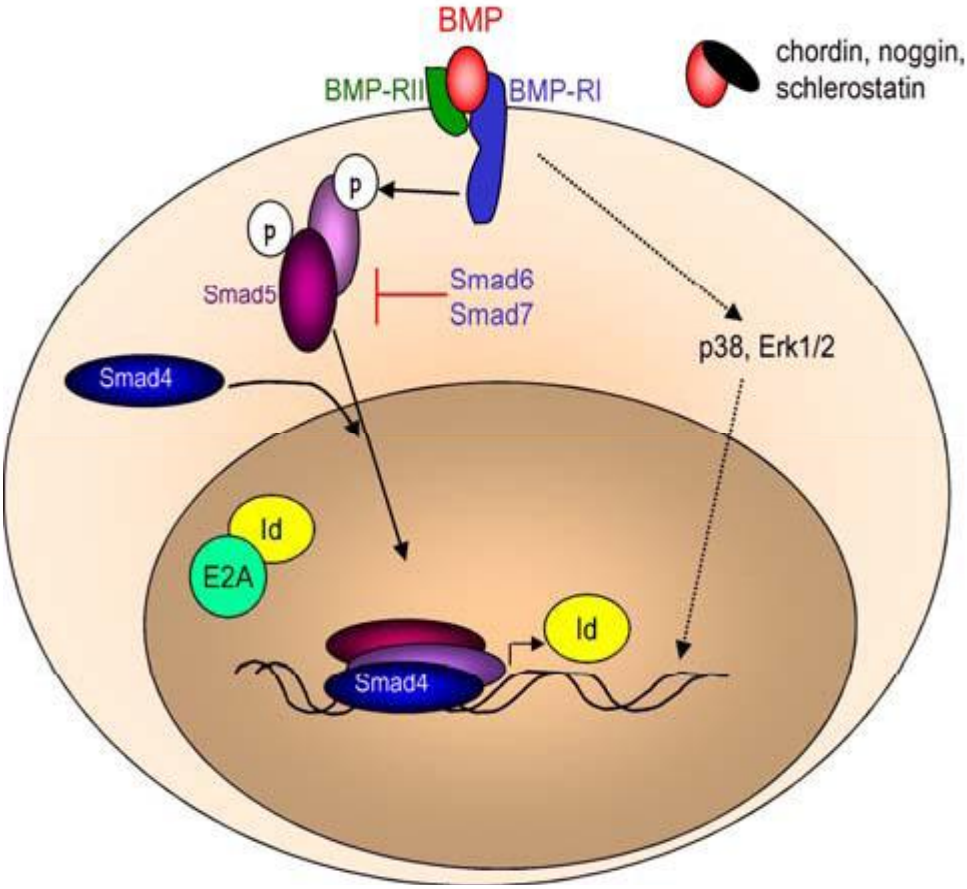

Figure 2.6: Bone morphogenetic proteins binds to bone morphogenetic proteins receptor type II (BMP-RII) that in turn activities bone morphogenetic proteins-RI (Ying *et al*., 2003; Qi *et al*., 2004).

2.8.4.4 Interaction between leukaemia inhibitory factor receptor (LIFR)-STAT3 and bone morphogenetic proteins signaling pathways

Similar to the situation of activation of the leukaemia inhibitory factor receptor, bone morphogenetic proteins binding with bone morphogenetic proteins receptor (BMPR) can activate both pro-self-renewal pathways, for example, inhibitor of differentiation gene activity and inhibitory pathways for self-renewal (for example, mitogen-activated protein kinases pathway). Thus, a balance may exist between STAT3 activation, SMAD activation and mitogen-activated protein kinases activity on stimulation of the respective receptors. Bone morphogenetic proteins acting through the SMAD pathway inhibits neurectoderm differentiation of embryonic stem cells, whilst leukaemia inhibitory factor activation of the STAT3 pathway blocks the bone morphogenetic proteins-induced endoderm and mesoderm differentiation that would occur in the absence of leukaemia inhibitory factor. Concomitant leukaemia inhibitory factor and bone morphogenetic proteins signalling through STAT3 and SMAD activation, helps to maintain pluripotency within the embryonic stem cell population. Interacting pathway between SMAD and STAT3 via formation of a tertiary complex between SMAD, STAT3, and the transcription factor p300, has been identified in neuroepithelial cells which reported by Nakashima *et al*. (1999). Ying *et al*. (2003) published data which showed that embryonic stem cells could be derived and maintained, even from single cells, in a serum-free medium supplemented with leukaemia inhibitory factor and bone morphogenic protein such as bone morphogenic protein 4 (Figure 2.7).

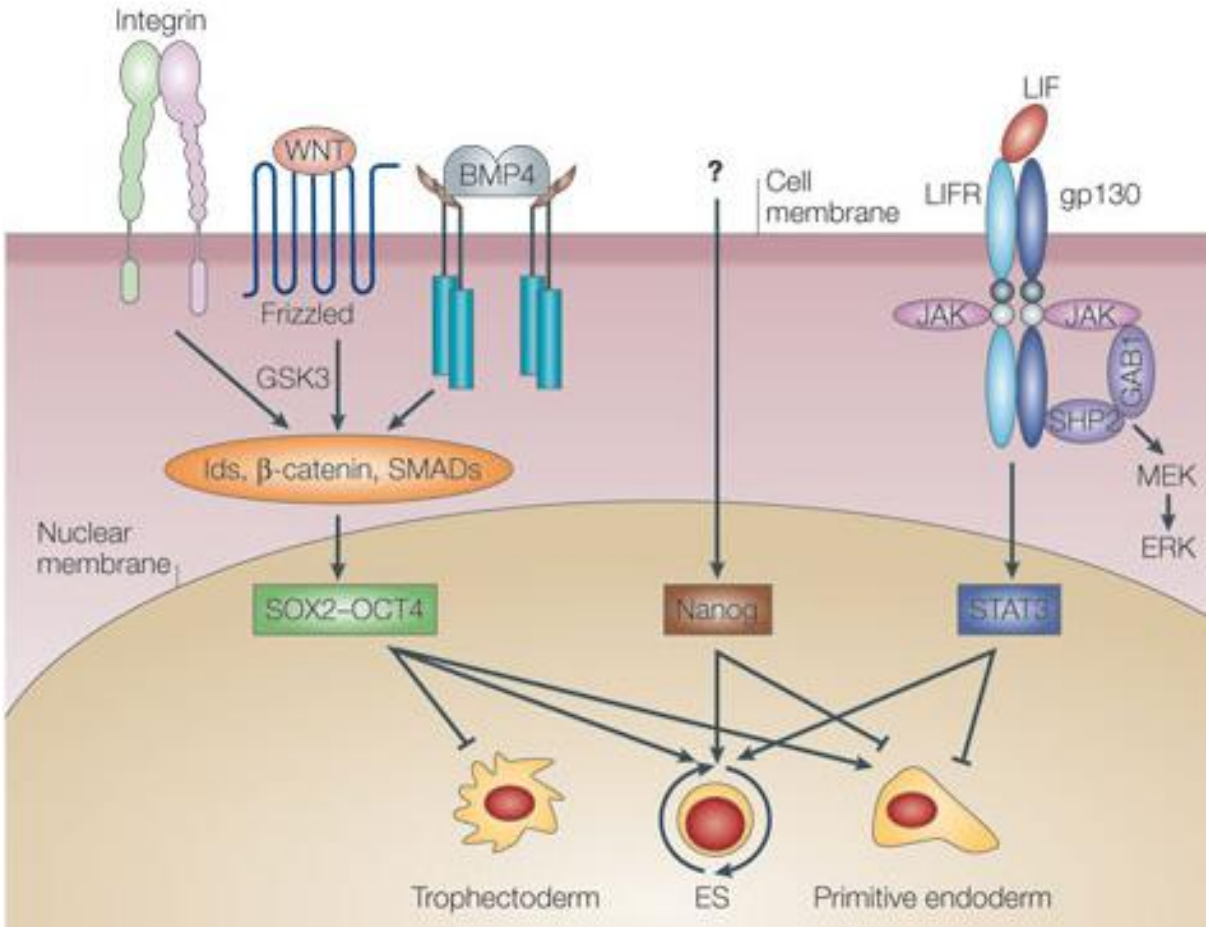

Figure 2.7: Interaction between luekaemia inhibitory factor receptor-STAT3 and bone morphogenetic proteins signaling pathways in controlling the self-renewal and maintaining the undifferentiated of embryonic stem cells (Boiani and Scholer, 2005).

2.8.4.5 The mitogen-activated protein kinase (MAPK)

Binetruy *et al*. (2007) reported that the mitogen-activated protein kinase family consist of mitogen-activated protein kinase 2 (MAPK2); extracellular regulated kinase 5(ERK5); c-Jun amino terminal kinases (JNK) 1, 2 and 3 as well as P38 mitogen-activated protein kinase (P38 MAPK) α, β, δ and γ, where each isoform is encoded by its own gene. The extracellular regulated kinase 1 or 2 (ERK1/2), c-Jun amino terminal kinases and P38 mitogen-activated protein kinase pathway were the most studied in stem cell research among the mitogen-activated protein kinase family (Binetruy *et al*., 2007). Besides involved in regulating apoptotic process, these kinases are important regulators of the proliferation and differentiation of the embryonic stem cells. Chung *et*

al. (2006) reported that the MEK-1 (I) has been used for deriving murine embryonic stem cells from embryonic blastomere, which was co-cultured with established murine embryonic stem cell. There are several mitogen-activated protein kinase inhibitors have been investigated for their roles in early embryo development, for example, MEK-1 (I) (Paliga *et al*., 2005; Chung *et al*., 2006) and P38 mitogen-activated protein kinase (I) (Natale *et al*., 2004; Maekawa *et al*., 2005; Paliga *et al*., 2005; Madan *et al*., 2006). Embryonic stem cell lines have been successfully established, but the role of MEK-1 (I) and embryonic stem cells co-culture on single blastomere development has not yet been determined. Maekawa *et al*. (2005) reported that P38 mitogen-activated protein kinase (I) has shown to have inhibiting effect on the development of trophectoderm cells in murine morula stage embryos, which may enhance the development toward the inner cell mass fate while trophectoderm is being suppressed.

2.8.4.6 Adrenocorticotropic hormone (ACTH) on embryonic stem cell establishment

Adrenocorticotropic hormone also known as corticotropin is a polypeptide hormone secreted and produced by the pituitary gland. Adrenocorticotropic hormone is also generates several other hormones in the pituitary gland such as proopiomelanocortin (POMC) (Bowen, 1998). Adrenocorticotropic hormone could induce the adenylyl cyclase (AC) activity (Dhanasekaran and colleagues, 1998). When the adenylyl cyclase activity is high, the cAMP level will also high since adenylyl cyclase converse ATP to cAMP. Currently, Faherty and colleagues (2007) studied about the role of cAMP/PKA pathway on murine embryonic stem cell self renewal where they suggested that using the forskolin, the adenylate cyclase agonist, could increase cAMP level. Therefore, high cAMP level could induce the embryonic stem cell self renewal but only if the culture medium did not supplemented with leulaemia inhibitory factor and foetal bovine serum (FBS).

2.9 EXPRESSION OF MARKERS IN UNDIFFERENTIATED, PLURIPOTENT CELLS OF EMBRYONIC STEM CELLS

Recently, stem cells have become the subject of extensive investigation where partly due to their therapeutic potential and because stem cells raise several fundamental issues concerning the regulation of proliferation and differentiation. Five widely adopted antibody markers, anti-Oct 4, anti-Sox 2, anti-SSEA 3, anti-TRA-1-60 and anti-TRA-1-81, are the protein marker that usually found on embryonic stem cells. Several transcription factors, including Oct 3/4 (Nichols *et al*., 1998; Niwa and Smith, 2000), Sox 2 (Avilion *et al*., 2003), and Nanog (Chambers *et al*., 2003; Mitsui *et a*l., 2003), function in the maintenance of pluripotency in both early embryos and embryonic stem cells. Several genes that are frequently upregulated in tumors, such as STAT3 (Matsuda *et al*., 1999; Niwa *et al*., 1998), E-Ras (Takahashi *et al*., 2003), c-myc (Cartwright *et al*., 2005), Klf4 (Li *et al*., 2005), and beta-catenin (Kielman *et al*., 2002; Sato *et al*., 2004), have been shown to contribute to the long term maintenance of the embrtonic stem cell phenotype and the rapid proliferation of embryonic stem cells in culture. In addition, it has been identified several other genes that are specifically expressed in embryonic stem cells (Maruyama *et al*., 2005; Mitsui *et al*., 2003). Oct 4 and Sox 2 are transcription factors highly expressed in undifferentiated embryonic stem cells and embryonic germ cells (Looijenga *et al*., 2003; Masui and Shinji, 2007; Zhao and Daley, 2008). Embryonic stem cells are continuous proliferating stem cell lines of embryonic origin first isolated from the inner cell mass. Two distinguishing features of embryonic stem cells are their ability to be maintained indefinitely in an undifferentiated state and their potential to develop into any cell within the body. Based on previous methods developed for murine embryonic stem cells, human embryonic stem cell lines were first established by Thomson and Marshal (1981). Like murine embryonic stem cells, human embryonic stem cells express high levels of membrane alkaline phosphatase (AP) and

Oct 4, a transcriptional factor critical to inner cells mass and germline formation. However, unlike murine embryonic stem cells, human embryonic stem cells do not express stage-specific embryonic antigen (SSEA 1). In addition, prolonged propagation of human embryonic stem cells is typically achieved by co-culture with primary mouse embryonic fibroblasts serving as feeder cells. Human embryonic stem cell lines are not able to maintain their undifferentiated state in the absence of supporting feeder layer cells, even when exogenous cytokines such as leukemia inhibitory factor and gelatin-coated plates are used.

The undifferentiated state of embryonic stem cells can be characterised by high level of expression of alkaline phosphatase (AP) (Pease *et al*., 1990), the expression surface markers including SSEA and TRA antigens and the transcription factor Octamer 4 (Oct 4). Undifferentiated state of the embryonic stem cells will express high level of the POU transcription factor Oct 4 (Pesce *et al*., 1998a). This relationship between Oct 4 and plurioptency has seen this transcription factor emerge as a marker of pluripotent stem cells. Undifferentiated murine and human pluripotent embryonic stem cells and embryonic carcinoma cells express Oct 4. Additionally, murine embryonic germ cells also known to express Oct 4. Following stem cell differentiation, the level of Oct 4 expression decreases. However, the Oct 4 protein itself is insufficient to maintain embryonic stem cells in the undifferentiated state. Few years later, Chamber and colleagues (2003) and Mitsui and colleagues (2003) identified another transcription factor, Nanog, that is essential for the maintenance of the undifferentiated state of mouse embryonic stem cells. The expression of Nanog decreased rapidly if murine embryonic stem cells differentiated. Cooperative interaction between Oct 4 and Sox 2 forms a heterodimer. This synergistic interaction between the 2 drives transcription of target genes such as Nanog which is a transcription factor that functions in maintaining the pluripotent cells of the inner cell mass and in deriving embryonic stem cells from

these cells (Rodda *et al*., 2005). So, the set of Oct 4, Sox 2 and Nanog expression is considered as transcription factors involved in maintaining the pluripotent embryonic stem cell phenotype.

Pluripotent embryonic stem cells can be characterised by high level expression of Oct 3/4, (also termed Oct 3 or Oct 4) a member of the POU transcription factors, and Nanog. A critical amount of Oct 3/4 and Nanog expression is required to sustain stem cell pluripotency. When embryonic stem cells are induced to differentiate, Oct 3/4 and Nanog are down-regulated, which has proven to be essential for a proper and divergent developmental program (Solter and Knowles, 1978; Smith *et al*., 1988; Pease *et al*., 1990; Shamblott *et al*., 1998; Thomson and Marshall, 1998). Pluripotent stem cells can also be characterised by the expression of a number of cell surface antigens. SSEA 1, a carbohydrate antigen, is a fucosylated derivative of type-2 polyactosamine and appears during late cleavage stages of murine embryos. It is strongly expressed by undifferentiated, murine embryonic stem cells (Solter *et al*., 1978; Gooi *et al*., 1981). Undifferentiated, human embryonic stem cells also express the keratin sulphate-associated antigen, TRA-1-60 and TRA-1-81 (Andrews *et al*., 1984; Banting *et al*., 1984).

2.9.1 Octamer 4 (Oct 4)

In embryonic stem cells, a critical level of expression of Oct 4 is essential and important in maintaining embryonic stem cell renewal (Niwa and Smith, 2000). Less than a 2 fold increase causes embryonic stem cells to differentiate into mesoderm or endoderm, while reduction to less than 50% expression levels causes differentiation into trophectoderm. A classical marker of undifferentiated embryonic stem cells is expression of the POU class transcription factor Oct 4 gene. Oct 4 is very essential for embryo development throughout blastocyst stage (Nichols *et al*., 1998). Its expression is restricted to

totipotent and pluripotent cells of the mouse (Pesce *et al*., 1998a) and is down-regulated in the majority of adult tissue excluding the germ line (Teom *et al*., 1996). Normally, Oct 4 is express in all blastomeres up to morula stage then Oct 4 expression is more restricted in inner cell mass cells and very low express in trophectoderm cells. The establishment of embryonic stem cells in domestic species is the capability to identify truly pluripotent cells are limited. Henderson *et al*. (2002) reported that stem cell marker gene expression is generally limited to the inner cell mass and is not observed in the trophectoderm in murine and human blastocyts. Hence, the best approach is to use a panel of markers because any one marker may not be truly definitive. In the past, researchers have depended on only a few markers, such as Oct 4 (POU5F1), a single stage-specific embryonic antigen (for example, SSEA 1) or alkaline phosphatase (AP), without a clear understanding of the expression of these gene markers in embryos of different species. By using alone of the marker may not be sufficient as they are not specific to the inner cell mass in ungulates. As an example, different laboratories have now shown that Oct 4 protein is expressed in both the inner cell mass and trophectoderm of ungulates, such as porcine, bovine and caprine (van Eijk *et al*., 1999; Kirchhof *et al*., 2000; He *et al*., 2004). He *et al*. (2006) found that SSEA 1 and SSEA 4 genes are expressed by both inner cell mass and trophectoderm of caprine embryos. Similarly, the alkaline phosphatase gene appears to be expressed at different times by either epiblast or trophectoderm (Talbot *et al*., 1995; Vejlsted *et al*., 2005). Therefore, these markers should be considered characteristic of embryonic stem cells, but may not be used as definitive markers. Depending on the study, comparisons were made between either differentiated versus undifferentiated embryonic stem cells, murine versus human embryonic stem cells, or adult compared with embryonic stem cells (Bhattacharya *et al*., 2004; Ginis *et al*., 2004; Wei *et al*., 2005). By using microarrays, Sperger *et al*. (2003) showed the genes POU5F1 (Oct 4) and FLJ10713 (a homolog

highly expressed in murine embryonic stem cells) (Ramalho-Santos *et al*., 2002) are highly expressed in both the pluripotential human embryonic stem cell and embryonal carcinoma cells in seminomas. Apart from being a marker, Oct 4 is a key factor that regulates self-renewal of embryonic stem cells. Nichols *et al*. (1998) showed by targeted gene deletion the role of Oct 4 *in vivo* is to establish the pluripotent nature of cells within the inner cell mass. Oct 4 is not a direct target gene of STAT3 and therefore its expression is not directly regulated by leukaemia inhibitory factor. When Oct 4 is constitutively expressed within embryonic stem cells it does not prevent differentiation of embryonic stem cell induced by leukaemia inhibitory factor withdrawal (Niwa *et al*., 2000). Therefore, expression of Oct 4 alone is not sufficient to maintain pluripotency, but rather also needs the cytokine-induced action of STAT3. Oct 4 is thought to function in several ways within embryonic stem cells. If Oct 4 expression is inhibited in cultured embryonic stem cells, the cells generate trophectoderm (Niwa and Smith, 2005) whereas Oct 4 expression level is artificially increased, embryonic stem cells would differentiate into primitive endoderm and mesoderm. Therefore, the level of Oct 4 expression dictates a significant aspect of the developmental program of embryonic stem cell. Oct 4 can also repress or active other target genes by cooperating with various transcriptional co-factors. Sry-related factor Sox 2 (Avillion *et al*., 2003) and the Forkhead Box family member, FoxD3 are the 2 co-factors thought to play a key role in self-renewal (Hanna *et al*., 2002). Both co-factors cooperatively function with Oct 4 (Guo *et al*., 2002; Yuan *et al*.,1995). Oct 4 transcription factor has been used as one of the pluripotency markers in embryonic stem cells (Hay *et al*., 2004). Recent knowledge about mechanism of Oct 4 to maintain pluripotent state is not yet been clearly identified but it is possible that some of the components of signaling pathways in cultured mouse embryonic stem cells such as adaptor protein, Gab1, may suppress interactions of specific receptors to the Ras-ERK signaling pathway. Furthermore, the expression of

this altered form of Gab1 may be promoted by the transcription factor Oct 4. In murine embryonic stem cells, Oct 4 expression and increased synthesis of Gab1 may help suppress induction of differentiation by suppress extracellular regulated kinase down stream signaling pathway result in the inhibition of differentiate.

2.9.2 Nanog

Nanog usually detected in the morula stage, inner cell mass and early germ cells and in the proximal epiblast in the region of the presumptive primitive streak (Mitsui *et al*., 2003; Hart *et al*., 2004). It is expressed slightly later than Oct 4 during murine ontogeny. Nanog plays a role at the second stage when the primitive endoderm is formed. Nanog is specially expressed in embryonic stem cells, embryonic carcinoma cells and embryonic germ cells, but is not expressed in hematopoietic stem cells, adult tissues or differentiated cells (Chambers *et al*., 2003; Mitsui *et al*., 2003). During development in Nanog-deficient murine, the cells of the inner cell mass spontaneously differentiate into visceral and parietal endoderm (Mitsui *et al*., 2003). Oct 4 and Nanog act together during embryonic stem cell self-renewal with Nanog being downstream of Oct 4. Nanog does appear to be a specific marker of pluripotency for ruminants because Nanog mRNA and protein are found in the inner cell mass and strongly down-regulated in the trophectoderm of caprine blastocysts (He *et al*., 2005; 2006). Chambers *et al*. (2003) and Mitsui *et al*. (2003) described a divergent homeobox transcription factor; Nanog, which promotes embryonic stem cell self-renewal, pluripotency and epiblast formation. According to Chambers *et al*. (2003), Nanog and STAT3 signalling are not directly related. However, when leukaemia inhibitory factor was added to Nanog overexpressing cells it enhanced their proliferation. Thus self-renewal via the Nanog pathway acts in a STAT3 independent manner.

2.9.3 Sox 2

Sox 2 is a member of the Sox gene family that encode transcription factors with a single high mobility group (chromosomal proteins where known nuclear proteins which specifically bind to the nucleosome core particle and are implicated in the generation and/or maintenance of structural features specific to active chromatin) DNA-binding domain. Several lines of evidence indicate that Sox 2 may act to maintain or preserve developmental potential. For example, Sox 2 expression is associated with uncommitted dividing stem and precursor cells of the developing central nervous system (CNS) and indeed can be used to isolate such cells (Li *et al*., 1998; Zappone *et al*., 2000). Avilion *et al*. (2003) reported that Sox 2 is highly expressed in embryonic stem cells and is essential in pluripotentiality. Sox 2 belongs to the Sox B1 subgroup, which also includes Sox 1 and Sox 3, based on homology within and outside the HMG box (Kamachi *et al*., 2000). Sox 2 also marks the pluripotent lineage of the early murine embryo, so that like Oct 4 it is expressed in the inner cell mass, epiblast, and germ cells. Sox 2 was found in the inner cell mass at blastocyst stages, in which the protein was primarily nuclear as shown by antibody staining on isolated inner cell mass. The intact blastocysts also revealed the presence of Sox 2 in trophectoderm, but here the protein was only cytoplasmic. Oct 4 was seen in the layer of cells adjacent to the blastocoel, presumed to be primitive endoderm (Palmieri *et al*., 1994; Nichols *et al*., 1998), and absent from trophectoderm, unlike Sox 2. This suggests parallel roles for the 2 factors in the maintenance of pluripotentiality (Pesce and Scholer, 2000). The phenotypic consequences of the targeted disruption of Oct 4 reveal that it is required for the first cell fate decision, as all mutant inner cell mass cells are diverted to a trophectoderm fate (Nichols *et al*., 1998). The zygotic Sox 2 expression is also required for the inner cell mass/epiblast lineage, but this is not revealed until after implantation, presumably when maternal Sox 2 protein becomes diluted out through the rapid growth of the embryo. In

the absence of Sox 2, there is no equivalent rapidly proliferating cell population, the only surviving cells being trophoblast giant cells. Alternatively, Sox 2 could be essential for epiblast cell identity, and in its absence the cells could differentiate into another cell type.

2.9.4 Stage-specific embryonic antigen (SSEA 1, SSEA 2, SSEA 3 and SSEA 4)

There are several types of stage-specific embryonic antigen (SSEA) that found in pluripotent of embryonic stem cells, namely SSEA 1, SSEA 3 and SSEA 4. SSEA 1 is a globoseries carbohydrate antigen present on the surface of murine embryonic stem cells, but not human embryonic stem cells (Kohji *et al*., 2008). It was localised on the stem cells of differentiating solid teratocarcinomas and on the surface of core cells of solid embryoid bodies. At the egg cylinder stage the antigen is restricted to embryonic ectoderm and visceral endoderm. During subsequent development, SSEA 1 becomes localised to portions of the brain and primordial germ cells. In adult murine, the epithelium of the oviduct, the endometrium, and the epididymis are the cells most reactive with the monoclonal antibody to SSEA 1; although some areas of the brain and kidney tubules are weakly positive. SSEA 3 and SSEA 4 is a globoseries carbohydrate antigen present on the surface of human embryonic stem cell, but not murine embryonic stem cell (Solter and Knowles, 1978; Przyborski, 2001). Upon differentiation, murine embryonic stem cells are characterised by the loss of SSEA 1 expression and may be accompanied, in same instances, by the appearance of SSEA 3 and SSEA 4 (Solter *et al*., 1979). In contrast, human embryonic stem cells and embryonic carcinoma cells typically express SSEA 3 and SSEA 4 but not SSEA 1, while their differentiation is characterised by down regulation of SSEA 3 and SSEA 4 and an up regulation of SSEA 1 (Andrews 1984; Andrews *et al*., 1987; Fenderson *et al*., 1987).

2.9.5 Alkaline phosphatase (AP)

Alkaline phosphatase is a stem cell membrane marker. Elevated expression of alkaline phosphatase is associated with pluripotent status (Thomson *et al*., 1998). Alkaline Phosphatase is an enzyme in the blood, intestines, liver, and bone cells and exists as membrane-bound isoforms of glycoproteins sharing a common protein structure but differing in carbohydrate content. These enzymes are most active at alkaline pH-hence the name (Thomson *et al*., 1998). Undifferentiated human embryonic stem cells, embryonic carcinoma cells and embryonic germ cells have been shown to express a very high level of the liver/bone/kidney isozyme of alkaline phosphatase (Millan and Fishman, 1996; Thomson *et al*., 1995; Shamblott *et al*., 1998; Draper *et al*., 2004). Expression levels of alkaline phosphatase decrease following stem cell differentiation.

2.10 TIMELINE OF EMBRYONIC STEM CELLS

Table 2.3 shows the timeline of significant findings in murine and caprine embryonic stem cell.

Table 2.3: Timeline of significant findings in murine and caprine embryonic stem cell

Year	Author	Species	Significant finding
1981	Evans and Kaufman	Murine	First isolation and establishment in culture of pluripotent murine embryonic stem cells (mESC).
1981	Martin *et al*.	Murine	First successful *in vitro* culture of murine embryonic stem cell (mESC).
1984	Bradley *et al*.	Murine	Production of chimaeric murine using embryonic stem cells.
1985	Doetschman *et al*.	Murine	Consistent *in vitro* differentiation of murine embryonic stem cells (mESC) into various cells.
1986	Robertson	Murine	Murine embryonic stem cells (mESC) can enter the germ line.
1987	Suemori and Nakatsuji	Murine	Primary mouse embryonic fibroblasts (MEF) are better than STO cells as feeder layer for establishment of murine embryonic stem cells (mESC).
1988	Smith *et al.*	Murine	Identification of leukaemia inhibitory factor (LIF) as being crucial for maintaining the pluripotency of murine embryonic stem cells (mESC) *in vitro*.
1988	Williams *et al*.	Murine	Feeder layer releases leukaemia inhibitory factor (LIF) which inhibits differentiation of murine embryonic stem cells (mESC).

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1993	Maltsev *et al.*	Murine	Induced differentiation of murine embryonic stem cell (mESC) into various cardiomyocyte types.
1994	Kawase *et al.*	Murine	There are variations in embryonic stem cell (ESC) derived from different strains of murine.
1995	Bain *et al.*	Murine	Induced differentiation of murine embryonic stem cell (mESC) into neurons.
1997	Dani *et al*.	Murine	Induced differentiation of murine embryonic stem cell (mESC) into adipocytes.
2000	Munsie	Murine	Establishment of nuclear transfer of embryonic stem cell (ntES) lines via somatic cell nuclear transfer (SCNT).
2001	Buttery *et al*.	Murine	Induced differentiation of murine embryonic stem cell (mESC) into osteoblasts.
2001	Lumelsky *et al.*	Murine	Induced differentiation of murine embryonic stem cell (mESC) into pancreatic islets.
2005	Tesar	Murine	Murine embryonic stem cell (mESC) can be derived using embryos from 4-cell stage onwards.
2006	Yang *et al*.	Caprine	Mechanical cutting with or without the aid of enzyme digestion was an effective method for passaging caprine embryonic stem cell colonies.
2007	Hashemi-Tabar *et al.*	Murine	Differentiation of murine embryonic stem cell (mESC) depends on the dose of inhibitory factors.

(Continued)

(Continued)

2007	Jia *et al*.	Caprine	Production of chimaeric caprine derived by injecting caprine embryonic germ cells into host blastocyst.
2007	Wakayama *et al.*	Murine	Polar bodies can be used to derive murine embryonic stem cell (mESC).
2007	Masui *et al*.	Murine	Sox 2 regulates Oct 3/4 expression to maintain the pluripotency of murine embryonic stem cell (mESC).
2009	Pawar *et al*.	Caprine	Successfully established caprine embryonic stem cell-like outgrowths from *in vitro* produced caprine blastocyst.

2.11 TIMING OF ISOLATION AND INITIATION OF BLASTOCYST PRIMARY CULTURES

The optimal time for the initiation of blastocyst primary cell cultures for establishing ungulate embryonic stem cell lines is not known. The blastocysts of ungulates have an extended period of preimplantation development compared to murine and human. The blastocysts of the porcine, ovine and bovine first develop at approximately 6 to 7 days post-fertilisation. Then, the blastocyst increases in size relatively slowly over the next few days of development as a spherical form that increases in diameter (Chang, 1952). During this time there is probably only a modest increase in the number of epiblast cells in the inner cell mass of the blastocyst compared with the increase in trophectoderm and visceral endoderm cells (Carlson, 1981). At 11 to 12 days post-fertilisation, depending on the species, the blastocyst elongates by the rapid growth of the trophectoderm and visceral endoderm to form a long, thin, filamentous blastocyst greater than 100 mm in

length (Chang, 1952; Carlson, 1981). The epiblast is exposed to the uterine environment during this preimplantation development by the loss of the overlaying layer of trophectoderm cells, or Rauber's layer, and the inner cell mass is thereafter referred to as the embryonic or germinal disc (Stroband *et al*., 1984). Gastrulation, marked by the formation of the primitive streak in the embryonic disc, begins during this elongation phase and at this point mesoderm differentiation and migration from the epiblast is well underway (Talbot *et al*., 2000b; Guillomot *et al*., 2004). What point in the preimplantation development of ungulate blastocysts is best for the isolation and establishment of embryonic stem cell lines? Previous report have been used whole blastocyst culture of *in vivo* produced porcine embryos that were judged to be early blastocysts, having just emerged from the zona pellucida or blastocysts that had "hatched" from the zona pellucid some hours earlier (Chen *et al*., 1999). Early hatched blastocysts had many fewer trophectoderm cells and a less flattened inner cell mass. The success rate for the establishment of porcine embryonic stem-like cell cultures was decidedly greater [12 cultures (21%) versus none] from recently hatched blastocysts than from late hatched blastocysts (Chen *et al*., 1999). Three of the resulting cultures were composed of alkaline phosphatase positive cells, a marker of the undifferentiated state, and were purported to differentiate into multiple cell types *in vitro*, although no supporting evidence of this was shown. Another report used *in vivo* pig blastocysts from days 5 to 6 or 10 to 11 of gestation and found that days 10 to 11 blastocysts yielded embryonic stem-like cell cultures. Few or none were propagated from days 5 to 6 embryos or day 11 blastocysts that had elongated (Hochereau-de Reviers and Perreau, 1993). STO feeder cell layer were required for the survival of porcine and bovine epiblast cells in primary culture (Talbot *et al*., 1993, 1995). Without feeder cell support, cultures of primary porcine epiblast cells failed to grow, senesced and died over a 10 to 14 days period (unpublished observations). Similar results were reported with feeder

free, short term, primary cultures of porcine inner cell mass, with or without the addition of leukemia inhibitory factor to the medium (Moore and Piedrahita, 1997). It is probable that ungulate embryonic stem cell line establishment will therefore require feeder cells, at least in their initial culture, as has been true for the establishment of most murine and primate embryonic stem cell lines. Although for both murine and human embryonic stem cell derivation STO feeder cell layer have been used successfully (Martin, 1981; Park *et al*., 2003), the use of primary or early secondary foetus derived feeder cell is often thought to be an advantage. Primary feeder cell populations presumably supply different kinds and amounts of factors for the maintenance and growth of embryonic stem cells compared with STO feeder cell layer. Moreover, the use of homologous primary feeder cells, for example, bovine fibroblast for bovine epiblast culture, would presumably remove the potential problem of species specificity that exists with some cell ligand or cell receptor systems. However, primary foetal fibroblasts of the bovine would be expected to produce bovine leukaemia inhibitory factor and basic fibroblast growth factor (bFGF), and most likely several other growth factors or cytokines, did not maintain bovine epiblast cells in the undifferentiated state in our experience (Keefer *et al*., 2007). Likewise, caprine foetal fibroblast could in some cases maintain caprine embryonic stem-like cells during short term culture but usually stimulated these cells to differentiate rapidly (Keefer *et al*., 2007). It should be hoped that through further research it might be possible to replace feeder-dependent culture conditions for establishment of ungulate embryonic stem cell lines as has been done in murine and human (Pease *et al*., 1990; Xu *et al*., 2001; 2005; Wang *et al*., 2005).

2.12 INNER CELL MASS ISOLATION TECHNIQUES

Inner cell mass isolation technique is an important step of the embryonic stem cell establishment successiveness. In intact embryo, trophoblastic cells induce inner cell mass differentiation to be 3 embryonic germ layers by suppress Oct 4 and Nanog expression level (Roberts *et al*., 2004). Therefore, completely removed trophoblastic cells provide more benefit to inner cell mass cells turn to be embryonic stem cells. The inner cell mass could be separated and isolated from the trophectoderm by 5 ways namely immunosurgery (Solter and Knowles, 1975), with mechanical processes (Bongso *et al*., 1994), with whole embryo culture of the blastocysts and partial embryo culture methods (Kim *et al*., 2005) or single blastomeres (Chung *et al*., 2006) and laser dissection (Turetsky *et al*., 2008).

2.12.1 Mechanical Dissection

The inner cell mass of the blastocyst was isolated by using a specially made flexible metal needle, made of tungsten, with a diameter of 0.125 mm (Susanne *et al*., 2007) where the tip was made thin and sharp using electrolysis. Another blunter needle was used to hold the blastocysts while cutting out the inner cell mass. According to Susanne *et al*. (2007), the needles were fixed to hand-pieces of pencil-thickness for manual operation under a stereomicroscope. The blastocyst was first cleaned in 3 or 4 drops of culture medium and the last drop were then drawn out with the needle so that the blastocyst became attached to the surface of the well. With the blastocyst attached to the plastic, it was possible to make a hole in the zone pellucida with the needle to open up the blastocyst and by 2 to 3 cuts removing the inner cell mass from the trophectoderm and place the inner cell mass onto a fresh feeder plate. The procedure took about 2 to 3 minutes. This technique is applied manually, and it does not require any expensive equipment. Mechanical isolation of the inner cell mass using a simple 2 needle system

proved successful. The isolation procedure was fast, and it did not require any major financial or time investments. The inner cell masses were isolated from hatched blastocysts using mechanical and enzymatic isolation techniques. Mechanical isolation was performed using 2 fine glass needles under stereozoom microscope (Olympus, SZ61) as described by Verma *et al*. (2007). Immunosurgery is not the optimal derivation method when poor quality, discarded embryos with hardly visible inner cell mass are used. By using mechanical isolation or dissection method, we could avoid the use of murine antibodies and guinea pig complement, and it was not necessary to use pronase or Tyrode's acid, as the zona pellucid could be cut away at the same time. The procedure was therefore also time-saving. The efficient, successful derivation of 5 cell lines from 19 blastocysts appeared to be better than using immunosurgery (16 cell lines from 100 blastocysts) in similar culture medium (Inzunza *et al*., 2005). However, it was not possible to carry out a comparative study and many other factors may have influenced the improved results in the latest series of derivations. Mechanical isolation of the inner cell mass has previously been successfully used in the derivation of 2 cell lines, as reported by Genbacev *et al*. (2005) and by Mummery, (2004) and van de Stolpe *et al*. (2010). The properties of inner cell mass and trophectoderm have been extensively studied after their microsurgical separation (Gardner and Papaioannou, 1975; Rossant, 1975a, b; Van Blerkom *et al*., 1976). However, the difficulty of the microsurgical procedure limits the rate at which blastocysts can be dissected. Furthermore, some inner cell mass cells may become damaged due to the use of needles to pull the inner cell mass away from the overlying trophectoderm. The embryo tended to rotate away from the advancing blade when it was cut towards one pole. This could be prevented by increasing suction or by using a needle to help immobilise the embryo. The choice of well expanded blastocysts enabled trophoblast to be obtained with little risk of inclusion of inner cell mass cells.

2.12.2 Immunosurgery

There are 4 inner cell mass isolation methods that include immunosurgery (Solter and Knowles, 1975), whole embryo culture, partial embryo culture (Kim *et al*., 2005) and laser dissection (Turetsky *et al*., 2008). Since trophoblast inhibits proliferation of inner cell mass, the isolation of pure inner cell mass with minimal contamination of trophoblast. Trophoblastic cells have tendency to grow faster than the inner cell mass representing a frequent source of contamination and often a stimulus to differentiation. Solter and Knowles (1975) have been established immunosurgery method to isolate inner cell mass from murine blastocyst and it is now used in many research groups for the same scope (Thomson *et al*., 1995, 1998; Dattena *et al*., 2006). The isolation of the inner cell mass prior to plating represents a preventive measure towards contamination and unplanned differentiation. Immunosurgery is based on destruction of trophoblast by reaction of antibody and complement. However, these reagents may contain animal pathogen and molecules (Martin *et al*., 2005). Therefore, this is a drawback of immunosurgery for future clinical application of embryonic stem cells. Kim *et al*. (2005) compared immunosurgery, partial inner cell mass isolation method and whole blastocyst seeding method using human *in vitro* fertilised derived blastocysts. Solter and Knowles (1975) described the immunosurgery methods, the zona pellucida was first removed from cultured blastocysts (3.5 to 4.0 days post-coitus) using pronase (0.5%) for 5 minutes. Before exposure to antiserum, blastocysts were incubated at 37 ℃ in CO_2 (5%) in incubator for 30 minutes. Then, the zona-free blastocysts were then exposed to 1:100 dilution of rabbit anti-mouse serum (Sigma) for 30 minutes and washed 3 times with PBS for 5 minutes. Washed blastocysts were then transferred to guinea pig complement (Sigma) at 37 ℃ in CO_2 (5%) for 20 minutes. The blastocysts were then washed and each inner cell mass is transferred into a single droplet of trypsin/EDTA (0.05%) solution and incubated for 5 minutes to allow disaggregation of the inner cell

mass cells. Peripheral lysing cells were detached by active pipetting after addition of complement to the damaged trophoblastic layer. Intact expanded blastocysts, hatching, or hatched; zona-free blastocysts, and inner cell mass isolated by immunosurgery were plated on mouse embryonic fibroblasts and cultured in a chemically defined media with 10% serum substituted enriched with recombinant LIF (1000 U/ml). Immunosurgery is a conventional efficient method to isolate inner cell mass cells from blastocyst but it cannot be applied efficiently to a low quality primate cloned blastocyst (Kim *et al*., 2005; Byrne *et al*., 2007). Solter & Knowles (1975) study the purity of recovered inner cell mass cells was assessed by the failure to detect trophoblastic-type outgrowths *in vitro.* To determine the purity of inner cell mass, first is no cells could be detected by immunofluorescent staining of bound antibody or by presence of melanin granules despite the fact that both these markers were present on or in all trophoblast cells before immunosurgery. Secondly, the patterns of protein synthesis of the inner cell masses showed none of the spots specific for microsurgically-isolated trophectoderm but did show inner cell mass specific spots (Van Blerkom *et al.,* 1976). Thirdly, chimaeras formed by injection of immunosurgically-isolated inner cell mass into host blastocysts showed donor inner cell mass contribution to trophoblast-derived tissues in only 2 cases. Additional evidence on the purity of immunosurgically isolated inner cell mass has come from the analysis of 3 interspecific chimaeras made by injecting immunosurgically isolated rat inner cell mass into mouse blastocysts (Gardner and Johnson, 1975). The rat inner cell mass contributed only to embryonic ectoderm and to extra-embryonic and embryonic endoderm, but not to trophoblast, ectoplacenta and extra-embryonic ectoderm, indicating have same developmental potential as microsurgically-isolated inner cell mass (Gardner and Johnson, 1975). Through these experiments, it can reduced doubts that the inner cell mass are deleteriously affected by the process of immunosurgery. The real advantages of immunosurgery are the ease with

which larger numbers of blastocysts can be dissected and the possibility of obtaining inner cell mass from strains which are difficult to handle microsurgically. Polzin *et al.* (2010) reported that the immunosurgery procedure in caprine species where caprine blastocysts were treated with pronase (5%) in Whitten's medium without bovine serum albumin (BSA) for 20 to 30 second to remove the zonae pellucidae. Then, zona free caprine blastocyst were gently rinsed and cultured in Whitten's medium for 1 to 2 hours to allow them to recover from the effects of pronase. Caprine blastocysts were then cultured in a 1:8 dilution of non-heat-treated rabbit anti-sheep antiserum for 20 to 30 minutes at which time the trophoblastic cells began to lyse. The embryos were then rinsed 3 times to remove excess antiserum, and finally drawn in and out of a small-bore glass pipette (approximately 75 µm) mounted on a Leitz micromanipulator. Lysed trophoblastic cells were removed, leaving an intact inner cell mass (Polzin *et al*., 2010).

2.12.3 Laser (XYclone)

The use of murine embryos would be a good model with which to do research to discover the best methodologies to use in order to derive new caprine embryonic stem cell lines. The aim of the present study was to evaluate a new method of isolation of the inner cell mass and derivation of embryonic stem cell lines in a murine and caprine blastocyst model using laser drilling to eliminate the trophectoderm cells and compare it with the usual control method consisting of culturing the whole murine and caprine blastocyst. Usually, laser technology is commonly used for assisted hatching with some applications. One of them consists of making the embryonic membrane weaker to make the exit of the future blastocyst easier and so favour the derivation (Antinori *et al*., 1996). Another consists of blastomere biopsy for preimplantational diagnosis (Sermon *et al*., 2004). Until now, this new laser dissection method has been suggested to derive stem cell lines by Wang and Sun (2005), although without presenting any conclusive

results. A recent paper, Tanaka *et al*. (2006) reported preliminary results for a murine model using this method; subsequent culture of embryonic stem cells in a serum or cell free culture system was achieved. In our opinion, some notion of which of these methods are the most efficient for the isolation of inner cell mass is very important for future research in this field. Accordingly, we have compared the inner cell mass isolation method using laser dissection in a murine and caprine blastocyst with one of the most commonly used methods, the whole blastocyst culture (Kim *et al.*, 2005). However, with good quality blastocysts with a large and distinct inner cell mass, we used the laser drill (Hamilton, USA). We did not use poor quality blastocysts in this method for this study because we cannot shoot the inner cell mass if we cannot see it. In our experiment, the murine and caprine blastocyst to be treated with laser shot was positioned at the centre of the field of view under magnification 40x. The blastocyst position was moved when necessary. Blastocysts can be secured by 2 holding pipettes with the inner cell mass positioned at '9 o'clock' if desired (Tanaka *et al*., 2006). After focusing on the trophectoderm cells, the object had to be moved so that the part of the trophectoderm to be treated was located at the cross-hair position displayed on the monitor as the impact location of the laser focus. Thus this new mechanical method destroyed the trophectoderm cells by shooting the laser over them carefully without damaging the inner cell mass. The length of radiation time of the laser and the number of laser shots were controlled by the embryologist. According to the adhesion and isolation of the inner cell mass, we observed that the whole embryo culture method of the blastocysts is more effective than the laser method (70% vs 52.4%) (Polzin *et al*., 2010). Taking into account that we had used the laser drill for the good quality blastocysts and that the concealment of the inner cell mass by the trophectoderm cells is the only disadvantage of the whole embryo culture method (Bongso *et al*., 1994), we must continue to improve the laser dissection technique so that the trophectoderm cells

are destroyed and do not interfere with inner cell mass adhesion (Tanaka *et al*., 2006). Curiously, the blastocysts treated with the laser at day 1 formed a pseudo-trophectoderm, with a few cells not destroyed by the laser because they were near the inner cell mass. Trophectoderm cells disappeared after repeated passages (Polzin *et al*., 2010).

2.12.4 Whole Blastocyst Culture

Only the whole blastocysts seeding method succeeds to establish embryonic stem cell lines when low quality blastocysts were obtained (Kim *et al*., 2005). A complete hatching process is essential to allow blastocysts to properly attach to feeder cells. To overcome this problem, establishment of a new method of inner cell mass isolation from whole primate cloned blastocysts, using whole bovine cloned blastocysts as a model system because bovine oocytes can be acquired easily and the somatic cell nuclear transfer protocols are similar to those of primates (Stojkovic *et al*., 2005; Jang *et al*., 2006; French *et al*., 2008). Production of cloned human blastocysts has been reported (Stojkovic *et al*., 2005; French *et al*., 2008), and inner cell mass isolation was reported in the rhesus monkey (Byrne *et al*., 2007). However, Byrne *et al*. (2007) reported that primate cloned blastocysts have lower quality than *in vitro* fertilisation derived blastocysts. The whole embryo culture tends to manifest an abundance of both trophectoderm and differentiated cells, and the isolation of murine embryonic stem cell-like colonies from differentiated cells requires a great deal of care and caution. However, the poor quality of the blastocysts often results in failures in the attachment of the inner cell mass to the dish. Therefore, this procedure runs a much greater risk of trophectoderm overgrowth than other methods because the entire trophectoderm is cultured along with the inner cell mass. In 1988, murine blastocysts at day 3.5 post-coitus flushed from uteri of C57Bl/10 murine were cultured by Hollands either as

singletons or in groups (Hollands, 1988). Many blastocysts lost their morphology as they expanded and attached to plastic within 24 hours. According to Hollands (1988), outgrowths of their cells formed cystic embryoid bodies and occasional blood islands, sometimes characterised by aggregates of pigmented cells. According to Robertson *et al*, (2004), the embryonic starting material can either be normal 3.5 days post-coitus expanded blastocyst or delayed" blastocyst (which are normally collected 4 to 6 days). Both groups the culture procedures are identical, with the only difference being the timing of the first disaggregation, as delayed blastocyst will initially grown more slowly (Robertson *et al*., 2004). Approximately 90% of surviving embryos were used to prepare as stem cells for injection into recipients. Controls included cell lines from blastocysts cultured *in vitro* for less than 3 days and standard embryonic stem cells as described by other investigators (Evans and Kaufman, 1981). Clinical implications of these studies are apparent. In a sense, the demonstrable colonising properties of these embryo stem cells seemed to resemble those of murine embryonic stem cells injected singly into recipient blastocysts. Therefore, such evidence implies that human cell outgrowths from blastocysts may retain their multipotency during their period in culture. An alternative approach to making embryonic stem cells was tested in Glasgow by culturing the intact rabbit inner cell mass of day 6 blastocysts trimmed of its trophectoderm or disaggregating the inner cell mass and culturing its component cells (Cole and Paul, 1965; Cole *et al*., 1966). They were characterised by high activities of alkaline phosphatase or arginase, characteristics persisting over many generations and had a varying morphology with large nuclei and many nucleoli. Nowsaday, many investigators routinely apply similar methods, using cultures of intact embryos to produce either highly differentiated tissues or culturing disaggregated cells to prepare embryonic stem cell lines seemingly suspended in an early phase of differentiation. Once embryo stem cell lines could be made *in vitro,* new experimental approaches were

needed to assess developmental capacities of single, marked embryonic stem cells. The murine was the ideal species, with many well characterised marker genes enabling cells to be traced as they migrated through recipient tissues. Roberts *et al*. (2004) develop microsurgical methods for injecting single embryonic stem cells into blastocoelic cavities of recipient blastocysts.

2.13 PASSAGING EMBRYONIC STEM CELLS (ESC)

Routinely passage embryonic stem cells every 2 to 3 days, otherwise cells will spontaneously differentiate (Boris, 2000). Challenge for the establishment of embryonic stem cells is the ability to dissociate effectively inner cell mass cells from one another. Usually, embryonic stem cells are separated from one another by treatment with enzymes such as trypsin, collagenase and pronase or in combination with chemical agents such as ethylenediaminetetraacetic acid (EDTA), citrate or Ca^{2+}/Mg^{2+} free phosphate buffered saline (PBS). With these combination treatments, a suspension of individual cells is created so that the culture can be subdivided for continued growth, and growth inhibition due to crowding can be reduced. The dissociation into single cells breaks down the cell-to-cell signaling that fosters stem cell differentiation. Thus, the routine passage of the cell population helps maintain the pluripotency of the cell population over time. Species differences appear to exist in the ease with which embryonic stem cells can be separated into a single cell or near single cell suspension. The dissociation of primate embryonic stem cells into single cell suspensions is a complicating factor in the culture of these cell lines. Enyzmatic and chemical dissociation of human or monkey embryonic stem cells typically give re-plating efficiencies of less than 1% (Thomson and Marshall, 1998). In contrast, murine embryonic stem cells are commonly dissociated by treatment with trypsin/EDTA for routine passage and maintenance of pluripotency, and their plating efficiencies are

usually 20% or greater (Robertson, 1987). This also makes murine embryonic stem cells more amenable to techniques fundamental to their use in creating genetically engineered murine, such as efficient colony-cloning and blastocyst injection (Bradley, 1987). Primary colony outgrowths of murine inner cell masses or blastocysts are usually dissociated with trypsin/EDTA treatment in combination with mechanical manipulation to initiate the secondary passage and establishment of embryonic stem cell lines (Robertson, 1987). Primary cultures of alkaline phosphatase positive, undifferentiated, embryonic stem cells prepared by the successive immunodissection, culture and physical dissection method are extremely sensitive to lysis by either physical manipulation, withdrawal of calcium, or exposure to trypsin/EDTA (Talbot *et al*., 1993, 1995; Talbot and Garrett, 2001). Check the embryonic stem cells under the microscope for sub-confluence and refeed cells 3 hours before passing them (very important), warm up reagents briefly before use. Aspirate medium off and wash once or twice with PBS (without bivalent cations), add about 1 ml (2 ml) of trypsin/EDTA to each 5 cm (10 cm) dish and incubate at 37℃ until colonies float off when flicking the plate. Carefully transfer trypsin/cell suspension to a sterile falcon which enrich the embryonic stem cell or feeder mix for embryonic stem cells and trypsinise for a few more minutes at 37℃. Then, dissociate colonies into single cells by "Gilson pipetting", then add several ml of embryonic stem cell medium without leukaemia inhibitory facotr to inactivate the trypsin, pellet cells by low-speed centrifugation for 5 minutes. Then, remove supernatant and resuspend cells in appropriate volume of embryonic stem cell medium depending on plate format and splitting ratio. According to Boris (2000), splitting ratios for embryonic stem cells can vary from 1:1 to 1:10. Return plates to the 37℃ incubator. Feeder cell density is very important to achieve optimal growth conditions where they should be almost confluent, but each feeder cell should have enough space around itself

to spread. Use feedes that are at least one day old because then they will have settled nicely and flattened.

2.14 CRYOPRESERVATION OF EMBRYONIC STEM CELLS (ESC)

Shaw and Jones (2003) reported that cryopreservation method is long term storage by cooling of samples to low sub-zero temperatures. There are few reasons for cryopreserved the stem cells. Firstly, stem cell will be easier to undergo senescence for long term cultured. Secondly, it is possible to cause contamination after prolonged culture *in vitro*. Therefore, it avoids the need to maintain the cell lines in continuous *in vitro* culture and allows the cells to be distributed to other users (Freshney, 1994). According to Freshney (1994), the stem cells are typically cooled to a low temperature and stored in liquid nitrogen (LN_2) at temperature -196 ℃. This freezing method is allowing equilibrium to be established between the rate of water loss from the cells and the rate of extracellular ice crystallisation (Shaw and Jones, 2003). Reduce the time the embryonic stem cells are in culture before freezing and freeze at a density that allows recovery of the culture even if 90% of the cells die during the freezing and thawing process (Boris, 2000). Depending on the size and permeability of the particular cell type, cryopreservation of embryonic stem cells involves the lowering of the temperature at a relatively slow rate (Shaw and Jones, 2003). Dehydration occurred where water leaves the cells and thus minimising intracellular ice crystallisation as well as reducing cryogenic damage from the highly concentrated solutes (Freshney, 1994).

2.15 THAWING EMBRYONIC STEM CELLS (ESC)

Always have feeder plates prepared (mitomycin C treatment at least one day before use) when thawing embryonic stem cells. Remove embryonic stem cells from freezer or liquid nitrogen and quickly thaw in a 37 ℃ waterbath and transfer cell suspension (cell

concentration is not very important) to a sterile tube containing several ml of warm medium. Then gently mix and pellet the cells by centrifugation or low speed for 5 minutes. Aspirate off supernatant (removal of dimethyl sulfoxide (DMSO) in freezing medium) and resuspend cells into 12 ml (5 ml) of warm embryonic stem cell medium and plate out in a 10 cm (5 cm) feeder plate. Ideally, should refeed cells daily with fresh embryonic stem medium (Lorthongpanich, personal communication). Boris (2000) described the embryonic stem cells need to be passaged or frozen or used for doing experiments upon sub-confluence.

2.16 NUCLEAR TRANSFER OF EMBRYONIC STEM CELLS (ntES)

The first successful cloning experiments in which fully differentiated adult cells developed into complete organisms were conducted in plants (Steward *et al*., 1958) approximately 40 years ago. The earliest reports of mammalian embryo cloning attempted to address the issue of embryonic cell differentiation. According to Wilmut *et al*. (1997), researchers using vertebrate models overlooked this monumental achievement and it took another 39 years for the same feat to be accomplished in mammals. Illmensee and Hoppe (1981) reported success in cloning murien by directly injecting inner cell mass nuclei into enucleated pronuclear-stage oocytes using procedure similar to the amphibian studies (Briggs and King, 1952). Embryonic stem-like cell lines generated from somatic cells via nuclear transfer of embryonic stem cells (ntES) technique were first reported for the bovine (Munsie *et al*., 2000) and then the murine (Kawase *et al*., 2000; Munsie *et al*., 2000a). Some reported that nuclear transfer of embryonic stem cell lines are capable of differentiating into all 3 germ layers *in vitro* or into spermatozoa and oocytes in chimaeric murine (Wakayama *et al*., 2001). Cloned murine can be obtained from this nuclear transfer of embryonic stem cell lines using a second nuclear transfer (Wakayama *et al*., 2001). Although these techniques

have now been applied to basic research, such as to demonstrate irreversible changes to DNA in adult lymphocytes (Hochedlinger and Jaenisch, 2002) , not olfactory neurons (Eggan *et al*., 2004; Li *et al*., 2004b) and to examine the characteristics of different types of cancer cells (Hochedlinger *et al*., 2004; Blelloch *et al*., 2006). However, since nuclear transfer of embryonic stem cells are not yet fully characterised in terms of the effects of the animal strain, genotype or sex of the donor nucleus, therefore such factors often affect the successful full-term development of cloned animals (Wakayama and Yanagimachi, 2001; Inoue *et al*., 2003). Here, nuclear transfer of embryonic stem cells can be generated from the tissues of both males and females of different murine genotypes using different cell types, with higher success rates than for adult somatic cell nuclear cloning.

2.17 SPONTANEOUS DIFFERENTIATION OF EMBRYONIC STEM CELLS (ESC)

Differentiation is the process whereby an unspecialised early embryonic cell acquires the features of a specialised cell such as a muscle, liver or heart. Differentiation *in vitro* can be spontaneous or controlled. The most significant challenge hindering the establishment of embryonic stem cell lines is the unable to control the spontaneous differentiation of embryonic stem cells in *in vitro* culture (Talbot *et al*., 1993, 1995). Primary cultures of pure porcine, ovine and bovine inner cell mass cells plated on STO feeder cells “spontaneously” differentiate into multiple cell types when left undisturbed in culture (Talbot *et al*., 1993, 1994, 1995, 1996, 2002a). According to Talbot *et al*. (1993, 1995), embryonic stem cells loss of alkaline phosphatase activity showed that differentiation events begin 48 to 96 hour post-plating of the inner cell mass *in vitro* culture. Usually, larger initial colonies of inner cell mass cells tend to differentiate sooner and become senescent and slowly die off. Cytokines and growth factors that

inhibit spontaneous differentiation in murine and primate embryonic stem cell lines are leukaemia inhibitory factor and basic fibroblast growth factors but do not inhibit this differentiation in ungulate inner cell mass primary cultures (Moore and Piedrahita, 1997; Talbot *et al*., 1993, 1995). Within a week of passage, early morphological differentiation events may be observed in the cells within embryonic stem cells colonies (Sathananthan *et al*., 2002). A wide range of differentiating cell types can be observed in ectodermal neuroectoderm, mesodermal muscle, and endodermal organ tissue types (Conley *et al*., 2004; Reubinoff *et al*., 2000; Sathananthan and Trounson, 2005). The embryonic stem cell ball up into embryoid bodies, with differentiation occurring within 5 to 7 days into the primary embryonic germ lineages (Itskovitz-Eldor *et al*., 2000). Human embryoid bodies have a consistent vesicular appearance and structure (Sathananthan, 2003; Conley *et al*., 2004; Gertow *et al*., 2004), with a variety of cell types that appear to develop in a more random organisation than mouse embryoid bodies. In theory, embryonic stem cells can be induced to differentiate into any cell type *in vitro* by providing the correct signals. Embryonic stem cells were found to differentiate *in vitro* into many cell lineages including primitive ectoderm (Rathjen *et al*., 1999), parietal (Keller *et al*., 1993) and visceral (Soudais *et al*., 1995; Abe *et al*., 1996) endoderm, early mesoderm (Johansson and Wiles, 1995, Rohwedel *et al*., 1998) and into differentiated cell types such as cardiac (Wobus *et al*., 1991; Maltsev *et al*., 1993, 1994; Wobus and Guan, 1998), myogenic (Rohwedel *et al*., 1994, 1995, 1998), chondrogenic (Kramer and Irish, 2000), adipogenic (Dani *et al*., 1997), hematopoietic (Schmitt *et al*., 1991; Wiles and Keller, 1991; Helgason *et al*., 1996; Suwabe *et al*., 1998), epithelial (Bagutti *et al*., 1996), neuronal (Bain *et al*., 1995; Fraichard *et al*., 1995; Strubing *et al*., 1995; Okabe *et al*., 1996), endothelial (Risau *et al*., 1988; Vittet *et al*., 1996) and vascular smooth muscle (Drab *et al*., 1997) cells. In similar, embryonic germ cells also have the capacity to differentiate into many cell types when cultivated as

embryoid body *in vitro* (Rohwedel *et al*., 1996). Although spontaneous differentiation of embryonic stem cells revealed only low levels of neuronal differentiation (Strubing *et al*., 1995), transcripts of neuron-specific genes can be detected (Rohwedel *et al*., 1998; Schmidt *et al*., 2001). Retinoic acid (RA) (Fraichard *et al*., 1995; Stru ¨bing *et al*., 1995) and growth factor induced differentiation protocols (Okabe *et al*., 1996) clearly showed different expression patterns of neuronal genes (Guan *et al*., 2001; Rolletschek *et al*., 2001). The differentiation of embryonic stem cells *in vitro* provides an excellent model for studying cell commitment and the potential of stem cell technology (Nishikawa *et al*., 1998; Rathjen *et al*., 1998).

2.18 INDUCED OR DIRECTED DIFFERENTIATION OF EMBRYONIC STEM CELLS (ESC)

Induced diiferentiation of embryonic stem cells proofs have been common practice with both murine and primate embryonic stem cell lines since the first reports in the literature (Martin, 1981; Thomson *et al*., 1995, 1998). Bovine and porcine embryos have been shown to produce teratomas when injected into immunocompromised murine, and so it should be with any putative ungulate embryonic stem cell lines (Anderson *et al*., 1996). Although proofs of germline transmission are well established in the murine (Bradley, 1987; Nagy *et al*., 1993); however, have never been achieved with the putative embryonic stem cells of ungulates. It is more likely that the isolated embryonic cells quickly lose their embryonic competence following propagation under suboptimal *in vitro* conditions. The enhancement of differentiation toward a specific lineage can be achieved by activating endogenous transcription factors, by transfection of embryonic stem cells with ubiquitously expressing transcription factors, by exposure of embryonic stem cells to selected growth factors, or by co-culture of embryonic stem cells with cell types capable of lineage induction (Lavon *et al*., 2004; Pera and Trounson, 2004;

Trounson, 2005a, b). According to Loebel *et al*. (2003), embryonic stem cells may be induced to form cell types of interest by a combination of growth factors and/or their antagonists. Embryonic stem cells derived neurones respond to neurotransmitters and generate action potentials as well as make functional synapses (Carpenter *et al*., 2001). Oligodendrocytes derived from embryonic stem cells are able to remylinate neurones of the shiver murine model (Nistor *et al*., 2005). Doperminergic neurons can be formed from embryonic stem cells (Park *et al*., 2004; Perrier *et al*., 2004) and they are shown to be capable of reversing some motor behavioral abnormalities after transplantation into the brain of a parkinsonian rat model (Ben-Hur and Noble, 2004). Embryonic stem cells may be efficiently directed into neuroectoderm by culture in the presence of an antagonist to bone morphogenetic proteins signaling (Pera *et al*., 2004). Manipulation of culture conditions with bone morphogenetic proteins 4 induces epidermogenesis or neural crest and dorsal most central nervous system cells, and suppression of *sonic hedgehog* promotes motor neurone formation (Mizuseki *et al*., 2003; Trounson, 2004). Cultures of embryonic stem cells cultured together with mouse visceral endoderm cell type will preferentially form beating heart muscle cell colonies (Mummery *et al*., 2002, 2003). Similarly, differentiation strategies similar to those developed by Lumelsky *et al*. (2001), Rajagopal *et al*. (2003), and Sipione *et al*. (2004) in murine embryonic stem cells to produce insulin and C-peptide expressing cells have differentiated human embryonic stem cells into insulin producing cells that coexpress insulin and C-peptide and glucagon or somatostatin (Segev *et al*., 2004).

2.19 EMBRYOID BODIES (EB)

The most essential technique to form embryoid bodies where culture embryonic stem cells in suspension without antidifferentiation factors such as leukaemia inhibitory factor and feeder layer cells will spontaneously differentiate and form 3 dimensional

multicellular aggregates. Embryoid bodies play an important role in the differentiation of ES cells into a variety of cell types *in vitro*. There are several methods for inducing the formation of embryoid bodies from embryonic stem cells. The 3 basic methods namely, liquid suspension culture in bacterial-grade dishes, culture in methylcellulose semisolid media, and culture in hanging drops, are usually used for the formation of embryoid bodies to induce the formation of a variety of cell types from embryonic stem cells (Keller, 1995; Höpfl *et al*., 2004). Recently, the methods using a round-bottomed 96-well plate and a conical tube are adopted for forming embryoid bodies from predetermined numbers of embryonic stem cells. For the production of large numbers of embryoid bodies, stirred-suspension culture using spinner flasks and bioreactors is performed. Each of these methods has its own peculiarity. Therefore, the features of formed embryoid bodies depending on the method used. The formation of embryoid bodies is the principal step in the differentiation of embryonic stem cells. Thus, the features of formed embryoid bodies are considered to be not homogeneous embryologically and morphologically. Embryoid bodies are classified as simple embryoid bodies or cystic embryoid bodies according to the stage of differentiation (Abe *et al*., 1996; Magyar *et al*., 2001; Conley *et al*., 2004). In the case of murine embryonic stem cells, simple embryoid bodies show spherical embryonic stem cell aggregates with morula-like structures formed in 2 to 4 days in suspension culture. For cystic embryoid bodies formation, a central cavity forms in embryoid bodies in 4 to 5 days in suspension culture; resemble an embryo in the blastula or egg-cylinder stage, consisting of a double-layered structure with an inner ectodermal layer and an outer of endoderm enclosing the cavity. Cystic embryoid bodies expand to larger cystic structures homologous to the visceral yolk sac of postimplantation embryos after 8 to 10 days in suspension culture. Doetschman *et al*. (1985) developed a technique of forming embryoid bodies from embryonic stem cells in suspension culture using bacterial- grade

dishes (60 mm or 100 mm in diameter). Seeded embryonic stem cells do not attach to the plastic surfaces of the bacterial-grade dishes, and they stick to each other, and form aggregates without any movement or shaking. By this method, the differentiation of embryonic stem cells into a variety of differentiated cell types, for example, neural progenitors (Plachta *et al*., 2004), vascular cells (Risau *et al*., 1988, 2001), cardiomyocytes (Klug *et al*., 1996), chondrocytes (Hwang *et al*., 2006), hepatic cells (Jones *et al*., 2002), insulin-producing cells (Soria *et al*., 2000; Morita *et al*., 2003), and germ cells (Toyooka *et al*., 2003) are induced from embryoid bodies formed in the bacterial-grade dishes for mouse embryonic stem cells. As for human embryonic stem cells, neural cells (Itskovitz-Eldor *et al*., 2000), hematopoietic cells (Itskovitz-Eldor *et al*., 2000), cardiomyocytes (Itskovitz-Eldor *et al*., 2000; Kehat *et al*., 2001), insulin-producing cells (Assady *et al*., 2001), and endothelial cells (Levenberg *et al*., 2002) are also induced from embryoid bodies. The size and shape of the resulting embryoid bodies tend to be heterogeneous (Wartenberg *et al*., 1998). Rotating suspension culture was introduced to improve the homogeneity of embrtoid bodies formed in bacterial-grade dishes (Zweigerdt *et al*., 2003). Rundnick *et al*. (1987) introduced methylcellulose culture to form cell aggregates of a clonal origin. Methylcellulose culture has been used for the study of hematopoietic differentiation of embryonic stem cells (Wiles and Keller, 1991; Keller *et al*., 1993; Kennedy *et al*., 1997; Potocnik *et al*., 1994). Hanging drop culture is the embryoid body formation induction method that has been frequently and widely used to differentiate embryonic stem cells into a variety of cell types. Hanging drops provide embryonic stem cells a good environment for forming embryoid bodies. Embryoid bodies formed by the hanging drop method have been used to generate a broad spectrum of cell types, including neuronal cells (He *et al*., 2006), lymphoid (Potocnik *et al*., 1997), hematopoietic cells (Dang *et al*., 2004), cardiomyocytes (Wobus *et al*., 1991; Metzger *et al*., 1994; Takahashi *et al*., 2003;),

smooth muscle cells (Drab *et al.*, 1997; Yamada *et al*., 2002), chondrocytes (Kramer and Irish, 2000; Hegert *et al*., 2002) and gametes (Geijsen *et al*., 2004) (Figure 2.8).

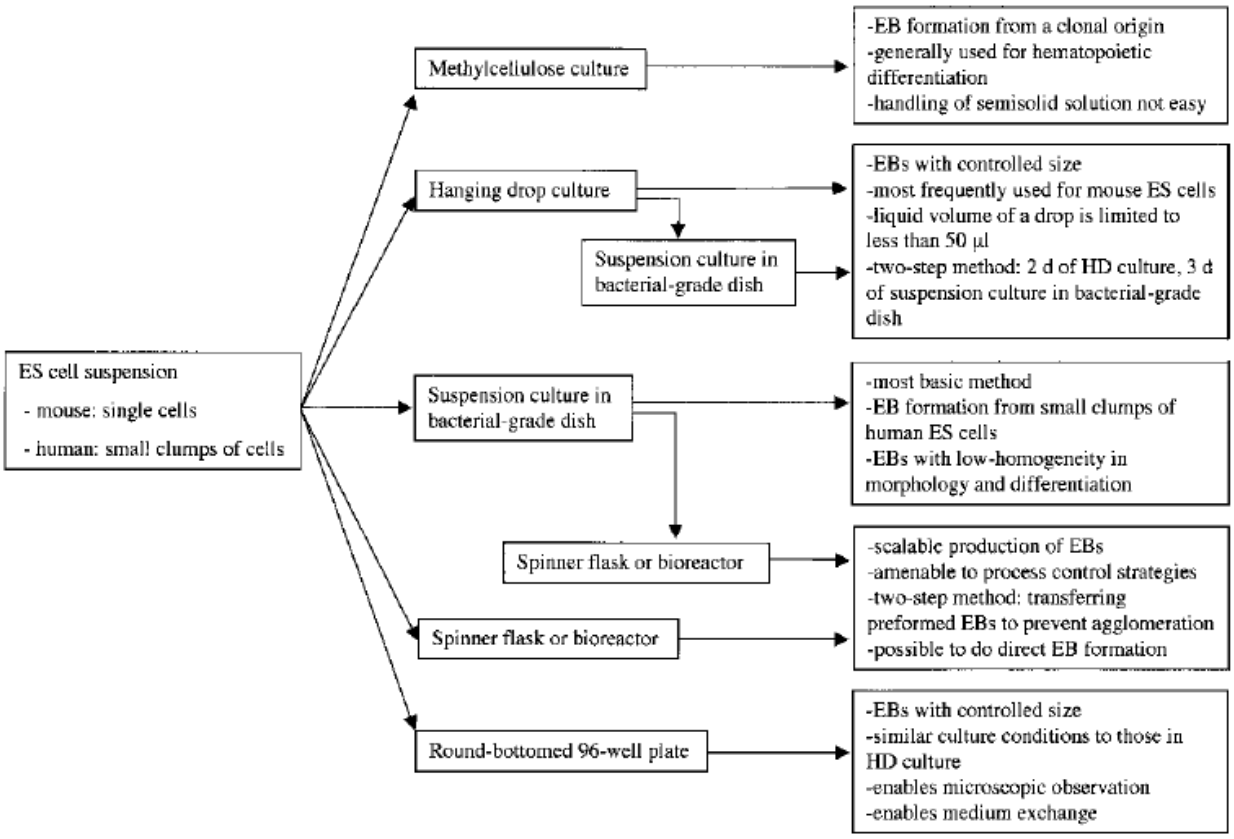

Figure 2.8: Outlines of methods for inducing embryoid bodies formation (Kurosaw *et al*., 2007).

2.20 POTENTIAL OF EMBRYONIC STEM CELLS APPROACHES

Embryonic stem cells which derived from blastocysts are capable of long-term self-renewal and differentiation into most tissues while retaining a normal genomic structure. It has been shown that human embryonic stem cells can proliferate for 2 years, including 300 cell cycles, a characteristic that is not shared with any adult stem cell (Donovan and Gearhart, 2001). Embryonic stem cells also can be directed towards differentiation pathways by changing growth conditions, leading to development of specialised cells such as heart muscle cells, neurons or insulin-secreting cells, opening the way to the development of new therapies. Examples of the functional correction of

the neuro-degenerative diseases in different animal models by the transplantation of a low member of differentiated or undifferentiated embryonic stem cells is particularly encouraging in this direction (Reubinoff *et al*., 2001; Bjorklund *et al*., 2002; Liu *et al*., 2002). Bsides that, embryonic stem cells can been modified by homologous recombination, nuclear transplantation (Vogelstein *et al*., 2002) or retroviral transduction, allowing researchers to dispose of *in vitro* models to study developmental pathways that are mutated in genetic diseases. The best example of the potentiality of these combined strategies has been recently reported by Rideout *et al*. (2002), whose work provides the proof of principle for nuclear transplantation combined with gene therapy to treat a form of severe combined immune deficiency in murine. Through somatic nuclear transfer, it allowed the isolation of embryonic stem cells genetically identical to diseased individuals, therefore, avoiding rejection, which remains the main obstacle to conventional organ transplantation. Recently, these genetically matched embryonic stem cells can then be corrected by homologous recombination (Rideout *et al*., 2002) and induced to differentiate into cells of several different developmental lineages. The proliferative capacity of undifferentiated embryonic stem cell is a matter of concern. Undifferentiated embryonic stem cells are not suitable for transplantation purpose due to the risk of unregulated growth. Thus, the identification of differentiation steps at which the risk of tumour formation is minimised becomes a critical point for further of research. A fruitful research period has begun all around the world focused on the biology of stem cells. Two medical perspectives are around the corner. In perspective of developmental medicine, discoveries in this field can help to understand physiopathology of both infertility and developmental genetic diseases. The second one deals with the so-called ‘regenerative medicine’ that could bypass some therapeutical barriers caused by donor organ scarcity and immunological constraints. Therefore, basic research should provide further information of importance for future therapeutic usage

of stem cells. An alternative approach to assessing gene function during development is to randomly create heterozygous phenotypes by insertional mutagenesis of genes using gene trapping. Another potent application is to transfect embryonic stem cells to express GFP either constitutively, or targeted under the control of a lineage-restricted gene. During differentiation *in vitro*, embryonic stem cells were found to be able develop into specialised somatic cells types and to recapitulate processes of early embryonic development. These properties allow us to use embryonic stem cells as model system for studying early embryonic development by gain or loss of function approaches, or to investigate the effects of drugs and environmental factors on differentiation and cell function in embryotoxicity and pharmacology. The establishment of ungulate embryonic stem cell lines, either from *in vivo* or *in vitro* produced blastocysts, carries many known and unknown challenges. Approaches to the problem may involve innovative genetic manipulation techniques for targeted cell ablation (McWhir *et al*., 1996), ectopic gene expression (Chambers *et al*., 2003) or gene expression knock-down. Embryonic stem cell has the potential for treating a variety of major diseases. Patients with Type I diabetes mellitus suffer from the autoimmune destruction of beta cells, the insulin-producing cells of the pancreas (Chapman *et al.*, 1999). Currently, the treatment involves transplantation of pancreatic cells and Islet of Langerhans from donated pancreas, which is limited by the scarcity of donated organs (Chapman *et al.*, 1999; Assady *et al.*, 2001). This approach is considered a superior alternative to the use of pancreatic stem cells to form the islet cells, as only a low number of cells can be derived from the pancreas (Bonner-Weir *et al.*, 2000; Assady *et al.*, 2001). Another major disease that can be potentially treated with embryonic stem cells therapy is disease of the nervous system. A significant problem in treatment of the nervous system is that in many cases, the cause of the disease is due to loss of nerve cells, in which most mature nerve cells cannot replicate to replace lost cells (Chapman *et al.*, 1999). The use of

embryonic stem cells to regenerate these cells circumvents this problem. In the case of Parkinson's disease whereby the patients lack dopaminergic neurons, these neurons have been successfully derived from human embryonic stem cells (Kim *et al.*, 2002; Schulz *et al.*, 2004; Zeng *et al.*, 2004). Besides the brain region, the clinical potential of embryonic stem cells also expands to spinal cord injuries, which was explored by Keirstead *et al.* (2005). The therapeutic potential of embryonic stem cells for regenerative medicine will depend on their ability to direct their differentiation into cell lineages and pure homogeneous progenitor cell types that can be screened for drug discovery, their reliance that they will remain of the tissue type required and not form undesirable teratomas or other tissue derivatives (Brustle *et al*., 1997; Deacon *et al*., 1998). It is difficult to identify and monitor the appearance of specific cell types in culture because the changes in gene expression may not be visibly identifi able or the cell morphology may not be easily recognisable, especially during the early stages of differentiation. Clonal derivation of embryonic stem cells is difficult, and hence the efficiency of identifying homologous recombination for gene "knock-in" or "knock-out" has been extremely low. Conventional transfection methods have been successful established by Eiges *et al*. (2001) as have lentiviral methods (Gropp *et al*., 2003; Ma *et al*., 2003). Zwaka and Thomson (2005) have shown that it is possible to electroporate embryonic stem cells to achieve homologous recombination of embryonic stem cells colony fragments. Gene function may be determined in embryonic stem cells by using small inhibitory RNAs (siRNA) to explore renewal, differentiation, apoptosis and oncogenesis (Trounson, 2006).

2.21 CHALLENGES IN THE ESTABLISHMENT OF EMBRYONIC STEM CELL LINES

There are many problems and challenges in establishing embryonic stem cell lines. An initial problem in the isolation and culture of embryonic stem cells is in recognising contaminating cell types in the primary culture of blastocysts or inner cell masses that may be mistaken for embryonic stem cells. When entire blastocyst is used to initiate a primary culture, trophectoderm, endoderm and inner cell mass cells may all survive and grow in the culture medium together. Although the 3 cell types present, only inner cell mass cells are the source of embryonic stem cell lines (Brook and Gardner, 1997). According to Solter and Knowles (1975), the blastocyst's outer trophectoderm cells can be eliminated by immunodissection, but the isolated inner cell mass is coednsist of both epiblast tissue and a visceral endoderm cell layer (Talbot *et al*., 1993). Thus, physical dissection methods for isolating the inner cell mass should be assumed to always leave viable trophectoderm cells attached to the inner cell mass. As with murine and human primary blastocyst or inner cell mass cell cultures, trophectoderm and endoderm cells may be mistaken for embryonic stem cells and are in one sense "weeds" in the primary culture (Robertson, 1987; Talbot *et al*., 1993). Ungulate embryonic stem cells since their trophectoderm and visceral endoderm are very resilient epithelial cells that grow rapidly in feeder cell co-cultures (Talbot *et al*., 1993, 1995, 2000a, 2005; Flechon *et al*., 1995; Shimada *et al*., 2001; Miyazaki *et al*., 2002). It is important that putative embryonic stem cells of ungulates be tested for markers of trophectoderm or visceral endoderm cells. For example, a definitive marker of bovine, caprine and ovine trophectoderm is the expression of interferontau (Talbot *et al*., 2000a; Shimada *et al*., 2001; Miyazaki *et al*., 2002). A specific marker is the presence of serum proteins such as alpha-fetoprotein and transferrin (Talbot *et al*., 2000a) for visceral endoderm. It is important to appreciate the fact that these 2 cell types, like other polarised or "dome-

forming" epithelial cells, will make "embryoid-like" bodies if grown without attachment to a solid cell culture substrate. Therefore, these and other properties of ungulate trophectoderm and endoderm should be carefully evaluated. The spontaneous differentiation of the primary epiblast cells to epithelial cell types is rapid and common (Talbot *et al*., 1993; 1995). Many epithelial cells grown on fibroblast feeder cell monolayers can look "embryonic stem-like", especially shortly after passage, for example, foetal kidney epithelial cells are often confused with primordial germ cells for this reason and also because kidney epithelial cells express the alkaline phosphatase gene to a greater extent (Gibson-D'Ambrosio *et al*., 1987). Talbot *et al*. reported that pig epiblast-derived epithelial cell line exhibits an embryonic stem-like morphology shortly after passage and in some cases these epithelial cells may also be alkaline phosphatase positive (Talbot *et al*., 1993, 1995). Finally, the feeder cells themselves may be a source of confusion in the identification and proof of ungulate embryonic stem cell lines. Mostly, it is common for STO feeder cell layer to adopt the morphology of oligodendrocytes, astrocytes or neurons, particularly if they are exposed to various members of the fibroblast growth factor family. The use of rodent or oprimary fetal fibroblast as feeder cell layer necessarily introduces many different types of cells into the embryonic stem cell derivation culture system. For example, macrophages can comprise as much as 50% of the "fibroblast" population in the early passage mouse fibroblast cultures that are routinely used for preparing feeder cell layer (Talbot *et al*., 2004).

Chapter 3

3.0 MATERIALS AND METHODS

Chapter 3

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3.1 GENERAL INTRODUCTION

In the present study, the research was conducted at 3 main laboratories, namely Animal Biotechnology-Embryo Laboratory (ABEL), Embryo Micromanipulation Laboratory (EMiL) (Institute of Biological Sciences, ISB), and Nuclear Transfer and Reprogramming Laboratory (NaTuRe) (Institute of Postgraduate Studies, IPS) at the University of Malaya, Kuala Lumpur, Malaysia. The experimental murine and caprine were sourced from Institute of Biological Sciences (ISB) Mini Farm (University of Malaya) located approximately 1 km from the laboratories. For caprine sample, oocytes were retrieved from live female caprine (donor does) by oocyte retrieval surgery (laparoscopic ovum pick-up, LOPU) or ovariectomy was conducted at the NaTuRe laboratory once week. Each surgery was carried out either 1 or 2 caprine donors per surgery session. The immature oocytes were transported to the EMiL laboratory after the oocytes retrieval surgery for conducting the subsequent *in vitro* maturation, fertilisation and culture (IVMFC) experiments. Besides that, the caprine oocytes also retrieved from local abattoir (slaughter house) in Shah Alam, Senawang or ISB Mini Farm, University of Malaya by slicing technique. For murine, it was carried out to produce viable lines of murine embryonic stem cell line using mouse embryonic fibroblast as feeder cell layer. The source of embryonic stem cells was produced using preimplantation embryos obtained via embryo flushing or somatic cell nuclear transfer cloned-murine embryos. Three different murine strains and passage numbers of primary mouse embryonic fibroblast were isolated and cryopreserved as bank storage for future use. The duration of this study was from November 2008 to May 2011.

3.2 MATERIALS

The materials that used in this study included various facilities, study experimental animals, equipment and instruments; glasswares or labwares or disposables, chemicals, reagents and media.

3.2.1 Facilities

In the present study, the facilities used included EMiL laboratory (for preliminary experiment on murine; *in vitro* maturation, fertilisation and culture and nuclear transfer experiments in caprine species), NaTuRe laboratory (for caprine oocyte retrieval surgery), ISB Mini Farm (sources of live experimental caprine and murine species).

3.2.2 Experimental Animals

In this study, 2 experimental animals had been used, caprine and murine. For caprine, Boer crossbred, local mixed-breed caprine and Jamnapari caprine were used as caprine oocyte donors. The caprine age for surgery ranged from 12 to 50 months old. These caprine underwent oestrus synchronisation and hyperstimulation. For murine, 3 different strains were used, which were ICR (white), CBA/ca (brown) and C57BL/6J (black) strains. Two different sizes of cages were used: large (43.5 cm x 28.5 cm x 16.0 cm) and medium (29.5 cm x 22.5 cm x 13.5 cm) sizes. The murine were separated in cages according to gender, strain and status of pregnancy. The murine were fed with commercial murine pellets and sawdust was used for bedding as well as provided *ad libitum* using water bottles. The murine cages were cleaned and sawdust changed once a week. The environment was controlled under natural day and night cycle, with the natural lighting period approximately 12 hours at room temperature. For breeding purposes, 8 to 12 weeks male murine were mated with female murine (6 to 10 weeks) to produce offspring and embryos as needed in this study.

3.2.3 Equipment and Instruments

Equipment and instruments used in present study with manufacture's and supplier's names as well as model numbers are listed in Appendix Table 1.1 (Appendix 1). Common used equipment such as CO_2 incubator (Kendro Laboratory Products, Germany), autoclave (Hirayama Manufacturing Corporation, Tokyo, Japan), flushing and aspiration system (Cook Australia, Australia), inverted microscope (Olympus Optical CO., Ltd., Japan) with micromanipulators (Narishige Co., Ltd., Japan), laminar flow (Gelman Sciences Pty. Ltd., Australia), oven (Memmert GmbH, Germany), water bath (Memmert GmbH, Germany), ultrapure water system (Millipore, USA), pH meter (Hanna Instruments, Singapore), stage warmer (Linkam Scientific Instrument, England), microgrinder (Narishige Co., Ltd., Japan), micropuller (Sutter Instrument Co., USA), microforge (Technical Products Internationals, USA), osmometer (Wescor, Inc., USA), stereomicroscope (Olympus Optical Co., Ltd., Japan), laparoscopic system (Aesculap A.G. and Company, Germany), centrifuge machine (Kendro Laboratory Products, Germany), fluorescent microscope (Carl-Zeiss Inc., Germany), stereomicroscope microscope (Nikon, Japan), liquid nitrogen tank (MVE, USA), La banana (self-assembled), surgical set (Aesculap A.G. and Company, Germany) micropipette dispenser (Eppendorf, Germany) and laser ablation system, Xyclone (Halmiton Thorne Research, Belverly, MA, USA).

3.2.4 Glassware/Labware/Disposable

Glassware/labware/disposables used are listed in Appendix Table 1.2 (Appendix 1). For oocyte retrieved, graduated test tubes (14 ml, Becton Dickinson Lanware, USA) were used. Nunc 4-well dishes (Nunc, Denmark) were used for staining and culture embryonic stem cells. For *in vitro* maturation, activation, *in vitro* culture of oocytes and embryos in polystyrene culture dishes (35 mm; Becton Dickinson Labware, USA). The

lids of polystyrene culture dishes (35 mm; Becton Dickinson Labware, USA) were used for somatic cell nuclear transfer procedure.

3.2.5 Sample Sources

Caprine and murine oocytes as well as blastocysts were the sample of this present study. Caprine oocytes were obtained either by laparoscopic ovum pick-up technique or slicing of ovaries collected by ovariectomy or from abattoir source. Uterine flushing of in caprine also carried out to obtain blastocyst as a source for goat embryonic stem cell production. For murine sample, the oocytes obtained by superovulated with pregnant mare serum gonadotrophin and human chorionic gonadotrophin hormones to obtain more sample to carry out somatic cell nuclear transfer procedure to produce blastocyst as a source for production of embryonic stem cells. Besides *in vitro* produced embryos from somatic cell nuclear transfer technique, *in vivo* embryos also obtained through *in vivo* flushing at 2-cell stage of murine embryo (oviductal flushing) and subsequently *in vitro* culture until blastocysts and *in vivo* flushing blastocyst (uterine flushing) as source of murine embryonic stem cells production.

3.2.5 (a) Laparoscopic ovum pick-up derived caprine oocytes

The laparoscopic ovum pick-up procedure was a big project which involved a group of people who worked as a team. During the laparoscopic ovum pick-up procedure, a team of researchers was involved to carry out various activities such as administration of anesthetics, surgery and oocytes pick-up. The surgery was conducted by the supervisors and oocytes pick-up was carried out by the embryologists including the author. The caprine oocytes obtained from laparoscopic ovum pick-up were subsequently subdivided to other researchers for various experiments.

3.2.5 (b) Caprine oocyte retrieval via ovariectomy

Usually, the caprine donor failed to be hyperstimulated after repeated oocyte retrieved surgeries or when the ovary was physically abnormal, for example, adhesion, polycystic ovary syndrome, degenerative ovary, these ovaries were surgically removed (ovariectomy) from the caprine donor. These animals were not hormonally treated for oestrus syncronisation and superovulation. A middle skin incision (longitudinal abdominal incision) was made (3 to 5 cm) at the abdomen part of the caprine donor. The ovaries were then removed and collected in a polystyrene culture dish (35 mm; Becton Dickinson Labware, USA) with the warmed flushing medium. The ovary was held with the help of forceps and was sliced by using a scalpel blade. The Petri dish was then examined under a stereomicroscope (magnification 20x or 40x). Oocytes were then washed in flushing medium and cultured according to oocyte grades for subsequent *in vitro* maturation-somatic cell nuclear transfer (SCNT) procedures.

3.2.5 (c) Abattoir-derived caprine ovaries

Caprine ovaries from abattoir were collected within 1 hour of slaughter in sterile saline (0.9% NaCl) supplemented with penicillin-G (100000 IU) and streptomycin sulphate (100 mg/liter) and transported to the laboratory at 35 ℃ to 37 ℃ within 1 to 2 hours of slaughter in a thermos flask (Megatrade Instumental Inc., USA). The breed, origin and health status of the does were unknown. Therefore, the collected ovaries were regardless of the phase of oestrus cycle and pregnancy.

3.2.5 (d) Caprine blastocyst derived from uterine flushing

A 4 cm long incision was made on the skin along the mid-abdominal wall which 2 cm away from the udder by using a scalpel blade (Size 15, BB515, Aesculap, Germany). The incision was continued further on the underlying tissues and peritoneum layer along

the linea alba. The uterine horns along with the ovaries were taken out and the number of corpora luteum in each ovary was recorded. Using the modified technique of flushing of embryos in sheep, a puncture wound was made on each uterine horn near the bifurcation of the uterus using a small pair of haemostatic forceps (BH 104, Aesculap, Germany). A 2 way size 8 Foley Catheter (Inmed Paediatric Balloon Catheter, USA) was then inserted through the puncture to a deep of 3 cm. The balloon of the catheter was sufficiently inflated to completely obstruct the lumen at the caudal end of each uterine horn. A Teflon intravenous (I.V.) Catheter placement unit fixed with a 20 gauge needle (Vasocan, B. Braun, Germany) was introduced closer to the anterior end of the uterine horn. Then, the Teflon intravenous Catheter needle was removed and 20 ml sterile disposable syringe (Terumo, Japan) fixed to the end of the Teflon Catheter. The fluid contents of the uterine horn were collected through the free end of the Foley Catheter. This was repeated with a further 20 ml of the flushing medium. Subsequently the same procedure was used for the flushing of the other uterine horn if at least one corpus luteum was present in the respective ovary. After flushing completed, the surgical incision was sutured with catgut (size 2, Aesculap, Germany). The incised skin was closed with size 2 surgical grade silk suture material (B. Braun, Germany) and sprayed with Gusanex aerosol (1% w/w Dichlofenthione; Coopers, Pitman Moore, Malaysia) as a fly repellent (Figure 3.1).

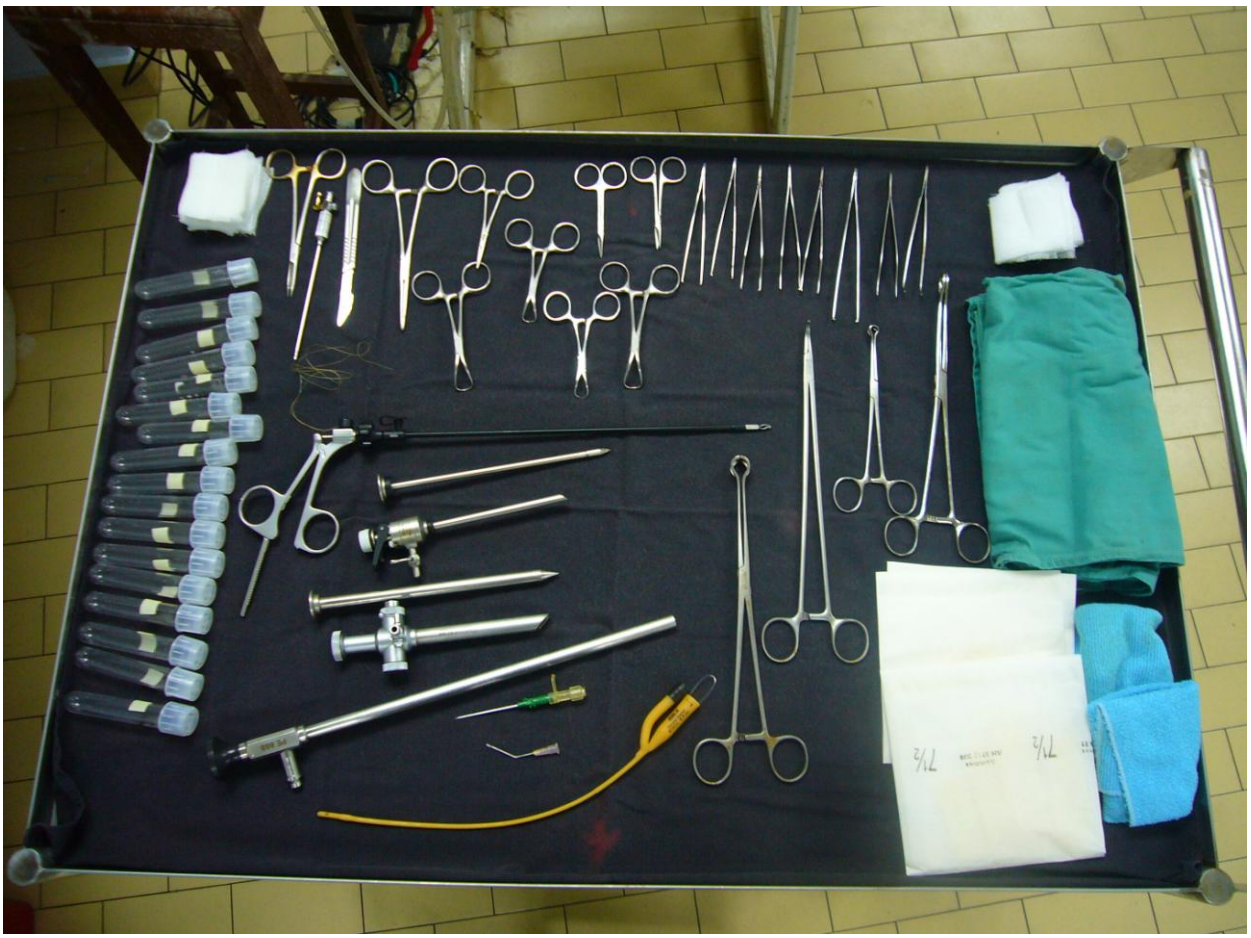

Figure 3.1: Surgical instruments for uterine flushing in caprine.

3.2.5 (e) Recovery of murine oocytes

The superovulated female murine were sacrificed at the time of ovulation was conducted by 13-15 hours post hCG injection. The female murine was sacrificed by cervical dislocation. The coils of female murine gut were pushed out of the way and the 2 horns of the uterus that was ovaries and oviducts were located. The upper end of one of the uterine horns was grasped fine forceps and gently pulls the uterus, oviduct, ovary and fat pad taut and away from the body cavity. This will reveal a fine membrane as the mesometrium, which connects the reproductive tract to the body wall and carries a prominent blood vessel. In the membrane, a hole was poked that close to the oviduct with the closed tips of a pair of fine forceps or scissors. The oviduct was transferred into

Petri dish with Hepes Whitten's medium (50 µl). Oviducts from several murine can be collected in the same dish.

The freshly ovulated oocytes as cumulus oocyte complexes (COC) that surrounded by cumulus cells, are found in the upper part of the oviduct (ampulla), which at this time (12 hours post-ovulation) is much enlarged. The fimbriae end of the oviduct (infundibulum) is also swollen during ovulation and can be easily be located under 20x magnifications in the stereomicroscope. A pair of needle (26 G) was used to hold the swollen infundibulum firmly on the bottom of the dish and another to tear the ampulla where the cumulus oocyte complexes are located. The cumulus oocyte complexes (eggs surrounded with cumulus mass) were released. The cumulus oocyte complexes were immediately placed into hyaluronidase (0.2%) to remove the cumulus surrounding. The matured murine oocytes with polar bodies were selected for further cloning experiment.

3.2.5 (f) Recovery of *in vivo* produced murine embryos from superovulated female Murine

In vivo produced murine embryos at 2-cell stage and blastocyst were used. Briefly, murine were housed in an environmentally controlled room (25 ℃) with a constant photoperiod (12 light: 12 dark), with commercial pellets and water available *ad libitum*. Female murine (6 to 8 weeks old) were superovulated with an intraperitoneal (i.p.) injection of pregnant mare serum gonadotrophin (PMSG; 10 IU) followed by an intraperitoneal injection of human chorionic gonadotrophin (hCG; 10 IU) at 46 to 48 hours later. Each female murine was then placed in a cage with a stud male and copulation plug was checked on the next morning (day 1 pregnancy).

3.2.5 (f) (i) Recovery 2-cell stage murine embryos

To collect 2-cell stage murine embryos, oviducts of superovulated female murine were excised from day 2 pregnant female that had mated with the male murine. The oviducts were flushed with Hepes Whitten's medium using a flushing needle (32 G) connected to a syringe (1 ml). Collected embryos were washed 3 times in equilibrated Whitten's medium and cultured under purified mineral oil at 37.5℃ in CO_2 (5%) in humidified air for *in vitro* development prior to the embryonic stem cell procedures.

3.2.5 (f) (ii) Recovery of murine blastocysts

Generally, murine blastocyst stage will be located in the uterus. Blastocysts were flushed from the uterus between days 3.5 to 4.5 post-coitus. After exposing the abdomen of the murine and locating the uterus, the uterus was removed. The uterus was held with forceps just above the cervix which was located directly above the bladder. The cervix was cut across using fine scissors. The uterus was completely removed by cutting below the junction with the oviduct on both horns. After removing the uterus, it was place in a Petri dish containing modified Hepes Whitten's medium. Both horns were flushed with Hepes Whitten's medium using 26 G, ½ inch needles attached to 1 ml syringes. The blastocysts will be flushed out, detected using stereomicroscope and picked-up using puller pipettes. The embryos were then washed with Hepes Whitten's medium for 3 times and Whitten's medium for 2 times and subsequently *in vitro* cultured in Whitten's medium at 37ºC incubator.

3.2.6 Chemicals

All chemicals, reagents and media used were purchased mainly from Sigma-Aldrich Co. from USA. A detailed complete list of all the chemicals and reagents with their

catalogue numbers, supplier's and manufacturer's names are found in Appendix Table 1.3.

3.3 METHODS

There are 4 main subsections has been divided in methodology section, namely 1) preparations for a successful *in vitro* produced environment, 2) preparation of stock solutions and media, 3) preparation of microtools and 4) experimental procedures.

3.3.1 Preparations for a Successful *In Vitro* Produced Environment

For successful cloning and stem cell research, it is very important to ensure that facilities and laboratory activities adhere to strict cleanliness regimes throughout all experimental procedures. Cleanliness and sterilisation are the main priority when each time an experiment is carried out to prevent contamination by microorganisms such as bacteria and fungus. A small amount of contaminant will ruin the entire cell culture even very little amount where the microorganisms are able to multiply rapidly in the nutrient-rich media. It is the responsibility of the laboratory user to maintain the laboratory in sterile to optimise the output of cloning and stem cell culture works. It is utmost important to minimise the potential introduction of contaminants to the *in vitro* produced work space, especially for oocyte or embryo handling and culture systems.

3.3.1.1 Water quality

In present study, all the media is mainly prepared using ultrapure water as based medium. Therefore, a reliable source of ultrapure water system is necessary to prevent bacterial contamination from the environment. 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 and prevent bacterial contamination.

3.3.1.2 Cleanliness and sterilisation for general laboratory research

All the solution and media were prepared inside a laminar flow work station (Gelman Sciences, Australia). The surface of the laminar flow and work bench were also cleaned and wiped with 70% ethanol (HmbG Chemicals, Germany) before and after used. For the bottles containing reagents and media, and apparatus such as pipettes were flamed before used to make it more sterile. All the Pyrex glass wares and surgical instruments were initially soaked in 7x detergent (1% v/v) (Libro, Flow Laboratories, Australia Pte. Ltd.), scrubbed thoroughly inside-out using a brush or sponge and then washed under running tap water. Then, they were rinsed thoroughly with reversed-osmosis (RO) water (Milli-Q System, Millipore Products, USA). After finished rinsed, the cap of glassware was places loosely and covered snugly with a layer of aluminium foil. Autoclave tape was placed onto the aluminum cover for identification of autoclaved items later. Alternatively, conical tubes were placed in an autoclave bag and sealed tightly; a piece of autoclave tape was placed on the autoclave bag. They were autoclaved ("Hiclave" Model HA-300M11, Hirayama, Tokyo, Japan) at 121℃ for 25 minutes and dried in an oven at 60℃ before stored in a cool, dry plastic container. After autoclaving was done, the glassware cap was tightened a little bit to prevent any contaminants from entering. The cap was not tightened completely until the glassware had cooled to prevent a vacuum forming in the glassware.

For the reusing pasteur pipettes, they were first thoroughly cleaned by soaking them in diluted 7x detergent for at least an hour. The inner walls of the pipettes were washed by pipetting up and down with 7x detergent, and then rinsed with tap water until

the bubbles from the detergent were gone. The pipettes were further cleaned through the same procedure using 70% ethanol. The outer walls were sprayed with 70% ethanol and finally rinsed with reversed osmosis water, before being autoclaved. Besides cleaned with 70% ethanol before and after used, all apparatus were irradiated using ultra-violet (UV) light for 15 to 30 minutes prior to each experiment.

3.3.1.3 Maintenance of carbon dioxide (CO_2) incubator

For culturing the embryos development *in vitro* in CO_2 incubator, 5% concentration of CO_2 is required to maintain the correct pH (pH 7.2 to 7.4) in bicarbonate-buffered culture media systems. The CO_2 incubator (HERAcell 240; Kendro Laboratory Products, Germany) was monitored regularly and LED display of temperature checked with independent readings. It is important to ensure the CO_2 incubator must maintain at 38.5℃ for caprine embryo whereas for murine sample, the temperature for CO_2 incubator should maintain at 37℃ to obtain the optimum cleavage rate. 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*.

Generally, CO_2 incubator was cleaned very month involved wiping inside walls, doors and racks with sterile reverse osmosis water and sterile towel or gauze. The tray was always filled or contained with sterile reverse osmosis water and changes in every cleaning regime for providing humidity environment in CO_2 incubator. Kept the minimum repeated opening and closing of CO_2 incubator to stabilise the culture environment.

3.3.1.4 Mineral oil

There were various types of oil that were used to overlay the microdroplets for culturing oocytes and embryos, namely mineral oil, paraffin oil or silicone oil. In current study, mineral oil (M8410; Sigma-Aldrich Co., USA) was used to overlay *in vitro* maturation, somatic cell nuclear transfer and *in vitro* culture microdroplets. Mineral oil are best for this purpose compared to silicone oil because it less toxic to oocytes and embryos as well as clear in appearance. The main purpose of using mineral oil is to prevent evaporation and delay gas diffusion as well as stabilises the pH, temperature and osmolarity of the microenvironment surrounding the oocytes and embryos during taking outside the CO_2 incubator.

3.3.2 Preparation of Stock Solutions and Media

All stock solutions and media were prepared before experiment were carried out. The methods of preparations were shown as below:

3.3.2 (a) Preparation of Stock and Solutions and Media

All fundamental stock solutions prepared were filter-sterilised using syringe-driven Millex©-GS filter (0.22 mm; Milipore, Ireland), aliquot and stored in the refrigerator (2 to 8 ℃) or freezer (-20 ℃) as appropriate. Usually, fresh solution and culture media were prepared weekly. For aseptic purposes, all medium and solution preparations were carried out in the laminar flow. The purified Milli-Q water was used as the base solution for preparing the medium. All chemicals in powder form were measured using a digital analytical balance and dissolved in Milli-Q water whereas chemicals in liquid form were measured using sterile disposable plastic pipettes. The pH of medium was measured using pH meter (7.2 to 7.4) while an osmometer was used to adjust the

osmolarity (280 to 285 mOsm) of the media. The entire prepared medium was filtered by filtration using disposable Millipore filter (0.22 μm).

3.3.2 (a) (i) Preparation of normal saline

Generally, normal saline was prepared in a 1 L bottle (Duran, Germany) by adding 9 g sodium chloride (0.9% w/v; Sigma-Aldrich Co., USA) in 1 L of Mili-Q water for surgery without any antibacterial chemicals. Normal saline was autoclaved to make it sterile and could kept for 3 months in the refrigerator (2 to 8 ℃) for future use.

3.3.2 (a) (ii) Preparation of Dulbecco's phosphate buffered saline

Dulbecco's phosphate buffered saline (DPBS) was usually prepared in 1 L bottle by dissolving 10 phosphate buffered saline tablets (Dulbeccos A, BR0014G; Oxoid Ltd., England) in 1 L of Mili-Q water. This mean 1 tablet phosphate buffered saline in each 100 ml of Mili-Q water. Same as normal saline, the Dulbecco's phosphate buffered saline was sterilsed by autoclaving and could kept for 3 months in the refrigerator (2 to 8 ℃) for future use .

3.3.2 (a) (iii) Preparation of ovary collection medium

Ovary collection medium was prepared as normal saline except it supplemented with gentamycin sulphate (G1264; Sigma-Aldrich Co., USA). The purpose of ovary collection medium is used for washing and collecting ovaries from abattoir. Gentamycin sulphate (30 ug/ml) was weighed and dissolved in normal saline solution that was prepared earlier and kept in the refrigerator to obtain the final volume (300 ml) (Table 3.1). The ovary collection medium was warmed in water bath at 38.5 ℃ before being used.

Table 3.1: Composition of ovary collection medium

Components	Concentration	Volume/weight
Autoclaved normal saline	1x	300 ml
Gentamycin sulphate	30 μg/ml	9 mg
	Total volume	300 ml

3.3.2 (a) (iv) Preparation of ovary slicing medium

Ovary slicing medium also known as TL-hepes was used for rinsing, washing and slicing of ovaries collected either from ovariectomised or abattoir-derived ovaries before transferring into the maturation medium. The stock solution of TL-hepes was prepared as shown in Table 3.2.

Table 3.2: Composition of TL-hepes stock solution

Components		Sigma Cat. No.	Final Conc. (mM)	g/1000 ml
NaCl		S 5886	114	6.6600
KCl		P 5405	3.2	0.2400
$NaHCO_3$		S 5761	2.0	0.1680
$NaH_2PO_4.H_2O$		S 9638	0.4	0.0560
Na Lactate (60%Syrup)		L 7900	10.0	1.8600 ml
$CaCl_2.2H_2O$		C 3881	2.0	0.3000
$MgCl_{2.}6H_2O$		M 2393	0.5	0.1000
HEPES	$C_8H_{17}N_2O_4SNa$	H 0763 or H 3784	10.0	1.2000
	$C_8H_{18}N_2O_4S$	H 3375 or H 6147		1.2000
Penicillin G		PEN-NA	100 IU/ml	0.0650
Phenol Red		P 3532		0.0050

The TL-hepes stock solution was check for pH (7.4 ± 0.5) and osmolarity (255 to 270 mOsm) The TL-hepes stock was filtered (0.2 μm) into sterile bottles and store at 4^oC for 1 to 2 weeks. Before using as ovary slicing medium, TL-hepes stock solution should supplemented with gentamycin sulphate, sodium pyruvate (P4562) and bovine serum albumin-fraction V (A7030) as shown in Table 3.3.

Tab le 3.3: Composition of TL-hepes working solution

Chemicals	Volume/weight
TL-hepes stock solution	100 ml
Gentamycin stock solution	50 µl
Sodium pyruvate (Sigma; P4562)	0.0022 g
BSA-fraction V (Sigma; A7030)	0.1000 g

Prepare the working solution on the day of use and were filtered (0.2 µm) and used freshly. However, TL-hepes working solution can be prepared directly and whole solution can be used within 3 to 4 days.

3.3.2 (a) (v) Preparation of flushing medium for laparoscopic ovum pick-up

Flushing medium (300 ml) was prepared within 12 hours before oocytes retrieval by laparoscopic ovum pick-up. The flushing medium was used for flushing microvolumes of fluid into the ovarian follicle and aspirated with the oocytes together with fluid from the follicle was connected to an aspiration system (a vacuum pump). The aspirated medium was collected in test tube to be searched for oocytes under stereomicroscope. The flushing medium consisted of Dulbecco's phosphate buffered saline supplemented with gentamycin sulphate (50 µ/ml; Sigma-Aldrich Co., USA) and heparin (50 µ/ml; H0777; Sigma-Aldrich Co., USA) as depicted in Table 3.4. The flushing medium was filter-strilised using syringe-driven Millex©-GS filter (0.22 mm; Milipore, Ireland), aliquot into syringe (50 ml; Terumo Corporation, Japan) maintained at 38.5 ℃ prior to oocytes retrieval (Table 3.4).

Table 3.4: Composition of flushing medium (1000 ml or 1 L)

Chemical component (Catalogue number)	Final concentration	Quantity/1000 ml
Phosphate-buffered saline (PBS) tablets (P4417)	1 tablet/100 ml	PBS (10 tablets) were dissolved in Mili-Q water (100 ml), sterilised by autoclaving.
Gentamycin sulphate (G3632)	50 µg/ml	Gentamycin sulphate (50 mg) was dissolved in phosphate-buffered saline solution (1000 ml) prior to use.
Heparin (H0777)	50 µg/ml	Heparin (50 mg) was dissolved in phosphate-buffered saline solution (1000 ml) prior to use.

3.3.2 (a) (vi) Blood collection and preparation of oestrus goat serum (OGS)

The caprine blood samples were collected aseptically through jugular vein from caprine synchronised for oestrus using vacutainer tubes (without heparin) with a needle (21 G). The fresh collected blood was distributed into sterile conical tube (15 ml) and kept in laminar flow at room temperature for approximately 3 hours to obtain the serum from clotted blood.

3.3.2 (a) (vii) Heat-inactivation

The conical tubes containing clotted blood were centrifuged (3000 rpm) for 10 minutes at 25 ℃. The supernatant (serum) was aspirated out the placed in a sterile conical flask and heat-inactivated in a water bath for 30 minutes at 56 ℃ to destroy components that might lead to cell lysis by antibody binding. The serum was removed from the water bath after 30 minutes of treatment at 56 ℃ to let it cooling at room temperature (25 ℃). Prolong heat treatment could cause deterioration of some components of the serum. The serum was centrifuged again at 1000 rpm, for 10 minutes at 25 ℃ to sediment residual erythrocytes after cooling to room temperature. The oestrus goat serum was aliquot into small microcentrifuge tubes (1.5 ml) and stored at -20 ℃ for 6 months.

3.3.2 (a) (viii) Preparation of oestrus goat serum (OGS)

Oestrus goat serum (OGS) was supplemented in the culture media to provide additional growth factors, hormones and pipettes which may be present in the serum.

3.3.2 (a) (viiii) Preparation of *in vitro* maturation (IVM) medium

In vitro maturation (IVM) medium was prepared a day before and equilibrated overnight in the CO_2 incubator (5%) prior to oocyte retrieval. The maturation medium used was TCM 199 supplemented with cystein (0.0085 g; Sigma-Aldrich, Co., USA, C2529), bFSH (10 μg/ml; Folltropin-V^R, Vetripharm, L032-B053), 17 β-oestradiol (1 μg/ml; Sigma-Aldrich, Co., USA, E8875), sodium pyruvate (0.22 Mm; Sigma- Aldrich Co., USA, P-4562), gentamycin sulphate (50 μg/ml; Sigma-Aldrich Co., USA, G3632) and foetal bovine serum (10%; Gibco BRL,16000-044) as depicted in Table 3.5.

Table 3.5: Composition of *in vitro* maturation medium (10 ml)

Components	Volume/ml
TCM-199	8.9 ml
Foetal bovine serum	1.0 ml
TCM-pyruvate	100 μl
Bfsh	10 μl
Gentamycin sulphate	5 μl

After added with all the chemicals above, the *in vitro* maturation medium was filtered (0.2 μm) in a 15 ml centrifuge tube. After that, the 17 β-oestradiol stock (1.0 μl/ml) was added to the above solution. Pre-incubated the *in vitro* maturation medium at least 3 hours in CO_2 incubator set at CO_2 (5%), 38.5^{o}C in humidified air.

3.3.2 (a) (x) Preparation of hyaluronidase solution (0.2%)

Hyaluronidase solution was used to denude cumulus complexes oocytes after maturation. A type IV-S hyaluronidase from bovine testes (H4272; Sigma-Aldrich Co.,

USA) was used to prepare hyaluronidase solution. The preparation of mDPBS was depicted as Table 3.6. The hyaluronidase solution was prepared by added polyvinylpyrrolidone (PVP) (0.05 g) and hyaluronidase (0.10 g) into 50 ml mDPBS (Table 3.7). The prepared solution was filter-sterilesed by Millex©-GS filter (0.22 mm; Millipore. Ireland) and aliquot (100 µl) in microcentrifuge tubes (0.5 ml; Eppendorf GmbH, Germany) and stored for 6 months in the freezer (-20 ℃) (Tables 3.6 and 3.7).

Table 3.6: Based medium for hyaluronidase solution (mDPBS)

Components	Volume/weight
NaCl	4.0000 g
KCl	0.1000 g
KH_2PO_4	0.1000 g
Na_2HPO_4	0.5750 g
Glucose	0.5000 g
Pyruvic acid	0.0180 g
$CaCl_2$.2HZO	0.0687 g
MgCl2.6H2O	0.0500 g
Penicillin-streptomycin (stock 100 x)	500 µl
Ultrapure water	500 ml

Table 3.7: Composition of hyaluronidase solution (0.2%)

Components	Volume/weight
Hyaluronidase	0.1000 g
Polyvinylpyrrolidone (PVP360)	0.0500 g
mDPBS	50 ml

3.3.2 (a) (xi) Preparation of polyvinylpyrrolidone (PVP) (10%)

The polyvinylpyrrolidone (PVP 360; Sigma-Aldrich Co., USA) was used in somatic cell nuclear transfer during donor cell injection. The function of polyvinylpyrrolidone is to make the donor cell became swollen and easier to break the membrane of donor cell during nuclear transfer procedure. Usually 10% of polyvinylpyrrolidone was used in this study. The polyvinylpyrrolidone was prepared as shown in Table 3.8.

Table 3.8: Composition of polyvinylpyrrolidone

Components	Volume/weight
Polyvinylpyrrolidone	0.1000 g
TL-HEPES working solution	1000 ml

3.3.2 (a) (xii) Preparation of cytochalasin B (CB) stock

Preparation of cytochalasin B stock was in 100x which cytochalasin B (1 mg) was mixed with dimethyl sulfoxide (DMSO) (2 ml) to make sure that cytochalasin B is completely dissolved and aliquot into small microcentrifuge tube (10 µl) and could be stored at -20 ℃ (Table 3.9). Before the experiment, the working solution of cytochalasin B was prepared by adding with holding medium (990 µl) (Table 3.10) and mixed well and it will be ready to use.

Table 3.9: Preparation of cytochalasin B (CB) stock

Components	Volume/weight
Cytochalsain B (CB)	1 mg
DMSO	2 ml

Table 3.10 Preparation of cytochalasin B (CB) working solution

Components	Volume/weight
TL-hepes working solution	990 µl
Cytochalasin B (stock)	10 µl

3.3.2 (a) (xiii) Preparation of calcium ionophore solution

Calcium ionophore (Ca^{2+} ionophore; I0634; Sigma-Aldrich Co., USA) was used to activate caprine oocytes after the somatic cell nuclear transfer. A solution containing dimethyl sulfoxide and absolute ethanol in 3:1 ratio was prepared and the whole content of calcium ionophore vial (1 mg) was dissolved into dimethyl sulfoxide-ethanol solution (1.34 ml),labeled as ‘Stock A’. The concentration of ‘Stock A’ solution was 1

mM. The ‘Stock A’ was aliquot (10 µl) into each microcentrifuge tube (0.2 µl; Eppendorf GmbH, Germany) and wrapped in aluminium foil and could be stored up to 6 months in the freezer (-20°C). On the day of experiment, one tube of ‘Stock A’ was taken out from freezer and *in vitro* culture medium (90 µl) was added. The solution with (100 µl) was mixed properly and labeled as ‘Stock B’ with concentration 100 µM. From ‘Stock B’, 10 µl was withdrawn and diluted again with *in vitro* culture medium (90 µl) to make it final concentration (10 µM) to activate caprine oocytes.

3.3.2 (a) (xiiii) Preparation of 6-dimethylamino pyridine (6-DMAP) solution

In present study, 0.2 M concentration is the final concentration for activation in caprine oocytes. 0.25 mg of 6-dimethylamino pyridine was diluted with 7.7 ml to make 0.2 M concentration. After diluted, the 6-dimethylamino pyridine was aliquot into small microcentrifuge tubes (10 µl) and stored at -20°C as ‘Stock A’. The day before treatment, 10 µl of ‘Stock A’ was withdrawn and diluted again with *in vitro* culture medium (990 µl) to use for activated caprine oocytes.

3.3.2 (a) (xv) Preparation of k simplex optimisation medium (KSOM) stock solution for caprine

The k simplex optimisation medium (KSOM) stock solution was prepared as shown in Tables 3.11 and 3.12

Table 3.11: Composition of KSOM stock solution for caprine

Component	Cat.No.	Mol.wt	Conc. (mmol/l)	g/100ml	g/200ml
NaCl	S-5886	58.45	95	0.5553	1.1106
KCl	p-5405	74.56	2.5	0.0186	0.0373
KH_2PO_4	P-5655	136.09	0.35	0.0048	0.0095
$MgSO_4$	M-7506	120.4	0.2	0.0024	0.0048
Lactate	L-7900	112.1	10	0.1860 ml	0.3720 ml
Pyruvate	P-4562	110.04	0.2	0.0022	0.0044
D-Glucose	G-6152	179.86	0.2	0.0036	0.0072
$NaHCO_3$	S-5761	84.02	25	0.2101	0.4201
$CaCl_2$	C-5670	111	1.71	0.0190	0.0380
L-Glutamine	G-3126	146.1	1	0.0146	0.0292
EDTA	E-9884	292.2	0.01	0.0004 (10 µl of 0.5 M EDTA, pH 8.0)	0.0007 (10 µl of 0.5 M EDTA, pH 8.0)

Filter (0.2 µ) and store at 4^0C for up to 4 weeks.

Table 3.12: Composition of KSOM working solution

Component	Cat.No.	Amount/10 ml
Stock	-	9.85 ml
BSA	A-6003	0.0400 g
MEM	M-7145	50 µl
BME	B-6766	100 µl

After prepared the working solution of KSOM, the medium was filtered (0.2 µm) and preincubate for at least 2 hours in CO_2 incubator before used. An additional D-glucose (0.004g) was added into KSOM working solution for culturing later stage of bovine and caprine embryos (P.J. Kwong, personal communication).

3.3.2 (b) Preparation of Media and Solution for Murine Sample

Various medium were used in the present study in murine experiment. These media were the *in vitro* culture medium (modified Whitten's Medium), embryo handling medium (modified Hepes Whitten's medium), mouse embryonic fibroblasts culture medium, mouse embryonic fibroblasts washing medium, mouse embryonic fibroblasts cryopreservation medium and embryonic stem cell culture medium. Media were prepared and stored in scotch bottles (100 ml and 250 ml) and could kept at 4 ℃ in the refrigerator. Media without the addition of bovine serum albumin (BSA) can be stored for up to 3 months. After the addition of bovine serum albumin to media (Whitten's medium and Hepes Whitten's medium), they must be used within a week.

3.3.2 (b) (i) Preparation of Modified Whitten's medium (WM medium)

Modified Whitten's medium was prepared based on Whitten's medium with a few modifications such as the addition of sodium ethylenediaminetetraacetic acid (EDTA). This medium was used for the *in vitro* culture of murine embryos develop to the blastocyst stage. The Whitten's medium that supplemented with ethylenediaminetetraacetic acid enhances embryo development into blastocysts (Abramczuk *et al.*, 1977). Sodium ethylenediaminetetraacetic acid stock (10 mM) was prepared separately beforehand by dissolving a measured amount of sodium ethylenediaminetetraacetic acid in Milli-Q water. Along with other chemical ingredients except bovine serum albumin was dissolved in Milli-Q water to make up 100 ml of stock WM medium. The pH of medium was checked using the pH meter and adjusted to pH 7.25. The osmolarity of was checked by using the osmometer and adjusted to 285 mOsm. The stock medium was then filtered using sterile filters (0.22 μm pore size) into Scott bottle (250 ml). Bovine serum albumin (3%) was added to the stock medium before used for experiment (Table 3.13).

Table 3.13: Chemicals used in the preparation of modified WM medium

Chemicals	g / 100 ml
NaCl	0.5140
KCl	0.0356
KH_2PO_4	0.0162
$MgSO_4.7H_2O$	0.0294
$NaHCO_3$	0.1900
Glucose	0.1000
Calcium lactate	0.0338
Lactic acid	0.37 ml
Natrium pyruvate	0.0029
Penicillin	0.0075
Streptomycin	0.0050
L-glutamine	0.0146
Taurine	0.0125
Phenol red	0.0010
Na_2 EDTA	0.10 ml
BSA	0.3000

3.3.2 (b) (ii) Preparation of Modified Hepes Whitten's medium (HWM medium)

Hepes Whitten's medium was used for embryo manipulation outside the incubator, such as embryo washing and embryo pick-up. All chemicals except for bovine serum albumin were diluted in Milli-Q water (50 ml). After all the chemicals had dissolved, additional Milli-Q water was added to make a stock solution of 100 ml. pH of this medium was checked and adjusted to 7.25 and osmolarity adjusted to 275 to 325 mOsm. The medium was then filtered with sterile filter and stored at 4℃ in the refrigerator. Same as the Whitten's Medium, the stock medium were aliquot and bovine serum albumin (3%) was added. The chemicals used in the preparation of modified HWM medium as given in Table 3.14.

Table 3.14: Chemicals used in the preparation of modified HWM medium

Chemicals	g / 100 ml
NaCl	0.5140
KCl	0.0356
KH_2PO_4	0.0162
$MgSO_4.7H_2O$	0.0294
$NaHCO_3$	0.1900
Glucose	0.1000
Calcium lactate	0.0338
Lactic acid	0.37 ml
Natrium pyruvate	0.0029
Penicillin	0.0075
Streptomycin	0.0050
L-glutamine	0.0146
Taurine	0.0125
Phenol red	0.0010
Na_2 EDTA	0.10 ml
BSA	0.3000

3.3.2 (b) (iii) Preparation of strontium chloride (Sr^{2+}) for oocyte activation

Strontium chloride is used for murine oocyte activation after nuclear transfer. $SrCl_2.6H_2O$ powder was weight 2.666 g and directly put into 100 ml of Milli-Q water. Then, let the powder completely dissolved and aliquot small volume into the vials (10ul/vial) and keep 4 ℃.

3.3.2 (b) (iv) Preparation of tissue culture medium for mouse embryonic fibroblasts

There were 2 types of tissue culture medium for mouse embryonic fibroblasts, namely alpha minimum essential medium (αMEM*)* and Dulbecco's modified eagle medium (DMEM). For alpha minimum essential medium, the tissue culture medium was prepared by adding alpha minimum essential medium (αMEM*)* (M0644- 1L; Sigma-Aldrich Co., USA) with sodium bicarbonate ($NaHCO_3$, 2.2 g; Sigma-Aldrich Co., USA) into 1 L sterile Mili-Q water and mixed well by magnetic stirrer. The Dulbecco's modified eagle medium (D5796-1L) component comes prepared in solution form which

purchase from ready-made stock solution from Sigma-Aldrich Co., USA consisted 4500 mg glucose/L, L-glutamine, $NaHCO_3$, pyridoxine and HCL. Thus only foetal bovine serum and antibiotics need to be added. The foetal bovine serum must be heat-inactivated in the water bath at 56 ℃ for 30 minutes before used. The medium was then filtered with sterile filter and stored at 4 ℃ in the refrigerator and can last for 1 month (Tables 3.15, 3.16 and 3.17).

Table 3.15: Alpha minimum essential medium **(**αMEM) stock solution (1 L)

Chemicals	Amount
αMEM (powder)	Whole bottle
$NaHCO_3$	2.2 g
Mili-Q water	1 L

Table 3.16: Alpha minimum essential medium **(**αMEM) working solution (100 ml)

Chemical	Amount
αMEM (Stock solution)	90 ml
Foetal bovine serum	10 ml
Penicillin-streptomycin	300 µl (3X) or 100 µl (1X)

Table 3.17: Dulbecco's modified eagle medium (DMEM) working solution (100 ml)

Chemical	Amount
DMEM (Stock solution)	90 ml
Foetal bovine serum	10 ml
Penicillin-streptomycin	300 µl (3X) or 100 µl (1X)

3.3.2 (b) (v) Freezing medium for mouse embryonic fibroblasts

In freezing medium, the percentage of foetal bovine serum is higher compared to tissue culture medium which is 20%. Besides that, the freezing medium also with the addition of dimethyl sulfoxide (20%) which is sensitive to light. Therefore, the prepared freezing medium should be wrapped with aluminium foil after being filtered and was stored at 4 ℃ in the refrigerator. The freezing medium could last for 1 to 2 weeks.

3.3.2 (b) (vi) Embryonic stem cells culture medium

Embryonic stem cells culture medium (Table 3.18) was used to culture both the inner cell mass outgrowth from the blastocyst as well as subsequent passages of embryonic stem cells. Alpha minimum essential medium and Dulbecco's modified eagle medium (αMEM and DMEM) were used as the base medium to campare the growth rate of embryonic stem cells in different medium. The only differences from the mouse embryonic fibroblast culture medium are the addition of leukaemia inhibitory factor and beta-mercaptoethanol to maintain the embryonic stem cells in an undifferentiated state. Filtering was done and was stored in 4℃ refrigerator for 1 month. The medium was warmed up to 38℃ before used. The components of embryonic stem cells culture medium are given in Table 3.18.

Table 3.18: Embryonic stem cell culture medium (50 ml)

Chemicals	Volume	Concentration
α-MEM/DMEM	38.5 ml	
Foetal bovine serum	10 ml	20%
Penicillin-streptomycin	50 µl	Standard
L-glutamine	0.5 ml	1mM
MEM-NEAA	0.5 ml	0.1 mM
β-merchaptoethanol	0.5 ml	0.1 mM
Leukaemia inhibitory factor	10 µl	1000 IU/ml

3.3.2 (b) (vii) Freezing medium for embryonic stem cells

For cryopreserved the embryonic stem cells, freezing medium was prepared as depicted in Table 3.19. Foetal bovine serum (10%) was added as well as dimethyl sulfoxide (20%) into freezing medium. Besides that, penicillin and streptomycin were also added to prevent bacteria growth. Since the dimethyl sulfoxide was sensitive to light, the prepared freezing medium was wrapped with aluminium foil after being filtered and was stored at 4℃ in the refrigerator. The freezing medium could last for 1 to 2 weeks. The components of freezing medium for embryonic stem cells as given in Table 3.19.

Table 3.19: Freezing medium for embryonic stem cells (10 ml)

Chemicals	Volume
Embryonic stem cell culture medium	6 ml
Foetal bovine serum (Hyclone; SH30070.03)	2 ml
DMSO	2 ml

3.3.3 Preparation of Solutions in Murine

In the present study, there were several types of hormones and solutions used. For example, superovulation hormones (pregnant mare's serum gonadotrophin and human chorionic gonadotrophin), cell washing solution (PBS-; phosphate buffered saline without Ca^{2+} and Mg^{2+}), trypsinisation solution, gelatin (0.1%), mitomycin C and pronase (0.5%).

3.3.3 (a) Preparation of hormone solutions

There were 2 types of hormones namely, pregnant mare's serum gonadotrophin and human chorionic gonadotrophin were administered to the female murine to induce superovulation and control the time of ovulation for timed mating. This will allow a large number of embryos to be obtained. The hormones were prepared from stock vials and filtered using sterile filters (0.22 μm pore size). Saline solution (0.9%) was used as the base solution, and was prepared beforehand by diluting 0.9 g NaCl in 100 ml Milli-Q water and filtered with sterile filter. They were stored in sterile syringes (1 ml) in the freezer (-20 ℃). Before using, the frozen hormones were thawed to room temperature. The prepared hormones can last for 3 months.

3.3.3 (a) (i) Preparation of Pregnant mare's serum gonadotrophin (PMSG)

A bottle of pregnant mare's serum gonadotrophin vials contain 1000 IU of white, freeze-dried crystalline plug. Generally, they were dissolved in 20 ml of filtered saline (0.9%) and made up to 50 IU/ml. Aliquot the pregnant mare's serum gonadotrophin

hormone into 1 ml syringe and labelled properly as well as stored in freezer (-20 ℃) for future use.

3.3.3 (a) (ii) Preparation of Human chorionic gonadotrophin (hCG)

A vial of human chorionic gonadotrophin contain 5000 IU of white, freeze-dried crystalline plug and they were dissolved in 100 ml of filtered saline (0.9%) and made up to 50 IU/ml. Same as preparation of pregnant mare's serum gonadotrophin, the human chorionic gonadotrophin hormone was aliquot into 1 ml syringe and labeled properly and kept in freezer (-20 ℃) for future use.

3.3.3 (b) Preparation of Modified phosphate buffered saline (PBS-)

Phosphate buffered saline with Ca^{2+} and Mg^{2+} free solution (Table 3.20) was used as the base medium for trypsin and for washing cell cultures. The phosphate buffered saline with Ca^{2+} and Mg^{2+} free solution was prepared by adding NaCl (Sigma-Aldrich Co., USA), KCl (Sigma-Aldrich Co., USA), Na_2HPO_4 (Sigma-Aldrich Co., USA), KH_2PO_4 (Sigma-Aldrich Co., USA) into ultrapure water (1 L) as shown at Table 3.20. The solution could be stored in the refrigerator (4 ℃) for up to 6 months. The constituents of PBS (-) as given in Table 3.20.

Table 3.20: Preparation of phosphate buffered saline with Ca^{2+} and Mg^{2+} free (PBS-)

Chemicals	Volume/weight
NaCl	10 g
KCl	0.25 g
Na2HPO4	1.44 g
KH2PO4	0.25 g
Ultra pure water	1 L

3.3.3 (c) Preparation of Trypsin/EDTA (0.25%)

The trypsinisation of stem cell medium was prepared by adding the trypsin (Sigma-Aldrich Co., USA; from porcine pancrease) and EDTA powders into 100 ml of phosphate buffered saline with Ca^{2+} and Mg^{2+} free solution as described in Table 3.21 and stirred using a magnetic stirrer. The solution could be stored after filtering using a sterile filter.

Table 3.21: Prepration of trypsin/EDTA (0.25%)

Chemicals	Volume/ weight
Trypsin	0.25 g
EDTA	0.04 g
PBS (-)	100 ml

3.3.3 (d) Preparation of Gelatin (0.1%)

The preparation of 0.1% gelatin solution involve the simple addition of gelatin powder (0.100 g; Sigma-Aldrich Co., USA, G-8150) into phosphate buffered saline without calcium and magnesium solution and mixed thoroughly and stirred by using a magnetic stirrer. After dispensing into a scotch bottle, the solution was autoclaved and subsequently stored in the refrigerator (-4 ℃) (Table 3.22).

Table 3.22: Composition of gelatin (0.1%)

Chemicals	Volume/weight
Gelatin	0.1 g
PBS (-)	100 ml

3.3.3 (e) Preparation of Mitomycin C (MTC) stock

To prepare stock mitomycin C solution, diluted 2 mg of mitomycin C (Sigma-Aldrich Co., USA, M-4287) with 2 ml of Dulbecco's modified eagle medium or alpha minimum essential medium was as described in Table 3.23. After that, the stock mitomycin C was aliquots into small vials which each vials contained 55 µl/vial were made and stored at -

20℃ and could last for 2 to 3 months. For mitotic inactivation, 50 µl of stock mitomycin C was diluted in 5 ml of Dulbecco's modified eagle medium or alpha minimum essential medium culture medium in a conical tube to obtain a final concentration (10 µg/ml). The solution was shaken gently to mix well. It could be used up to 1 week and kept at 4℃.

Table 3.23: Composition of mitomycin C stock

Chemicals	Volume/weight
Mitomycin C	2 mg
DMEM or αMEM	2 ml

3.3.3 (f) Preparation of Pronase (0.5%)

Pronase powder (0.5 g; Sigma-Aldrich Co., USA, P-8811) was weighed and diluted in mDPBS solution (50 ml) and stirrer using a magnetic stirrer. The solution was then filtered and aliquot into 1.5 ml centrifuge tubes. It could be kept for up to 6 months at -20℃. The chemical composition is detailed in Table 3.24.

Table 3.24: Composition of pronase (0.5%)

Chemicals	Volume/weight
Pronase	0.5000 g
mDPBS	50 ml

3.3.4 Preparation of Microtools and Accessories

Microtools that were used in present study included holding pipette, cutting needle, biopsy needle, injection needle and La banana (oocytes or embryos picking and denudation pipette). All the glass pipette were prepared 'in-house' in the laboratory.

3.3.4.1 Preparation of hand-controlled micropipette

The purpose of a hand-controlled pipette was for handling the oocytes and embryos and the pulled-haematocrit capillary attached to silicone tubing at one end. The other end of the silicone tubing was completely sealed with silicone glue. The glass capillary 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 smoothly to produce a tube with an internal diameter of approximately 200 µm and 400 µm for embryos and cumulus oocyte complexes, respectively. The pulled portion of the capillary was scribed with a diamond-tip pen and snapped at the scribed portion for a neat break to obtain evenly snapped straight tip because a jagged capillary end may cause damage to the zona pellucida during manipulation of oocyte or embryo. Besides that, the pipette with sharp edges were easily caught by the plastic surface of the culture dish and causing the pipette to break and tend to collect more debris during manipulation, particularly in handling the cumulus oocytes complex. The tip of the pipette was fire polished by touching the flame quickly to achieve a smooth edge of the micropipette tip.

3.3.4.2 Capillary cleaning and sterilisation

The capillary tubes were soaked overnight in 7x (FlowLab^{TM}, Australia). Then, they were washed thoroughly and rinsed vigorously 5 times first with tap water followed by reverse osmosis water. The washed capillary tubes and Borosilicate glass tubing for making holding pipette, cutting needle, biopsy needle and injection needle were soaked in hydrochloric acid (10%; HmbG Chemicals, Germany) overnight before being washed 20 times in Milli-Q water. Lastly, the capillary for La banana were sterilized by autoclaving at 121 ℃ for 20 minutes and were dried in the oven at 60 ℃. The capillaries

for holding pipettes and other injection needles were kept properly in a sterile container followed by drying and sterilisation in the oven at 180 ℃ for 4 hours.

3.3.4.3 Preparation of holding pipettes

Capillary tube were placed and fixed into the micropuller. According to the need, the parameters were set to a programme. For this study, the parameters were set at programme 0 (heat: 780, pull=80, velocity=130 and time=20). The pull button (green) was switched on. The filament is gradually heated up, became red hot and produced 2 uniform sized pulled pipettes or needles. The pulled micropipette was placed into the holder of the microforge horizontal to the glass bead. The pipette was squared and broke at a diameter between 150 to 180 μm (Figure 3.2). After squaring, the pipette placed vertically; the opening of the pipette should be on the top of the glass bead. The filament was fired polished in such a way that the inner diameter (ID) of the opening becomes approximately 25 μm. The pipette was placed again horizontal to the glass bead. The pipette was bent to an angle of 19˚C. The holding pipette was cleaned 3 times with HFL (10%) and rinsed 10 times with Mili-Q water (blown only). After preparing holding pipettes, sterilised these are at 150˚C for 2 hours in an oven before cloning procedure. Sterilisation can also be done by applying ultra-violet light inside the laminar flow work station for 30 minutes. Holding pipettes was stored in a stainless steel container that is wrapped up with a layer of aluminium foil or in sterilised recycled needle and pipette holders.

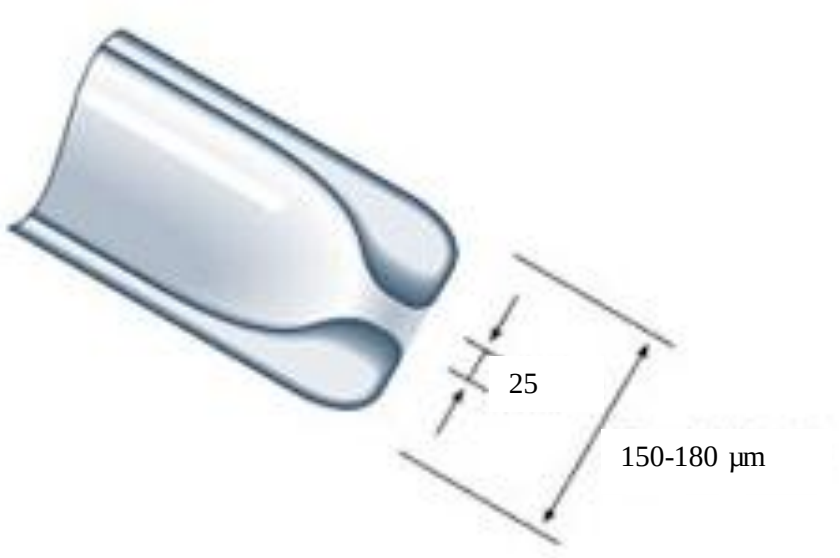

Figure 3.2: The inner and outer diameter of the holding pipette.

3.3.4.4 Preparation of cutting needle

The pulled micropipette was placed into the holder of the microforge horizontal to the glass bead (in the same procedure for a holding pipette). The needle was squared and broke and placed vertically; the opening of the needle should be on the top of the glass bead. The filament was switched on and brought towards the needle. The filament was pulled it up and down to make a sharp end without a hole. The cutting needle was placed again horizontal to the glass bead. The cutting needle was bent to an angle of 19˚C. The holding pipette was cleaned 3 times with HFL (10%) and rinsed 10 times with Mili-Q water (blown only). After preparing cutting needle, it was sterilised at 150˚C for 2 hours in an oven before cloning procedure. Sterilisation can also be done by applying ultraviolet light inside the laminar flow work station for 30 minutes. Cutting needle (Figure 3.3) was stored in a stainless steel container that was wrapped up with a layer of aluminium foil or in sterilised recycled needle and pipette holders.

Figure 3.3: Cutting needle.

3.3.4.5 Preparation of biopsy needle

The pulled micropipette was placed into the holder of the microforge horizontal to the glass bead (in the same procedure for a holding pipette). The needle was squared and broke is at a diameter 30 to 35 µm. After squaring, the pipette was placed vertically; the opening of the pipette should be on the top of the glass bead. The filament was switched on and fire polished was (just to make it in blunt shape) in such a way that the ID of the opening becomes approximately 30 µm. The pipette was placed again horizontal to the glass bead and bended to an angle of 19˚C. The pipette was cleaned 3 times with HFL (10%) and rinsed 10 times with Mili-Q water (blown only). After preparing biopsy needle, it was sterilised at 150˚C for 2 hours in an oven before cloning procedure. Sterilisation can also be done by applying ultraviolet light inside the laminar flow work station for 30 minutes. Biopsy needles (Figure 3.4) were stored in a stainless steel container that is wrapped up with a layer of aluminium foil or in sterilised recycled needle and pipette holders.

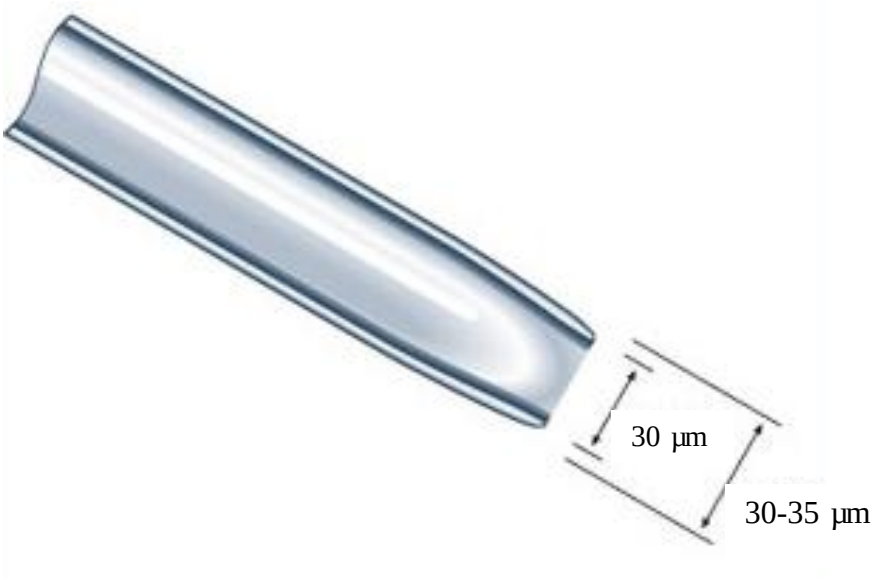

Figure 3.4: Biopsy needle.

3.3.4.6 Preparation of injection needle

The pulled micropipette was placed into the holder of the microforge horizontal to the glass bead (in the same procedure for a holding pipette). After squaring, the pipette placed vertically; the opening of the pipette should be on the top of the glass bead. The filament was fired polished in such a way that the inner diameter (ID) of the opening becomes approximately 10 µm. The pipette was placed again horizontal to the glass bead. Injection needle were bended to an angle of 19˚C for easy and better cloning performance. The injection needle with blunt end was cleaned 3 times with HFL (10%) and rinsed 10 times with Mili-Q water (blown only). After preparing injection needle, sterilised these are at 150˚C for 2 hours in an oven before cloning procedure. Sterilisation can also be done by applying ultra-violet light inside the laminar flow work station for 30 minutes. Injection needle was stored in a stainless steel container that is wrapped up with a layer of aluminium foil or in sterilised recycled needle and pipette holders.

3.3.5 Experimental Procedures

Following are different experimental procedures mainly employed to obtain murine and caprine blastocysts derived from different embryo techniques (parthenogenesis, somatic cell nuclear transfer and *in vivo* flushing) in an attempt to produce caprine and murine embryonic stem cells lines using mouse embryonic fibroblasts as a feeder layer with special reference to inner cell mass isolation techniques.

3.3.5.1 Sources of blastocyst obtained in caprine and murine species

Caprine and murine blastocysts could be obtained from 2 sources, namely *in vitro* or *in vivo* techniques.

3.3.5.1 (A) *In vitro*-derived blasotcyst in caprine species

Caprine blastocysts *in vitro* were derived from somatic cell nuclear transfer (SCNT) and parthenogenetic activation of oocytes. The cloned-caprine blastocysts and pathenogenetic blastocysts were used as a source for production of caprine embryonic stem cell lines.

3.3.5.1 (A) (a) Caprine oocyte retrieval

Different methods of oocytes retrieval techniques were carried out in caprine and murine. In caprine oocytes retrieval, the oocytes were collected from laparoscopic ovum pick-up, ovariectomy (via laparotomy) and ovary slicing from abattoir at Shah Alam and Senawang, whereas the oocyte retrieval from murine by superovulation and subsequently retrieved oocyte from ampulla.

3.3.5.1 (A) (b) Caprine oocyte retrieval through laparoscopic ovum pick-up (LOPU) procedure

Several essential steps were carried out to retrieve caprine oocytes through laparoscopic ovum pick-up procedure, namely oestrus synchronisation, superovulation, anaesthesia and sedation, disinfection of surgical instruments and skin area of the donor does.

3.3.5.1 (A) (b) (i) Oestrus synchronisation of caprine donor

Before retrieving the oocytes through laparoscopic ovum pick-up procedure, the experimental does underwent oestrus synchronisation to manipulate the oestrous cycle of the does to facilitate planning the time and date of oocytes retrieval during laparoscopic ovum pick-up. In order to synchronise the oestrous cycle, a Controlled Intravaginal Drug Release device (CIDR, 0.3 g progesterone; EAZI BREED™, CIDR®, Pharmacia and Upjohn Limited, New Zealand) was inserted into the vagina of the caprine donor with the help of a clean and sterile controlled intravaginal drug release (CIDR) applicator (Pharmacia and Upjohn Limited, New Zealand) with water-based lubricant (K-Y Jelly, Pharmedica Lab Pty Ltd., South Africa). The controlled intravaginal drug release device remained in the vagina for 14 days (at 0900 hours on day 0 was the day of CIDR insertion) before being removed. The controlled intravaginal drug release 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 of Estrumate® (125 μg cloprostenol; Schering-plough, Australia) was injected intramuscularly (at 0900 hours on day 12) to regress the corpus luteum that facilitates initiation of pro-oestrus and eventually resulted in oestrogen surge for the onset of oestrus (heat). Upon removal of controlled

intravaginal drug release device (at 2100 hours on day 13), a gonadotrophin treatment of Ovidrel™ (250 IU) was injected intramuscularly 60 hours post-Ovidrel™ to stimulate multiple follicular development prior to oocyte retrieval surgery (at 0900 hours on day 16) (Figure 3.5).

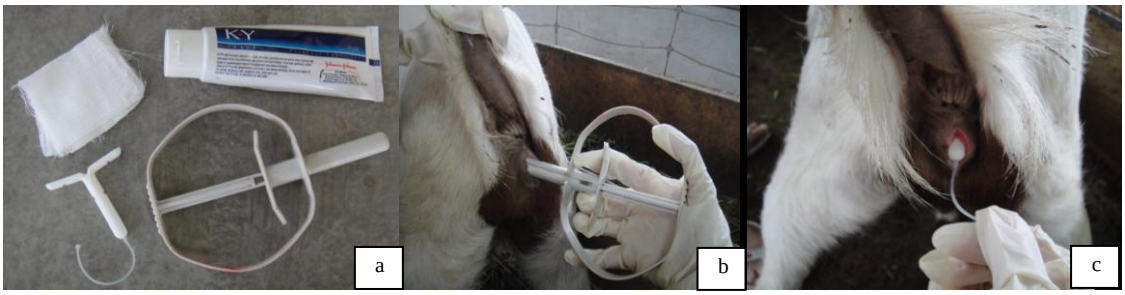

Figure 3.5: (a) Controlled intravaginal drug release device (CIDR), controlled intravaginal drug release device applicator, sterile gauze and K-Y Jell. (b) Insertion of CIDR. (c) Removal of CIDR.

3.3.5.1 (A) (b) (ii) Superovulation of caprine donor

On day 0 (day of CIDR insertion), controlled intravaginal drug release device was inserted on the selected female caprine at 0900 hours. On day 9, Estrumate (0.5 ml) was injected to the same female caprine at 0900 hours. On day 10, controlled intravaginal drug release device was removed at 2100 hours. After the controlled intravaginal drug release device removal, Ovidrel was injected as well. Two days after the controlled intravaginal drug release device removal, the female caprine was prepared for laparoscopic ovum pick-up. The oestrus was detected by the twigling of its tail and by using a teaser buck.

3.3.5.1 (A) (b) (iii) Sedation and anaesthetisation of caprine donor

Identification tag of the caprine was determined and the donor doe was off-feed and water (at 0900 hours on day 15) for 18 to 24 hours before to oocyte retrieval surgery (at 0900 hours on day16), the caprine donors were prepared in an area separated from

where surgery was performed. Anaesthesia to a caprine with mixture of xylazine hydrochloride (0.22 mg/kg body weight; Ilium Xylazine-20; Troy Laboratorries Pty Ltd, Australia) and ketamine hydrochloride (11 mg/kg body weight; Ketamil; Troy Laboratorries Pty Ltd, 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) (b) (iv) Disinfection of surgical instruments and skin area of female caprine

The anaesthetised caprine donor was placed on a clean small ruminant restraining cradle. The restraining cradle was set at a 45 º 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%; ZENECA Limited, 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 that revealed 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.

3.3.5.1 (A) (b) (v) Laparoscopic ovum pick-up equipment and surgical instruments

All the surgical instruments and washable laparoscopic ovum pick-up equipment (CO_2 tube, grey endoscope tube, endoscope, trocar, cannula, verrus needle) were washed and soaked into 20% Gigasept FF (Schulke and Mary GmbH, Germany) for 10 minutes and rotated periodically after 15 minutes. Then, they were rinsed and soaked for 15 minutes

in autoclaved reverse osmosis water to rinse off the antiseptic. After rinsing, all surgical instruments and the CO_2 tube were packed and sealed in the autoclaved bag and autoclaved at 120℃ for 20 to 25 minutes (Hiclave, Model HA-300M II, Hirayama, Tokyo, Japan). Then, the autoclaved instruments were transferred into the oven (Memmert, Germany) to dry. The other laparoscopic ovum pick-up equipment were dried and packed in sterile plastic bags. The day before surgery, all surgical instruments and the CO_2 tube were put under ultra-violet light overnight.

3.3.5.1 (A) (b) (vi) Preparation of surgical instruments on surgical trolley

On the day before the surgery, non-autoclavable surgical instruments, such as atraumatic grasper, trocar and cannula, fibre optic cable, light probe for endoscope, flushing system tubing, CO_2 gas tubing, vacuum pump tubing and collection bottle for vacuum pump, were disinfected by immersing completely in Gigasapt solution (10%; Schulke and Mary GmbH, Germany) for 10 minutes (fully submerged, opening of instruments were flushed using a 50 ml syringe), then rinsed twice in sterile autoclaved distilled water, and subsequently drained dry before placing in a sterile surgical table-cum-trolley. Autoclavable surgical instruments in the autoclave bag were ready to use on the day of surgery after being autoclaved and dried completely in the oven (56℃) for overnight. Before opening a sterilised surgical pack (for example, surgical set, scalpel blade, catgut suture, sterile gauze, sterile towels, sterile gloves, sterile drapes, round bottom tubes and aspiration needle), the sterilisation date and adhesive indicator tape (appropriate colour change for autoclaved instruments) and pack description (for example, surgical sets for laparoscopic ovum pick-up or laparotomy, size of drapes for animal and size of sterile gloves) must be checked properly. The outer wrapping of the surgical pack was opened and carefully unfolded without touching the sterilised instruments inside. All the surgical instruments were assembled and arranged in a

consistent order on a sterile surgical trolley were routinely placed in a sequential order so that items used first were placed on top (Figure 3.6).

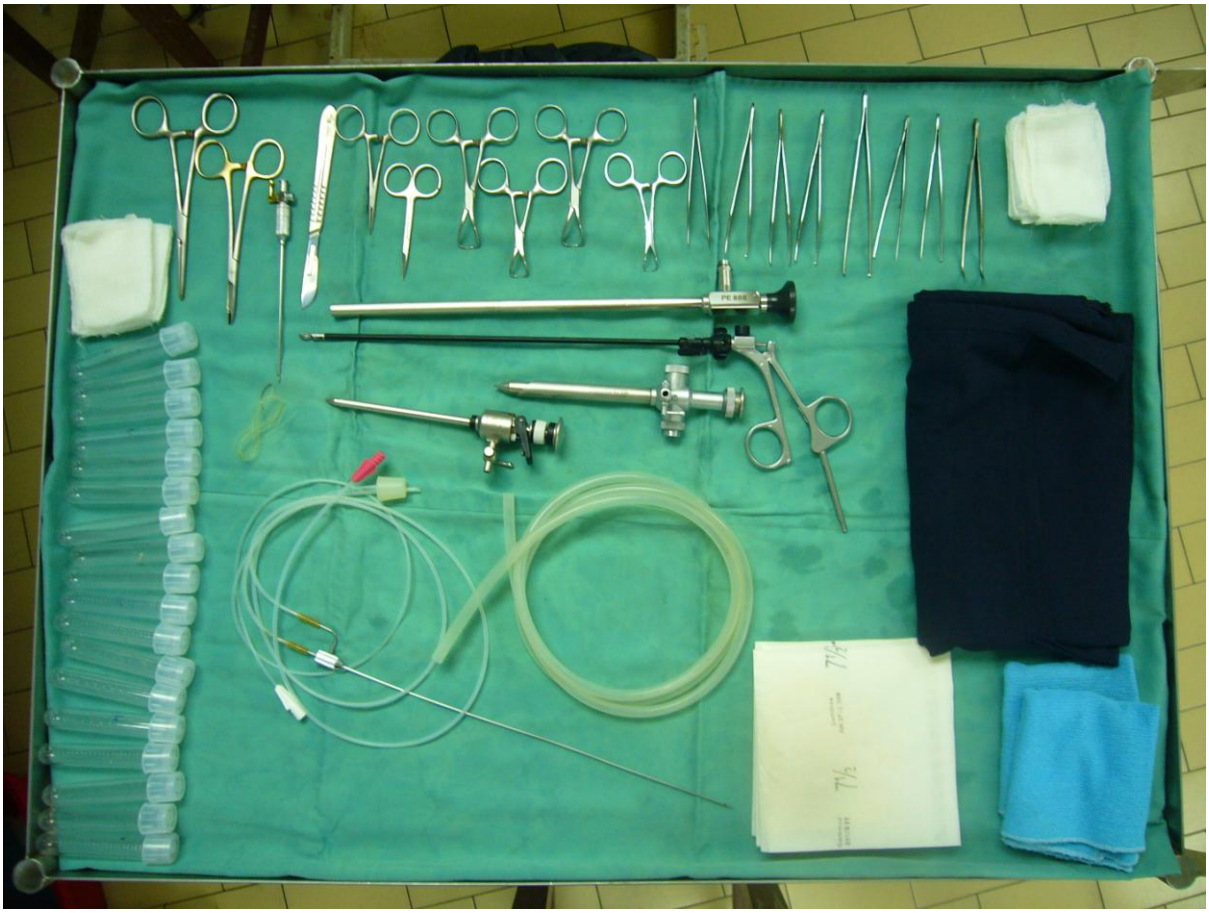

Figure 3.6: Surgery instruments which were used in laparoscopic ovum pick-up.

3.3.5.1 (A) (b) (vii) Responsibility of the surgery team

In our laboratory setting, all members involved in the surgery paid strict attention to aseptic techniques. However, with semi-equipped surgical facilities, only certain sophisticated sterile procedures were performed, such as the requirements of all members to wear an appropriate clean laboratory coat (dedicated for surgery purpose only), face mask and sterile gloves. Each member had his/her own duty during the surgery carried out such as embryologist, surgeon, passing embryos or oocytes tubes, recording the condition and history of animal, anaesthetic, antibiotic, setting up CCD

camera and CO_2 gas insufflators, cleaning as well as arrangement of surgical trolley to ensure the surgery ran smoothly.

3.3.5.1 (A) (b) (viii) Laparoscopic ovum pick-up (LOPU)

The laparoscopic system and the accessories used were purchased from Aesculap A.G. and Company, Germany. The laparoscopic system composed of a paediatric Storz laparoscope (7.0 mm; PE 688A), an Aesculap endoscopic camera system (PV431), a 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 plastic 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 flushing and aspiration system was purchased from Cook, Australia which composed of a pedal operated aspiration pump (Aspiration system, K-MAR-5100) for proving a constant vacuum for laparoscopic ovum pick-up, a flushing pump (a microprocessor controlled pump for automated follicle flushing) (Flushing system; K-MAR-4000), a test tube heater (K-FTH-1012) for maintaining a constant aspirated temperature during laparoscopic ovum pick-up, a connecting tubing that connects an ovum pick-up 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 ovum pick-up needle (FAS Set C2) was purchased from Gynetics Medical Product, Belgium. The collection tubes were graduated test tubes (14 ml; Becton Dickinson Labware, USA) with inlet and outlet ports in the stopper. The vacuum pressure was regulated with a flow valve (-100 mm Hg) and measured as drops of flushing medium per minute entering the collection tube and was adjusted to 50 to 70 drops per minute. 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 and a filter was inserted into the incision to insufflate the abdominal cavity with CO_2. The uterine horns were gently manipulated by the grasper to allow visualisation of each stimulated ovary. Once an ovary was indentified, the ovarian ligament was grasped using a forceps without damaging the oviduct, to effectively stabilise the ovary for follicular puncture. When flushing the follicles, extra care was taken to avoid leakage of follicular fluid and the inadvertent oocytes loss from the follicle. The collection tube containing aspirated fluid was dispensed into a sterile Petri dish for oocytes isolation and searched by embryologist under a stage warmer stereomicroscope (38.5℃). Oocytes were then washed in flushing medium and cultured according to oocyte grades for subsequent *in vitro* maturation-somatic cell nuclear transfer procedures. After aspiration, the ovary was rinsed repeatedly with warm heparinised (38.5℃) physiological saline 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 animal was monitored for 24 to 40 hours after the surgery. In general, 30 to 40 minutes were needed to complete the oocytes retrieval procedure, depending on the quality of the ovary and number of follicles to be aspirated.

3.3.5.1 (A) (b) (viiii) Post-surgical treatment of the doe

The incisions on the abdomen were sutured and the doe was carefully removed from the cradle. The sutured incision area was sprayed with antiseptic and insecticide containing cyphenothrin. The doe was injected intramascularly (i.m.) with antibiotic (Tetracyclines; 20 mg/kg body weight) once in 4 days within the duration of 2 weeks to prevent possible post-surgery infection.

3.3.5.1 (A) (c) Oocyte retrieval through ovariectomy

Ovariectomy was carried out when the doe failed to be hyperstimulated after repeated oocytes retrieval surgeries or when the ovary was having minor or major adhesion, and consequently the doe was not suitable for laparoscopic ovum pick-up procedure. The ovaries were surgically removed from the doe using laparotomy technique. Briefly, a mid-ventral abdominal incision was made (3 to 5 cm) at the abdomen part on the anaesthetised doe. The uterine horns were exposed through mid-ventral incision and blood vessel leading to each ovary was clamped with a forceps. The blood vessels were tied off with suture and the ovary was excised anterior to the sutures. After removal of the ovaries, muscle layers and the skin layer were sutured with strong synthetic sutures. A wound dressing was sprayed onto the incison. After completion of surgery, the doe received antibiotic to prevent infection.

3.3.5.1 (A) (c) (i) Ovary slicing

Under the laminar flow, each ovary was freed from the surrounding tissues and overlaying bursa using surgical scissors and forceps. The ovary was placed in a Petri dish (60 mm) containing TL-hepes working solution (5 ml) on a stage warmer at 38.5 ℃. The ovary was held with the help of forceps and each ovary was sliced individually. Systematic slices were given along the whole ovarian surface using a sharp razor blade. While retrieving the oocytes by slicing method, the ovary was kept completely dipped in the TL-hepes working solution. The Petri dish (60 mm) was kept undisturbed for 3 minutes (38.5 ℃), allowing the oocytes to settle. After sedimentation, excess medium was taken out using a pulled Pasteur pipette, without disturbing the oocytes at the bottom of the Petri dish. The Petri dish was then examined under a warm stereomicroscope (38.5 ℃) under magnification 20x or 40x. Oocytes were then washed

in TL-hepes working solution and cultured according to oocyte grades for subsequently *in vitro* maturation-cloning procedures.

3.3.5.1 (A) (d) Oocyte retrieval from abattoir-derived ovaries

The caprine 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 (38.5 ℃) saline supplemented with penicillin and streptomycin sulphate. A pair of surgical scissors was brought over and used to cut the ovaries from the slaughtered caprine. The excess tissues and some blood of the ovaries were trimmed and rinsed with the warmed saline before transferred to 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. In laboratory, the ovaries were washed thoroughly with warm saline medium (37˚C) and were placed in a beaker containing fresh saline medium. The caprine ovaries were placed in a Petri dish (60 mm) containing TL-hepes working solution (5 ml) on a stage warmer (38.5 ℃). The ovary was held with the help of forceps and each ovary was sliced individually. The whole ovarian surface was sliced using a quarter sections of a stainless steel razor blade held with a sterile haemostat (Figure 3.7). While retrieving the oocytes by slicing method, the ovary was kept completely dipped in the TL-hepes working solution. After finished slicing, the ovaries were rinsed with TL-hepes working solution in a small beaker to ensure that all oocytes were retrieved. The contents of the beaker were poured into a Petri dish was then examined under a stereomicroscope (magnification 20x to 40x). The beaker was rinsed thoroughly to ensure that all the oocytes were transferred into Petri dish. Since the caprine oocytes were very sensitive to temperature so it was important to monitor carefully the

temperature throughout the collection procedures as fluctuations could easily occur. Caprine oocytes were then washed with TL-hepes working solution followed by *in vitro* maturation medium prior cultured according to oocytes grading (if sufficient number of oocytes available) for subsequent experiment. Caprine oocytes were classified according to the character of the cumulus cells and cytoplasm morphology under a stereomicroscope. The oocytes were washed several times with maturation medium to ensure that the oocytes used for maturation were free from unwanted chemical substances which may affect the maturation process. The cumulus oocyte complexes were cultured in a microdroplets (50 µl) of maturation medium overlaid with mineral oil and then it was culutred at 38.5˚C in CO_2 (5%) incubator in humidified atmosphere.

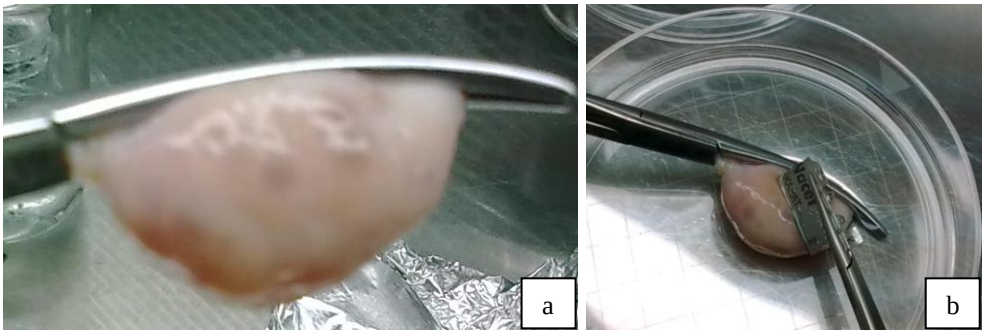

Figure 3.7: (a) Caprine ovary was collected from slaughterhouse. (b) Slicing of ovary.

3.3.5.1 (A) (e) Grading of retrieved caprine oocytes

The caprine oocytes were graded and cultured in separated droplets based on the criteria stated in Table 3.25.

Table 3.25: Oocyte grading based on cumulus cell layers and cytoplasm uniformity

Oocyte grade	Cumulus cell layers and cytoplasm uniformity
Grade A	≥5 multi-layered compact cumulus cells with evenly granulated cytoplasm
Grade B	3 to 4 layers of cumulus cells with evenly granulated cytoplasm
Grade C	1 to 2 layers of cumulus cells with evenly granulated cytoplasm
Grade D	No layers of cumulus cells (denuded) with evenly granulated cytoplasm
Grade E	Pale-coloured or heterogeneous cytoplasm*

(Adapted from Rahman, 2008)

*Heterogeneous cytoplasm: non-homogenous cytoplasm, in which the oocyte cytoplasm showed pigmentation in some areas.

Cumulus oocyte complexes of all grades were selected for subsequent use and each *in vitro* maturation droplet (50 µl) contained not more than 10 cumulus oocyte complexes according to the oocyte grades. *In vitro* maturation was performed at 38.5 ℃ in humidified atmosphere with CO_2 (5%) in air subjected to different oocyte maturation duration. At the end of the maturation culture, cumulus expansion was scored into 2 categories, namely either complexes floating in the maturation medium and surrounded by a light colour fully expanded cumulus mass which was defined as "cumulus-expanded" (matured oocyte) or vice versa defined as "cumulus-unexpanded" (immature oocyte).

3.3.5.1 (A) (f) *In vitro* maturation procedure in caprine oocytes

In vitro maturation medium was prepared in microdroplets (50 µl) and overlaid with equilibrated mineral oil in a small polystyrene culture dish (35 mm; Becton Dickinson Labware, USA). The prepared dish was placed in the CO_2 incubator (5%) and equilibrated at least for 3 hours. After finished washed and graded, the selected caprine oocytes were placed in *in vitro* maturation microdroplets (10 oocytes/50 µl). The carpine oocytes were cultured for 18 to 21 hours (laparoscopic ovum pick-up derived oocytes) and 24 to 27 hours (ovariectomy and abattoir derived oocytes) at 38.5 ℃ in the presence of CO_2 (5%) in air in humidified atmosphere of a CO_2 incubator (HERAcell 240; Kendro Laboratory Products, Germany).

3.3.5.1 (A) (g) Preparation of recipient caprine oocytes

After *in vitro* maturation, the caprine cumulus oocyte complexes were treated with hyaluronidase (0.2%; 100 µl). All the caprine oocytes were transferred into hyaluronidase in a microcentrifuge tube (1.5 ml) and using the disposable yellow tips (100 µl), attached with micropipettor (10 to 100 µl) to suck up and down for 80 times to make the cumulus cells dispersed and removed totally from the caprine oocytes. After denuding, if the caprine oocytes were still surrounded cumulus cells, the oocyte were denuded again by gentle pipetting in and out using a narrow-bored Pasture pipette (80 to 100 µm) in hyaluronidase (0.2%) covered with mineral oil (< 5 minutes) and washed 5 times in TL-hepes working solution (100 µl/microdroplet). After denuding, the caprine oocytes were washed in TL-hepes working solution, supplemented with cytochalasin B droplets to observe the present of first extruding polar body (M II) (< 5 minutes). Only the metaphase II caprine oocytes with first extruding polar body were choosen as recipient oocytes (cytoplasts). The metaphase II caprine oocytes were transferred into TL-hepes holding medium with cytochalasin B (7.5 µg/ml) droplet covered with

mineral oil on the enucleation Petri dish (35 mm) (Figure 3.8). The caprine oocytes without first polar bodies were considered immature and discarded.

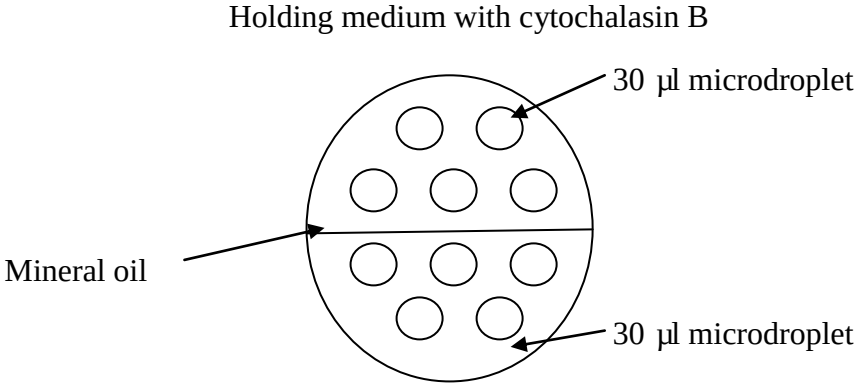

Figure 3.8: Washing Petri dish for enucleated oocytes.

3.3.5.1 (A) (h) Somatic cell nuclear transfer procedure

The somatic cell nuclear transfer technique included dish preparation, micromanipulation system, needle alignment, oocytes enucleation, donor cells preparation and nuclear transfer.

3.3.5.1 (A) (h) (i) Preparation of blank and somatic cell nuclear transfer dish

The blank dish was prepared to align the nuclear transfer needle and the holding pipette (OD: 80 to 100 µm; ID: 25 µm) by using cover lid of Petri dish (35 mm). After preparing a blank dish, nuclear transfer dish was prepared. Three microdroplets (20 µl) of TL-hepes solution with cytochalasin B were prepared and overlaid with mineral oil for align the holding pipette and cutting needle or biopsy needle.

3.3.5.1 (A) (h) (ii) Micromanipulation system

In the present study, an Olympus inverted microscope (IX71; Olympus Optical Co., Ltd, Japan) with Normarski optics using Hoffman contrst system and fitted with Narishige hydraulic micromanipulators (Narishige Co. Ltd., Japan) to perform somatic cell nuclear transfer. The micromanipulation system was comprised: a set of left and right that could be controlled both mechanically and manually by their respective joysticks. Usually, the holding pipette holder was set on left hand side, while the injection holder was set on right hand side. The joysticks allowed the control of coarse and fine 3 dimensional positioning as well as also gaveprecise linear displacement simultaneously. The joysticks had a hydraulic (de Fonbrune's system). The holding and injection needles were filled entirely with air. Both the holding and injection pipettes were comprised of completely airtight syringes connected to the micropipette holder by Teflon plastic tubing with airtight fittings.

3.3.5.1 (A) (h) (iii) Micromanipulator and micropipette alignment

The nuclear transfer was performed on the lid of a small polystyrene culture dish (35 mm; Becton Dickinson Labware, USA) under the inverted microscope fitted with hydraulic micromanipulators (Narishige, Japan). All the knobs and the knob syringes were adjusted to the centre of the scale. A blank Petri dish (35 mm) was placed on the inverted microscope with stage warmer heated to 38.5◦C. The holding pipette was inserted to the needle holder (right micromanipulator) and tightened well. Alignment was done following by the injection needle, biopsy needle or cutting needle. The tip of both needles were touched in the mineral oil and was focused which parallel to the microscope stage under low magnification. The end of both pipettes was filled with oil-medium-oil to maintain the pressure for easy to control and manipulated the oocytes.

Finally, both needles were checked under high magnification (40x) to assure the accurate alignment which sharply in focus and parallel.

3.3.5.1 (A) (h) (iv) Caprine oocyte enucleation

There were 2 enucleation techniques applied to caprine matured oocytes, namely squeezing technique and laser shooting technique. The holding needle and cutting needle or biopsy needle were aligned in the washing droplet (30 µl) containing holding medium with cytochalasin B (7.5 µg/ml) which overlaid with equilibrated mineral oil on the enucleation Petri dish (35 mm) (Figure 3.9). The oocytes were focused and aligned in one line. The matured caprine oocytes were held using holding needle (OD: 75 to 100 µm; ID: 25 µm), placing the polar body at 12 o'clock position. Subsequently, cutting needle was used to pierce the zona pellucida and the polar body was squeezed out or using laser to make a small hole and pushed out together with 10% of cytoplast. The whole enucleation process must be completed within 30 minutes for each batch of oocytes (10 to 15 oocytes per batch) due to cytochalasin B will cause damage to the oocyte if expose too long in cytochalasin B (7.5 µg/ml) medium. After enucleation, the enucleated oocytes were washed in TL-hepes holding medium (without cytochalasin B). The enucleated caprine cytoplasts were allowed to recover at 38.5℃ in *in vitro* culture (KSOM) medium for at least 30, 60, 90 or 120 minutes (pre-incubated) prior to injection with donor cells.

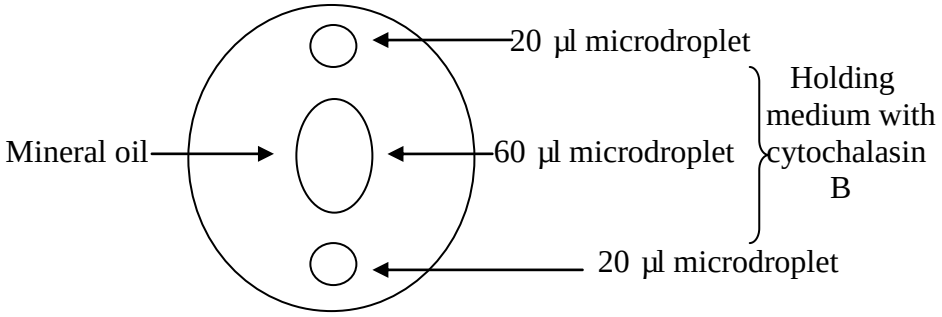

Figure 3.9: Enucleation Petri dish (35 mm).

3.3.5.1 (A) (h) (iv) (a) Squeezing technique for enucleation in caprine cytoplast

The matured caprine oocytes were held by holding pipette and the first polar body was positioned at 12 o'clock. A slit at the matured oocyte zona pellucida was made and the polar body as a guide to remove the recipient nucleus by squeezing it with holding pipette and cutting needle.

3.3.5.1 (A) (h) (iv) (b) Laser shoots technique for enucleation in caprine cytoplast

Laser shoot is a new and advance technique that has been used to enucleate recipient oocytes (cytoplast). In comparasion with squeezing technique, laser shoot technique is easier in enucleating the caprine oocytes, as it uses the power (100%), pulse (190 µs), laser beam (red colour) and 140 ℃ (hole size, orange colour) to make a hole on zona pellucida. Briefly, a hole was made near the first polar body as a guide to remove the recipient nucleus. The nucleus and first polar bodies were pushed out by holding pipette (left side) and biopsy needle (right side) on the both side together. Around 10% of the cytoplasm was removed.

Isotherm Rings™ (Figure 3.10) appeared on the screen as a series of six concentric circles of varying colours and indicate the maximum temperature reached at the ring diameter at various laser pulse durations. At longer pulse duration, it is apparent that temperatures radiate farther into the centre of the embryo, increasing the likelihood of blastomere damage. The orange ring (second from center) is also a useful indicator of the drill hole size at the selected pulse duration. With this interactive Isotherm Rings™ feature, a user could now "see" the heating and drill hole size and eliminate the temperature "guesswork."

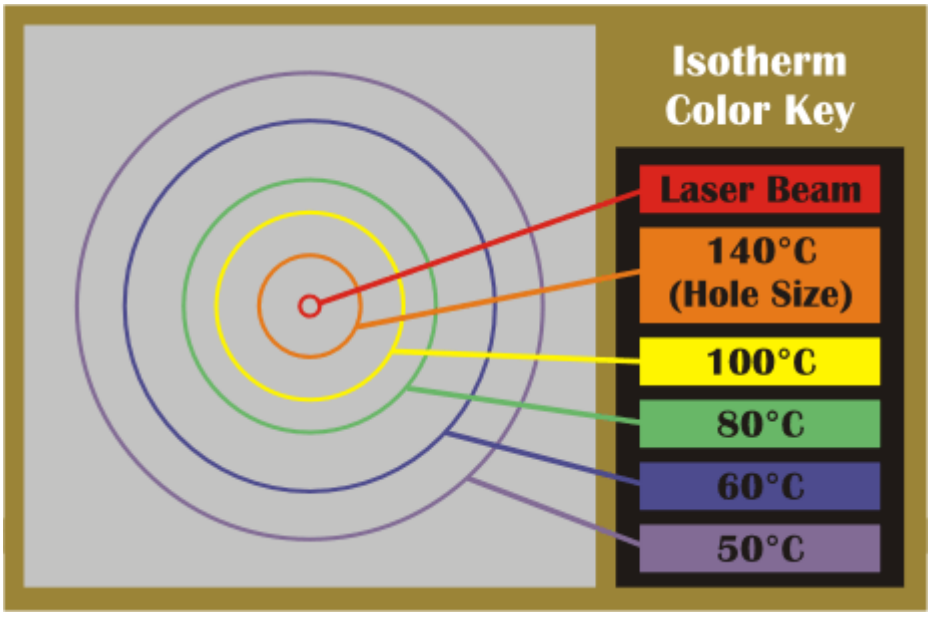

Figure 3.10: Isotherm Ring™ shown is laser system. (Adapted from http://www.hamiltonthorne.com/products/lasers/lykos/safety.htm).

The heat conduction into the embryo, as shown by the Isotherm Rings™ (Figure 3.10), is based on published scientific algorithms that are built into the software. For increased embryo safety, the Isotherm Rings™ are hardwired and their specifications cannot be altered or deleted by users. With the Isotherm Rings™ it is accurate and determined according to scientific definitions. The heat penetrates further into the embryo as the pulse length increases. So even if the actual drill hole (orange ring) does not come close to the blastomeres, the blastomeres are exposed to the heat generated by the laser beam at longer pulse lengths. By using the Isotherm Rings™ feature of the ZILOS-tk, the heat conduction prior to treatment and the pulse length are adjusted and the embryo is position accordingly.

Isotherm Rings™ were derived by calculation of the heat diffusion based on the assumption that the zona pellucida thermal conductivity was close to that of water. Since the zona pellucida is composed of approximately 80% water, this was most likely a very good assumption. This assumption was even on the conservative side, since the presence of proteins would lessen the conductivity and the resultant rings would even

be smaller. Lasers produce light beams of varying intensity, and are categorized in accordance with the emitted power. There are several vital components inherent to a laser microsurgery apparatus. In addition to the laser itself, a collimating lens, a dichroic mirror, and an objective that can transmit the beam are required:

a) Laser: 1450 μm, Infrared Class 1
b) Collimating Lens: A lens used to produce a beam of parallel light rays
c) Dichroic Mirror: An optical device which acts like an optical gate to split light into two colors ("di" = two, "chroic" = pertaining to color). The dichroic mirror reflects the infrared laser beam up through the objective, while the visible light passes through.
d) Objective: Must allow transmission of laser beam to the sample.

Once the optimum translational and angular alignments and beam quality are achieved, the laser is "locked" into place, and does not move position. This "locking" process is similar to that used for aligning and locking microscope objectives. It is similar to aligning the inside components of microscope objectives. Therefore, it is not need to align the internal components of the XYClone, even the microscope is switch on-off (Figure 3.11).

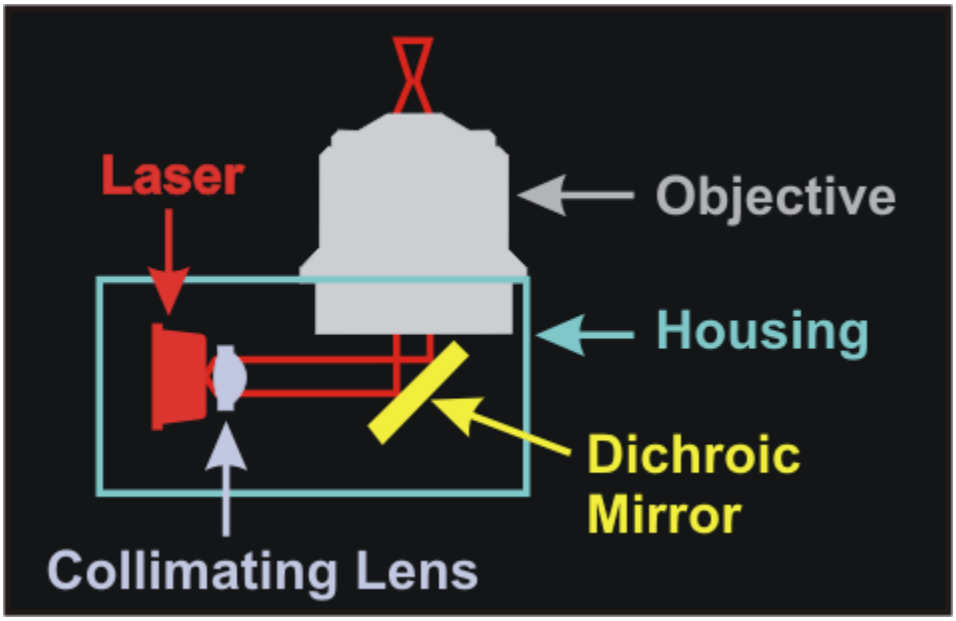

Figure 3.11: Components of XYclone laser system (Adapted from http://www.hamiltonthorne.com/products/lasers/xyclone/module.htm).

3.3.5.1 (A) (i) Caprine donor cell preparation

Nuclear donor cells were obtained from 2 different cell types in this study, namely fresh caprine cumulus cells and caprine ear fibroblast cells.

3.3.5.1 (A) (i) (i) Preparation of caprine donor cells (fresh caprine cumulus cells)

The fresh cumulus cells were usually obtained from a pool of *in vitro* matured oocytes harvested either from laparoscopic ovum pick-up or abattoir ovaries. After 24 hours onset of maturation, the cumulus cells were isolated by pipetting in hyaluronidase (0.2%) within 5 minutes at 37^{o}C. All fresh cumulus cells were collected and washed in holding medium (TL-hepes working solution) and centrifuged for 5 minutes. Cumulus cell pellet was formed and the supernatant was removed. TL-hepes working solution (100 µl) was added into the pellet and sucked up and down to make the fresh cumulus cells became single cell (7 to 8 µm) and were ready for use as donor cells (Figure 3.12).

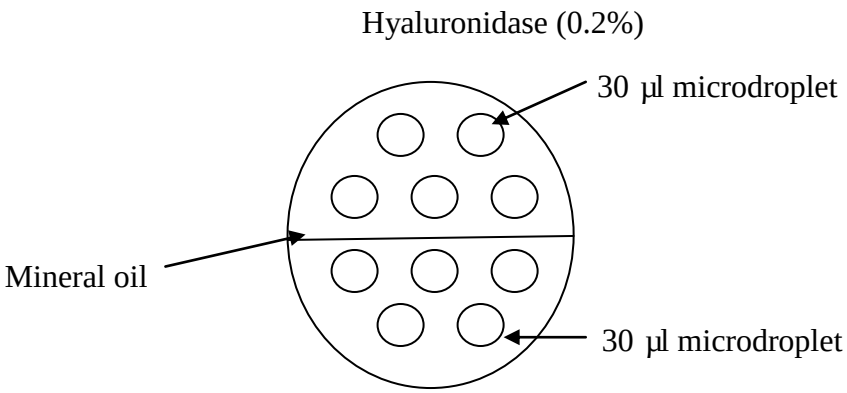

Figure 3.12: Denuding Petri dish (35 mm).

3.3.5.1 (A) (i) (ii) Preparation of caprine ear fibroblast cells

For preparing the caprine eat fibroblast cells, Katjang caprine's ear tissue cells were collected. Hair of caprine ear tissues was removed. Coverslip was sterilised rinsed in 70% ethanol and it was flamed it until it dry and sterile. Epidermis of caprine ear tissues was cut into small pieces. Each small piece of caprine ear epidermis tissues was placed on the culture dish (60 mm). The caprine ear fibroblast cells were covered with coverslip. The culture dish was filled with αMEM+FBS+PS (300 µl). The culture dish was covered and kept in the incubator at 37˚C, 5% CO_2. After 8 to 10 days, the caprine ear fibroblast cells reached >80% confluency and further sub-cultured and cyroperserved for future use. Caprine ear fibroblast cells were thawed, washed and plated in Petri dish prior to oocyte reconstruction. Donor cells were trypsinised 1 to 2 hours before being transferred to enucleated caprine oocytes. A single caprine ear fibroblast cell (10 to 15 µm) was ready to be used as donor cell for nuclear transfer.

3.3.5.1 (A) (j) Caprine somatic cell nuclear transfer

In this study, whole cell intracytoplasmic injection (WCICI) technique was used for nuclear transfer in production of cloned-caprine embryos.

3.3.5.1 (A) (k) Injection of caprine donor cells by whole cell intracytoplasmic injection (WCICI) technique in caprine species

After pre-incubated (30, 60, 90 or 120 minutes), the enucleated oocytes were washed for 5 times in TL-hepes holding medium (Figure 3.13).

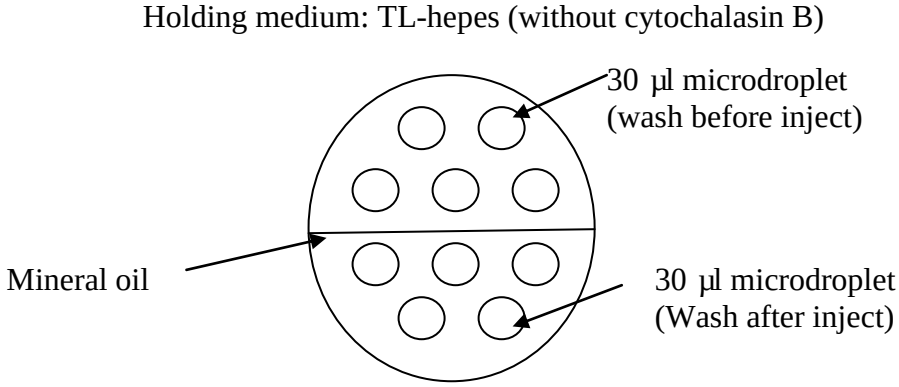

Figure 3.13: Washing medium for injected oocytes.

Subsequently, the holding needle and injection needle were aligned in the washing droplet containing holding medium (without cytochalasin B) on the enucleation Petri dish. The oocytes were transferred in the injection droplet as well as the donor cell in 10% polyvinylpyrrolidone (PVP) droplet on the injection Petri dish. The oocytes were focused and aligned in one line. A single fresh cumulus cell (7 to 8 µm) or caprine ear fibroblast cell (10 to 15 µm) was injected as a donor cell into enucleated caprine oocyte by the direct insertion of the whole somatic donor cell content into the cytoplast. (care was taken to transfer the donor cell content with the minimum amount of the media and pressure.) The nuclei of cumulus cell or ear fibroblast cell was isolated just before injection with shear stress using a blunt end pipette 7 to 10 µm in diameter which

sucked in and out to remove the cell membrane of donor cell in the polyvinylpyrrolidone (10%) to make the donor cell swollen. Then, single nuclei were aspirated into the pipette and injected into a enucleated caprine oocyte in TL-hepes holding medium without cytochalasin B (Figure 3.14). After injection, the enucleated oocytes were washed in TL-hepes holding medium. The reconstructed caprine oocytes were washed in KSOM medium 3 times and pre-incubated at least 60 minutes before activation. No fusion was needed to fuse the cell-cytoplast couplets.

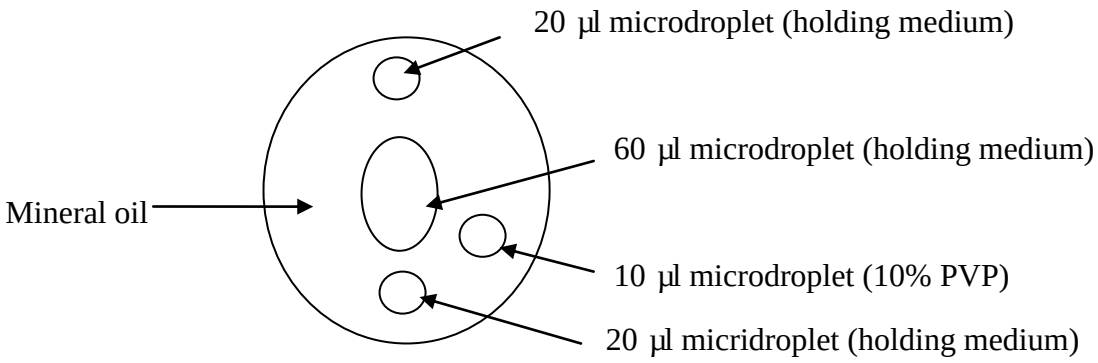

Figure 3.14: Injection Petri dish (35 mm).

3.3.5.1 (A) (l) Caprine oocyte activation

The reconstructed oocytes were chemically activated by incubating for 5 minutes in KSOM working solution containing calcium ionophore (5 µm), washed thoroughly in KSOM working solution and then incubates for 4 hours in KSOM working solution containing 6-dimethylaminopurine (2 mM) at 38.5^{o}C under CO_2 (5%). Following, the activated caprine embryos were cultured in groups of embryos (10 to 15) in KSOM medium (50 µl microdroplets) covered with mineral oil.

3.3.5.1 (A) (m) *In vitro culture* (IVC) in caprine embryos

The *in vitro* culture medium was incubated for at least 3 hours before culture of oocytes. Generally, 7 microdroplets (100 µl) was prepared on a small culture dish (35 mm) and overlaid with equilibrated mineral oil. Caprine oocytes were reconstructed and *in vitro* cultured in KSOM medium in CO_2 incubator at 38.5 ℃ for further development. The *in vitro* culture medium was changed at days 3 and 5 with KSOM working solution with supplementation of glucose (0.004 g). The caprine embryos were checked under stereomicroscope on days 3 and 5, and accordingly their developmental stages were recorded. The cloned-caprine blastocyst obtained (inner cell mass) was used as a source for producing the caprine embryonic stem cell lines.

3.3.5.1 (B) *In vitro*-derived blastocyst in murine species

The murine blastocysts were *in vitro* derived from somatic cell nuclear transfer (SCNT) technique to become a source of blastocyst for production of murine embryonic stem cell lines.

3.3.5.1 (B) (a) Superovulation of female murine

In order to obtain the optimum quality and quantity of murine oocytes to conduct the cloning procedure, the mature female of the 3 pure strains murine were superovulated by intraperitoneal injection of pregnant mare's serum gonadotrophin (PMSG) (10 IU) on day 1 at 1800 hours and followed by intraperitoneal injection of human chorionic gonadotrophin (hCG) (10 IU), 48 hours apart. The time of ovulation normally occurred by 13 to 15 hours post-hCG injection.

3.3.5.1 (B) (a) (i) Intraperitoneal injection

In intraperitoneal injection technique, the murine was picked up by the scruff of its neck which was close to the ears. Enough skin was hold up to prevent the murine from turning its head and biting. 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 by twisting it around the little finger to hold properly to prevent the murine struggle and move while being injected. Hormones were injected intraperitoneally at the centre point between the 4 tits of the abdomen by using 26 G, ½ inch hypodermic needles. After injected, the needle stayed for 10 seconds before withdrawn out to prevent the solution from seeping out.

3.3.5.1 (B) (b) Oocyte collection in murine

The superovulated female murine was sacrificed at the time of ovulation that is 13 to 15 hours post-hCG injection. The female murine's coils of gut were pushed out of the way and located the 2 horns of the uterus after which the ovaries and oviducts were identified. The upper end of one of the uterine horns was grasped with fine forceps and gently pulled the uterus, oviduct, ovary and fat pad taut and away from the body cavity. In the membrane, a hole that was near to the oviduct was poked with the closed tips of a pair of fine forceps or scissors. The oviduct was transferred and attached segment of uterus into the silicone oil overlay in Petri dish with Hepes Whitten's medium. The freshly ovulated oocytes as cumulus oocyte complexes that surrounded by cumulus cells are found in the upper part of the oviduct (ampulla), which at this time (12 hours post-ovulation) is much enlarged. The fimbriae end of the oviduct (infundibulum) is also swollen during ovulation and can be easily be located under stereomicroscope (20x magnification). A pair of needle (26 G) was used to hold the swollen infundibulum firmly on the bottom of the dish and another to tear the ampulla where the cumulus

oocyte complexes are located. The cumulus oocyte complexes were released. If the cumulus oocyte complexes did not flow out, a fine forceps was used to push them out by gently squeezing the oviduct. The cumulus oocyte complexes were placed immediately in the hyaluronidase (0.2%).

3.3.5.1 (B) (c) Preparation of murine recipient oocytes

After denuding, the murine oocytes were washed in holding medium (Hepes Whitten's medium) supplemented with cytochalasin B droplets to observe the present of first extruding polar body (metaphase II) and pre-incubated 5 minutes (37^{o}C).

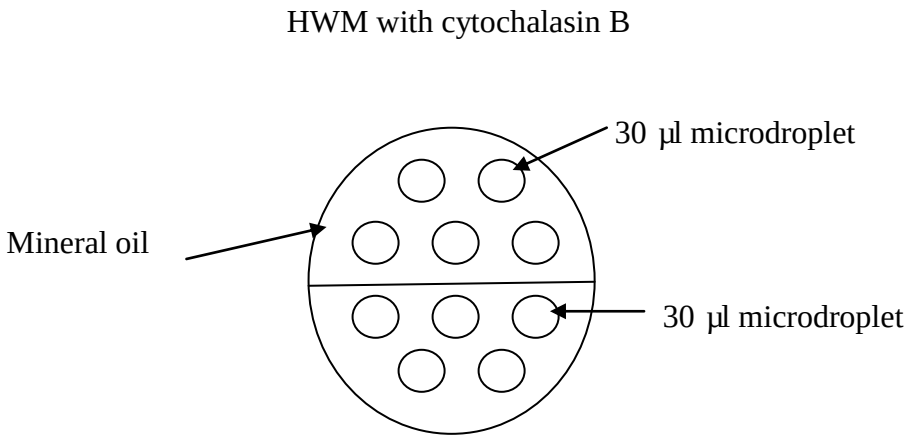

Figure 3.15: Preparation of murine emucleation Petri dish (35 mm).

Only the metaphase II oocyte with first extruding polar body will be choose as murine recipient oocyte. The metaphase II oocytes were transferred into holding medium with cytochalasin B droplet covered with mineral oil on the enucleation Petri dish (Figure 3.15).

3.3.5.1 (B) (d) Enucleation of murine oocyte

The holding needle and cutting needle or biopsy needle were aligned in the washing droplet containing holding medium with cytochalasin B (7.5 µg/ml) on the enucleation

Petri dish (35 mm) (Figure 3.16). The oocytes were focused and aligned the oocyte in one line. The oocytes were held using holding needle where placing the spindle at 12 o'clock position. Following, cutting needle or biopsy needle was used to pierce the zona pellucida and squeeze out or pushed out the murine spindle. The whole enucleation process must finish within 30 minutes for each batch of oocytes (10 to 15 oocytes per batch).

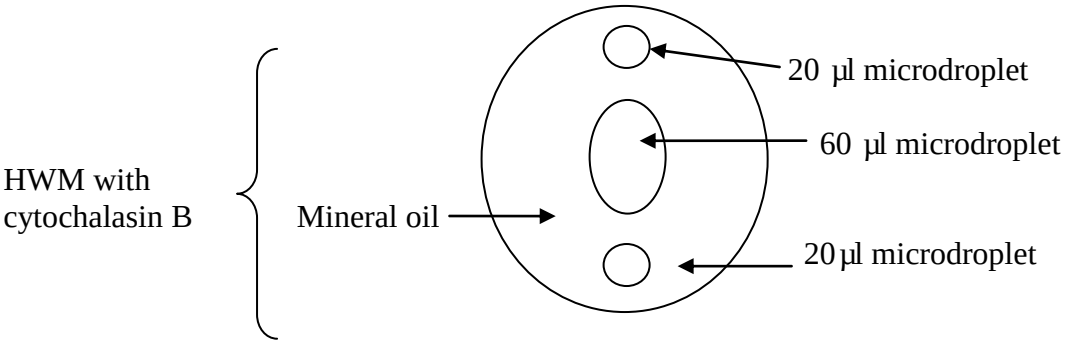

Figure 3.16: Murine enucleation Petri dish (35 mm).

After enucleation, the enucleated oocytes were washed in holding medium (without cytochalasin B). The oocytes were washed in Whitten's medium 3 times and incubate 30 to 120 minutes before injection.

3.3.5.1 (B) (e) Preparation of fresh murine donor cells (fresh murine cumulus cells)

The cumulus cells were isolated by pipetting the cumulus oocyte complexes in and out using a narrow-bored pasture pipette in 0.2% hyaluronidase within 5 minutes at 37^{o}C. All single cumulus cells was collected and washed in holding medium (Hepes Whitten's medium) (Figure 3.17). The isolated cumulus cells kept in the last droplet of holding medium and were ready for use as donor cells.

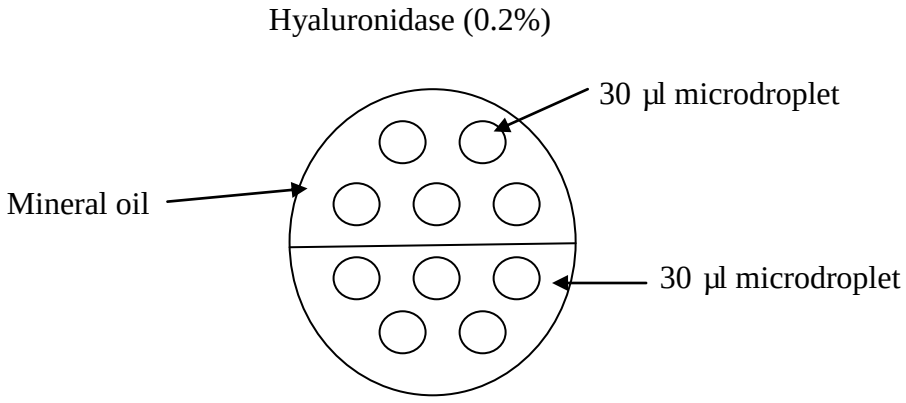

Figure 3.17: Denuding murine Petri dish (35 mm).

3.3.5.1 (B) (f) Injection of murine donor cells

After 30, 60, 90 or 120 minutes, the enucleated murine oocytes were washed 5 times in holding medium.

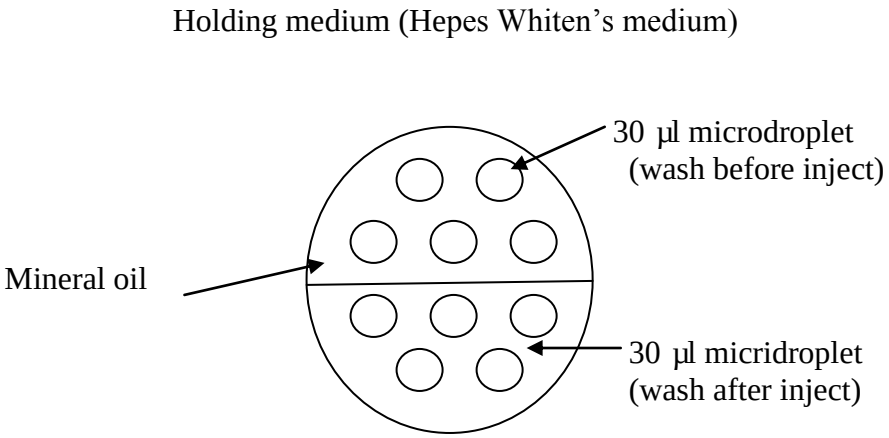

Figure 3.18: Washing of enucleated murine Petri dish.

Following, the holding needle and injection needle were aligned in the washing droplet containing holding medium (without cytochalasin B) on the enucleation Petri dish

(Figure 3.18). The oocytes were transferred in the injection droplet as well as the donor cell in PVP (10%) droplet on the injection Petri dish (Figure 3.19). The murine oocytes were focused and aligned in one line. Tthe donor cell was injected into enucleated murine oocyte by the direct insertion of the whole somatic donor cell content into the cytoplast.

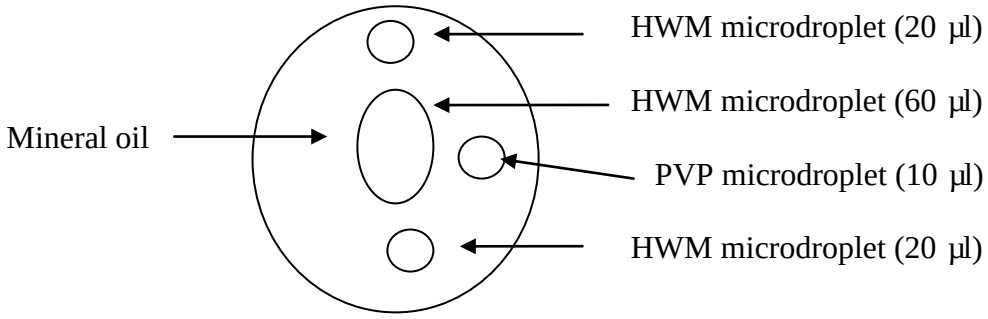

Figure 3.19: Injection of murine Petri dish.

After injection, the enucleated murine oocytes were washed in holding medium. The oocytes were washed in Whitten's medium 3 times and incubate at least 60 minutes before activation.

3.3.5.1 (B) (g) Activation and i*n vitro* culture murine embryos

The reconstructed murine embryos were chemically activated by incubation for 6 hours activation medium containing $SrCl_2$+CB+CZB (free of Ca^{2+}) at 38.5^{o}C under CO_2 (5%). Following, culture the activated embryos in groups of around 10 to 15 embryos in 50 µl droplets of Whitten's medium covered with mineral oil.

3.3.5.2 *In vivo*-derived blastocysts in caprine and murine species

The caprine and murine blastocysts could be derived through *in vivo* flushing technique either oviductal or uterine flushing. Theoretically, the *in vivo* derived blastocysts were

produced higher quality than *in vitro* derived blastocysts due to more stabilised in the body condition development rather than *in vitro* environment development.

3.3.5.2 (A) Uterine flushing in caprine species

The caprine donor was superovulated with FSH (160 mg) for 4 days consecutively. The caprine donor was inserted with Controlled Intravaginal Drug Release device for 17 days. The Controlled Intravaginal Drug Release device was removed out on day 18 and heat check was carried out to detect the oestrus. Once the caprine donor had oestrus, she was mated by fertile buck 3 times per day (0800 hours to 0900 hours; 1300 hours to 1400 hours; 1700 hours to 1800 hours) for 2 to 3 days until the end of oestrus in caprine donor. Fertile buck was changed for every mating session to produce good quality and quantity of sperm to fertilise the oocytes once ovulation happened. The caprine donor was off-fed and water the day before the surgery (18 to 24 hours). The blastocyst recovery was on day 7 through uterine flushing. On the day of surgery, a 2-way size 8 Foley Catheter (Inmed Paediatric Balloon Catheter, USA) was then inserted through the puncture to a deep of 3 cm. The balloon of the catheter was sufficiently inflated to completely obstruct the lumen at the caudal end of each uterine horn. A Teflon intravenous (I.V.) Catheter placement unit fixed with a 20 gauge needle (Vasocan, B. Braun, Germany) was introduced closer to the anterior end of the uterine horn. Then, the Teflon intravenous Catheter needle was removed and 20 ml sterile disposable syringe (Terumo, Japan) fixed to the end of the Teflon Catheter. The fluid contents of the uterine horn were collected through the free end of the Foley Catheter. This was repeated with a further 20 ml of the flushing medium. Subsequently the same procedure was used for the flushing of the other uterine horn if at least one corpus luteum was present in the respective ovary. After flushing completed, the surgical incision was sutured with catgut (size 2, Aesculap, Germany). The incised skin was closed with size

2 surgical grade silk suture material (B. Braun, Germany) and sprayed with Gusanex aerosol (1% w/w Dichlofenthione; Coopers, Pitman Moore, Malaysia) as a fly repellent (Figure 3.20).

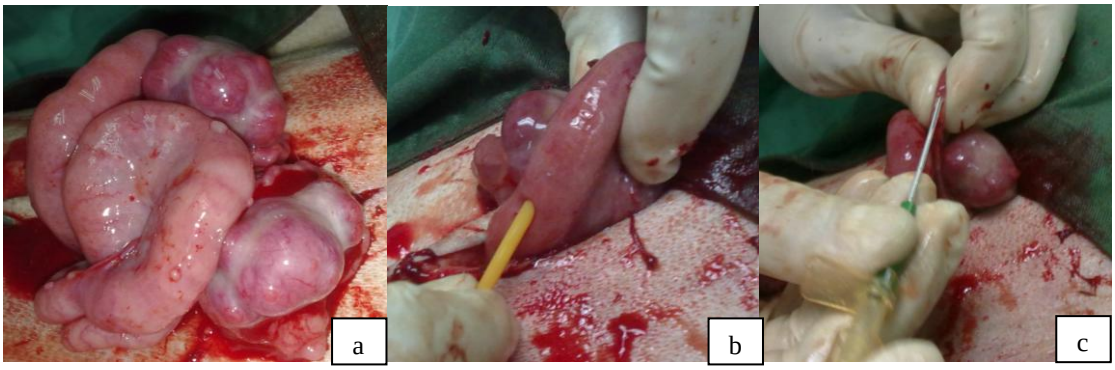

Figure 3.20: (a) Corpus luteum on caprine ovaries. (b) Insertion of Foley Catheter into uterus of caprine. (c) Insertion of intravenous catheter needle into oviduct of caprine.

3.3.5.2 (A) (a) Mouse embryonic fibroblast (MEF) feeder cell preparation

Requirement of mouse embryonic fibroblasts are vital in production of embryonic stem cell lines to maintain embryonic stem cells in an undifferentiated state and to support its growth. The techniques preparation of mouse embryonic fibroblasts included (1) isolation of primary mouse embryonic fibroblast, (2) harvesting of mouse embryonic fibroblast, (3) passage of mouse embryonic fibroblast cultures, (4) cryopreservation of mouse embryonic fibroblast, (5) thawing of mouse embryonic fibroblast and (6) preparation of feeder layers through the mitotic inactivation of mouse embryonic fibroblast.

3.3.5.2 (A) (a) (i) Isolation of primary mouse embryonic fibroblasts

Mouse embryonic fibroblasts were prepared from 13.5 to 14.0 days post-coitus murine foetuses. The mouse embryonic fibroblasts were cultured in Dulbecco's modified eagle medium or alpha minimum essential medium supplemented with 10% foetal bovine serum (FBS; Invitrogen), 200 mM L-glutamine (Invitrogen) and 3x

Penicillin/steptomycin (Sigma). The whole uterus was surgical removed from the murine and kept in PBS-. The uterus was surgical open with scissor then each foetal sac was removed out of the uterus. The foetuses were washed in PBS-, head limbs and internal organ was removed from the foetuses. The foetuses were minced into the small pieces and the mincing tissues were taken to a beaker. The optimal volume of trypsin/EDTA was added in the beaker and stir on the magnetic stirrer for 30 minutes. After that, it was filtered through nylon mesh into the conical tube (50 ml). Culture media (3x) was added to the supernatant (1.5 fold of supernatant volume) and centrifuged at 3000 rpm for 5 minutes (2 times). The supernatant was removed, re-suspended the pellet with culture media and seed to the medium culture dish (60 mm). The primary mouse embryonic fibroblasts were cultured at 37℃, CO_2 (5%) until the cell reaching 80 to 90% confluent. At 80 to 90% confluent, subculture the mouse embryonic fibroblast cell for expanded (split ratio is ~ 1:5) and then freezing as the feeder cell bank for future use.

3.3.5.2 (A) (a) (ii) Passages of mouse embryonic fibroblasts

Mouse embryonic fibroblasts cell cultures can be harvested after they reach 80% confluency. All media were sucked out and removed using a pasteur pipette. The cells were washed with PBS- solution (around 2 ml) twice to remove the remaining media. This is because presence of excessive culture media would inhibit the subsequent trypsinisation step. Next, the cells were detached by adding 2 to 3 ml of trypsin/EDTA into each culture dish. The dishes were then incubated in an incubator for 2 to 3 minutes to allow the enzymatic reaction to take place. After that, the dishes were returned to the laminar flow and the solution was sucked in and out using pasteur pipette to physically detached the cells. This can be observed as a layer “peeling-off” the bottom of the culture dish. The cell suspension was then transferred to a conical tube (15 ml). Mouse

embryonic fibroblast washing media (supplement with only 3% FBS) were added at a ratio of 1:1.5 media and the tubes were then centrifuged at 2000 rpm for 5 minutes. After centrifugation has completed, a pellet would form at the bottom of the conical tube. If there were still pellets attached to the walls, centrifugation was repeated for another 5 minutes. The supernatant was removed and culture media (1x) were added accurately using a micropipettor (100 to 1000 µl) to make up 2 to 4 ml. The solution containing cells were mixed gently by pipetting in and out while avoided the formation of bubbles to produce isolated, single cells. Vigorous pipetting may damage the protein of cells. Mouse embryonic fibroblasts were seeded at around $5.0x10^5$ viable cells per dish by dispersing droplets of the mouse embryonic fibroblasts mixture onto the dishes using Pasteur pipettes. The cells were grown until confluent (2 to 4 days) and the media were refreshed every subsequent day by replacing half of the media. The steps can be repeated for further passages.

3.3.5.2 (A) (a) (iii) Cryopreservation of mouse embryonic fibroblasts

The culture media was removed from the confluent culture dish. PBS- was added just to cover the cells and then sucked it out. Trypsin/EDTA was added just to cover the cells and incubated it for 2 to 3 minutes. The cell was pipetted by Pasteur pipette to break the cell into single cell. All the cells in trypsin were taken to conical tube and culture media (1.5 fold of trypsin/EDTA volume) was added as well as centrifuged at 3000 rpm for 5 minutes. After centrifuged, supernatant was removed and resuspended the pellet (1ml of culture media) and counted the cell to know the concentration. The volume of culture media was adjusted to the desire concentration (that the total volume was from 2 part evenly; one from culture media and another from freezing media). Freezing medium (0.5 ml) was dispensed into cryovials with the number of cryovials depending on the volume of mouse embryonic fibroblasts cells mixture. For each cryovial, 0.5 ml of

mouse embryonic fibroblasts mixture was added to give a final volume of 1.0 ml. The freezing medium were added slowly into the resuspended cell (small volume each time) in the same volume and shaked well. The cell in the cryo-tube was aliquot and labelled as well as inserted into cryocanes and the cryocanes labeled. The cryocanes were placed into the freezer (-20℃) for 15 minutes. They were sealed within autoclave bags and placed into insulated containers containing ice packs. The ice packs were pre-loaded into the containers to lower the temperature within the containers. The cryocanes were then carried to the lab at the Institute of Postgraduate Studies where the whole journey takes around 10 minutes. The cryocanes were then placed into -80℃ freezer for overnight. After that, the cryocanes were transported back to ABEL lab within insulated containers containing liquid nitrogen and removed from the autoclave bags and placed into liquid nitrogen tanks for storage.

3.3.5.2 (A) (a) (iv) Thawing of mouse embryonic fibroblasts

Cryovials were removed from cryocanes in liquid nitrogen tank without exposing the cryocanes for too long outside liquid nitrogen tanks. The cryovials were then immediately thawed by placing the lower portion of the vials into water bath, taking care to avoid contact between the cap and the water. After the mixture has thawed for about 2 minutes, the vials were removed from the water bath and sprayed with 70% alcohol to make it sterile. The cap was flamed and removed in the laminar flow. The contents were transferred into conical tubes (15 ml) containing 2 ml of mouse embryonic fibroblasts washing media. If many vials were thawed at once, vials from the same batch can be pooled together into the same conical tube, while maintaining the ratio to mouse embryonic fibroblasts washing media at around 1:1.5. The tubes were then centrifuged at 2000 rpm for 5 minutes. After completed centrifuged, a pellet was formed at the bottom of the conical tube. Culture media (1x) were added accurately

using a micropipettor (100 to 1000 µl) to make up 1 or 2 ml. The solution containing cells were mixed gently by pipetting in and out. They were then seeded onto small culture dishes containing 2 ml of MEF culture media according to the desired cell density. The cells were seeded at $3.0x10^5$ viable cells per small Petri dish (35 mm). The cells were cultured in the incubator at 37 ℃ to confluence and the media was changed on every alternate day.

3.3.5.2 (A) (b) Feeder cell management for caprine embryonic stem cells

The culture media was removed from the confluent culture dish. Mitomycin C (50 µl) was added to 5 ml of culture media (αMEM+10% FBS or DMEM+10% FBS) for a final concentration (10 µg/ml) and incubated for 3 hours in the culture incubator at 37 ℃. After 3 hours, the mitomycin C was removed and washed 3 to 5 times with PBS(-). Trypsin/EDTA was added just to cover the cells and leaved it for 3 minutes. Pipetting to break the cell into single cell and all the cell in trypsin were taken to conical tube, culture media (1.5 fold of trypsin/EDTA volume) was added and centrifuged at 3000 rpm for 5 minutes. The supernatant was removed, resuspended the pellet (1ml of culture media) and count the cell to know the concentration. Adjust to the optimal concentration for each culture dish type shown in Table 3.26.

Table 3.26: Concentration of mouse embryonic fibroblast cells seeding in different size of culture Petri dish

Size of culture Petri dish	Cell concentration
35 mm	$4x10^5$ cell/dish
60 mm	$1x10^6$ cell/dish
4-well dish	$1.2x10^5$ cell/well

The cell was seeded and cultured in the incubator (37 ℃, 5% CO_2). The culture Petri dish was shaked well in the direction shown below to make sure the cell will distributed cover all the bottom area of the culture Petri dish (Figure 3.21).

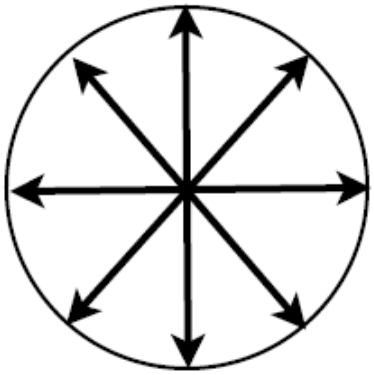

Figure 3.21: Direction of distributed mouse embryonic fibroblast in the culutre Petri dish.

The feeder cell should be prepared 1 day prior to use as the feeder layer for embryonic stem cell and should be incubated with embryonic stem cell culture media at least 3 hours before used. Before seeding the cell in to culture Petri dish, the feeder layers were prepared by expanding mouse embryonic fibroblast cultures on gelatinised culture Petri dishes. Culture dishes were gelatinised by covering the dish with 0.1% gelatin solution and left for 15 minutes in the laminar flow. After that, the gelatin solution was removed and the dishes let to dry prior to culturing mouse embryonic fibroblasts. The source of mouse embryonic fibroblasts could be obtained either from freshly passaged mouse embryonic fibroblasts or thawed from frozen stocks. Usually, they were seeded at $3.0x10^5$ viable cells per dish to allow confluency to be reached within 2 to 3 days. After the mouse embryonic fibroblasts culture reaches 80% confluency, the media was removed. 2 ml of mitomycin C (10 μg/ml; Sigma) solution was added and the cells were incubated for 3 hours. The solution was then aspirated, and the cells were washed twice times with PBS- solution. Around 2 ml of murine embryonic stem cells culture media were added to the culture Petri dishes and incubated for at least 3 hours.

3.3.5.2 (A) (c) Isolation of caprine embryonic stem cells

Embryonic stem cells were usually obtained by isolating undifferentiated outgrowths that originate from the inner cell mass of the blastocysts. The caprine embryonic stem cells were expanded, passaged, frozen and tested for pluripotency via differentiation. Outgrowths from the inner cell mass of caprine blastocysts can be derived through 3 techniques, namely culture of whole culture of caprine blastocysts, mechanical dissection or manual cut (30 G needle) and lasser dissection of inner cell mass from the caprine blastocysts and subsequently cultured to obtain primary outgrowths. In culture of whole caprine blastocysts technique, the zona pellucida of caprine blastocysts were removed by using pronase (0.5%). The caprine blastocysts were observed under stereomicroscope until the zona pellucida has degraded. The caprine blastocysts were then washed 3 to 5 times in embryonic stem cells culture medium (30 µl). The washed caprine blastocyst was isolated via different methods. After isolation of inner cell mass, inner cell masses were placed on inactivated mouse embryonic fibroblasts in a humidified atmosphere of CO_2 (5%) in air at 37˚C for culturing. After 5 to 12 days later, the growing colonies were individually dissociated into clumps after treating with a mixture of trypsin/EDTA (0.05%). The resultant small clumps containing approximately 20 to 50 cells were transferred to a new well with a fresh feeder layer and medium. The new colonies were inspected daily and subcultured at an interval of approximately 6 to10 days according to their size and growth rate. Culture medium was changed every other day. The washed caprine blastocysts were proceed to the next step.

3.3.5.2 (A) (c) (i) Culture of whole caprine blastocysts

The washed caprine blastocysts were then directly transferred onto feeder layers of inactivated feeder cell layer on a gelatinised 35 mm culture Petri dish. The free zona pellucida caprine blastocyst were placed directly on top of the feeder cells. Sufficient gap was left between each caprine blastocyst. A small Petri dish could be cultured up to 10 to15 caprine blastocysts. The culture dishes were carefully carried and placed back into the incubator, minimising shaking in the process and were left for 2 days without moving them because the caprine blastocysts were very sensitive in attachment onto the feeder cells once it moving. The culture media were changed on day 2 and every alternate day.

3.3.5.2 (A) (c) (ii) Manual cut (30 G) in isolation of caprine inner cell mass

After removing the zona pellucida of caprine blastocyst, a single blastocyst was transferred onto a droplet of embryonic stem cells culture media. A cut was performed on the caprine blastocyst by using 2 30 G needles, one needle to hold the caprine blastocyst in place and another to perform the cut. The inner cell mass was isolated via the cut. Care must be taken to prevent cutting too deep on the surface of the dish, or else the inner cell mass may get stuck on the Petri dish. After cut, the inner cell mass was carefully transferred onto a feeder layer of inactivated feeder cell layer. The caprine inner cell mass were placed onto the feeder layer with sufficient gap in between. A single dish may contain up to 10 to 15 caprine inner cell masses. After transferring all inner cell masses, the culture dishes were carefully placed back into the incubator. They were left for 2 days without any interference. The culture was observed on day 2 and media were changed on every other day.

3.3.5.2 (A) (c) (iii) Laser isolation of caprine inner cell mass

In laser isolation technique, the zona pellucida of caprine blastocyts was not removed. The inner cell mass isolation was carried out on the micromanipulator system where the holding pipette (left side) and biopsy needle (right side) were used in manipulated the caprine blastocyst. The inner cell mass was located at the 9 o'clock positioned. The caprine blastocyst was hold by holding pipette and the laser was shot along the way between the inner cell mass cells and trophectoderm cells. After shooting, the biopsy needle was sucked on the other side of the caprine blastocyst to remove the zona pellucida and trophectoderm from the inner cell mass. All the manipulation processed was done in PBS- medium. The isolated inner cell mass was washed with murine embryonic stem cell culture medium for several times before cultured onto the inactivated feeder cell layer. They were left for 2 days without any interference. The culture was observed on day 2 and media were changed on every other day.

3.3.5.2 (A) (d) Sub-culture of primary caprine inner cell mass outgrowths

The primary caprine inner cell mass outgrowths were sub-culture using 2 techniques, namely trypsinisation and mechanical dissection procedures by trypsin/EDTA (0.05%). After around 4 to 6 days of culture, the primary outgrowths from the caprine inner cell mass were picked. Suitable outgrowths were those of undifferentiated cells, characterised by slightly dark-pigmented clumps of cells with clearly defined borders. The inner cell mass that selected should have characters included dome shape colony surrounded with primitive endoderm, homogeneous and higher nuclear to cytoplasm ratio (bigger nucleus). Unwanted outgrowths were typically those of round, loosely arranged cells without a clear, defined border. They have a slightly refractal and were located directly next to the outgrowths. Outgrowths from the trophectoderm (TE) were characterised by the presence of giant cells, and these are not wanted. The suitable

outgrowths were picked before differentiation occurs. Outgrowths beginning to differentiate will exhibit dark pigmentation.

3.3.5.3 (A) (d) (i) Trypsinisation procedure for sub-culture caprine inner cell mass outgrowth

Culture Petri dish with feeder layer in murine embryonic stem cell culture medium at least 3 hours was incubated in incubator at 37 ℃, 5% CO_2 before use. For trypsinisation, the outgrowths were lift up from feeder cell and transferred to wash in drops of PBS- for 4 times before incubating in 0.05% trypsin/EDTA for 3 min at 37℃. Then gently pipetting up and down several times to break the outgrowth into small pieces which contains about 20 to 30 cells/piece. Then the small pieces were washed in murine embryonic stem cell culture medium for 4 times before spread onto a new feeder cell pre-incubated with murine embryonic stem cell culture medium. After 24 hours, change half of the media everyday. The cell colony will appear 3 to 4 days after trypsinisation (passage 1). Subculture when the colonies get bigger (should not let it bigger than 400 μm).

3.3.5.2 (A) (d) (ii) Mechanical sub-culture caprine inner cell mass outgrowth

The pulled Pasteur pipette connected with mouth piece will be used to cut the colony into smaller pieces (avoid the primitive endoderm). Using a micropipette (2 to 20 μl), the wanted outgrowths were detached and transferred onto a 30 μl droplet of PBS- for washing. Then, they were transferred to a droplet of 30 μl of trypsin/EDTA (0.05%) and gently disaggregated into smaller aggregates. The clumps were not disaggregated into single cells. The cell was transferred into prepared 4 well dish and incubated at 37 ℃, CO_2 (5%). After 24 hours, the media was changed half everyday. The colony will appear sooner when compared with trypsinisation procedure. Checked the cell carefully everyday and selected the one with caprine embryonic stem cell character for

subculture. For embryonic stem cell mechanical procedure were recommended in the first 3 to 4 passages. The good character colony for subculture was selected is highly recommended.

3.3.5.2 (B) Superovulation in female murine for embryos recovery

Female murine (6 to 8 weeks) were injected intraperitoneally with PMSG (10 IU). After 48 hours, female murine were injected intraperiotneally with hCG and mated suddenly with male murine (8 to 12 weeks) for 12 hours. The vaginal plug was observed in female murine at 12 hours post mated to make sure the female murine were successfully mated. The blastocyst collection from uterus will be run 72 to 78 hr after vaginal plug observation whereas *in vivo* flushing at 2-cell stage was carried out on day 1.5 after hCG injection (Table 3.27).

Table 3.27: Superovulation regime in murine species

Day	Time	Activities/embryo stages
Monday	1800 hours	PMSG
Wednesday	1800 hours	hCG+mating
Thursday	0600 hours	Vaginal plug observation (day 0.5)
Friday	0600 hours	2-cell stage embryo (day 1.5)
Saturday	0600 hours	Morula (day 2.5)
Sunday	0600 hours	Balstocyst (day 3.5)
Monday	2400 hours	Expanded blastocyst (day 4.0)

3.3.5.2 (B) (a) Recovery of preimplantation embryos (*in vivo* flushing)

In order to obtain blastocysts as source for production of murine embryonic stem cell, 2 approaches were carried out. First involves the recovery of 2-cell stage embryos via flushing of the oviduct and subsequent *in vitro* culture up to the blastocyst stage, while another approach involves direct recovery of blastocysts through uterine flushing.

3.3.5.2 (B) (a) (i) Recovery of 2-cell stage embryos through oviductal flushing

In between day 1.5 and 2.0 post-coitus, the female murine which mated and plugged were sacrificed by cervical dislocation. The murine oviducts were removed and placed into 20 µl droplets of warmed modified Hepes Whitten's medium to keep it moisture. The droplets were prepared on sterile Petri dishes before sacrificing the murine. Embryo flushing was prepared by attached a sterile blunt stainless steel hypodermic needle (32 G) a syringe (1 ml) containing modified Hepes Whitten's medium. The oviduct was held in place using sterile fine forceps, the flushing needle was then inserted into the infundibulum located among the coils of the oviduct. The modified Hepes Whitten's medium was injected into the infundibulum to flush out the murine embryos (Figure 3.22).

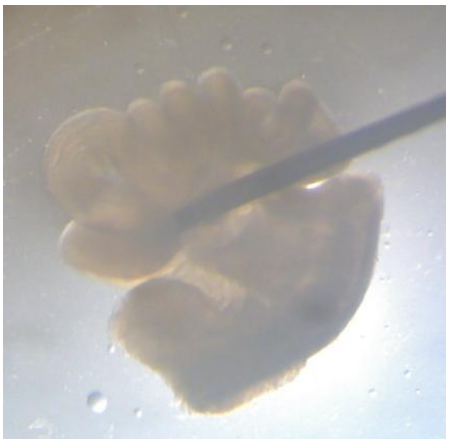

Figure 3.22 : Oviductal flushing through murine infundibulum.

The collected 2-cell stage embryos were observed under a stereomicroscope microscope and picked up using sterile puller pasteur pipette, controlled either by using a mouth piece or hand piece. The embryos were washed 3 times in modified Hepes Whitten's medium droplets until no debris was visible under the dissecting microscope, followed by 2 droplets of modified Whitten's medium (Figure 3.23). The 2-cell embryos were then transferred into equilibrated microdroplet cultures and *in vitro* culture up to

blastocyst stage. In this present study, 2-cell stage embryos can be obtained by flushing at 36 to 48 hours (1.5 to 2.0 days post-coitus).

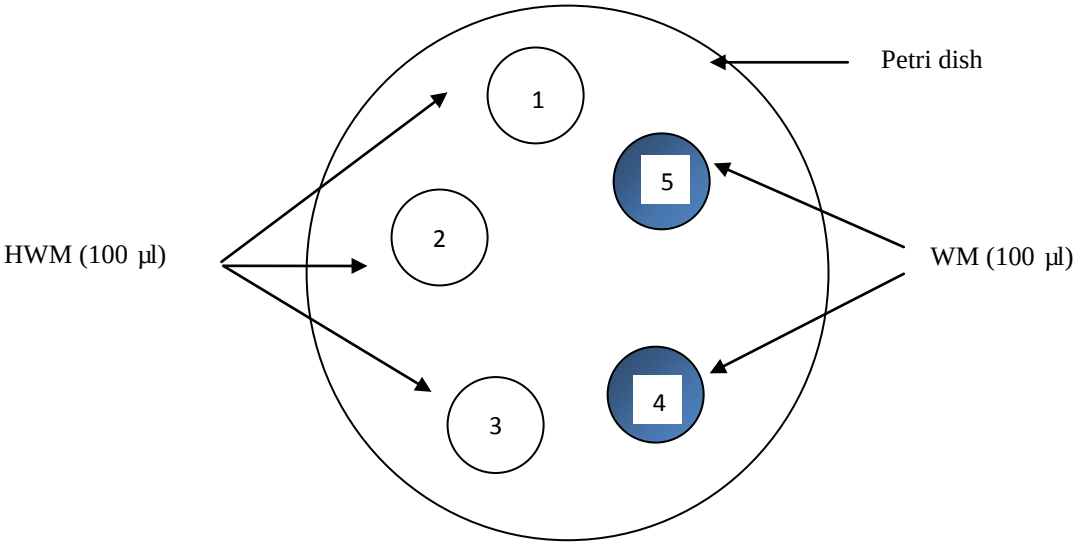

Figure 3.23: Washing medium for collected murine embryos.

3.3.5.2 (B) (a) (ii) Recovery of blastocysts

The flushed murine blastocysts were flushed at day 3.5 days post-coitus and cultured (day 4) were used as a source for production of murine embryonic stem cells according to the blastocyst stages (early blastocyst, mid blastocyst, expanded blastocyst, hatching blastocyst and hatched blastocyst). Murine blastocysts were flushed from the uterus between day 3.5 to 4.5 post-coitus as described by Hogan *et al*. (1986). The murine uterus was held with forceps just above the cervix which was located directly above the bladder. The cervix was cut across using fine scissors. The mesometrium holding the uterus to the body wall was trimmed by pulling the uterus upward to stretch the membrane. The uterus was completely removed by cutting below the junction with the oviduct on both horns. The uterus was placed in a Petri dish containing modified Hepes Whitten's medium. Both horns were flushed with Hepes Whitten medium using 26 G, ½ inch needles and 1 ml syringes. The blastocysts will be flushed out and detected

using an stereomicroscope microscope as well as picked up using puller pasteur pipettes. The blastocysts were then washed in droplets of Hepes Whitten's medium and Whitten medium (Figure 3.24) before *in vitro* culture in Whitten's medium in an incubator similar as recovery 2-cell stage embryos.

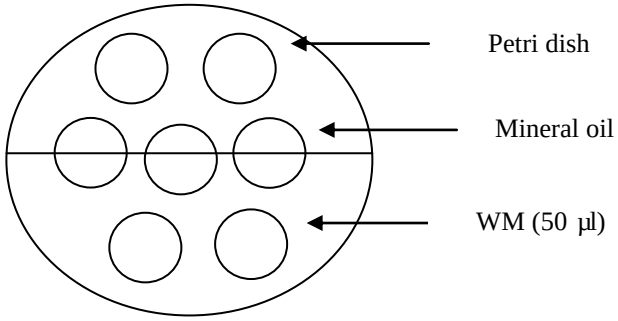

Figure 3.24: Culture medium for muirne embryos.

3.3.5.2 (B) (b) Mouse Embryonic Fibroblast (MEF) feeder cell preparation

Requirement of mouse embryonic fibroblasts are vital in production of embryonic stem cell lines to maintain embryonic stem cells in an undifferentiated state and to support its growth. The techniques preparation of mouse embryonic fibroblasts included (1) isolation of primary mouse embryonic fibroblast, (2) harvesting of mouse embryonic fibroblast, (3) passage of mouse embryonic fibroblast cultures, (4) cryopreservation of mouse embryonic fibroblast, (5) thawing of mouse embryonic fibroblast and (6) preparation of feeder layers through the mitotic inactivation of mouse embryonic fibroblast.

3.3.5.2 (B) (b) (i) Isolation of primary mouse embryonic fibroblasts

Mouse embryonic fibroblasts were prepared from 13.5 to 14.0 days post-coitus murine foetuses. The mouse embryonic fibroblasts were cultured in Dulbecco's modified eagle medium or alpha minimal essential medium supplemented with foetal bovine serum (10%, FBS; Invitrogen), 200 mM L-glutamine (Invitrogen) and 3x Penicillin/steptomycin (Sigma). The whole uterus was surgical removed from the murine and kept in PBS- (Figure 3.25). The uterus was surgical open with scissor then each foetal sac was removed out of the uterus. The foetuses were washed in PBS-, head limbs and internal organ was removed from the foetuses. The foetuses were minced into the small pieces and the mincing tissues were taken to a beaker. The optimal volume of trypsin/EDTA was added in the beaker and stir on the magnetic stirrer for 30 minutes (Figure 3.26). After that, it was filtered through nylon mesh into the conical tube (50 ml). Culture media (3x) was added to the supernatant (1.5 fold of supernatant volume) and centrifuged at 3000 rpm for 5 minutes (2 times) (Figure 3.27). The supernatant was removed, re-suspended the pellet with culture media and seed to the medium culture dish (60 mm) (Figure 3.28). The primary mouse embryonic fibroblasts were cultured at 37℃, CO_2 (5%) until the cell reaching 80 to 90% confluent. At 80 to 90% confluent, subculture the mouse embryonic fibroblast cell was sub-cultured for expanded (split ratio is ~ 1:5) and then was frozen as the feeder cell bank for future use.

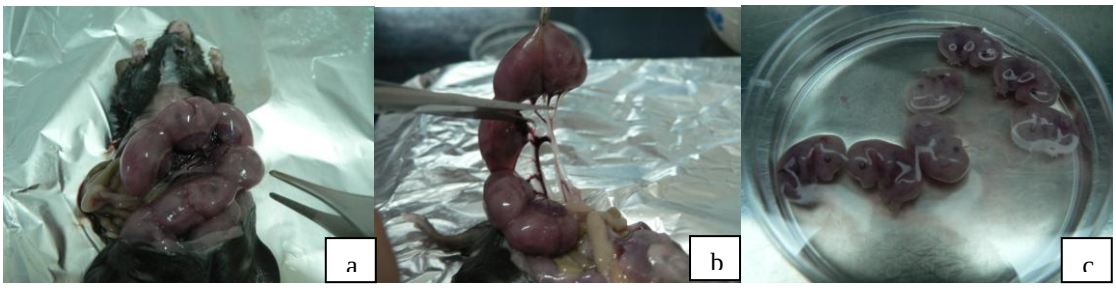

Figure 3.25: (a) Murine foetuses contained in the uterine sac. (b) Isolation of foetus from the uterine sac. (c) Washing of foetuses in PBS- solution.

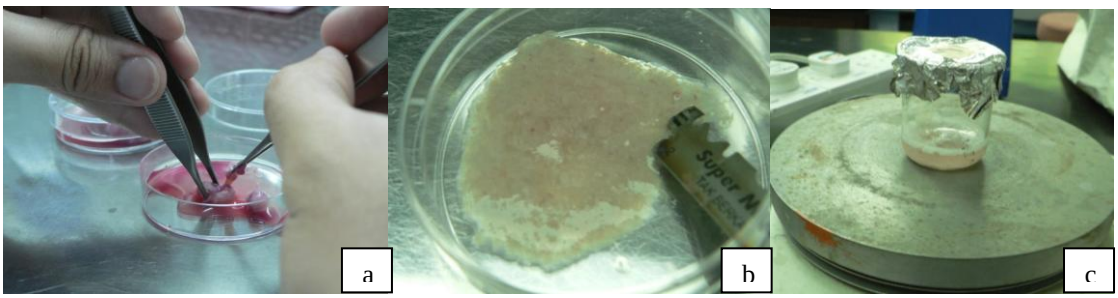

Figure 3.26: (a) Removal of limbs, organs and red blood cells from the foetus. (b) Mincing and trysinisation of foetuses into small pieces. (c) Further trysinisation and breaking down of the pieces of foetus using a magnetic stirrer.

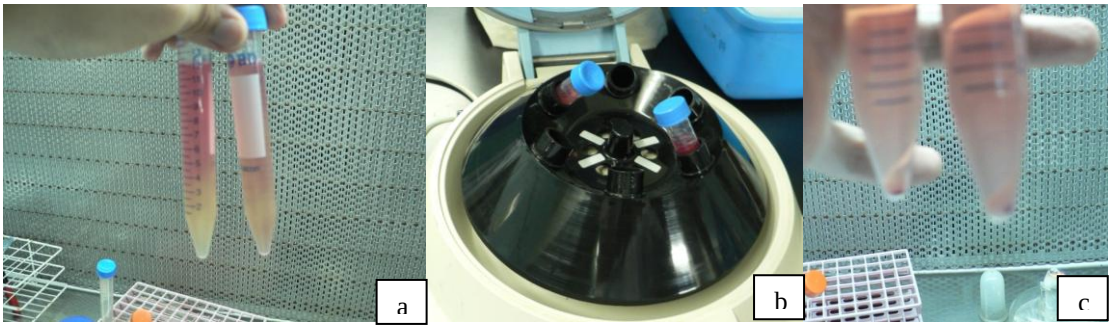

Figure 3.27: (a) Adding DMEM solution to the cell mixture at a ratio of 1.5 to 1.0. (b) Centrifugation for 5 minutes at 5000 rpm. (c) Formation of cell pellet after centrifugation.

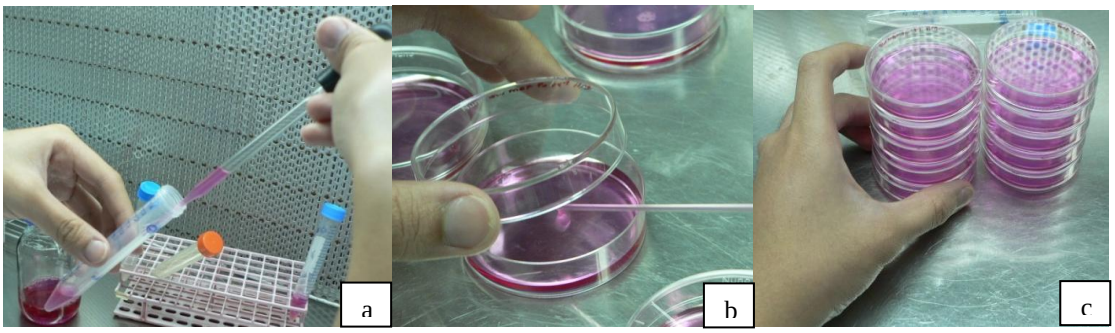

Figure 3.28: (a) The cell pellet was diluted to the desired cell concentration and sucked in and out to obtain a single cell suspension. (b) Dispensing of mouse embryonic fibroblast into culture dishes. (c) Gently shaking of the culture dishes to spread the mouse embryonic fibroblast evenly before culture in the CO_2 incubator.

3.3.5.2 (B) (b) (ii) Passages of mouse embryonic fibroblasts

Mouse embryonic fibroblasts cell cultures can be harvested after they reach 80% confluency. All media were sucked out and removed using a pasteur pipette. The cells were washed with PBS- solution (around 2 ml) twice to remove the remaining media.

This is because presence of excessive culture media will inhibit the subsequent trypsinisation step. Next, the cells were detached by adding 2 to 3 ml of trypsin/EDTA into each culture dish. The dishes were then incubated in an incubator for 2 to 3 minutes to allow the enzymatic reaction to take place. After that, the dishes were returned to the laminar flow and the solution was sucked in and out using pasteur pipette to physically detached the cells. This could be observed as a layer "peeling-off" the bottom of the culture dish. The cell suspension was then transferred to a conical tube (15 ml). Mouse embryonic fibroblast washing media (supplement with only 3% FBS) were added at a ratio of 1:1.5 media and the tubes were then centrifuged at 2000 rpm for 5 minutes. After centrifugation completed, a pellet would form at the bottom of the conical tube. If there were still pellets attached to the walls, centrifugation was repeated for another 5 minutes. The supernatant was removed and culture media (1x) were added accurately using a micropipettor (100 to 1000 µl) to make up 2 to 4 ml. The solution containing cells were mixed gently by pipetting in and out while avoided the formation of bubbles to produce isolated, single cells. Vigorous pipetting may damage the protein of cells. Mouse embryonic fibroblasts were seeded at around $5.0x10^5$ viable cells per dish by dispersing droplets of the mouse embryonic fibroblasts mixture onto the dishes using pasteur pipettes. The cells were grown until confluent (2 to 4 days) and the media were refreshed every subsequent day by replacing half of the media. The steps could be repeated for further passages.

3.3.5.2 (B) (b) (iii) Cryopreservation of mouse embryonic fibroblasts

The culture media was removed from the confluent culture dish. PBS- was added just to cover the cells and then sucked it out. Trypsin/EDTA was added just to cover the cells and incubated it for 2 to 3 minutes. The cell was pipetted by Pasteur pipette to break the cell into single cell. All the cells in trypsin were taken to conical tube and culture media

(1.5 fold of trypsin/EDTA volume) was added as well as centrifuged at 3000 rpm for 5 minutes. After centrifuged, supernatant was removed and resuspended the pellet (1 ml of culture media) and counted the cell to know the concentration. The volume of culture media was adjusted to the desire concentration (the total volume will be from 2 part evenly; one from culture media and another from freezing media). Freezing media (0.5 ml) was dispensed into cryovials with the number of cryovials depending on the volume of mouse embryonic fibroblasts cells mixture. For each cryovial, 0.5 ml of mouse embryonic fibroblasts mixture was added to give a final volume of 1.0 ml. The freezing media were added slowly into the resuspended cell (small volume each time) in the same volume and shaked well. The cell in the cryo-tube was aliquot and labelled as well as inserted into cryocanes and the cryocanes labeled. The cryocanes were placed into the freezer (-20 ℃) for 15 minutes. They were sealed within autoclave bags and placed into insulated containers containing ice packs. The ice packs were pre-loaded into the containers to lower the temperature within the containers. The cryocanes were then carried to the lab at the Institute of Postgraduate Studies where the whole journey took around 10 minutes. The cryocanes were then placed into -80 ℃ freezer for overnight. After that, the cryocanes were transported back to ABEL lab within insulated containers containing liquid nitrogen and removed from the autoclave bags and placed into liquid nitrogen tanks for storage.

3.3.5.2 (B) (b) (iv) Thawing of mouse embryonic fibroblasts

Cryovials were removed from cryocanes in liquid nitrogen tank without exposing the cryocanes for too long outside liquid nitrogen tanks. The cryovials were then immediately thawed by placing the lower portion of the vials into water bath, taking care to avoid contact between the cap and the water. After the mixture has thawed for about 2 minutes, the vials were removed from the water bath and sprayed with 70%

alcohol to make it steriled. The cap was flamed and removed in the laminar flow. The contents were transferred into conical tubes (15 ml) containing 2 ml of mouse embryonic fibroblasts washing media. If many vials were thawed at once, vials from the same batch can be pooled together into the same conical tube, while maintaining the ratio to mouse embryonic fibroblasts washing media at around 1:1.5. The tubes were then centrifuged at 2000 rpm for 5 minutes. After completed centrifuged, a pellet was formed at the bottom of the conical tube. Culture media (1x) were added accurately using a micropipetter (100 to 1000 µl) to make up 1 or 2 ml. The solution containing cells were mixed gently by pipetting in and out. They were then seeded onto small culture dishes containing 2 ml of MEF culture media according to the desired cell density. The cells were seeded at $3.0x10^5$ viable cells per small Petri dish (35 mm). The cells were cultured in the incubator at 37 ℃ to confluence and the media was changed on every alternate day.

3.3.5.2 (B) (c) Feeder cell management for murine embryonic stem cell

The culture media was removed from the confluent culture dish. Mitomycin C (50 µl) was added to 5 ml of culture media (αMEM+10% FBS or DMEM+10% FBS) for a final concentration (10 µg/ml) and incubated for 3 hours in the culture incubator at 37 ℃. After 3 hours, the mitomycin C was removed and washed 3 to 5 times with PBS(-). Trypsin/EDTA was added just to cover the cells and leaved it for 3 minutes. Pipetting to break the cell into single cell and the entire cell in trypsin were taken to conical tube, culture media (1.5 fold of trypsin/EDTA volume) was added and centrifuged at 3000 rpm for 5 minutes. The supernatant was removed, resuspended the pellet (1ml of culture media) and count the cell to know the concentration. The cell was seeded and cultured in the incubator (37 ℃, 5% CO_2). The culture Petri dish was shaked well to make sure the cell will distributed cover all the bottom area of the culture Petri dish. The feeder

cell should be prepared 1 day prior to use as the feeder layer for embryonic stem cell and should be incubated with embryonic stem cell culture media at least 3 hours before used. Before seeding the cell in to culture Petri dish, the feeder layers were prepared by expanding mouse embryonic fibroblast cultures on gelatinised culture Petri dishes. Culture dishes were gelatinised by covering the dish with gelatin (0.1%) solution and left for 15 minutes in the laminar flow. After that, the gelatin solution was removed and the dishes let to dry prior to culturing mouse embryonic fibroblasts. The source of mouse embryonic fibroblasts could be obtained either from freshly passaged mouse embryonic fibroblasts or thawed from frozen stocks. Usually, they were seeded at $3.0x10^5$ viable cells per dish to allow confluency to be reached within 2 to 3 days. After the mouse embryonic fibroblasts culture reaches 80% confluency, the media was removed. 2 ml of mitomycin C (10 μg/ml; Sigma) solution was added and the cells were incubated for 3 hours. The solution was then aspirated, and the cells were washed twice times with PBS- solution. Around 2 ml of murine embryonic stem cells culture media were added to the culture Petri dishes and incubated for at least 3 hours.

3.3.5.2 (B) (d) Isolation of murine embryonic stem cells

Embryonic stem cells were usually obtained by isolating undifferentiated outgrowths that originate from the inner cell mass of the blastocysts. The murine embryonic stem cells were expanded, passaged, frozen and tested for pluripotency via differentiation. Outgrowths from the inner cell mass of murine blastocysts can be derived through 3 techniques, namely culture of whole cell culture murine blastocysts, mechanical dissection or manual cut (30 G needle) and lasser dissection of inner cell mass from the murine blastocysts and subsequently cultured to obtain primary outgrowths. In culture of whole muirne blastocysts technique, the zona pellucida of muirne blastocysts was removed by using pronase (0.5%). The murine blastocysts were observed under

stereomicroscope until the zona pellucida has degraded. The murine blastocysts were then washed 3 to 5 times in murine embryonic stem cells culture medium (30 µl). The washed murine blastocyst was isolated via different methods. After isolation of inner cell mass, inner cell masses were placed on inactivated mouse embryonic fibroblasts in a humidified atmosphere of CO_2 (5%) in air at 37˚C for culturing. After 6 to 8 days later, the growing colonies were individually dissociated into clumps after treating with a mixture of trypsin/EDTA (0.05%). The resultant small clumps containing approximately 20 to 50 cells were transferred to a new well with a fresh feeder layer and medium. The new colonies were inspected daily and subcultured at an interval of approximately 6 to10 days according to their size and growth rate. Culture medium was changed every other day. The washed blastocysts were proceeded to the next step.

3.3.5.2 (B) (d) (i) Culture of whole murine blastocysts

The washed murine blastocysts were then directly transferred onto feeder layers of inactivated feeder cell layer on a gelatinised culture Petri dish (35 mm). The free zona pellucida murine blastocyst was placed directly on top of the feeder cells. Sufficient gap was left between each blastocyst. A small Petri dish could be used to culture 10 to15 murine blastocysts. Special care and precaution should be taken to ensure the culture dishes were carried and placed back into the incubator to minimise shaking and were left there for 2 days due to the murine blastocysts sensitiveness in attachment onto the feeder cells during culture dish movement. The culture medium was changed on day 2 and consecutive alternate days.

3.3.5.2 (B) (d) (ii) Manual cut (30 G) in isolation of murine inner cell mass

After removing the zona pellucida of murine blastocyst, a single murine blastocyst was transferred onto a droplet of embryonic stem cell culture medium. A cut was performed

on the murine blastocyst by using 2 needles (30 G), one needle to hold the murine blastocyst in place and another to perform the cut. The murine inner cell mass was isolated after the cutting. Care must be taken to prevent cutting too deep on the surface of the Petri dish, or else the inner cell mass may get stuck on the Petri dish. After cut, the murine inner cell mass was carefully transferred onto a feeder layer of inactivated feeder cell layer. The murine inner cell masses were placed onto the feeder layer with sufficient gap in between them. A single culture dish may contain up to 10 to 15 murine inner cell masses. After transferring all murine inner cell masses, the culture dishes were carefully placed back into the incubator. They were left for 2 days without any interference. The primary outgrowth of the culture was observed under an inverted microscope on day 2 and medium were changed on every other day.

3.3.5.2 (B) (d) (iii) Laser isolation of inner cell mass

In laser isolation technique, the zona pellucida of murine blastocyts was not removed. The inner cell mass isolation was carried out on the micromanipulator system where the holding pipette (left side) and biopsy needle (right side) were used in manipulated the murine blastocyst. The inner cell mass was located at the 9 o'clock positioned. The murine blastocyst was held by holding pipette and the laser was shot along the way between the inner cell mass cells and trophectoderm cells. After shooting, the biopsy needle was sucked on the other side of the murine blsatocyst to remove the zona pellucida and trophectoderm from the inner cell mass. The isolated murine inner cell mass was washed with murine embryonic stem cell culture medium for several times before cultured onto the inactivated feeder cell layer. They were left for 2 days without any interference. The primary outgrowth of the culture was observed under an inverted microscope on day 2 and medium were changed on every other day.

3.3.5.2 (B) (e) Sub-culture of primary murine inner cell mass outgrowth

The primary murine inner cell mass outgrowths were sub-culture using 2 techniques, namely trypsinisation and mechanical dissection procedures by trypsin/EDTA (0.05%). After around 4 to 6 days of culture, the primary outgrowths from the murine inner cell mass were picked. Suitable outgrowths were those of undifferentiated cells, characterised by slightly dark-pigmented clumps of cells with clearly defined borders. The inner cell mass that selected should have characters included dome shape colony surrounded with primitive endoderm, homogeneous and higher nuclear to cytoplasm ratio (bigger nucleus). Unwanted outgrowths were typically those of round, loosely arranged cells without a clear, defined border. They had a slightly refractal and were located directly next to the outgrowths. Outgrowths from the trophectoderm were characterised by the presence of giant cells, and these were not wanted and discarded. The suitable outgrowths were picked before differentiation occurred. Outgrowths beginning to differentiate will exhibit dark pigmentation.

3.3.5.2 (B) (e) (i) Trypsinisation procedure for sub-culture murine inner cell mass outgrowth

Culture Petri dish with feeder layer in murine embryonic stem cell culture medium at least 3 hours was incubated in incubator at 37 ℃, 5% CO_2 before use. For trypsinisation, the murine outgrowths were lift up from feeder cell and transferred to wash in drops of PBS- for 4 times before incubating in trypsin/EDTA (0.05%) for 3 minutes at 37 ℃. Then gently pipetting up and down several times to break the outgrowth into small pieces which contains about 20 to 30 cells/piece. Then the small pieces were washed in embryonic stem cell culture medium for 4 times before spread onto a new feeder cell pre-incubated with embryonic stem cell culture medium. After 24 hours, change half of the media everyday. The cell colony appeared 3 to 4 days after trypsinisation (Passage 1). Sub-culture when the colonies get bigger (should not let it bigger than 400 μm).

3.3.5.2 (B) (e) (ii) Mechanical sub-culture for murine inner cell mass outgrowth

The pulled Pasteur pipette connected with mouth piece will be used to cut the colony into smaller pieces (avoid the primitive endoderm). Using a micropipette (2 to 20 µl), the wanted outgrowths were detached and transferred onto a droplet of PBS- (30 µl) for washing. Then, they were transferred to a droplet of trypsin/EDTA (30 µl; 0.05%) and gently disaggregated into smaller aggregates. The clumps were not disaggregated into single cells. The cell was transferred into prepared 4 well dish and incubated at 37℃, 5% CO_2. After 24 hours, the medium was changed half everyday. The colony appeared sooner when compared with trypsinisation procedure. Checked the cell carefully everyday and selected the one with murine embryonic stem cell character for subculture. For murine embryonic stem cell mechanical procedure were recommended in the first 3 to 4 passages. The good character colony for subculture was selected is highly recommended.

3.3.5.3 Immunofluorescent staining on caprine and murine embryonic stem cells

The caprine and murine embryonic stem cells can be confirmed by immunofluorescent staining. All the media were removed out from culture Petri dish and washed with PBS(-) for 3 times to remove all the remaining protein out of the cell. Then, they were fixed with paraformaldehyde (4%) for 30 minutes. After 30 minutes, the cell was washed with PBS(-) for 5 times and blocking solution (10% FBS+PBS-) was added just cover the cell and leave it for 2 hours at room temperature. The primary antibody (such as Oct 4, SSEA1, SSEA3, SSEA4, TRA-1-80 and TRA-1-60) was prepared at the optimal concentration with the ratio (1: 250). After 2 hours of blocking solution, the blocking solution was removed and the diluted primary antibody was added just to cover the cell and incubated overnight at 4 ℃. The embryonic stem cell marker sample kit of primary antibody (Catalogue no. SCR002) was obtained from Millipore Company

from USA. After that, the primary antibody was removed out and washed 5 times with PBS(-). The washing step takes 10 to 15 minutes/time. Next, secondary antibody (diluted in ratio 1:1000) was added just to cover the cell and incubated 2 hours in dark environment due to its sensitivity to light. The second antibody (Alexa 488) that use in immunofluorescent staining depends on what species used for the primary antibody, for example, murine Oct 4 (primary antibody) must be used together with murine second antibody. After 2 hours, the secondary antibody was discarded out and washed again 5 times with PBS(-). The washing step also took 10 to 15 minutes/time. Lastly, Hoechst 33342 (5 μg/ml) was added just cover the cell and incubate 5 minutes in dark condition. The staining cell was observed under fluorescent microscope. The expression was detect by the glow of secondary antibody and the nucleus were be detected by the glow of Hoechst 33342. The colour of the protein markers detected depended on the second antibody used.

3.3.5.4 Alkaline phosphatase activity on caprine and murine embryonic stem cells

The alkaline phosphatase detection kit (Catalogue no. SCR004) was purchased from Millipore Company, USA. All the media was removed out from culture dish and washed with PBS(-) for 3 times to remove all the remaining protein out of the cell. The cell was fixed with paraformaldehyde (4%) for 30 minutes. After 30 minutes, they were washed with PBS(-) for 5 times the the alkaline phosphatase was added to the substrate just to cover the cell and incubated for 15 to 30 minutes in dark environment. After 15 to 30 minutes, washed again with PBS(-) for 2 times and PBS(-) was added just to cover the cell and observed under fluorescent microscope.

3.4 EXPERIMENTAL DESIGN

Several experiments were carried out in this study. This study was designed with the following specific goals: (1) to establish mouse embryonic fibroblasts as a feeder layer for caprine and murine embryonic stem cell lines production, (2) to evaluate the sources of caprine inner cell mass from blastocyst for production caprine embryonic stem cell lines, (3) to determine the effects of culture medium, mouse embryonic fibroblasts freezing and stages of blastocyst on production of embryonic stem cell lines using whole blasotcyst culture technique (4) to develop inner cell mass isolation techniques in production of caprine and murine embryonic stem cells lines and (5) to confirm caprine and murine embryonic stem cell lines by immunofluorescent staining of embryonic stem cells protein markers.

3.4.1 Establishment of Mouse Embryonic Fibroblast as Feeder Cell Layer for Production of Murine Embryonic Stem Cell Lines (Experiment1)

The objective of this experiment was to determine the suitable murine strain for producing mouse embryonic fibroblast cell lines for use as feeder layer for embryonic stem cells culture, based on the growth rate. Primary mouse embryonic fibroblasts were obtained using days 13.5 to 14 post-coitus pregnant murine from the ICR, C57/6J and CBA/ca strains. They were passaged up to Passage 2 (P2) and the cells were frozen at each passage. In this study, the mouse embryonic fibroblasts were frozen at density of $5.0x10^6$ cells/cryovial. The cells were thawed when there were embryos for embryonic stem cells culture. Feeder cell layer were prepared by seeding $4x10^5$ cell/dish in small culture Petri dish (35 mm). Both fresh and frozen-thawed mouse embryonic fibroblasts from both P1 and P2 were used. Their growth rate was compared, based on the number of days needed to reach 80% confluency. Confluent dishes were mitomitically inactivated and prepared for use as feeder layers. Two different culture media were also compared in order to obtain the good quality of embryonic stem cells culture. Data

obtained were analysed using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT).

3.4.2 Production of Blastocysts as a Source of Inner Cell Mass for the Establishment of Caprine Embryonic Stem Cell Lines (Experiment 2)

The objectives of this experiment were (a) to compare the quality and efficiency of embryonic stem cells growth from *in vitro* or *in vivo* derived caprine blastocysts, (b) to compare the squeezing and laser enucleation teachniques in deriving the blasotcyst as source for caprine embryonic stem cells production and (c) to compare the pre-injection duration for 30, 60, 90 and 120 minutes in production of high caprine blastocyst rates. In addition, bovine somatic cell nuclear transfer and parthenogenesis to produce blastocysts as a comparative study (control) to caprine species were included in this experiment. Data obtained were analysed using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT).

3.4.3 Effects of Culture Medium and Mouse Embryonic Fibroblast Freezing on Production of Murine Embryonic Stem Cell Lines Using Whole Blastocyst Culture Technique (Experiment 3)

This experiment was designed to compare the efficiency of early-, mid-, expanded-, hatching- and hatched blastocysts for deriving embryonic stem cell lines. The blastocysts obtained were segregated according to sources of blastocyts obtained and blastocyst stages. They were then cultured on mouse embryonic fibroblasts as feeder layers. The success rate of murine and caprine blastocyst attachment was compared. The inner cell mass outgrowths were then isolated and passaged up to Passage 3 (P3). The number of outgrowths that were successfully cultured in an undifferentiated state to Passage 1 (P1), Passage 2 (P2) and Passages (P3) were compared among the different culture media and the different passages number of mouse embryonic fibroblasts used.

Data obtained were analysed using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT).

3.4.4 Effects of Inner Cell Mass Isolation Techniques, Culture Medium and Mouse Embryonic Fibroblast Freezing on Production of Murine and Caprine Embryonic Stem Cell Lines Using Mouse Embryonic Fibroblast as Feeder Cell Layer (Experiment 4)

The aim of this experiment was to compare the efficiency of murine and caprine embryonic stem cells derived 3 different techniques, namely whole blastocyst culture, manual cut and laser dissection for deriving murine and caprine embryonic stem cells. They were then cultured on fresh or frozen mouse embryonic fibroblasts as feeder layers. The success rates of blastocyst attachment were compared. The inner cell mass outgrowths were then isolated and passaged up to passage 3 (P3). The number of outgrowths that can be successfully cultured in an undifferentiated state to P1, P2 and P3 were compared among the *in vitro* and *in vivo* derived blastocysts. Data obtained were analysed using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT).

3.4.5 Confirmation of Caprine and Murine Embryonic Stem Cells by Immunofluorescent Staining Protein Markers (Experiment 5)

The objective of this experiment was to confirm the embryonic stem cells derived from caprine and murine species. Embryonic stem cells protein markers (immunofluoresent staining) were used in determined the embryonic stem cells formation. Alkaline phosphatase staining was carried out to determine the alkaline phosphatase activities that found in the caprine and murine embryonic stem cell lines. Purplish colour confirmed the presence of true embryonic stem cells. As for embryonic stem cells protein markers staining, specific colours were shown for specific staining protein markers (Oct 4: green (murine); red (caprine), SSEA 1: green (murine), SSEA3: green

(caprine), SSEA4, TRA-1-60 and TRA-1-180) to indicate the presence of true embryonic stem cells. The results of the staining of the embryonic stem cells were observed and recorded.

3.5 STATISTICAL ANALYSIS

Using SPSS statistical programme (SPSS 16.0 version), means were obtained and analysed using one-way analysis of variance (ANOVA and differences among the means were determined using Duncan's Multiple Range Test (DMRT). The obtained data were presented as mean ± standard error of means (SEM). For Experiment 1, effects of factors (murine strain, culture medium, fresh and frozen mouse embryonic fibroblast) on parameters (growth rates and passages number of murine embryonic fibroblast cell lines) measured were determined. Also, effects of factors (pre-intracytoplasmic injection duration and murine strain) on parameters (cleavage rates and successful superovulation rates). For Experiment 2, effects of factors (enucleation techniques, pre-intracytoplasmic injection duration, oocyte grading and sources of oocytes and blastocysts) on parameters (cleavage rates) measured were determined in caprine species. Also, effects of factors (enucleation techniques, oocyte grading and sources of blastocysts) on parameters (cleavage rates). Comparison between caprine and bovine species, effects of factors (species, oocyte grading, enucleation techniques) on parameter (cleavage rates) measured were determined. For Experiment 3, effects of factors (murine strain, culture medium, fresh and frozen-thawed mouse embryonic fibroblasts, blastocyst stages) on parameters (inner cell mass attachment rate, primary inner cell mass outgrowths rate, successful rate of passages murine embryonic stem cell lines) measured were determined in murine species. For Experiment 4, effects of factors (inner cell mass isolation techniques, culture medium, fresh and frozen-thawed mouse embryonic fibroblast as well as *in vivo*- and *in vitro*-derived blastocyst) on parameters

(inner cell mass attachment rate, primary inner cell mass outgrowth rate and successful rate of passages caprine embryonic stem cell lines) measeured were determined in caprine species. Lastly, for Experiment 5, effects of factors (staining of embryonic stem cell protein markers and alkaline phosphatase) on parameters (expression of staining colour and alkaline phosphatase activity) were observed in caprine and murine species (Table 3.29).

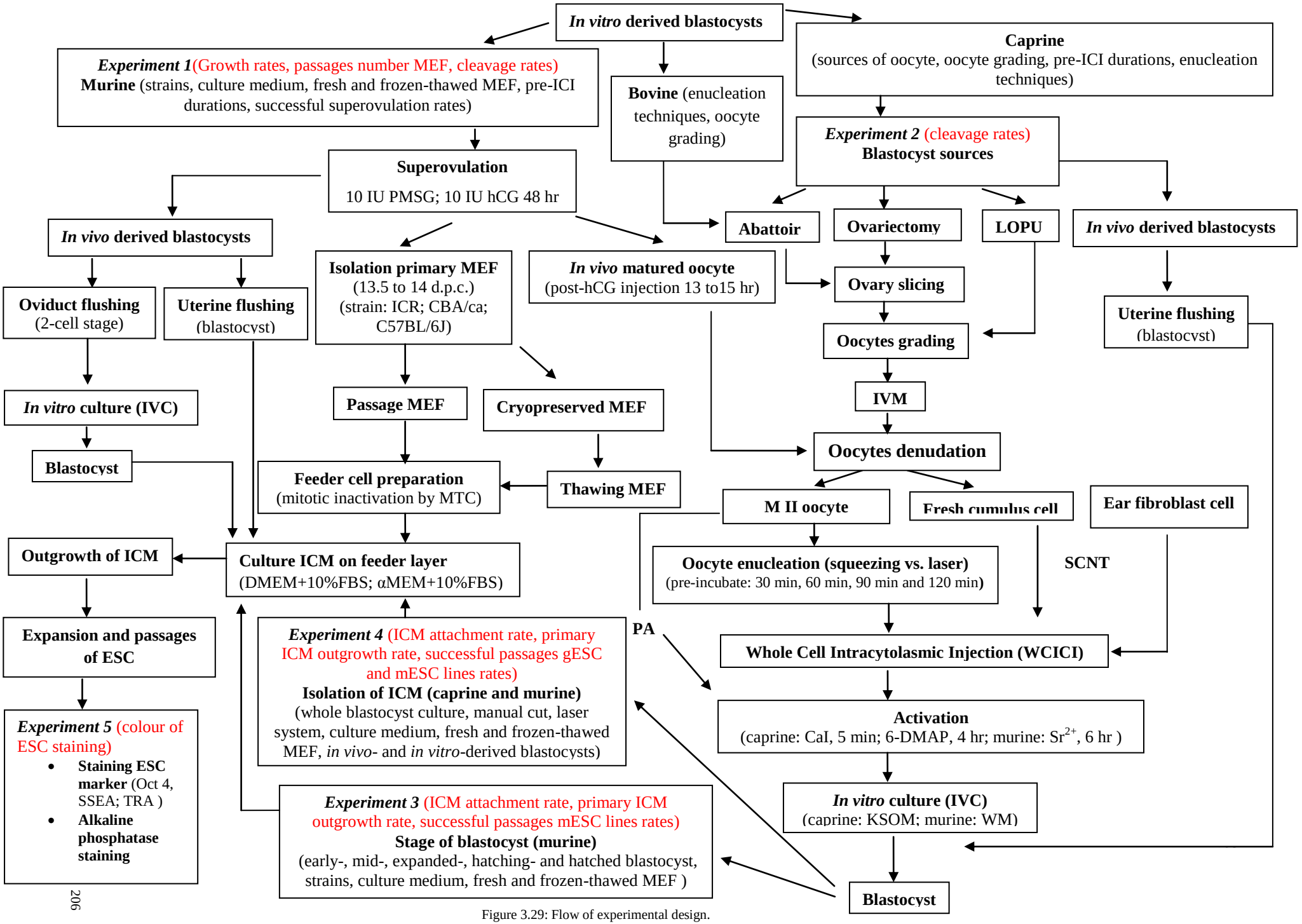

Figure 3.29: Flow of experimental design.

Chapter 4

4.0 RESULTS

Chapter 4

4.0 RESULTS

4.1 ESTABLISHMENT OF MOUSE EMBRYONIC FIBROBLAST AS FEEDER CELL LAYER FOR PRODUCTION OF MURINE EMBRYONIC STEM CELL LINES (EXPERIMENT 1)

Murine strains (ICR, CBA/ca and C57BL/6J) were evaluated to determine their suitability for producing mouse embryonic fibroblast cell lines for use as feeder cell layer for embryonic stem cells culture, based on their growth rate. The mouse embryonic fibroblast was passaged up to Passage 2 (P2) and the cells were frozen at each passage. The fresh and frozen-thawed mouse embryonic fibroblast at Passage 1 (P1) and Passage 2 (P2) were compared to determine their efficacies in producing murine embryonic stem cell lines. Besides that, 2 different culture media (DMEM and αMEM) were also compared to produce the good quality mouse embryonic fibroblast cell lines. Murine embryonic stem cells were obtained from 2 sources of blastocysts, namely *in vivo* superovulation-flushing embryos (2-cell stage embryos and blastocysts) and *in vitro* superovulated oocytes somatic cell nuclear transfer derived embryos.

4.1.1 Effects of Murine Pure-strains on Superovulation Responses

The production of embryos obtained from successful superovulation in 3 different murine pure-strains had been studied with the aim to determine the suitable strains of murine for use as the source of blastocysts for embryonic stem cells production.

Table 4.1: Percent successful superovulation (%, mean ±SEM) in 3 different pure-strains of murine

Murine strains	Number of murine injected	Number of murine successful superovulated	Percent successful superovulated female murine
ICR	329	281	75.34 ±3.66[x]
CBA/ca	302	243	77.00 ±3.81[x]
C57BL/6J	306	228	65.71 ±4.13[x]
Total	937	752	72.74 ±2.25

[x] Means with same superscript in a column were not significantly different (P>0.05).

Table 4.1 shows the percentage of successful superovulation in 3 different pure-strains of murine. A total of 752 out of 937 female murine were successfully superovulated. Out of total 329, 302, and 306 of ICR, CBA/ca and C57BL/6J, 281 (75.34 ±3.66%), 243 (77.00 ±3.81%) and 228 (65.71 ±4.13%) murine, respectively, were successful superovulated using PMSG and hCG. The successful superovulation was not significantly different (P>0.05) among the pure-strains of murine used in this experiment. Although no significant differences among the 3 different pure-strains murine were detected, the CBA/ca (77.00 ±3.81%) strain showed the highest percent successful superovulation followed by ICR (75.34 ±3.66%) and C57BL/6J (65.71 ±4.12%).

Table 4.2 shows the number of oocytes, 2-cell and blastocyst obtained from superovulation in 3 different pure-strains of murine. Total number of murine used in producing oocytes, 2-cell and blastocyst were 377, 864 and 212, respectively. There were significant differences (P<0.05) in number of oocytes for ICR and CBA/ca with C57BL/6J with values of 12.29 ±0.51 and 13.30 ±0.47 versus 14.98 ±0.53, respectively.

Table 4.2: Number of oocytes, 2-cell and blastocyst embryos obtained from superovulation (mean ±SEM) in 3 different pure-strains of murine

Murine strains	Oocytes			2-cell			Blastocyst		
	Total no. of selected females used	Total no. of oocytes obtained	No. of oocytes/female used	Total no. of selected females used	Total no. of 2-cell obtained	No. of 2-cell/female used	Total no. of selected females used	Total no. of blastocyst obtained	No. of blastocyst/female used
ICR	174	2048	12.29 ± 0.51^{x}	297	2999	10.62 ± 0.61^{x}	121	191	1.89 ± 0.18^{x}
CBA/ca	128	1660	13.30 ± 0.47^{x}	278	2402	11.07 ± 0.76^{x}	37	70	2.03 ± 0.23^{x}
C57BL/6J	75	1111	14.98 ± 0.53^{y}	289	2143	9.21 ± 0.71^{x}	54	72	1.52 ± 0.30^{x}
Total	377	4819	13.22 ± 0.30	864	7544	10.32 ± 0.40	212	333	1.81 ± 0.13

^{xy} Means with different superscript in a column were significantly different ($P<0.05$).

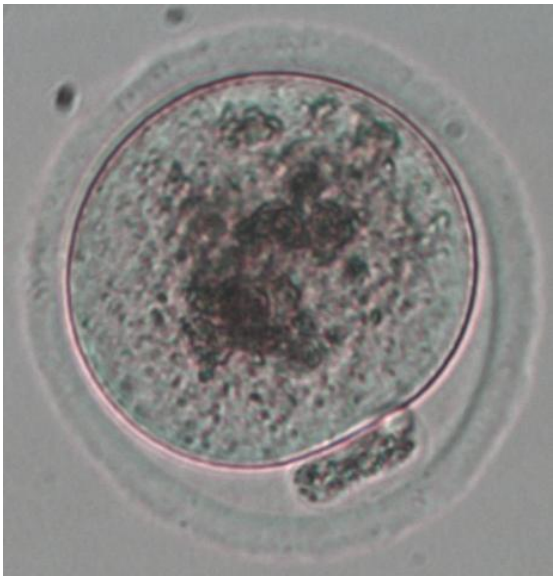

Figure 4.1: Murine oocytes with first polar body.

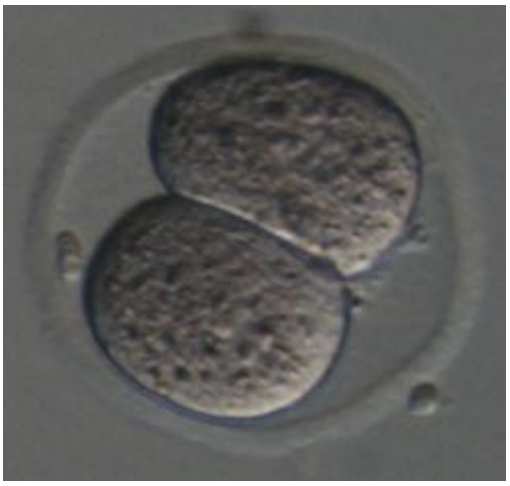

Figure 4.2: A 2-cell stage murine of embryo.

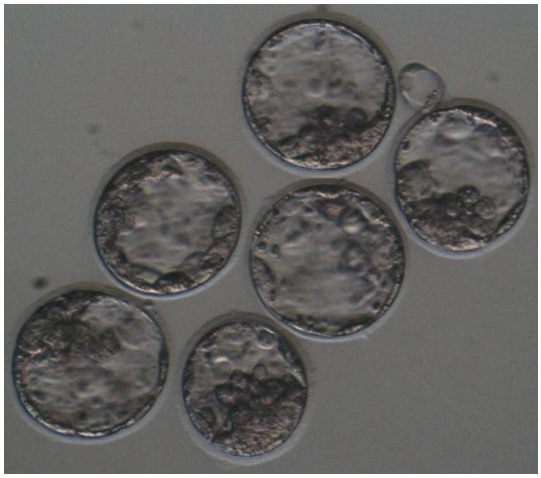

Figure 4.3: Blastocyst stage of murine embryos.

4.1.2 Effect of Murine Sources on the Percent Cleavage for the Production of Blastocysts

In this experiment, the murine blastocysts were obtained from *in vivo* and *in vitro* produced embryos. In the former, the murine embryos underwent *in vivo* oviduct (2-cell stage) or uterine (blastocyst) flushing. Somatic cell nuclear transfer was carried out in murine species to obtain *in vitro*-derived blastocysts. A total of 864 and 212 murine were used *in vivo* oviduct flushing and in *vivo* uterine flushing, whereas 377 murine were used *in vitro*-derived blastocyst through somatic cell nuclear transfer.

Table 4.3: Cleavage rates from 2-cell flushed embryos up to blastocyst stage (%, mean ±SEM) in 3 different pure-strains of murine

Murine strains	No. of selected females	No. of 2-cell	Percent cleavage (n)			
			4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)
ICR	297	2999	$89.69 \pm 1.41^{a,x}$ (2755)	$88.07 \pm 1.45^{a,x}$ (2673)	$87.19 \pm 1.48^{a,x}$ (2628)	$86.39 \pm 1.62^{a,x}$ (2589)
CBA/ca	278	2402	$89.65 \pm 0.85^{b,x}$ (2137)	$86.63 \pm 1.07^{ab,x}$ (2052)	$84.77 \pm 1.25^{a,x}$ (2000)	$84.26 \pm 1.33^{a,x}$ (1988)
C57BL/6J	289	2143	$87.53 \pm 1.95^{a,x}$ (1924)	$84.98 \pm 2.00^{a,x}$ (1843)	$82.86 \pm 2.07^{a,x}$ (1783)	$81.84 \pm 2.09^{a,x}$ (1747)
Total	864	7544	89.01 ±0.86	86.70 ±0.90	85.14 ±0.95	84.36 ±1.00

[ab] Means with different superscript in a row were significantly different (P<0.05).
[x] Means with same superscript in a column were not significantly different (P>0.05).

After *in vitro* culture, a total of 6324 (84.36 ±1.00%) murine blastocyst obtained from 7544 two-cell stage murine embryos through *in vivo* oviduct flushing. Out of 864 blastocysts obtained, 2589 (86.39 ±1.62%), 1988 (84.26 ±1.33%) and 1747 (81.84 ±2.09%) blastocysts were obtained from the ICR, CBA/ca and C57BL/6J murine pure-strains, respectively. Table 4.3 shows the results of percent cleavage among 3 different pure-strains of murine from 2-cell stage embryos up to blastocysts (Figure 4.4). There were no significant differences (P>0.05) among the 3 different pure-strains

of murine. However, there were significant differences ($P<0.05$) between 4-cell stage (89.65±0.85%) embryos versus the morula and blastocyst (84.77±1.25% and 84.26±1.33%) embryos in CBA/ca strain. The percent blastocyst obtained in ICR (86.39±1.62%) was the highest followed by CBA/ca (84.26±1.33%) and C57BL/6J (81.84±2.09%).

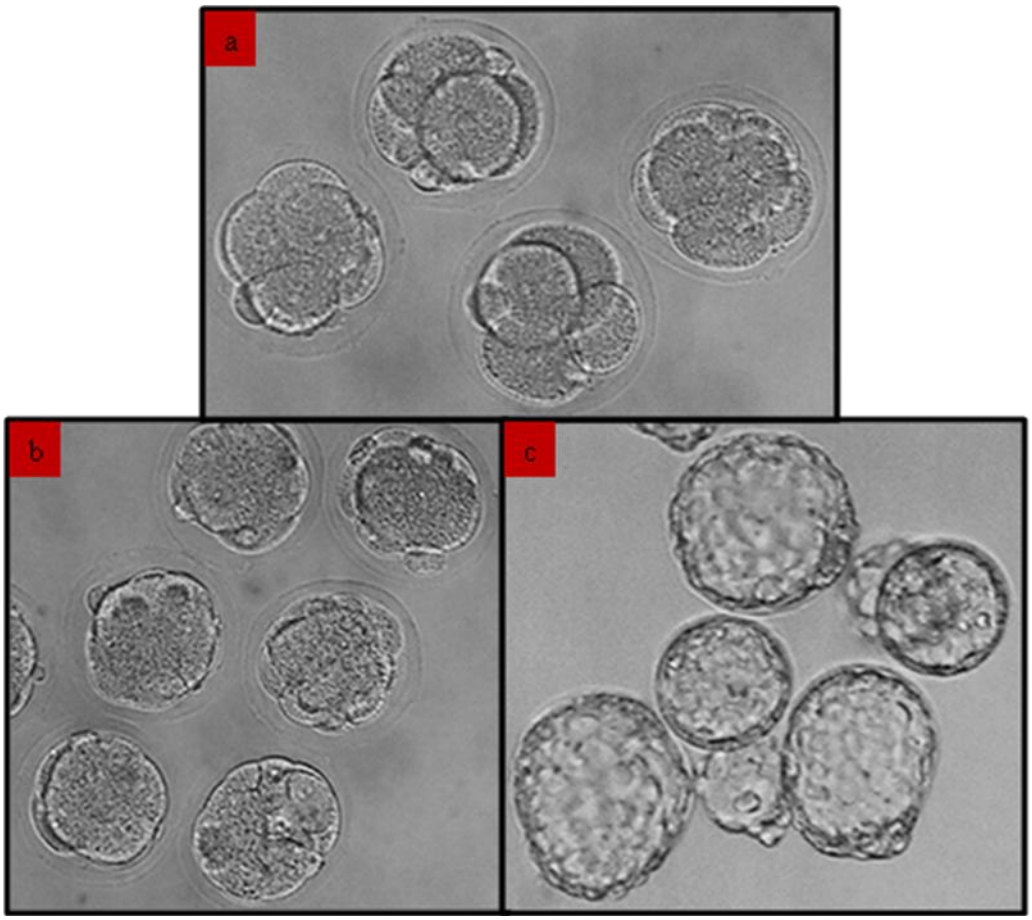

Figure 4.4: a) 4- and 8-cell murine embryos. b) Morula and early blastocyst murine embryos. c) Hatching and hatched murine blastocysts.

Table 4.4: Average number (mean ±SEM) of blastocyst obtained from 3 different pure-strains of murine through *in vivo* uterine flushing

Murine strains	No. of females	No. of blastocysts	Average (blastocyst) (n)
ICR	121	191	1.89 ± 0.18^{x}
CBA/ca	37	70	2.03 ± 0.23^{x}
C57BL/6J	54	72	1.52 ± 0.30^{x}
Total	212	333	1.81 ±0.13

[x] Means with same superscript in a column were not significantly different (P>0.05).

From Table 4.4, the data were collected from ICR (n=121), CBA/ca (n=37) and C57BL/6J (n=54) murine, which produced 191, 70 and 72 murine blastocysts, respectively. There were no significant differences (P>0.05) among the 3 different pure-strains murine which ICR (1.89 ±0.18), CBA/ca (2.03 ±0.23) and C57BL/6J (1.52 ±0.30).

Table 4.5 shows the cleavage rate of murine embryos from *in vitro* culture through somatic cell nuclear transfer. A total of 377 female murine from 3 different murine pure-strains were used in this experiment represented by 174, 128 and 75 for ICR, CBA/ca and C57BL/6J, respectively. There were no significant differences (P>0.05) in enucleation and injection rates from 3 different pure-strains of murine, except maturation rate, where C57BL/6J gave the highest maturation rate (61.00 ±2.26%) compared to ICR (57.15 ±1.73%) and CBA/ca (54.73 ±1.73%). It was observed that cleavage rates were decreasing from 2-cell stage embryos up to hatched blastocysts in 3 different pure-strains of murine. It was evident that ICR always showed the highest cleavage rates at 2-cell (42.79 ±3.00%), 4-cell (32.04 ±2.88%), 8-cell (25.69 ±2.67%), morula (22.46 ±2.50%) and blastocyst (18.02 ±2.30%) followed by C57BL/6J with the values of 39.53 ±3.95%, 25.89 ±3.21%, 17.50 ±2.76%, 12.57 ±2.32%, 10.10 ±1.81% and CBA/ca with the values of 35.43 ±3.03%, 20.99 ±2.43%, 13.48 ±2.09%, 9.55 ±1.69%, 7.35 ±1.39% for 2-, 4-, 8-cell, morula and blastocyst, respectively. However, C57BL/6J showed significant different (P<0.05) in percent

hatched blastocyst with the highest values (5.87±1.47%) followed by CBA/ca (1.22±0.51%) and ICR (1.28±0.28%).

Murine cleavage rates based on murine pure-strains and pre-intracytoplasmic injection (pre-ICI) durations through somatic cell nuclear transfer are shown in Table 4.6. Obviously, there were significant differences in pre-intracytoplasmic injection durations treatments at 90 minutes pre-intracytolasmic injection duration whereby it gave the highest cleavage rate in 3 different pure-strains murine compared to simultaneous injection as well as 30, 60 and 120 minutes pre-intracytoplasmic injection durations. Simultaneous injection right after enucleation showed the lowest cleavage rates in ICR (2-cell: 9.59±4.66%; 4-, 8-cell, morula, blastocyst and hatched blastocyst: 0.00±0.00%), CBA/ca (2-cell: 18.30±7.19%; 4-cell: 5.16±3.49%; 8-cell, morula, blastocyst and hatched blastocyst: 0.00±0.00%) and C57BL/6J (2-cell: 21.12±5.12%; 4-cell: 13.12±5.11%; 8-cell: 8.69±4.47%; morula: 1.43±1.43%; blastocyst and hatched blastocyst: 0.00±0.00%). Development of murine embryos from 2, 4-cell, morula, blastocyst and hatched blastocysts through somatic cell nuclear transfer technique as shown in Figure 4.5.

Table 4.5: Percent cleavage rate (%, mean±SEM) of murine embryos from *in vitro* culture through somatic cell nuclear transfer

Murine strains	No. of females	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
						2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
ICR	174	2048	57.15 ± 1.73^{xy} (1198)	61.10 ± 1.99^{x} (766)	65.22 ± 2.51^{x} (534)	$42.79 \pm 3.00^{e,x}$ (291)	$32.04 \pm 2.88^{d,y}$ (229)	$25.69 \pm 2.67^{cd,y}$ (159)	$22.46 \pm 2.50^{bc,y}$ (165)	$18.02 \pm 2.30^{b,y}$ (130)	$1.28 \pm 0.28^{a,y}$ (56)
CBA/ca	128	1660	54.73 ± 1.73^{x} (948)	57.63 ± 2.66^{x} (567)	61.82 ± 2.84^{x} (367)	$35.43 \pm 3.03^{e,x}$ (167)	$20.99 \pm 2.43^{d,x}$ (102)	$13.48 \pm 2.09^{c,x}$ (68)	$9.55 \pm 1.69^{bc,x}$ (46)	$7.35 \pm 1.39^{b,x}$ (35)	$1.22 \pm 0.51^{a,x}$ (8)
C57BL/6J	75	1111	61.00 ± 2.26^{y} (693)	63.13 ± 2.53^{x} (459)	67.55 ± 3.45^{x} (305)	$39.53 \pm 3.95^{d,x}$ (149)	$25.89 \pm 3.21^{c,xy}$ (99)	$17.50 \pm 2.76^{b,x}$ (69)	$12.57 \pm 2.32^{ab,x}$ (51)	$10.10 \pm 1.81^{ab,x}$ (40)	$5.87 \pm 1.47^{a,y}$ (25)
Total	377	4819	57.03±1.08	60.22±1.40	64.43±1.66	39.39±1.89	26.68±1.68	19.48±1.52	15.64±1.37	12.43±1.20	4.64±0.79

^{abcde} Means with different superscripts in a row within a group were significantly different ($P<0.05$).

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

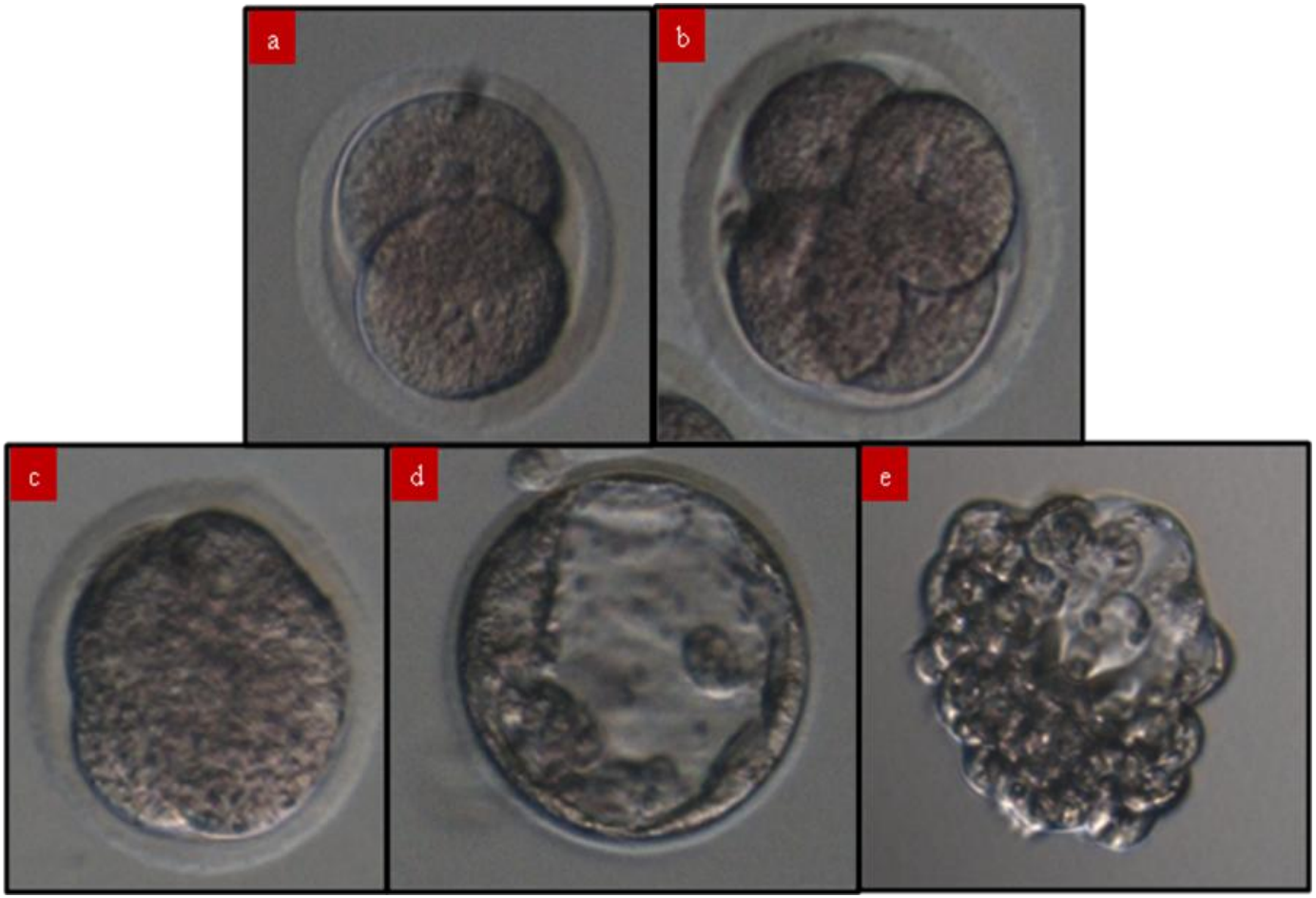

Figure 4.5: Development of murine embryos from 2-, 4-cell, morula, blastocyst and hatched blastocysts through somatic cell nuclear transfer technique.

Table 4.6: Percent murine cleavage (%, mean ±SEM) based on murine pure-strains and pre-intracytoplasmic injection (pre-ICI) durations through somatic cell nuclear transfer

Murine strains	Pre-ICI durations	Percent cleavage (n)					
		2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
ICR	Simultaneous	$9.59 \pm 4.66^{b,w}$ (7/52)*	$0.00 \pm 0.00^{a,w}$ (0/52)	$0.00 \pm 0.00^{a,w}$ (0/52)	$0.00 \pm 0.00^{a,w}$ (0/52)	$0.00 \pm 0.00^{a,w}$ (0/52)	$0.00 \pm 0.00^{a,w}$ (0/52)
	30 min	$39.25 \pm 6.62^{c,x}$ (24/56)	$23.85 \pm 6.41^{b,x}$ (15/56)	$19.97 \pm 5.75^{b,x}$ (13/56)	$17.91 \pm 5.46^{b,x}$ (11/56)	$14.73 \pm 4.29^{ab,x}$ (9/56)	$0.27 \pm 0.27^{a,w}$ (2/56)
	60 min	$55.54 \pm 6.21^{d,y}$ (58/97)	$43.76 \pm 5.94^{cd,y}$ (45/97)	$35.95 \pm 5.32^{bc,y}$ (36/97)	$32.91 \pm 5.03^{bc,y}$ (33/97)	$24.24 \pm 4.68^{b,y}$ (22/97)	$0.59 \pm 0.28^{a,w}$ (4/97)
	90 min	$67.76 \pm 2.87^{d,y}$ (160/237)	$60.14 \pm 3.11^{cd,z}$ (139/237)	$52.04 \pm 3.76^{bc,z}$ (122/237)	$47.26 \pm 3.84^{b,z}$ (111/237)	$42.85 \pm 3.92^{b,z}$ (99/237)	$4.14 \pm 0.74^{a,x}$ (50/237)
	120 min	$34.75 \pm 5.18^{b,x}$ (42/92)	$22.93 \pm 4.69^{c,x}$ (30/92)	$11.89 \pm 4.07^{b,x}$ (18/92)	$6.49 \pm 2.68^{ab,w}$ (10/92)	$0.00 \pm 0.00^{a,w}$ (0/92)	$0.00 \pm 0.00^{a,w}$ (0/92)
	Total	42.79 ± 3.00	32.04 ± 2.88	25.69 ± 2.67	22.46 ± 2.50	18.02 ± 2.30	7.05 ± 1.61
CBA/ca	Simultaneously	$18.30 \pm 7.19^{b,w}$ (11/29)	$5.16 \pm 3.49^{a,w}$ (3/29)	$0.00 \pm 0.00^{a,w}$ (0/29)	$0.00 \pm 0.00^{a,w}$ (0/29)	$0.00 \pm 0.00^{a,w}$ (0/29)	$0.00 \pm 0.00^{a,w}$ (0/29)
	30 min	$24.14 \pm 7.51^{b,w}$ (12/40)	$5.95 \pm 4.14^{a,w}$ (3/40)	$3.57 \pm 3.57^{a,wx}$ (2/40)	$1.79 \pm 1.79^{a,w}$ (1/40)	$0.00 \pm 0.00^{a,w}$ (0/40)	$0.00 \pm 0.00^{a,w}$ (0/40)
	60 min	$34.61 \pm 6.58^{c,w}$ (23/62)	$20.43 \pm 4.62^{b,x}$ (14/62)	$12.64 \pm 3.88^{ab,x}$ (9/62)	$10.73 \pm 3.42^{ab,x}$ (7/62)	$10.73 \pm 3.42^{ab,x}$ (7/62)	$0.00 \pm 0.00^{a,w}$ (0/62)
	90 min	$57.60 \pm 3.58^{e,x}$ (93/157)	$46.05 \pm 2.80^{d,y}$ (68/157)	$36.40 \pm 3.60^{c,y}$ (53/157)	$26.70 \pm 3.59^{b,y}$ (38/157)	$19.55 \pm 3.11^{b,y}$ (28/157)	$4.57 \pm 1.77^{a,x}$ (8/157)
	120 min	$28.81 \pm 6.17^{c,w}$ (28/79)	$12.68 \pm 4.14^{b,wx}$ (14/79)	$2.85 \pm 1.72^{a,w}$ (4/79)	$0.00 \pm 0.00^{a,w}$ (0/79)	$0.00 \pm 0.00^{a,w}$ (0/79)	$0.00 \pm 0.00^{a,w}$ (0/79)
	Total	35.43 ± 3.03	20.99 ± 2.43	13.48 ± 2.09	9.55 ± 1.69	7.35 ± 1.39	1.22 ± 0.51

C57BL/6J	Simultaneously	21.12 ±5.12^{c,w} (11/45)	13.12 ±5.11^{bc,w} (7/45)	8.69 ±4.47^{ab,w} (4/45)	1.43 ±1.43^{a,w} (1/45)	0.00 ±0.00^{a,w} (0/45)	0.00 ±0.00^{a,w} (0/45)
	30 min	37.33 ±8.84^{c,w} (18/44)	19.16 ±7.28^{b,w} (10/44)	10.84 ±4.31^{ab,w} (6/44)	7.09 ±3.48^{ab,w} (4/44)	5.84 ±3.01^{ab,w} (3/44)	0.00 ±0.00^{a,w} (0/44)
	60 min	35.07 ±8.93^{c,w} (15/36)	21.57 ±6.93^{bc,w} (9/36)	9.88 ±4.36^{ab,w} (5/36)	7.80 ±3.63^{ab,w} (4/36)	6.13 ±2.80^{ab,w} (3/36)	0.00 ±0.00^{a,w} (0/36)
	90 min	60.51 ±6.06^{d,x} (93/142)	44.19 ±4.92^{c,x} (66/142)	35.45 ±4.35^{bc,x} (52/142)	28.86 ±3.84^{ab,x} (41/142)	23.75 ±2.50^{ab,x} (33/142)	17.24 ±2.54^{a,x} (25/142)
	120 min	24.20 ±9.12^{c,w} (12/38)	13.69 ±5.35^{a,w} (7/38)	3.17 ±3.17^{a,w} (2/38)	1.59 ±1.59^{a,w} (1/38)	1.59 ±1.59^{a,w} (1/38)	0.00 ±0.00^{a,w} (0/38)
	Total	39.53 ±3.95	25.89 ±3.21	17.50 ±2.76	12.57 ±2.32	10.10 ±1.81	5.87 ±1.47

^{abcde} Means with different superscripts in a row within a group were significantly different (P<0.05).

^{wxyz} Means with different superscripts in a column within a group were significantly different (P<0.05).

4.1.3 Effect of 3 Different Pure-strains of Murine for Mouse Embryonic Fibroblast Cell Lines on Murine Embryonic Stem Cells Lines Performance

This experiment was to evaluate the suitable murine pure-strains for use to derive the mouse embryonic fibroblast cell lines used as feeder cell layer for murine embryonic stem cell lines production. A total of 3 mouse embryonic fibroblast cell lines were derived from 3 different pure-strain of murine (ICR, CBA/ca and C57BL/6J). There were no observable differences in terms of morphology of mouse embryonic fibroblasts from different strains and passages number, as well as before and after frozen-thawed.

Table 4.7: Growth rates of mouse embryonic fibroblast cell lines in 3 different pure-strains of murine

Murine strains	No. of females	No. of replicates	Growth rate (n)		
			P0 (n)	P1 (n)	P2 (n)
ICR	45	32	$95.31 \pm 2.62^{a,x}$ (42)	$100.00 \pm 0.00^{a,x}$ (42)	$96.88 \pm 2.17^{a,x}$ (40)
CBA/ca	23	22	$88.64 \pm 6.52^{a,x}$ (20)	$86.36 \pm 7.49^{a,x}$ (19)	$81.81 \pm 8.42^{a,x}$ (18)
C57BL/6J	21	18	$88.89 \pm 6.46^{a,x}$ (18)	$86.11 \pm 7.89^{a,x}$ (16)	$83.33 \pm 9.04^{a,x}$ (15)
Total	89	72	91.67 ± 2.80	92.36 ± 3.07	88.89 ± 3.60

[a] Means with same superscript in a row were not significantly different (P>0.05).
[x] Means with same superscript in a column were not significantly different (P>0.05).

Table 4.7 shows the growth rate of mouse embryonic fibroblast cell lines in 3 different pure-strains of murine (ICR, CBA/ca and C57BL/6J). There were no significant differences (P>0.05) in mouse embryonic fibroblast growth rates for P0, P1 and P2 as well as 3 different pure-strains of murine. Therefore, 3 different pure-strains of murine also could be used as feeder cell layers for production of murine embryonic stem cell lines. Growth rate and confluency of mouse embryonic fibroblast cells were shown in Figure 4.6.

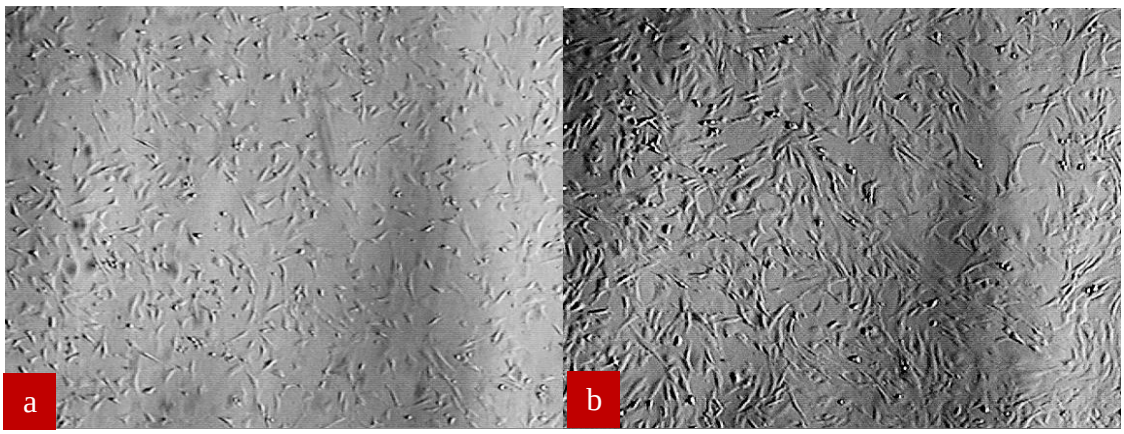

Figure 4.6: a) 50% growth rate mouse embryonic fibroblast cells. b) 80% confluency of mouse embryonic fibroblast cells.

Table 4.8: Successful growth rates of mouse embryonic fibroblast cell lines in 3 different pure-strains of murine after frozen-thawed at Passage 1 (P1)

Murine strains	No. of females	No. of replicates	P1 (n)	P2 (n)	Growth rates after frozen-thawed (n)	
					P1 (n)	P2 (n)
ICR	45	32	42	40	$93.75 \pm 3.72^{a,x}$ (39/42)	$92.19 \pm 3.96^{a,x}$ (36/40)
CBA/ca	23	22	19	18	$77.27 \pm 9.14^{a,x}$ (17/20)	$72.73 \pm 9.72^{a,x}$ (16/18)
C57BL/6J	21	18	16	15	$86.11 \pm 7.89^{a,x}$ (16/18)	$72.22 \pm 10.86^{a,x}$ (13/15)
Total	89	72	80	73	86.81 ± 3.83	81.25 ± 4.47

[a] Means with same superscript in a row were not significantly different (P>0.05).
[x] Means with same superscript in a column were not significantly different (P>0.05).

In Table 4.8, a total of 89 pregnant murine females from ICR (45), CBA/ca (23) and C57BL/6J (21) were used in this experiment to produce 77 (Passage 1) and 73 (Passages 2) mouse embryonic fibroblast cell lines as a bank store for used as feeder cell layers for cultured murine embryonic stem cells. There were no significant differences (P>0.05) in growth rates after frozen-thawed mouse embryonic fibroblast cells between Passage 1 (P1) and Passage 2 (P2) for 3 different pure-strains of murine.

Table 4.9: Growth rates of different culture media for mouse embryonic fibroblast cell lines

Culture media	No. of females	No. of replicates	Growth rates (n)		
			P0 (n)	P1 (n)	P2 (n)
DMEM+10% FBS	45	36	97.22 ±1.94[a,x] (43)	98.61 ±1.39[a,x] (42)	97.22 ±1.94[a,x] (40)
αMEM+10% FBS	44	36	95.83 ±3.07[a,x] (42)	94.44 ±5.19[a,x] (40)	90.28 ±4.37[a,x] (37)
Total	89	72	96.53 ±1.80	96.53 ±2.68	93.75 ±2.41

[a] Means with same superscript in a row were not significantly different (P>0.05).
[x] Means with same superscript in a column were not significantly different (P>0.05).

Table 4.9 shows the growth rates of mouse embryonic fibroblast cell lines in 2 different culture media (DMEM+10%FBS and αMEM+10%FBS). There were no significant differences (P>0.0.5) between DMEM+10% FBS and αMEM+10% FBS culture media for mouse embryonic fibroblast cells at P0 (97.22 ±1.94% versus 95.83 ±3.07%), P1 (98.61 ±1.39% versus 94.44 ±5.19%) and P2 (97.22 ±1.94% versus 90.28 ±4.37%), respectively. Therefore, both DMEM+10% FBS and αMEM+10% FBS culture media could be used in cultured the mouse embryonic fibroblast cell lines.

Table 4.10: Successful growth rates of different culture media for mouse embryonic fibroblast cell lines after frozen-thawed at Passage 1 (P1)

Culture media	No. of females	No. of replicates	P1 (n)	P2 (n)	Growth rates after frozen-thawed (n)	
					P1 (n)	P2 (n)
DMEM+10% FBS	24	18	24	23	94.44 ±5.56[a,x] (23/24)	97.22 ±2.78[a,x] (22/23)
αMEM+10% FBS	22	18	22	19	88.89 ±6.46[a,x] (19/22)	91.67 ±6.06[a,x] (17/19)
Total	46	36	46	42	91.67 ±4.23	94.44 ±3.32

[a] Means with same superscript in a row were not significantly different (P>0.05).
[x] Means with same superscript in a column were not significantly different (P>0.05).

Successful growth rates of different culture media for mouse embryonic fibroblast cell lines after frozen-thawed at Passage 1 (P1) was shown in Table 4.10. Similar as Table 4.9, there were no significant differences (P>0.0.5) between DMEM+10%FBS and αMEM+10%FBS culture media for mouse embryonic fibroblast cells after frozen-thawed at Passage 1 (P1) with the values of 94.44±5.56% versus 88.89±6.46% and 97.22±2.78% versus 91.67±6.06% for Passage 1 (P1) and Passage 2 (P2), respectively. However, culture medium for DMEM+10% FBS gave the highest growth rates (P1: 94.44±5.56%; P2: 97.22±2.78%) compared to αMEM+10% FBS culture medium (P1: 88.89±6.46%; P2: 91.67±6.06%).

4.2 PRODUCTION OF CAPRINE BLASTOCYSTS AS A SOURCE OF INNER CELL MASS FOR THE ESTABLISHMENT OF CAPRINE EMBRYONIC STEM CELL LINES (EXPERIMENT 2)

The objectives of this experiment were (a) to compare the efficiency and quality of embryonic stem cells growth from *in vitro* or *in vivo* derived caprine blastocysts, (b) to compare the squeezing and laser enucleation techniques in deriving the blasotcysts as source for caprine embryonic stem cells production and (c) to compare the pre-intracytoplasmic injection durations for simultaneous, 30 and 60 minutes in production of high caprine blastocysts. In addition, bovine somatic cell nuclear transfer and parthenogenesis to produce blastocysts as a comparative study (control) to caprine species were included in this experiment.

4.2.1 Production of Bovine Blastocysts Through Somatic Cell Nuclear Transfer and Parthenogenesis (Control)

Table 4.11 shows the cleavage rates of bovine embryos through somatic cell nuclear transfer by 2 different enucleation techniques, namely squeezing and laser enucleation. There were no significant differences (P>0.05) in maturation, enucleation and injection

rates between squeezing and laser enucleation techniques. However, embryos development at various stages were significantly higher in laser enucleation technique compared to squeezing enucleation technique with values of 63.72±4.04% versus 20.12±5.35%, 50.30±4.33% versus 13.72±4.38%, 37.74±4.31% versus 7.95±3.79%, 23.84±3.79% versus 1.71±1.33%; 7.01±1.42% versus 0.20±0.20% and 2.98±0.77% versus 0.10±0.10% for 2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst, respectively. For both enucleation techniques, the cleavage rates were decreasing as the stages of development progressing. No blastocysts were obtained for squeezing enucleation technique.

Table 4.12 shows the cleavage rate of bovine embryos based oocytes grading regardless of enucleation techniques. Grade A oocytes showed the highest maturation (49.77±3.21%), enucleation (84.34±3.12%) and injection (86.73±3.03%) rates followed by Grades B (33.62±3.66%; 57.60±5.39%; 57.19±5.40%), C (21.61±3.75%; 30.47±5.23%; 29.37±5.08%), D (8.44±2.39%; 13.27±3.83%; 13.61±3.88%) and E (9.79±2.38%; 17.89±4.38%; 17.25±4.28%), respectively. Similarly, cleavage rates for each developmental stage decreased as the grades of oocytes decreased (2-cell: 5.51 to 49.16%; 4-cell: 2.92 to 40.55%; 8-cell: 2.92 to 29.93%; morula: 2.08 to 18.04%; blastocyst: 0.14 to 5.78% and hatched blastocyst: 0.00 to 2.56%).

Table 4.11: Cleavage rates of bovine embryos (%, mean ±SEM) from *in vitro* culture through different enucleation techniques

Enucleation techniques	No. of ovaries	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
						2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Squeezing	72	1420	37.00 ±3.53^{x} (512)	82.73 ±3.81^{x} (375)	87.87 ±2.86^{x} (320)	20.12 ±5.35^{c,x} (94)	13.72 ±4.38^{bc,x} (71)	7.95 ±3.79^{ab,x} (45)	1.71 ±1.33^{a,x} (19)	0.20 ±0.20^{a,x} (2)	0.10 ±0.10^{a,x} (1)
Laser	148	1734	46.02 ±3.76^{x} (806)	88.64 ±3.21^{x} (699)	92.38 ±1.97^{x} (644)	63.72 ±4.04^{e,y} (413)	50.30 ±4.33^{d,y} (327)	37.74 ±4.31^{c,y} (257)	23.84 ±3.79^{b,y} (195)	7.01 ±1.42^{a,y} (71)	2.98 ±0.77^{a,y} (33)
Total	220	3154	42.01 ±2.65	86.01 ±2.47	90.38 ±1.69	44.35 ±4.14	34.04 ±3.76	24.50 ±3.40	14.01 ±2.53	3.89 ±0.89	1.65 ±0.46

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

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.12: Percent cleavage of bovine embryos (%, mean ±SEM) based on oocyte grading

Oocyte Grades	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
A	1417	49.77 ±3.21^{z} (690)	84.34 ±3.12^{z} (589)	86.73 ±3.03^{z} (538)	49.16 ±4.51^{d,y} (317)	40.55 ±4.29^{d,y} (267)	29.93 ±4.11^{c,x} (209)	18.04 ±3.11^{b,x} (152)	5.78 ±1.31^{a,y} (50)	2.56 ±0.70^{a,y} (24)
B	821	33.62 ±3.66^{y} (341)	57.60 ±5.39^{y} (256)	57.19 ±5.40^{y} (223)	24.45 ±4.27^{c,x} (105)	16.06 ±3.21^{b,x} (77)	7.46 ±2.09^{a,w} (51)	4.84 ±1.68^{a,w} (36)	2.70 ±1.01^{a,x} (17)	1.13 ±0.43^{a,x} (9)
C	452	21.61 ±3.75^{x} (155)	30.47 ±5.23^{x} (123)	29.37 ±5.08^{x} (110)	9.98 ±2.95^{c,w} (47)	5.85 ±2.02^{bc,w} (30)	4.89 ±1.89^{abc,w} (23)	3.35 ±1.55^{ab,w} (14)	0.56 ±0.35^{ab,w} (4)	0.14 ±0.14^{a,wx} (1)
D	141	8.44 ±2.39^{w} (57)	13.27 ±3.83^{w} (50)	13.61 ±3.88^{w} (42)	6.02 ±2.31^{b,w} (20)	3.40 ±1.41^{ab,w} (12)	2.06 ±1.00^{ab,w} (7)	3.70 ±2.01^{ab,w} (5)	0.23 ±0.23^{a,w} (1)	0.00 ±0.00^{a,w} (0)
E	323	9.79 ±2.38^{w} (75)	17.89 ±4.38^{w} (56)	17.25 ±4.28^{wx} (51)	5.51 ±2.37^{b,w} (18)	2.92 ±1.65^{ab,w} (12)	2.92 ±1.65^{ab,w} (12)	2.08 ±1.47^{ab,w} (7)	0.14 ±0.14^{a,w} (1)	0.00 ±0.00^{a,w} (0)
Total	3154	24.65 ±1.62	40.72 ±2.44	40.83 ±2.44	18.52 ±1.74	13.76 ±1.43	9.45 ±1.20	6.40 ±0.97	1.88 ±0.36	0.77 ±0.17

^{abcd} Means with different superscripts in a row within a group were significantly different (P<0.05).

^{wxyz} Means with different superscripts in a column within a group were significantly different (P<0.05).

Maturation rates of bovine oocytes based on enucleation techniques for Grades A, B, C, D and E are shown in Tables 4.13, 4.14, 4.15, 4.16 and 4.17. In all cases (even though majority insignificance), laser enucleation technique apparently gave higher maturation rates in all the grades of oocytes studied, except Grade E. The values were 60.10±3.68% versus 36.87±4.71%, 36.53±5.22% versus 29.98±5.04%, 21.78±5.23% versus 21.41±5.42%, 10.12±3.69% versus 6.35±2.79% and 7.22±2.73% versus 13.00±4.12% for Grades A, B, C, D and E, respectively. Enucleation rates of bovine oocytes based on enucleation techniques for Grades A, B, C, D and E are shown in Tables 4.13, 4.14, 4.15, 4.16 and 4.17. There were no significant differences ($P>0.05$) between squeezing and laser enucleation techniques for Grades B, C, D and E, except Grade A where the laser percent enucleation was higher than squeezing enucleation (91.93±2.50% versus 74.85±5.93%). Also, there were significant differences ($P<0.05$) in percent injection between squeezing (78.11±6.21%) and laser (93.63±1.65%) enucleation for Grade A bovine oocytes. Percentage of 2-cell stage embryos up to hatched blastocyst using laser enucleation technique showed the highest cleavage rate for all the oocytes grading compared to squeezing enucleation, except Grade E (Tables 4.13, 4.14, 4.15, 4.16 and 4.17). It was clearly evident shown that laser enucleation technique apparently gave the highest cleavage rate for all the grades compared to squeezing enucleation technique. The cleavage rates from 2-cell up to hatched blastocyst stages were significantly decreasing from Grades A to E. In overall, Grades A, B and C were better than Grades D and E on embryo developmental at various stages.

Table 4.18 shows cleavage rates of bovine embryos in somatic cell nuclear transfer and parthenogenesis treatments. There were significant differences ($P<0.05$) between parthenogenesis and somatic stem cell nuclear transfer, where the parthenogenesis showed higher cleavage rates with the values of 62.24±4.96%,

49.49±4.94%, 40.70±4.21%, 31.41±3.28%, 12.66±2.18% and 2.15±0.71% for 2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst stages compared to somatic cell nuclear transfer with the values of 44.35±4.14%, 34.04±3.76%, 24.50±3.40%, 14.01±2.53%, 3.89±0.89% and 1.65±0.46%, respectively. The cleavage rates consecutively decreased from 2-cell stage embryos to hatched blastocysts for both parthenogenesis and somatic cell nuclear transfer. Figure 4.7 shows the development of bovine embryos from 2-cell stage up to hatching blastocyst through somatic cell nuclear transfer technique.

Table 4.13: Percent maturation, enucleation, injection and cleavage rates of bovine oocytes (%, mean ±SEM) based on enucleation techniques for Grade A oocytes

Enucleation techniques	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Squeezing	708	36.87 ±4.71^{x} (283)	74.85 ±5.93^{x} (220)	78.11 ±6.21^{x} (190)	21.12 ±5.49^{c,x} (71)	14.61 ±4.57^{bc,x} (52)	7.83 ±3.87^{ab,x} (31)	2.15 ±1.55^{a,x} (15)	0.20 ±0.20^{a,x} (2)	0.10 ±0.10^{a,x} (1)
Laser	709	60.10 ±3.68^{y} (407)	91.93 ±2.50^{y} (369)	93.63 ±1.65^{y} (348)	71.59 ±4.31^{d,y} (246)	61.30 ±4.72^{d,y} (215)	47.61 ±5.28^{c,y} (178)	30.76 ±4.57^{b,y} (137)	10.25 ±2.09^{a,y} (48)	4.53 ±1.17^{a,y} (23)
Total	1417	49.77 ±3.21	84.34 ±3.12	86.73 ±3.03	49.16 ±4.51	40.55 ±4.29	29.93 ±4.11	18.04 ±3.11	5.78 ±1.30	2.56 ±0.70

^{abcd} Means with different superscripts in a row within a group were significantly different ($P<0.05$).

^{xy} Means with different superscripts in a column within a group were significantly different ($P<0.05$).

Table 4.14: Percent maturation, enucleation, injection and cleavage rates of bovine oocytes (%, mean ±SEM) based on enucleation techniques for Grade B oocytes

Enucleation techniques	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Squeezing	339	29.8 ±5.04^{x} (132)	55.56 ±7.83^{x} (93)	59.34 ±8.07^{x} (77)	12.03 ±5.04^{b,x} (15)	5.39 ±2.91^{ab,x} (12)	2.92 ±2.12^{a,x} (7)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)
Laser	482	36.53 ±5.22^{x} (209)	59.23 ±7.49^{x} (163)	55.47 ±7.34^{x} (146)	34.39 ±6.17^{b,y} (90)	24.59 ±4.92^{b,y} (65)	11.09 ±3.26^{a,x} (44)	8.71 ±2.90^{a,y} (36)	4.85 ±1.76^{a,y} (17)	2.03 ±0.74^{a,y} (9)
Total	821	33.62 ±3.66	57.60 ±5.39	57.19 ±5.40	24.45 ±4.27	16.06 ±3.21	7.46 ±2.09	4.84 ±1.68	2.70 ±1.01	1.13 ±0.43

^{ab} Means with different superscripts in a row within a group were significantly different (P<0.05).

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.15: Percent maturation, enucleation, injection and cleavage rates of bovine oocytes (%, mean ±SEM) based on enucleation techniques for Grade C oocytes

Enucleation techniques	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Squeezing	150	21.41 ±5.42^{x} (44)	31.77 ±8.07^{x} (25)	28.39 ±7.36^{x} (20)	0.00 ±0.00^{x} (0)	0.00 ±0.00^{x} (0)	0.00 ±0.00^{x} (0)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)
Laser	302	21.78 ±5.23^{x} (111)	29.43 ±6.94^{x} (98)	30.16 ±7.07^{x} (90)	16.31 ±4.83^{c,y} (47)	10.53 ±3.48^{bc,y} (30)	8.80 ±3.28^{abc,y} (23)	6.03 ±2.74^{ab,x} (14)	1.00 ±0.62^{a,x} (4)	0.25 ±0.25^{a,x} (1)
Total	452	21.61 ±3.75	30.47 ±5.23	29.37 ±5.08	9.98 ±2.95	5.85 ±2.02	4.89 ±1.89	3.35 ±1.55	0.56 ±0.35	0.14 ±0.14

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

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.16: Percent maturation, enucleation, injection and cleavage rates of bovine oocytes (%, mean ±SEM) based on enucleation techniques for Grade D oocytes

Enucleation techniques	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Squeezing	43	6.35 ±2.79^{x} (10)	10.31 ±5.07^{x} (7)	10.94 ±5.38^{x} (5)	0.00 ±0.00^{x} (0)	0.00 ±0.00^{x} (0)	0.00 ±0.00^{x} (0)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)
Laser	98	10.12 ±3.69^{x} (47)	15.64 ±5.61^{x} (43)	15.75 ±5.54^{x} (37)	10.83 ±4.03^{b,y} (20)	6.13 ±2.47^{ab,y} (12)	3.71 ±1.76^{a,x} (7)	3.75 ±2.63^{a,x} (5)	0.42 ±0.42^{a,x} (1)	0.00 ±0.00^{a,x} (0)
Total	141	8.44 ±2.39	13.27 ±3.83	13.61 ±3.88	6.02 ±2.31	3.40 ±1.41	2.06 ±1.00	2.08 ±1.47	0.23 ±0.23	0.00 ±0.00

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

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.17: Percent maturation, enucleation, injection and cleavage rates of bovine oocytes (%, mean ±SEM) based on enucleation techniques for Grade E oocytes

Enucleation techniques	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Squeezing	180	13.00 ±4.12^{x} (43)	22.92 ±7.25^{x} (30)	22.40 ±7.16^{x} (28)	5.63 ±3.62$^{a.x}$ (8)	5.00 ±3.36^{a,x} (7)	5.00 ±3.36^{a,x} (7)	3.75 ±3.17^{a,x} (4)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)
Laser	143	7.22 ±2.73^{x} (32)	13.88 ±5.33^{x} (26)	13.13 ±5.14^{x} (23)	5.42 ±3.18^{b,x} (10)	1.25 ±1.25^{ab,x} (5)	1.25 ±1.25^{ab,x} (5)	0.75 ±0.75^{a,x} (3)	0.25 ±0.25^{a,x} (1)	0.00 ±0.00^{a,x} (0)
Total	323	9.79 ±2.38	17.89 ±4.38	17.25 ±4.28	5.51 ±2.37	2.92 ±1.65	2.92 ±1.65	2.08 ±1.47	0.14 ±0.14	0.00 ±0.00

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

^{x} Means with same superscripts in a column within a group were not significantly different (P>0.05).

Table 4.18: Percent cleavage of bovine embryos (%, mean ±SEM) on 2 different treatments

Treatments	No. of ovaries	No. of oocytes	Maturation rate (n)	Percent cleavage (n)					
				2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
PA	75	759	61.50 ± 3.65^{y} (471)	$62.24 \pm 4.96^{e,x}$ (274)	$49.49 \pm 4.94^{d,x}$ (220)	$40.70 \pm 4.21^{cd,x}$ (178)	$31.41 \pm 3.28^{c,y}$ (133)	$12.66 \pm 2.18^{b,y}$ (59)	$2.15 \pm 0.71^{a,x}$ (12)
SCNT	220	3175	42.01 ± 2.65^{x} (1315)	$44.35 \pm 4.14^{e,x}$ (506/964)*	$34.04 \pm 3.76^{d,x}$ (398/964)*	$24.50 \pm 3.40^{c,x}$ (302/964)*	$14.01 \pm 2.53^{b,x}$ (211/964)*	$3.89 \pm 0.89^{a,x}$ (70/964)*	$1.65 \pm 0.46^{a,x}$ (33/964)*
Total	295	3934	45.37 ±2.41	47.43 ±3.60	36.71 ±3.27	27.29 ±2.97	17.01 ±2.28	5.40 ±0.89	1.74 ±0.40

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

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

*n=number of embryos (2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst)/number of injected oocytes.

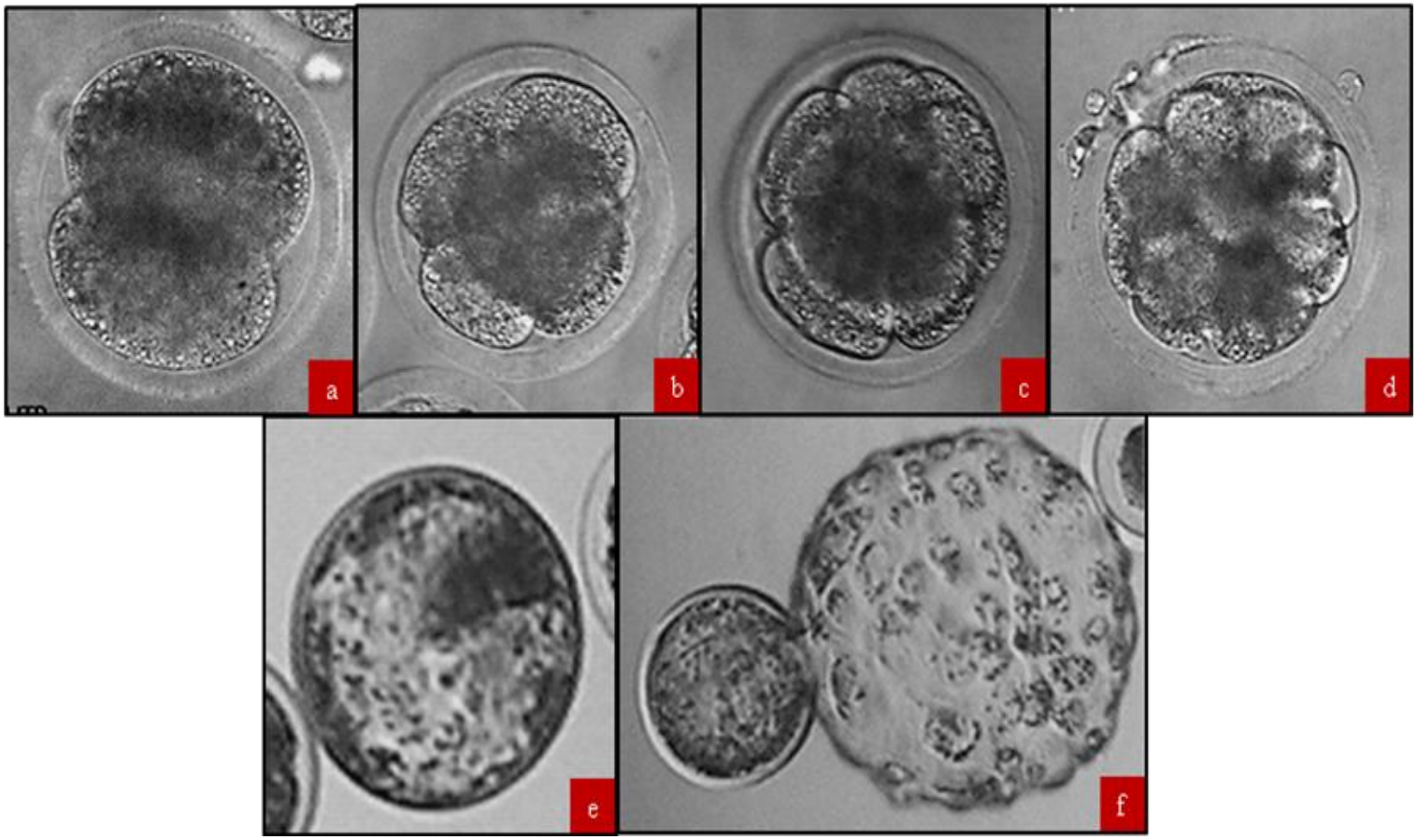

Figure 4.7: (a-f) Development of bovine embryos from 2-, 4-, 8-cell, morula, blastocyst and hatching blastocyst through somatic cell nuclear transfer technique.

4.2.2 Production of Caprine Blastocysts Through Somatic Cell Nuclear Transfer and Parthenogenesis (control)

Table 4.19 shows the cleavage rates of caprine embryos through somatic cell nuclear transfer by 3 different sources of oocytes, namely laparoscopic ovum pick-up (LOPU), ovariectomy and abattoir. There were significant differences ($P<0.05$) in maturation and enucleation rates for these 3 different sources with the values of 61.99±3.12% and 66.16±5.21% versus 51.23±3.37% as well as 97.85±1.23% and 98.32±1.16% versus 90.27±2.72% for laparoscopic ovum pick-up, ovariectomy and abattoir sources, respectively. Even though insignificance, the embryo development at 2-, 4-, 8-cell and morula was higher in laparoscopic ovum pick-up, followed by ovariectomy and abattoir with values of 74.12±5.30% versus 65.86±7.87% versus 66.61±6.64%; 63.40±5.48% versus 56.83±6.51% versus 51.46±7.33%; 53.30±5.84% versus 50.18±9.05% versus 43.48±8.14%; 48.56±5.38% versus 44.68±8.94% versus 26.28±8.19%, respectively. However, ovariectomy showed the highest blastocysts and hatched blastocysts rates (24.52±8.53% and 18.42±6.79%), followed by laparoscopic ovum pick-up (13.33±3.68% and 8.12±2.29%) and abattoir (10.06±5.83% and 5.01±3.19%), respectively.

Table 4.20 shows cleavage rates of caprine embryos based on caprine oocyte grading from Grades A to E. A total of 1296 caprine oocytes obtained from Grade A (366), Grade B (362), Grade C (306), Grade D (111) and Grade E (151). There were no significant differences ($P>0.05$) in percent cleavage at Grades A, B and C with values of 46.40±5.26%, 42.89±5.11%, 34.63±4.91%; 31.06±4.76%, 13.91±3.56% and 6.88±1.90%; 48.34±5.08%, 40.43±4.85%, 33.57±4.70%; 29.79±4.48% and 9.86±2.95%, 5.99±1.92%; 40.15±5.17%, 32.78±5.01%, 30.25±5.00%, 26.62±4.76%, 8.21±2.80% and 5.38±2.01% for 2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst, respectively. However, Grades A, B and C showed better cleavage rates than Grades D and E for all stages of embryo development. The development of caprine-

cloned embryos stained hatching blastocyst with Hoechst 3342 are shown in Figures 4.8 and 4.9. Fiigure 4.10 shows the grading of caprine oocytes from Grade A to Grade E.

Table 4.19: Cleavage rates of caprine embryos (%, mean ±SEM) from *in vitro* culture through somatic cell nuclear transfer by 3 different sources of oocytes

Source of oocytes	No. of ovaries	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
						2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
LOPU	91	487	61.99 ±3.12^{xy} (297)	97.85 ±1.23^{y} (292)	98.26 ±0.92^{x} (287)	74.12 ±5.30^{d,x} (227)	63.40 ±5.48^{cd,x} (207)	53.30 ±5.84^{bc,x} (186)	48.56 ±5.38^{b,x} (169)	13.33 ±3.68^{a,x} (66)	8.12 ±2.29^{a,xy} (43)
Ovariectomy	19	168	66.16 ±5.21^{y} (115)	98.32 ±1.16^{y} (111)	97.78 ±2.22^{x} (110)	65.86 ±7.87^{c,x} (75)	56.83 ±6.51^{c,x} (65)	50.18 ±9.05^{c,x} (54)	44.68 ±8.94^{bc,x} (45)	24.52 ±8.53^{ab,x} (29)	18.42 ±6.79^{a,y} (21)
Abattoir	99	641	51.23 ±3.37^{x} (355)	90.27 ±2.72^{x} (312)	94.61 ±2.25^{x} (289)	66.61 ±6.64^{e,x} (216)	51.46 ±7.33^{de,x} (190)	43.48 ±8.14^{cd,x} (166)	26.28 ±8.19^{bc,x} (136)	10.06 ±5.83^{ab,x} (75)	5.01 ±3.19^{a,x} (35)
Total	209	1296	59.76 ±2.28	95.99 ±1.10	97.28 ±0.86	71.23 ±3.83	59.59 ±3.99	50.43 ±4.32	42.45 ±4.20	13.85 ±2.93	8.57 ±1.86

^{abcde} Means with different superscripts in a row within a group were significantly different (P<0.05).

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

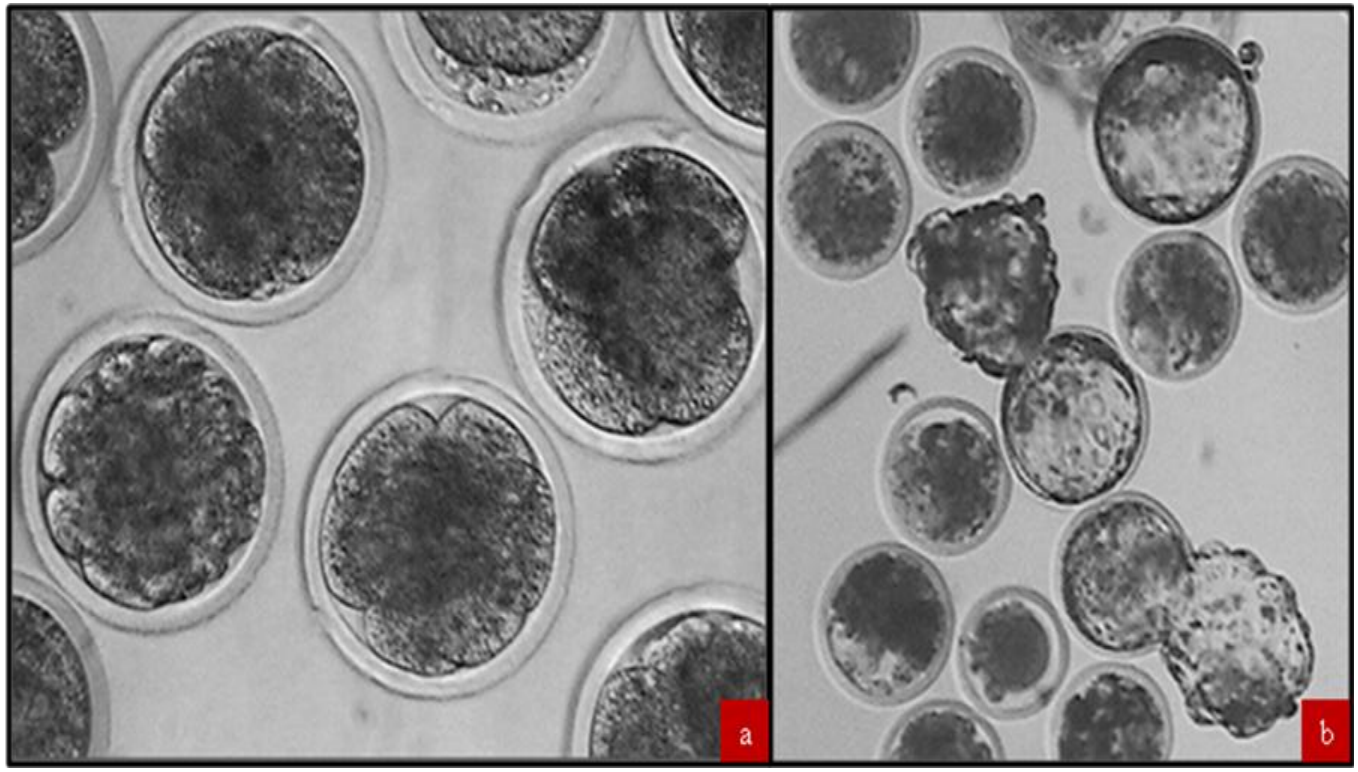

Figure 4.8: (a-b) Development of caprine-cloned embryos at day 3 (4- to 8-cells) and day 5 (blastocysts).

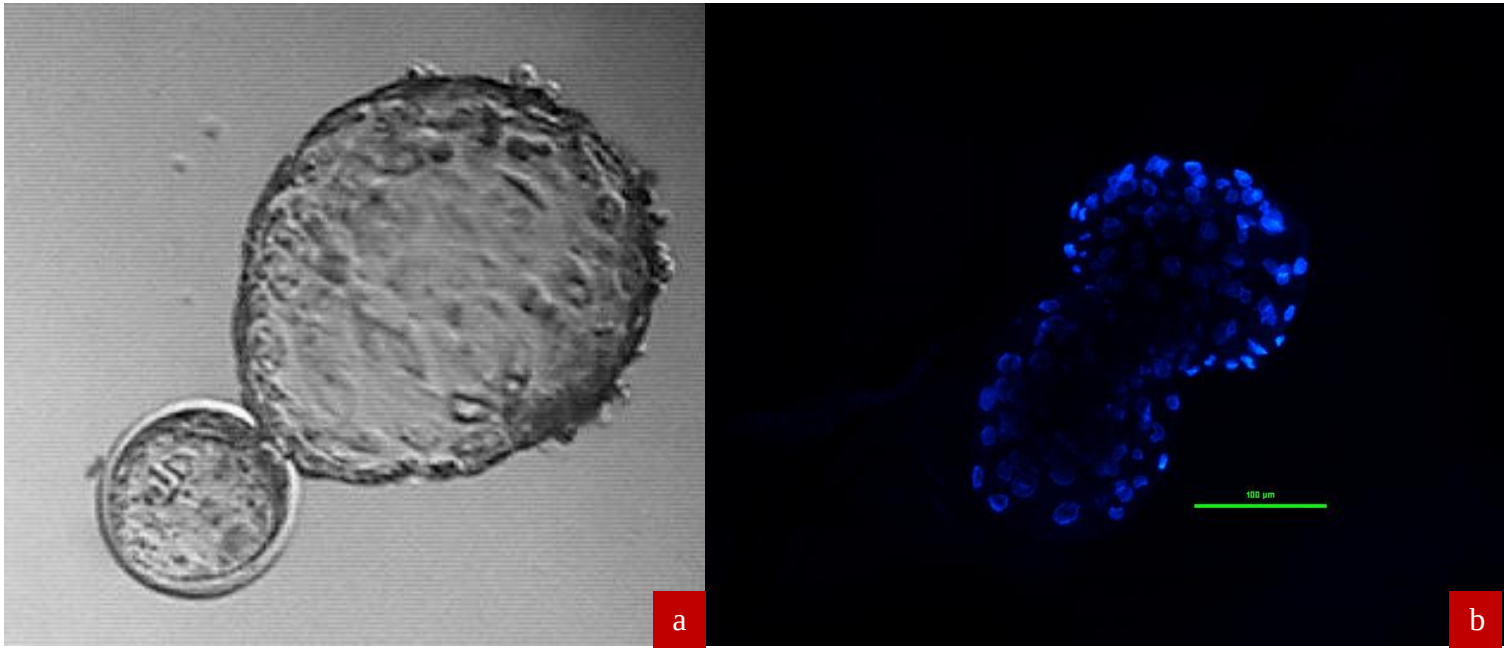

Figure 4.9: a) Hatching cloned caprine-blastocyst observed under steromicroscope (magnificantion 20x). b) hatching cloned-caprine blastocyst staining with Hoechst 3342 and observed under fluorescent microscope (magnificantion 20x).

Table 4.20: Percent cleavage of caprine embryos (%, mean ±SEM) based on oocyte grading

Oocyte Grades	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
A	366	51.73 ±4.83^{y} (253)	64.09 ±5.47^{y} (246)	63.53 ±5.45^{y} (240)	46.40 ±5.26^{d,y} (185)	42.89 ±5.11^{bc,y} (171)	34.63 ±4.91^{bc,y} (149)	31.06 ±4.76^{b,y} (133)	13.91 ±3.56^{a,y} (67)	6.88 ±1.90^{a,y} (39)
B	362	46.94 ±4.48^{y} (202)	63.22 ±5.43^{y} (190)	63.31 ±5.54^{y} (185)	48.34 ±5.08^{c,y} (144)	40.43 ±4.85^{bc,y} (129)	33.57 ±4.70^{b,y} (114)	29.79 ±4.48^{b,y} (99)	9.86 ±2.95^{a,y} (48)	5.99 ±1.92^{a,y} (28)
C	306	44.71 ±5.07^{y} (180)	54.73 ±5.67^{y} (166)	54.74 ±5.70^{y} (154)	40.15 ±5.17^{c,y} (116)	32.78 ±5.01^{bc,y} (101)	30.25 ±5.00^{bc,y} (87)	26.62 ±4.76^{b,y} (74)	8.21 ±2.80^{a,xy} (39)	5.38 ±2.01^{a,y} (21)
D	111	12.40 ±3.30^{x} (67)	15.14 ±4.06^{x} (60)	16.00 ±4.26^{x} (60)	12.33 ±3.46^{c,x} (47)	7.67 ±2.71^{bc,x} (38)	7.67 ±2.71^{bc,x} (38)	3.96 ±2.06^{ab,x} (28)	2.33 ±1.64^{ab,x} (14)	1.83 ±1.29^{a,xy} (11)
E	151	14.21 ±3.34^{x} (65)	19.67 ±4.47^{x} (53)	19.13 ±4.39^{x} (47)	9.44 ±3.00^{b,x} (26)	8.33 ±2.83^{b,x} (23)	6.67 ±2.63^{ab,x} (18)	5.33 ±2.15^{ab,x} (16)	1.33 ±0.94^{a,x} (2)	0.00 ±0.00^{a,x} (0)
Total	1296	34.00 ±2.09	43.37 ±2.51	43.34 ±2.52	31.33 ±2.18	26.42 ±2.05	22.56 ±1.95	19.35 ±1.82	7.13 ±1.16	4.02 ±0.73

^{abcd} Means with different superscripts in a row within a group were significantly different (P<0.05).

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

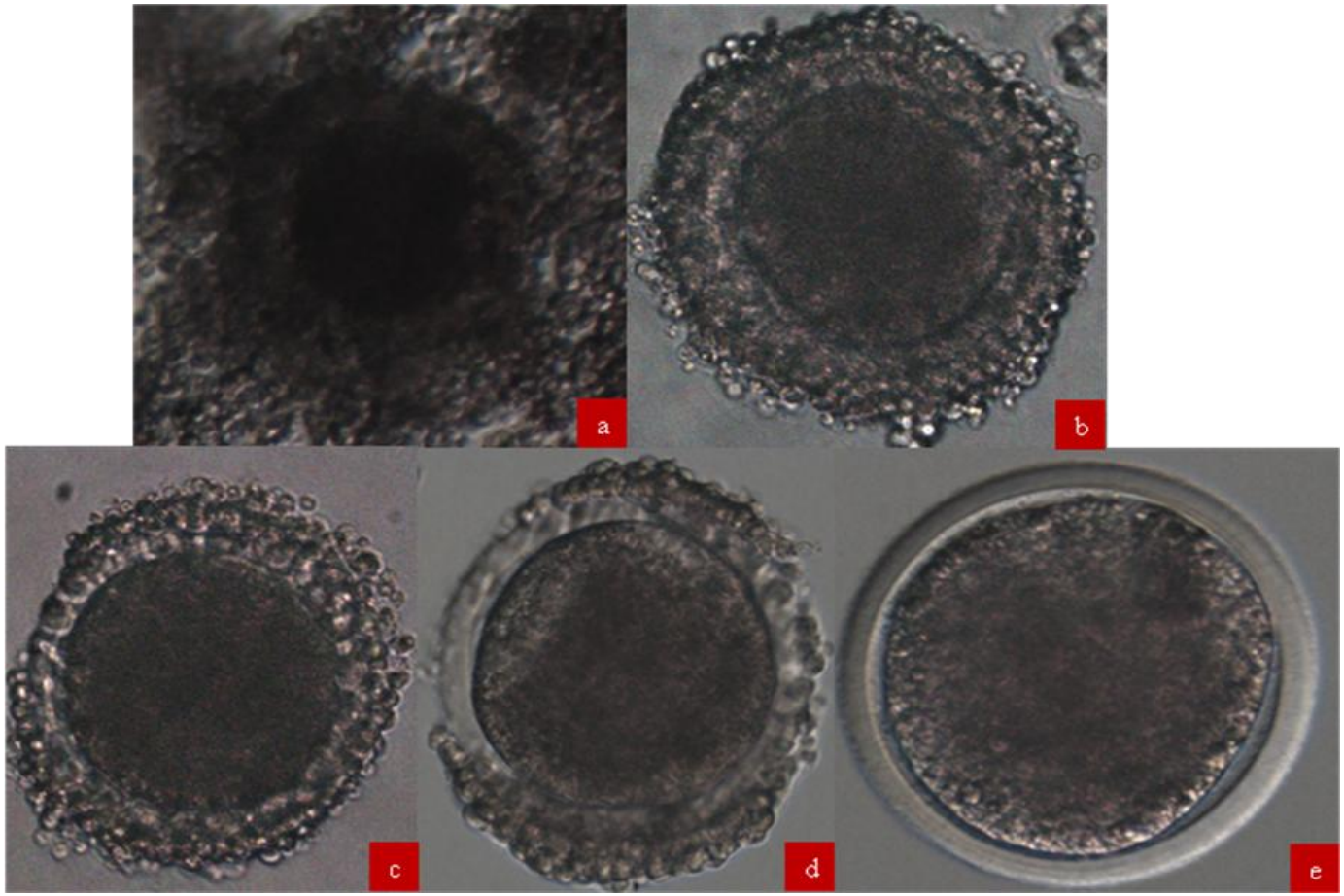

Figure 4.10: a) Grade A, b) Grade B, c) Grade C, d) Grade D and d) Grade E of caprine oocytes.

Table 4.21: Cleavage rates of caprine embryos (%, mean ±SEM) from *in vitro* culture through different enucleation techniques

Enucleation techniques	No. of ovaries	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
						2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Squeezing	89	516	51.74 ±3.19^{x} (267)	95.23 ±1.73^{x} (236)	94.71 ±1.76^{x} (215)	58.15 ±6.76^{d,x} (125)	42.04 ±6.53^{c,x} (95)	25.73 ±6.05^{b,x} (57)	16.36 ±5.22^{b,x} (35)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)
Laser	120	780	66.42 ±2.87^{y} (500)	96.61 ±1.42^{x} (479)	99.41 ±0.38^{y} (471)	82.07 ±3.45^{c,y} (393)	74.14 ±3.62^{bc,y} (367)	70.93 ±3.87^{b,y} (349)	64.08 ±3.91^{b,y} (315)	25.33 ±4.66^{a,y} (170)	15.68 ±2.99^{a,y} (99)
Total	209	1296	59.76 ±2.28	95.99 ±1.10	97.28 ±0.86	71.23 ±3.83	59.59 ±3.99	50.43 ±4.32	42.45 ±4.20	13.85 ±2.93	8.57 ±1.86

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

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.21 shows the cleavage rates of caprine embryos through somatic cell nuclear transfer by 2 different enucleation techniques, namely squeezing and laser enucleation. There were no significant differences (P>0.05) in enucleation rates between squeezing and laser enucleation techniques. However, embryos development at various stages were significantly higher in laser enucleation technique compared to squeezing enucleation technique with values of 82.07±3.45% versus 58.15±6.76%, 74.14±3.62% versus 42.04±6.53%, 70.93±3.87% versus 25.73±6.05%, 64.08±3.91% versus 16.36±5.22%; 25.33±4.66% versus 0.00±0.00% and 15.68±2.99% versus 0.00±0.00% for 2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst, respectively. For both enucleation techniques, the cleavage rates were decreasing as the stages of development progressing. No blastocysts were obtained for squeezing enucleation technique.

Percent maturation, enucleation and injection of caprine oocytes based 2 different enucleation techniques, namely squeezing and laser enucleations for Grades A, B, C, D and E are shown in Tables 4.22, 4.23, 4.24, 4.25 and 4.26. In overall, laser enucleation gave the highest maturation, enucleation and injection rates for Grade B (62.09±5.49%, 81.45±5.92%, 82.68±5.94%), Grade D (18.24±5.35%, 21.20±6.34%, 21.95±6.54%) and Grade E (16.94±4.79%, 22.68±6.37%, 23.78±6.65%), except for Grades A and C. There were significant differences (P<0.05) between squeezing and laser enucleation techniques for percent maturation, enucleation and injection rates for Grades A and B. Also, in all cases, laser enucleation technique showed significantly higher percent 2-cell up to hatched blastocyst for all oocytes grades, except Grade A compared to squeezing enucleation technique. Conversely, the squeezing enucleation at 2-cell stages embryos showed higher percent cleavage compared to laser enucleation technique with the values of 47.84±7.79% versus 445.21±7.21% for Grade A. In

overall, embryo developmental at various stages for Grade A, B and C were better than Grades D and E.

Table 4.27 shows percent cleaved caprine embryos based on different pre-intracytoplasmic injection (pre-ICI) durations. It was vividly shown that for each embryo developmental stages from 2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst the cleavage rates were significantly higher ($P<0.05$) for 60 minutes pre-intracytoplasmic injection duration followed by simultaneous injection and 30 minutes pre-intracytoplasmic injection (pre-ICI) durations with the values of 83.33±3.10% versus 61.17±10.65% versus 45.39±9.03%, 74.42±4.20% versus 42.42±8.77% versus 33.82±7.47%, 62.42±5.47% versus 37.12±8.97% versus 28.93±7.11%, 53.10±5.61% versus 29.39±7.74% versus 24.83±7.37%, 20.49±4.16% versus 7.68±5.93% versus 0.45±0.45% and 12.95±2.71% versus 4.29±3.41% versus 0.00±0.00%, respectively.

Average number of caprine blastocyst obtained from *in vivo* uterine flushing was shown in Table 4.28. Two different hormones were used in this experiment, namely PMSG and hCG. A total of 27 blastocysts were obtained through *in vivo* uterine flushing from 23 does. Obviously, the average number of blastocysts obtained in FSH treatment gave higher than that of PMSG treatment with the values of 41.54 and 0.00, respectively. Figure 4.11 shows the *in vivo*-derived caprine blastocyst from uterine flushing.

Table 4.22: Percent maturation, enucleation, injection and cleavage rates of caprine oocytes (%, mean ±SEM) based on enucleation techniques for Grade A oocytes

Enucleation techniques	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Squeezing	150	65.73 ±6.31^{y} (101)	79.61 ±6.57^{y} (94)	79.12 ±6.60^{y} (90)	47.84 ±7.79^{c,x} (56)	42.13 ±7.51^{c,x} (48)	24.63 ±6.38^{b,x} (29)	19.04 ±5.67^{b,x} (20)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)
Laser	216	40.95 ±6.73^{x} (152)	51.22 ±7.90^{x} (152)	50.61 ±7.83^{x} (150)	45.21 ±7.21^{b,x} (129)	43.52 ±7.06^{b,x} (123)	42.93 ±7.07^{b,x} (120)	41.03 ±7.00^{b,y} (113)	25.44 ±5.95^{ab,y} (67)	12.58 ±3.23^{a,y} (39)
Total	366	51.73 ±4.83	64.09 ±5.47	63.53 ±5.45	46.40 ±5.26	42.89 ±5.11	34.63 ±4.91	31.06 ±4.76	13.91 ±3.56	6.88 ±1.90

^{abc} Means with different superscripts in a row within a group were significantly different (P<0.05).

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.23: Percent maturation, enucleation, injection and cleavage rates of caprine oocytes (%, mean ±SEM) based on enucleation techniques for Grade B oocytes

Enucleation techniques	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Squeezing	127	28.66 ±6.05^{x} (52)	41.25 ±8.25^{x} (45)	39.95 ±8.35^{x} (41)	25.74 ±6.81^{c,x} (26)	19.80 ±6.22^{bc,x} (21)	8.43 ±4.24^{ab,x} (9)	6.37 ±4.10^{a,x} (5)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)
Laser	235	62.09 ±5.49^{y} (150)	81.45 ±5.92^{y} (145)	82.68 ±5.94^{y} (144)	67.09 ±6.01^{c,y} (118)	57.53 ±6.08^{bc,y} (108)	54.42 ±6.20^{bc,y} (105)	49.21 ±5.96^{b,y} (94)	18.04 ±5.07^{a,y} (48)	10.96 ±3.33^{a,y} (28)
Total	362	46.94 ±4.48	63.22 ±5.43	63.31 ±5.54	48.34 ±5.08	40.43 ±4.85	33.57 ±4.70	29.79 ±4.48	9.86 ±2.95	5.99 ±1.92

^{abc} Means with different superscripts in a row within a group were significantly different (P<0.05).

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.24: Percent maturation, enucleation, injection and cleavage rates of caprine oocytes (%, mean ±SEM) based on enucleation techniques for Grade C oocytes

Enucleation techniques	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Squeezing	154	44.07 ±7.41^{x} (82)	56.81 ±8.35^{x} (73)	57.27 ±8.48^{x} (64)	29.22 ±6.65^{c,x} (37)	14.24 ±5.04^{b,x} (23)	11.99 ±4.82^{ab,x} (16)	7.40 ±3.98^{ab,x} (10)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)
Laser	152	45.25 ±7.02^{x} (98)	53.01 ±7.80^{x} (93)	52.64 ±7.78^{x} (90)	49.22 ±7.46^{b,x} (79)	48.16 ±7.39^{b,y} (78)	45.39 ±7.48^{b,y} (71)	42.57 ±7.20^{b,y} (64)	15.01 ±4.90^{a,y} (39)	0.25 ±0.25^{a,y} (21)
Total	306	44.71 ±5.07	54.73 ±5.67	54.74 ±5.70	40.15 ±5.17	32.78 ±5.01	30.25 ±5.00	26.62 ±4.76	8.21 ±2.80	5.38 ±2.01

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

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.25: Percent maturation, enucleation, injection and cleavage rates of caprine oocytes (%, mean ±SEM) based on enucleation techniques for Grade D oocytes

Enucleation techniques	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Squeezing	29	5.36 ±3.05^{x} (12)	7.84 ±4.46^{x} (10)	8.82 ±4.94^{x} (10)	4.41 ±2.47^{a,x} (5)	2.94 ±2.05^{a,x} (3)	2.94 ±2.05^{a,x} (3)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)
Laser	82	18.24 ±5.35^{x} (55)	21.20 ±6.34^{x} (50)	21.95 ±6.54^{x} (50)	18.90 ±5.83^{b,y} (42)	11.59 ±4.61^{ab,x} (35)	11.59 ±4.61^{ab,x} (35)	7.24 ±3.70^{ab,x} (28)	4.27 ±2.98^{a,x} (14)	1.52 ±1.52^{a,x} (11)
Total	111	12.40 ±3.30	15.14 ±4.06	16.00 ±4.26	12.33 ±3.46	7.67 ±2.71	7.67 ±2.71	3.96 ±2.06	2.33 ±1.64	0.83 ±0.83

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

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.26: Percent maturation, enucleation, injection and cleavage rates of caprine oocytes (%, mean ±SEM) based on enucleation techniques for Grade E oocytes

Enucleation techniques	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Squeezing	56	10.91 ±4.59^{x} (20)	16.04 ±6.21^{x} (14)	13.53 ±5.37^{x} (10)	1.47 ±1.47$^{a.x}$ (1)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)
Laser	95	16.94 ±4.79^{x} (45)	22.68 ±6.37^{x} (39)	23.78 ±6.65^{x} (37)	16.06 ±5.16^{c,y} (25)	15.24 ±4.95^{c,y} (23)	12.19 ±4.66^{bc,y} (18)	9.76 ±3.82^{abc,y} (16)	2.44 ±1.70^{ab,x} (2)	0.00 ±0.00^{a,x} (0)
Total	151	14.21 ±3.34	19.67 ±4.47	19.13 ±4.39	9.44 ±3.00	8.33 ±2.83	6.67 ±2.63	5.33 ±2.15	1.33 ±0.94	0.00 ±0.00

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

^{xy} Means with different superscripts in a column within a group were not significantly different (P>0.05).

Table 4.27: Percent cleaved caprine embryos (%, mean ±SEM) based on different pre-intracytoplasmic injection (pre-ICI) durations

Pre-ICI durations	No. of ovaries	No. of oocytes	Percent cleavage (n)					
			2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Simultaneous	68	322	$61.17 \pm 10.65^{d,x}$ (96/125)*	$42.42 \pm 8.77^{cd,x}$ (76/125)	$37.12 \pm 8.97^{c,x}$ (69/125)	$29.39 \pm 7.74^{bc,x}$ (59/125)	$7.68 \pm 5.93^{ab,xy}$ (30/125)	$4.29 \pm 3.41^{a,xy}$ (17/125)
30 min	31	194	$45.39 \pm 9.03^{c,x}$ (49/89)	$33.82 \pm 7.47^{bc,x}$ (36/89)	$28.93 \pm 7.11^{bc,x}$ (26/89)	$24.83 \pm 7.37^{b,x}$ (18/89)	$0.45 \pm 0.45^{a,x}$ (1/89)	$0.00 \pm 0.00^{a,x}$ (0/89)
60 min	110	780	$83.33 \pm 3.10^{d,y}$ (383/472)	$74.42 \pm 4.20^{cd,y}$ (350/472)	$62.42 \pm 5.47^{bc,y}$ (309/472)	$53.10 \pm 5.61^{b,y}$ (273/472)	$20.49 \pm 4.16^{a,y}$ (139/472)	$12.95 \pm 2.71^{a,y}$ (82/472)
Total	209	1296	71.23 ± 3.83	59.59 ± 3.99	50.43 ± 4.32	42.45 ± 4.20	13.85 ± 2.93	8.57 ± 1.86

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

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

*n=number of embryos (2-, 4-, 8-cell, morula blastocyst and hatched blastocyst)/number of injected oocytes.

Table 4.28: Average number of caprine blastocysts (mean ±SEM) obtained from *in vivo* uterine flushing

Hormone treatments	No. of caprine	No. of ovaries	No. of CL	No. of blastocyst	Average no. of blastocyst
PMSG	12	24	12	0	0.00
FSH	11	22	65	27	41.54
Total	23	46	77	27	35.06

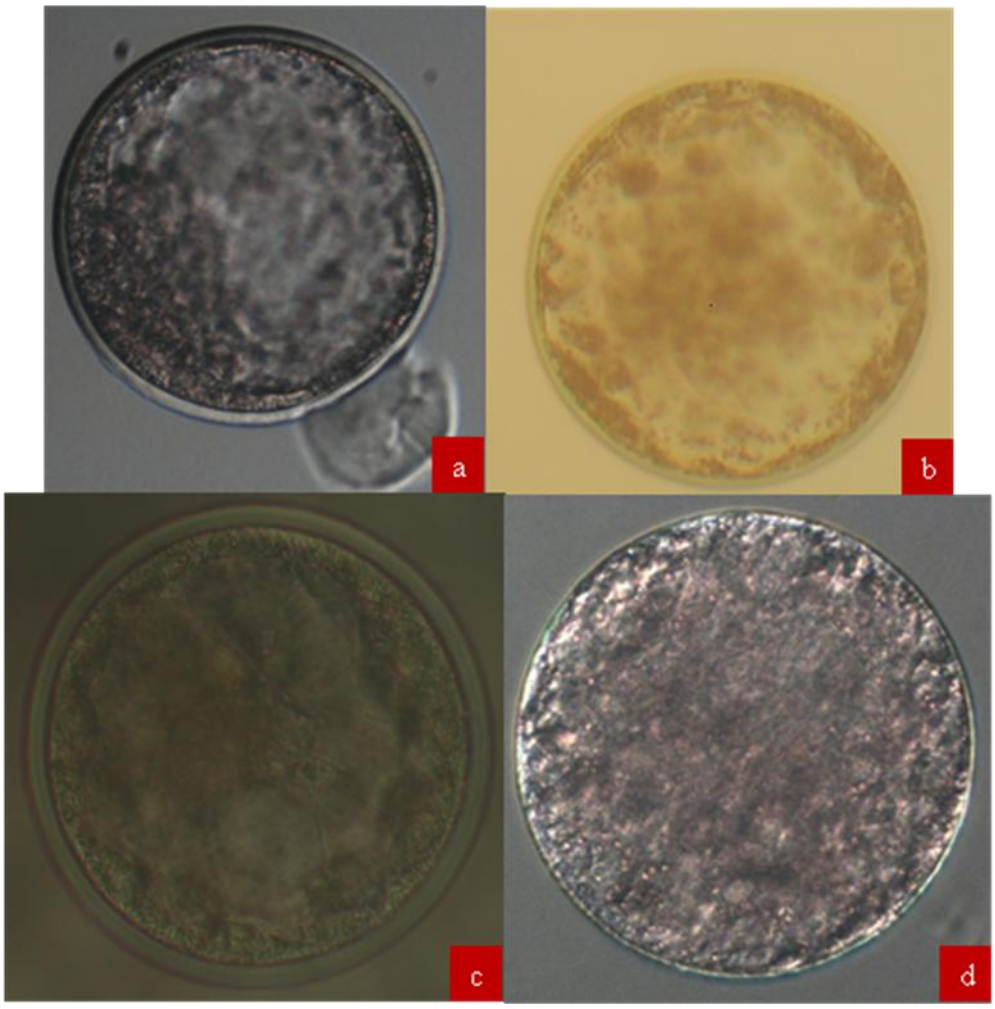

Figure 4.11: (a-d) *In vivo*-derived caprine blastocyst from uterine flushing.

In Table 4.29, parthenogenesis and somatic cell nuclear transfer have been compared for their cleavage rates. There were no significant differences (P>0.05) between the parthenogensis and somatic cell nuclear transfer treatments in percent cleavage. Although insignificance, the percent cleavage of somatic cell nuclear transfer were higher than with parthenogenesis respective values of 71.23 ±3.83% versus 65.95 ±5.08%, 59.59 ±3.99% versus

56.66±5.36%; 50.43±4.32% versus 49.37±6.18%; 42.45±4.20% versus 30.27±5.96% and 8.57±1.86% versus 4.67±2.51 for 2-, 4- and 8-cell, morula and hatched blastocyst, except blastocyst (13.85±2.93% versus 17.18±5.38%), respectively.

Table 4.29: Percent cleavage of caprine embryos (%, mean ±SEM) on 2 different treatments

Treatments	No. of ovaries	No. of oocytes	Maturation rate (n)	Percent cleavage (n)					
				2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
PA	51	438	63.73 ± 4.25^{x} (255)	$65.95 \pm 5.08^{d,x}$ (152)	$56.66 \pm 5.36^{cd,x}$ (125)	$49.37 \pm 6.18^{c,x}$ (96)	$30.27 \pm 5.96^{b,x}$ (54)	$17.18 \pm 5.38^{ab,x}$ (26)	$4.67 \pm 2.51^{a,x}$ (4)
SCNT	209	1296	59.76 ± 2.28^{x} (767)	$71.23 \pm 3.83^{d,x}$ (528/686)*	$59.59 \pm 3.99^{c,x}$ (462/686)*	$50.43 \pm 4.32^{bc,x}$ (404/686)*	$42.45 \pm 4.20^{b,x}$ (350/686)*	$13.85 \pm 2.93^{a,x}$ (170/686)*	$8.57 \pm 1.86^{a,x}$ (99/686)*
Total	260	1734	60.42 ±2.03	70.35 ±3.30	59.10 ±3.44	50.26 ±3.73	40.42 ±3.66	14.40 ±2.59	7.92 ±1.61

^{abcd} Means with different superscripts in a row within a group were significantly different ($P<0.05$).

^{x} Means with same superscripts in a column were not significantly different ($P>0.05$).

*n=number of embryos (2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst)/number of oocytes injected.

4.2.3 Comparison Between Caprine and Bovine Species on Production of Blastocysts Through Somatic Cell Nuclear Transfer and Parthenogenesis

Table 4.30 shows the percent cleavage between caprine and bovine embryos through somatic cell nuclear transfer. There were significant differences ($P<0.05$) in maturation, enucleation and injection rates between caprine and bovine species with values of 59.76±2.28% versus 42.01±2.65%, 95.99±1.10% versus 86.01±2.47% and 97.28±0.86 versus 90.83±1.69%, respectively. There were obviously significant differences ($P<0.0.5$) between caprine and bovine in percent embryos development for 2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst with values of 71.23±3.83% versus 44.35±4.14%, 59.59±3.99% versus 34.04±3.76%, 50.43±4.32% versus 24.50±3.40%, 42.45±4.20% versus 14.01±2.53%, 13.85±2.93% versus 3.89±0.89% and 8.57±1.86% versus 1.65±0.46%, respectively.

Percent maturation, enucleation, injection and cleavage rates between caprine and bovine oocytes based on oocyte grading are shown in Tables 4.31, 4.32, 4.33, 4.34 and 4.35. There were significant differences ($P<0.05$) in caprine and bovine maturation rates between Grades B and C with values of 46.94±4.48% versus 33.62±3.66% and 44.71±5.07% versus 21.61±3.75%, respectively. However, percent maturation in caprine was higher than bovine species (51.73±4.83% versus 49.77±3.21%, 46.94±4.48% versus 33.62±3.66%, 44.71±5.07% versus 21.61±3.75%, 12.40 ±3.30% versus 8.44±2.39% and 14.21±3.34% versus 9.79±2.38%) for Grades A, B, C, D and E, respectively. Also, there were significant differences ($P<0.05$) in percent enucleation and injection between caprine and bovine at Grades A and C with represented values of 64.09±5.47% versus 84.34±3.12% and 54.73±5.67% versus 30.47±5.23% for percent enucleation as well as 63.53±5.45% versus 86.73±3.03% and 54.74±5.70% versus 29.37±5.08% for percent injection, respectively. Obviously, caprine species showed the highest percent embryo development for all cell stages in all the oocyte grading from

Grades A to E. Most of the values showed decreasing trend from Grades A to E as well as from 2-cell up to hatched blastocyst, consecutively.

Table 4.30: Percent cleavage between caprine and bovine embryos (%, mean ±SEM) through somatic cell nuclear transfer

Species	No. of ovaries	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
						2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Caprine	260	1296	59.76 ±2.28^{y} (767)	95.99 ±1.10^{y} (715)	97.28 ±0.86^{y} (686)	71.23 ±3.83^{d,y} (528)	59.59 ±3.99^{c,y} (462)	50.43 ±4.32^{bc,y} (404)	42.45 ±4.20^{b,y} (350)	13.85 ±2.93^{a,y} (170)	8.57 ±1.86^{a,y} (99)
Bovine	220	3175	42.01 ±2.65^{x} (1315)	86.01 ±2.47^{x} (1074)	90.38 ±1.69^{x} (964)	44.35 ±4.14^{e,x} (506)	34.04 ±3.76^{d,x} (398)	24.50 ±3.40^{c,x} (302)	14.01 ±2.53^{b,x} (211)	3.89 ±0.89^{a,x} (70)	1.65 ±0.46^{a,x} (33)
Total	480	4471	51.07 ±1.89	91.10 ±1.39	93.90 ±0.98	58.06 ±3.02	47.08 ±2.93	37.73 ±2.95	28.52 ±2.73	8.97 ±1.60	5.18 ±1.01

^{abcde} Means with different superscripts in a row within a group were significantly different (P<0.05).

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.31: Percent maturation, enucleation, injection and cleavage rates between caprine and bovine oocytes (%, mean ±SEM) based on Grade A oocytes

Speices	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Caprine	366	51.73 ±4.83^{x} (253)	64.09 ±5.47^{x} (246)	63.53 ±5.45^{x} (240)	46.40 ±5.26^{c,x} (185)	42.89 ±5.11^{bc,x} (171)	34.63 ±4.91^{bc,x} (149)	31.06 ±4.76^{b,y} (133)	13.91 ±3.56^{a,y} (67)	6.88 ±1.90^{a,y} (39)
Bovine	1417	49.77 ±3.21^{x} (690)	84.34 ±3.12^{y} (589)	86.73 ±3.03^{y} (538)	49.16 ±4.51^{d,x} (317)	40.55 ±4.29^{d,x} (267)	29.93 ±4.11^{c,x} (209)	18.04 ±3.11^{b,x} (152)	5.78 ±1.30^{a,x} (50)	2.56 ±0.70^{a,x} (24)
Total	480	50.77 ±2.91	74.01 ±3.28	74.89 ±3.28	47.75 ±3.47	41.74 ±3.34	32.33 ±3.21	24.69 ±2.91	9.93 ±1.95	4.76 ±1.04

^{abcd} Means with different superscripts in a row within a group were significantly different (P<0.05).

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.32: Percent maturation, enucleation, injection and cleavage rates between caprine and bovine oocytes (%, mean ±SEM) based on Grade B oocytes

Species	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Caprine	362	46.94 ±4.48^{y} (202)	63.22 ±5.43^{x} (190)	63.31 ±5.54^{x} (185)	48.34 ±5.08^{c,y} (144)	40.43 ±4.85^{bc,y} (129)	33.57 ±4.70^{b,y} (114)	29.79 ±4.48^{b,y} (99)	9.86 ±2.95^{a,y} (48)	5.99 ±1.92^{a,y} (28)
Bovine	821	33.62 ±3.66^{x} (341)	57.60 ±5.39^{x} (256)	57.19 ±5.40^{y} (223)	24.45 ±4.27^{c,x} (105)	16.06 ±3.21^{b,x} (77)	7.46 ±2.09^{a,x} (51)	4.84 ±1.68^{a,x} (36)	2.70 ±1.01^{a,x} (17)	1.13 ±0.43^{a,x} (9)
Total	1183	40.41 ±2.95	60.47 ±3.82	60.31 ±3.86	36.64 ±3.46	28.49 ±3.09	20.78 ±2.81	17.57 ±2.63	6.35 ±1.61	3.61 ±1.02

^{abc} Means with different superscripts in a row within a group were significantly different (P<0.05).

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.33: Percent maturation, enucleation, injection and cleavage rates between caprine and bovine oocytes (%, mean ±SEM) based on Grade C oocytes

Species	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Caprine	306	44.71 ±5.07^{y} (180)	54.73 ±5.67^{y} (166)	54.74 ±5.70^{y} (154)	40.15 ±5.17^{c,y} (116)	32.78 ±5.01^{bc,y} (101)	30.25 ±5.00^{bc,y} (87)	26.62 ±4.76^{b,y} (74)	8.21 ±2.80^{a,y} (39)	5.38 ±2.01^{a,y} (21)
Bovine	452	21.61 ±3.75^{x} (155)	30.47 ±5.23^{x} (123)	29.37 ±5.08^{x} (110)	9.98 ±2.95^{d,x} (47)	5.85 ±2.02^{bc,x} (30)	4.89 ±1.89^{abc,x} (23)	3.35 ±1.55^{ab,x} (14)	0.56 ±0.35^{ab,x} (4)	0.14 ±0.14^{a,x} (1)
Total	758	33.40 ±3.30	42.85 ±3.98	42.32 ±3.95	25.37 ±3.25	19.59 ±2.95	17.83 ±2.90	15.22 ±2.71	4.46 ±1.47	2.81 ±1.05

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

^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.34: Percent maturation, enucleation, injection and cleavage rates between caprine and bovine oocytes (%, mean ±SEM) based on Grade D oocytes

Species	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Caprine	111	12.40 ±3.30^{x} (67)	15.14 ±4.06^{x} (60)	16.00 ±4.26^{x} (60)	12.33 ±3.46^{c,x} (47)	7.67 ±2.71^{bc,x} (38)	7.67 ±2.71^{bc,x} (38)	3.96 ±2.06^{ab,x} (28)	2.33 ±1.64^{ab,x} (14)	1.83 ±1.29^{a,xy} (11)
Bovine	141	8.44 ±2.39^{x} (57)	13.27 ±3.83^{x} (50)	13.61 ±3.88^{x} (42)	6.02 ±2.31^{b,x} (20)	3.40 ±1.41^{ab,x} (12)	2.06 ±1.00^{ab,x} (7)	3.70 ±2.01^{ab,x} (5)	0.23 ±0.23^{a,x} (1)	0.00 ±0.00^{a,x} (0)
Total	252	10.46 ±2.05	14.23 ±2.79	14.83 ±2.88	9.24 ±2.11	5.58 ±1.55	4.92 ±1.48	3.83 ±1.43	1.30 ±0.85	0.94 ±0.66

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

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

Table 4.35: Percent maturation, enucleation, injection and cleavage rates between caprine and bovine oocytes (%, mean ±SEM) based on Grade E oocytes

Species	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Caprine	151	14.21 ±3.34^{x} (65)	19.67 ±4.47^{x} (53)	19.13 ±4.39^{x} (47)	9.44 ±3.00^{b,x} (26)	8.33 ±2.83^{b,x} (23)	6.67 ±2.63^{ab,x} (18)	5.33 ±2.15^{ab,x} (16)	1.33 ±0.94^{a,x} (2)	0.00 ±0.00^{a,x} (0)
Bovine	323	9.79 ±2.38^{x} (75)	17.89 ±4.38^{x} (56)	17.25 ±4.28^{x} (51)	5.51 ±2.37^{b,x} (18)	2.92 ±1.65^{ab,x} (12)	2.92 ±1.65^{ab,x} (12)	2.08 ±1.47^{ab,x} (7)	0.14 ±0.14^{a,x} (1)	0.00 ±0.00^{a,x} (0)
Total	474	12.04 ±2.07	18.80 ±3.12	18.21 ±3.06	7.52 ±1.92	5.68 ±1.67	4.83 ±1.57	3.74 ±1.31	0.75 ±0.48	0.00 ±0.00

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

^{x} Means with same superscripts in a column were not significantly different (P>0.05).

Table 4.36 shows the percent enucleation by 2 different enucleation techniques, namely squeezing and laser enucleations between caprine and bovine species. There were significant differences (P<0.05) between caprine and bovine species in percent enucleation for squeezing and laser enucleation techniques with the values of 95.23±1.73% versus 82.73±3.81% and 96.61±1.42% versus 88.64±3.21, respectively. The percent enucleation was higher in the caprine compared to bovine in both enucleation techniques.

Table 4.36: Percent enucleation (%, mean±SEM) by 2 different enucleation techniques between caprine and bovine species

Species	No. of ovaries	No. of oocytes	Percent enucleation (n)	
			Squeezing (n)	Laser (n)
Caprine	209	1296	95.23±1.73[a,y] (236/267)	96.61±1.42[a,y] (479/500)
Bovine	220	3175	82.73±3.81[a,x] (375/509)	88.64±3.21[a,x] (699/806)
Total	429	4471	89.17±2.18	92.67±1.79

[a] Means with same superscripts in a row were not significantly different (P>0.05).
[xy] Means with different superscripts in a column were significantly different (P<0.05).
*n=number of enucleated oocytes/number of matured oocytes used.

The cleavage rates between caprine and bovine species are shown in Tables 4.37, 4.38, 4.39, 4.40, 4.41 and 4.42 for 2-, 4-,8-cell, morula, blastocyst and hatched blastocyst embryos in 2 different enucleation techniques (squeezing and laser enucleation). Most of the cleavage values were significantly higher in laser enucleation technique for both species. Caprine species always showed higher percent cleavage than bovine species for both enucleation techniques.

Table 4.37: Percent 2-cell embryos (%, mean ±SEM) and enucleation techniques between caprine and bovine species

Species	No. of ovaries	No. of oocytes	Percent 2-cell (n)	
			Squeezing (n)	Laser (n)
Caprine	209	1296	$58.15 \pm 6.76^{a,y}$ (135/215)*	$82.07 \pm 3.45^{b,y}$ (393/471)
Bovine	220	3175	$20.12 \pm 5.35^{a,x}$ (94/320)	$63.7 \pm 4.04^{b,x}$ (412/644)
Total	429	4471	39.71 ±4.91	73.01 ±2.83

[ab] Means with different superscripts in a row were significantly different (P<0.05).
[xy] Means with different superscripts in a column were significantly different (P<0.05).
*n=number of 2-cell embryos/number of injected oocytes.

Table 4.38: Percent 4-cell embryos (%, mean ±SEM) and enucleation techniques between caprine and bovine species

Species	No. of ovaries	No. of oocytes	Percent 4-cell (n)	
			Squeezing (n)	Laser (n)
Caprine	209	1296	$42.04 \pm 6.53^{a,y}$ (95/215)*	$74.14 \pm 3.62^{b,y}$ (367/471)
Bovine	220	3175	$13.72 \pm 4.38^{a,x}$ (71/320)	$50.30 \pm 4.33^{b,x}$ (327/644)
Total	429	4471	28.31 ±4.32	62.37 ±3.10

[ab] Means with different superscripts in a row were significantly different (P<0.05).
[xy] Means with different superscripts in a column were significantly different (P<0.05).
*****n=number of 4-cell embryos/number of injected oocytes.

Table 4.39: Percent 8-cell embryos (%, mean ±SEM) and enucleation techniques between caprine and bovine species

Species	No. of ovaries	No. of oocytes	Percent 8-cell (n)	
			Squeezing (n)	Laser (n)
Caprine	209	1296	$25.73 \pm 6.05^{a,y}$ (57/215)*	$70.93 \pm 3.87^{b,y}$ (347/471)
Bovine	220	3175	$7.95 \pm 3.79^{b,x}$ (45/320)	$37.74 \pm 4.31^{b,x}$ (257/644)
Total	429	4471	17.11 ±3.76	54.54 ±3.42

[ab] Means with different superscripts in a row were significantly different (P<0.05).
[xy] Means with different superscripts in a column were significantly different (P<0.05).
*****n=number of 8-cell embryos/number of injected oocytes.

Table 4.40: Percent morula embryos (%, mean ±SEM) and enucleation techniques between caprine and bovine species

Species	No. of ovaries	No. of oocytes	Percent morula (n)	
			Squeezing (n)	Laser (n)
Caprine	209	1296	$16.36 \pm 5.22^{a,y}$ (35/215)*	$64.08 \pm 3.91^{b,y}$ (315/471)
Bovine	220	3175	$1.71 \pm 1.33^{a,x}$ (16/320)	$23.84 \pm 3.79^{b,x}$ (195/644)
Total	429	4471	9.26 ±2.89	44.21 ±3.52

[ab] Means with different superscripts in a row were significantly different (P<0.05).
[xy] Means with different superscripts in a column were significantly different (P<0.05).
*****n=number of morula embryos/number of injected oocytes.

Table 4.41: Percent blastocyst embryos (%, mean ±SEM) and enucleation techniques between caprine and bovine species

Species	No. of ovaries	No. of oocytes	Percent blastocyst (n)	
			Squeezing (n)	Laser (n)
Caprine	209	1296	$0.00 \pm 0.00^{a,x}$ (0/215)*	$25.33 \pm 4.66^{b,y}$ (170/471)
Bovine	220	3175	$0.00 \pm 0.00^{a,x}$ (0/320)	$7.01 \pm 1.42^{b,x}$ (70/644)
Total	429	4471	0.00 ±0.00	16.28 ±2.65

[ab] Means with different superscripts in a row were significantly different (P<0.05).
[xy] Means with different superscripts in a column were significantly different (P<0.05).
*****n=number of blastocyst embryos/number of injected oocytes.

Table 4.42: Percent hatched blastocyst embryos (%, mean ±SEM) and enucleation techniques between caprine and bovine species

Species	No. of ovaries	No. of oocytes	Percent hatched blastocyst (n)	
			Squeezing (n)	Laser (n)
Caprine	209	1296	$0.00 \pm 0.00^{a,x}$ (0/215)*	$15.68 \pm 3.00^{b,y}$ (99/471)
Bovine	220	3175	$0.00 \pm 0.00^{a,x}$ (0/320)	$2.98 \pm 0.77^{b,x}$ (33/644)
Total	429	4471	0.00 ±0.00	9.40 ±1.71

[ab] Means with different superscripts in a row were significantly different (P<0.05).
[xy] Means with different superscripts in a column were significantly different (P<0.05).
*****n=number of hatched blsatocyst embryos/number of injected oocytes.

Comparisons of cleavage rates between caprine and bovine embryos in parthenogenesis treatment are shown in Table 4.43. A total of 1197 oocytes obtained from caprine and bovine were 438 and 759, respectively. There were no significant differences (P>0.05) in maturation rates between caprine and bovine with the values of 63.73 ±4.25% versus 61.50 ±3.65%, respectively. Similarly, cleavage rates for caprine and bovine showed no significant differences (P>0.05) (65.95 ±5.08% versus 62.24 ±4.96%, 56.66 ±5.36% versus 49.49 ±4.94%, 49.37 ±6.18% versus 40.70 ±4.21%,

30.27±5.96% versus 31.41±3.28%, 17.18±5.38% versus 12.66±2.18% and 4.67±2.51% versus 2.15±0.71%) for 2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst, respectively. There was a significant decrease in percent cleavage from 2-cell embryos up to hatched blastocyst for caprine and bovine species.

Table 4.43: Percent cleavage between caprine and bovine embryos (%, mean ±SEM) in parthenogenesis

Species	No. of ovaries	No. of oocytes	Maturation rate (n)	Percent cleavage (n)					
				2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Caprine	51	438	63.73 ±4.25^{x} (255)	65.95 ±5.08^{d,x} (152)	56.66 ±5.36^{cd,x} (125)	49.37 ±6.18^{c,x} (96)	30.27 ±5.96^{b,x} (53)	17.18 ±5.38^{ab,x} (26)	4.67 ±2.51^{a,x} (4)
Bovine	75	759	61.50 ±3.65^{x} (471)	62.24 ±4.96^{e,x} (274)	49.49 ±4.94^{d,x} (220)	40.70 ±4.21^{cd,x} (178)	31.41 ±3.28^{c,x} (133)	12.66 ±2.18^{b,x} (59)	2.15 ±0.71^{a,x} (12)
Total	126	1197	62.62 ±2.76	64.09 ±3.51	53.07 ±3.64	45.03 ±3.76	30.84 ±3.34	14.92 ±2.88	3.41 ±1.30

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

^{x} Means with same superscripts in a column within a group were not significantly different ($P>0.05$).

4.3 EFFECTS OF STRAINS, CULTURE MEDIUM AND MOUSE EMBRYONIC FIBROBLAST FREEZING ON PRODUCTION OF MURINE EMBRYONIC STEM CELL LINES USING WHOLE BLASTOCYST CULTURE TECHNIQUE (EXPERIMENT 3)

The aims of this experiment were to a) determine the effects of murine strains, b) culture medium and c) fresh and frozen-thawed mouse embryonic fibroblast on production of murine embryonic stem cell lines performance by using whole blastocyst culture technique. Five different stages of murine blastocyst have been studied, namely early-, mid-, expanded-, hatching- and hatched blastocyst to produce good quality of murine embryonic stem cell lines.

4.3.1 Effects of Murine Strains on Production of Murine Embryonic Stem Cells Lines Using Whole Blastocyst Culture Technique

Table 4.44 shows the percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells up to Passage 3 (P3) derived from 3 different pure-strains of murine (ICR, CBA/ca and C57BL/6J). There were no significant differences ($P>0.05$) among the 3 different pure-strains of murine in percent successful consecutive passages murine embryonic stem cell lines up to Passage 3 (P3). However, CBA/ca gave the highest percent attachment of blastocysts and successful consecutive passages murine embryonic stem cell lines with the values of $62.68 \pm 20.90\%$ (attachment rate), $41.32 \pm 3.49\%$ (P1), $31.00 \pm 3.56\%$ (P2) and $19.81 \pm 3.51\%$ (P3), respectively.

Table 4.44: Percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells (%, mean ±SEM) up to Passage 3 (P3) from 3 different pure-strains of murine

Strain	No. of females	No. of replicates	No. of blastocysts	Percent attachment (n)	Percent successful consecutive passages (n)		
					P1 (n)	P2 (n)	P3 (n)
ICR	63	35	971	56.74 ±3.18^{d,x} (777)	35.20 ±2.61^{c,x} (443)	23.77 ±2.48^{b,x} (207)	9.00 ±1.65^{a,x} (71)
CBA/ca	34	18	758	62.68 ±20.90^{b,x} (429)	41.32 ±3.49^{ab,x} (237)	31.00 ±3.56^{ab,x} (109)	19.81 ±3.51^{a,y} (38)
C57BL/6J	39	18	709	44.68 ±2.53^{c,x} (359)	40.71 ±4.49^{c,x} (160)	22.10 ±3.75^{a,x} (64)	8.50 ±2.59^{a,x} (22)
Total	136	71	2438	55.18 ±5.55	38.15 ±1.93	25.18 ±1.80	11.62 ±1.39

^{abcd}Means with different superscripts in a row within a group were significantly different (P<0.05).
^{xy}Means with different superscripts in a column within a group were not significantly different (P>0.05).

Table 4.45: Percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cell lines up to passage 3 (P3) (mean ±SEM) from 5 different blastocyst stages

Blastocyst stage	No. of females	No. of replicates	No. of blastocysts	Percent attachment (n)	Percent successful consecutive passages (n)		
					P1	P2	P3
Early	82	71	256	65.17 ±24.88^{b,x} (198)	27.88 ±3.79^{ab,x} (105)	20.28 ±3.70^{a,xy} (46)	9.18 ±2.98^{a,x} (14)
Mid	115	71	488	48.08 ±4.00^{c,x} (278)	45.35 ±5.60^{c,y} (164)	28.11 ±4.29^{b,yz} (81)	15.16 ±3.47^{a,x} (30)
Expanded	129	71	666	68.78 ±3.38^{d,x} (465)	52.79 ±3.92^{c,y} (262)	37.55 ±4.36^{b,z} (138)	17.24 ±3.67^{a,x} (55)
Hatching	122	71	682	57.01 ±3.65^{d,x} (418)	43.01 ±3.67^{c,y} (224)	28.02 ±3.81^{b,yz} (89)	11.60 ±2.95^{a,x} (25)
Hatched	97	71	346	37.45 ±4.58^{c,x} (206)	24.53 ±3.53^{b,x} (85)	11.92 ±3.26^{a,x} (26)	8.08 ±2.94^{a,x} (7)
Total	545	355	2438	55.44 ±5.48	38.71 ±1.94	25.18 ±1.80	12.27 ±1.44

^{abcd}Means with different superscripts in a row within a group were significantly different (P<0.05).
^{xyz}Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.46 shows percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells derived from 3 different pure-strains of murine and 5 different blastocyst stages. A total of 971, 758 and 709 blastocysts were used in this experiment for ICR, CBA/ca and C57BL/6J. Expanded blastocyst stage always showed the highest percent successful attachment of blastocysts and consecutive passages up to passage 3 (P3) throughout the 3 different pure-strains of murine compared to other blastocyst stages. The percent successful consecutive passages from Passage 1 (P1) up to Passage 3 (P3) showed significant decrease for ICR, CBA/ca and C57BL/6J. Murine blastocyst stages are shown in Figure 4.12.

Percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cell lines up to Passage 3 (P3) from 5 different blastocyst stages (early-, mid-, expanded-, hatching- and hatched blastocyst) are shown in Table 4.45. There were no significant differences ($P>0.05$) in percent attachment for early-, mid-, expanded-, hatching- and hatched blastocyst with the values of $65.17 \pm 24.88\%$, $48.08 \pm 4.00\%$, $68.78 \pm 3.38\%$, $57.01 \pm 3.65\%$ and $37.45 \pm 4.58\%$, respectively. Although each blastocyst stage could passage up to Passage 3 (P3) in producing murine embryonic stem cell lines, expanded blastocyst stage showed the highest percent successful attachment of blastocysts and successful consecutive passages compared to other blastocyst stages with the values of $68.78 \pm 3.38\%$ (attachment rate), $52.79 \pm 3.92\%$ (P1), $37.55 \pm 4.36\%$ (P2) and $17.24 \pm 3.67\%$ (P3), respectively.

Table 4.46: Percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells (%, mean ±SEM) derived from 3 different pure-strains of murine and 5 different blastocyst stages

Murine strains	No. of replicates	Blastocyst stages	No. of blastocysts	Percent attachment (n)	Percent successful consecutive passages (n)		
					P1 (n)	P2 (n)	P3 (n)
ICR	35	Early	96	35.78 ±7.35^{c,x} (76)	28.80 ±5.88^{bc,xy} (54)	19.05 ±5.03^{ab,xy} (23)	7.38 ±3.53^{a,xy} (6)
	35	Mid	187	57.93 ±6.96^{c,y} (145)	41.64 ±6.62^{bc,yz} (92)	26.92 ±5.67^{ab,xy} (44)	11.90 ±4.44^{a,xy} (16)
	35	Expanded	288	78.01 ±5.26^{c,z} (250)	49.28 ±5.93^{b,z} (143)	35.17 ±6.19^{b,y} (80)	16.68 ±4.87^{a,y} (36)
	35	Hatching	240	63.76 ±6.52^{d,yz} (181)	39.64 ±5.86^{c,yz} (103)	24.07 ±5.48^{b,xy} (42)	6.67 ±2.74^{a,xy} (9)
	35	Hatched	160	48.20 ±7.52^{c,xy} (125)	22.35 ±4.18^{b,x} (51)	13.62 ±4.85^{ab,x} (18)	2.38 ±8.03^{a,x} (4)
Total	175		971	56.74 ±3.18	36.34 ±2.64	23.77 ±2.48	9.00 ±1.65
CBA/ca	18	Early	85	10.50 ±7.66^{a,x} (76)	22.97 ±6.38^{a,x} (31)	21.85 ±6.89^{a,xy} (13)	15.74 ±7.91^{a,x} (4)
	18	Mid	160	35.05 ±5.27^{a,x} (69)	45.07 ±7.34^{a,yz} (40)	36.30 ±9.36^{a,xy} (21)	25.19 ±8.10^{a,x} (8)
	18	Expanded	208	65.14 ±5.13^{c,x} (125)	61.37 ±6.59^{bc,z} (73)	43.15 ±7.88^{b,y} (36)	18.89 ±7.78^{a,x} (10)
	18	Hatching	260	50.38 ±4.67^{b,x} (140)	49.17 ±5.82^{b,yz} (80)	38.71 ±6.30^{ab,y} (34)	25.37 ±8.03^{a,x} (13)
	18	Hatched	45	20.75 ±7.90^{a,x} (19)	28.04 ±9.40^{a,xy} (13)	15.00 ±7.85^{a,x} (5)	13.89 ±7.89^{a,x} (3)
Total	90		758	62.68 ±20.90	41.32 ±3.49	31.00 ±3.56	19.81 ±3.51

C57BL/6J	18	Early	75	43.16 ±7.38^{c,xy} (46)	31.00 ±7.44^{bc,x} (20)	21.11 ±8.74^{ab,xy} (10)	6.94 ±4.87^{a,x} (4)
	18	Mid	141	41.96 ±4.58^{ab,xy} (64)	52.84 ±16.74^{b,x} (32)	22.26 ±8.95^{ab,xy} (16)	11.48 ±6.68^{a,x} (6)
	18	Expanded	170	54.49 ±5.36^{b,y} (90)	51.03 ±7.94^{b,x} (46)	36.57 ±9.77^{ab,y} (22)	16.67 ±8.08^{a,x} (9)
	18	Hatching	182	50.52 ±4.39^{c,y} (97)	43.40 ±6.95^{b,x} (41)	25.00 ±8.37^{ab,xy} (13)	7.41 ±5.75^{a,x} (3)
	18	Hatched	141	33.25 ±5.35^{b,x} (62)	25.25 ±6.69^{b,x} (21)	5.56 ±4.04^{a,x} (3)	0.00 ±0.00^{a,x} (0)
Total	90		709	44.68 ±2.53	40.71 ±4.49	22.10 ±3.75	8.50 ±2.59

^{abcd}Means with different superscripts in a row within a group were significantly different (P<0.05).
^{xyz}Means with different superscripts in a column within a group were significantly different (P<0.05).

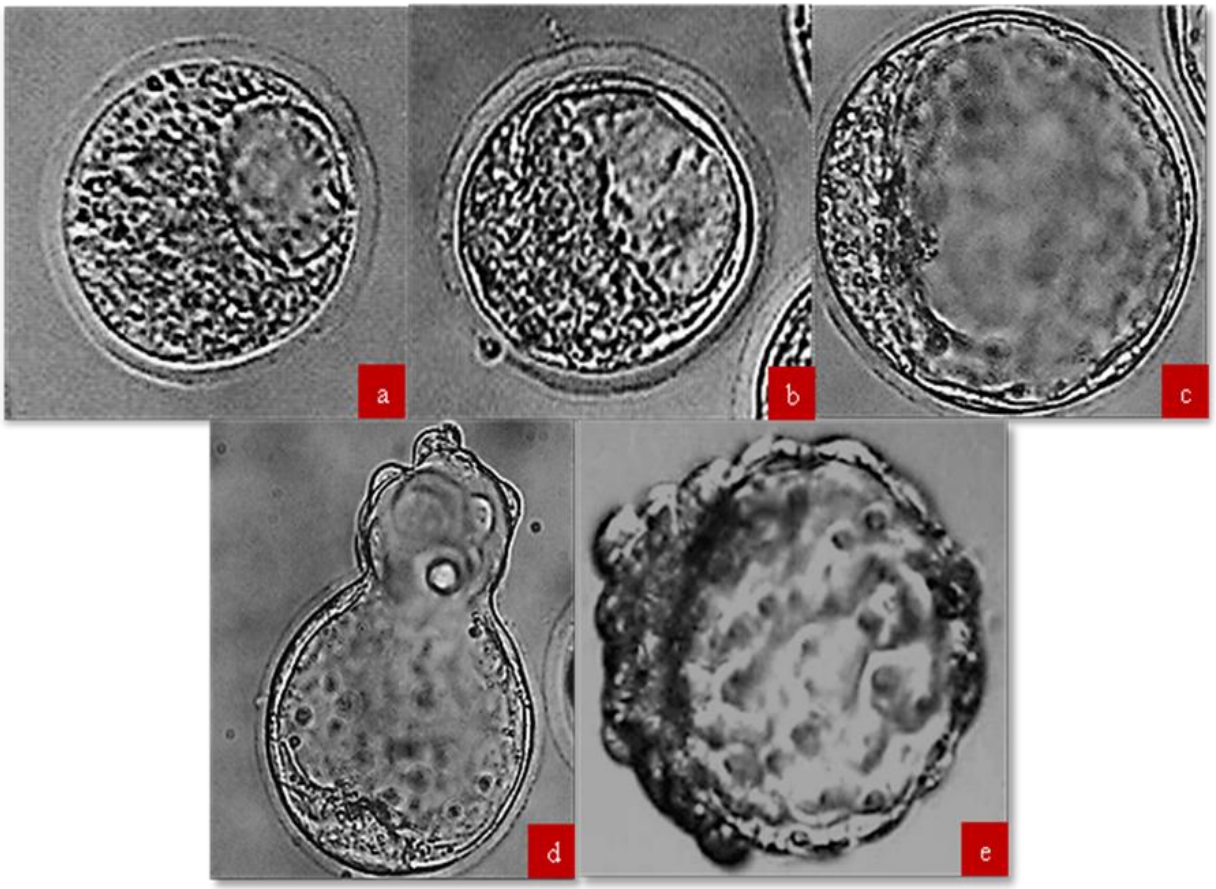

Figure 4.12: Blastocyst stages: a) early blastocyst, b) mid-blastocyst, c) expanded blastocyst, d) hatching blastocyst and e) hatched blastocyst.

4.3.2 Effects of Culture Medium on Production of Murine Embryonic Stem Cell Lines Using Whole Blastocyst Culture Technique

Table 4.47 shows percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells up to Passage 3 (P3) on 2 different culture media, namely DMEM+10% FBS and αMEM+10% FBS. There were no significant differences ($P>0.05$) between DMEM+10% FBS and αMEM+10% FBS with the values of 56.78±13.59% versus 53.13±2.51%, 41.60±3.64% versus 37.05±2.23%, 29.85±3.20% versus 22.78±2.22% and 15.75±2.50% versus 9.36±1.68% for percent attachment, Passage 1 (P1), Passage 2 (P2) and Passage 3 (P3), respectively. However, the DMEM+10% FBS culture medium showed higher percent successful attachment and consecutive passages than αMEM+10% FBS culture medium.

Percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells on DMEM+10%FBS and αMEM+10%FBS culture media based on 5 different blastocyst stages are shown in Table 4.48. A total of 881 and 1442 of murine blastocyst for DMEM+10%FBS and αMEM+10%FBS culture media were used in this experiment, respectively. There were no significant differences ($P>0.05$) for percent successful attachment of blastocysts in Passages 1, 2 and 3 at early blastocyst stage for DMEM+10%FBS culture medium only with the values of 34.60±7.27%, 28.78±7.01%, 22.26±6.62% and 14.29±5.81%, respectively.

Table 4.47: Percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells (%, mean ±SEM) up to Passage 3 (P3) on 2 different culture media

Culture medium	No. of females	No. of replicates	No .of blastocysts	Percent attachment (n)	Percent successful consecutive passages (n)		
					P1 (n)	P2 (n)	P3 (n)
DMEM+10% FBS	51	140	881	$56.78 \pm 13.59^{d,x}$ (563)	$41.60 \pm 3.64^{c,x}$ (333)	$29.85 \pm 3.20^{b,x}$ (185)	$15.75 \pm 2.50^{a,x}$ (80)
αMEM+10% FBS	77	205	1442	$53.13 \pm 2.51^{c,x}$ (935)	$37.05 \pm 2.23^{bc,x}$ (471)	$22.78 \pm 2.22^{ab,x}$ (189)	$9.36 \pm 1.68^{a,y}$ (51)
Total	128	345	2356	54.61 ± 5.70	38.89 ± 1.98	25.65 ± 1.86	11.95 ± 1.43

^{abcd}Means with different superscripts in a row within a group were significantly different ($P<0.05$).
^{xy}Means with different superscripts in a column within a group were significantly different ($P<0.05$).

Table 4.48: Percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells (%, mean ±SEM) on 2 different culture media based on 5 different blastocyst stages

Culture medium	No. of replicates	Blastocyst stages	No. of blastocysts	Percent attachment (n)	Percent successful consecutive passage (n)		
					P1 (n)	P2 (n)	P3 (n)
DMEM+10% FBS	28	Early	96	34.60 ±7.27^{a,xy} (69)	28.78 ±7.01^{a,x} (35)	22.26 ±6.62^{a,xy} (19)	14.29 ±5.81^{a,xy} (9)
	28	Mid	199	43.69 ±5.99^{ab,xy} (112)	61.26 ±11.60^{b,z} (83)	41.50 ±7.87^{ab,yz} (50)	26.90 ±6.86^{a,y} (23)
	28	Expanded	275	65.65 ±5.31^{b,z} (177)	55.63 ±6.53^{b,yz} (110)	47.57 ±7.50^{b,z} (71)	21.99 ±6.32^{a,y} (34)
	28	Hatching	249	49.47 ±6.42^{c,yz} (145)	37.60 ±6.61^{bc,xy} (73)	28.77 ±6.80^{ab,xyz} (37)	14.40 ±5.56^{a,xy} (12)
	28	Hatched	62	26.22 ±6.52^{b,x} (61)	27.15 ±6.23^{b,x} (32)	10.95 ±5.38^{a,x} (8)	3.57 ±2.48^{a,x} (2)
Total	140		881	43.93 ±3.02	42.09 ±3.66	30.21 ±3.23	16.23 ±2.57
αMEM+10% FBS	41	Early	153	39.46 ±6.06^{c,x} (104)	29.28 ±4.93^{bc,x} (58)	19.51 ±4.94^{ab,xy} (24)	8.13 ±3.78^{a,x} (5)
	41	Mid	278	50.11 ±5.54^{c,xy} (158)	34.66 ±4.87^{b,xy} (76)	19.86 ±4.69^{a,xy} (30)	7.89 ±3.28^{a,x} (7)
	41	Expanded	373	69.62 ±4.53^{d,z} (271)	50.45 ±5.14^{c,z} (138)	33.73 ±5.70^{b,y} (66)	14.84 ±4.63^{a,x} (21)
	41	Hatching	422	61.45 ±4.42^{d,yz} (265)	47.83 ±4.31^{c,yz} (149)	27.65 ±4.68^{b,xy} (51)	10.24 ±3.33^{a,x} (13)
	41	Hatched	216	45.00 ±6.21^{c,x} (137)	23.02 ±4.39^{b,x} (50)	13.17 ±4.32^{ab,x} (18)	5.69 ±3.47^{a,x} (5)
Total	205		1442	53.13 ±2.51	37.05 ±2.23	22.78 ±2.22	9.36 ±1.68

^{abcd}Means with different superscripts in a row within a group were significantly different (P<0.05).
^{xyz}Means with different superscripts in a column within a group were significantly different (P<0.05).

4.3.3 Effects of Fresh and Frozen-thawed Mouse Embryonic Fibroblast on Production of Murine Embryonic Stem Cell Lines Using Whole Blastocyst Culture Technique

In Table 4.49 shows percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells up to Passage 3 (P3) based on fresh and frozen-thawed mouse embryonic fibroblast. A total of 128 number of female murine, 70 (fresh mouse embryonic fibroblast) and 58 (frozen-thawed mouse embryonic fibroblast) were used in this experiment. There were no significant differences ($P>0.05$) between fresh and frozen-thawed mouse embryonic fibroblast for percent successful attachment of blastocysts, consecutive passages with the values of $50.35 \pm 2.93\%$ versus $48.22 \pm 2.41\%$, $38.18 \pm 2.61\%$ versus $39.78 \pm 3.05\%$, $26.59 \pm 2.53\%$ versus $24.63 \pm 2.72\%$ and $13.82 \pm 2.06\%$ versus $10.10 \pm 2.01\%$, respectively.

Percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells derived from 5 different blastocyst stages based on fresh and frozen-thawed mouse embryonic fibroblast are shown in Table 4.50. Obviously, majority of expanded blastocysts gave the highest percent attachment of blastocysts and successful consecutive passages in both fresh and frozen-thawed mouse embryonic fibroblast with the values of $76.05 \pm 4.24\%$ (attachment rate), $44.34 \pm 6.05\%$ versus $31.11 \pm 6.41\%$ (P2) and $21.68 \pm 5.43\%$ versus $12.90 \pm 5.02\%$ (P3), respectively.

Table 4.49: Percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells (%, mean ±SEM) up to Passage 3 (P3) on fresh and frozen-thawed mouse embryonic fibroblasts

MEF	No. of females	No. of replicates	No .of blastocysts	Percent attachment (n)	Percent successful consecutive passages (n)		
					P1 (n)	P2 (n)	P3 (n)
Fresh	70	190	1117	$50.35 \pm 2.93^{d,x}$ (758)	$38.18 \pm 2.61^{c,x}$ (458)	$26.59 \pm 2.53^{b,x}$ (232)	$13.82 \pm 2.06^{a,x}$ (90)
Frozen-thawed	58	155	1264	$48.22 \pm 2.41^{d,x}$ (741)	$39.78 \pm 3.05^{c,x}$ (354)	$24.63 \pm 2.72^{b,x}$ (142)	$10.10 \pm 2.01^{a,x}$ (41)
Total	128	345	2381	49.39 ± 1.94	38.90 ± 1.99	25.71 ± 1.85	12.15 ± 1.45

[abcd]Means with different superscripts in a row within a group were significantly different ($P<0.05$).
[x]Means with same superscripts in a column were within a group not significantly different ($P>0.05$).

Table 4.50: Percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells (%, mean ±SEM) derived from 5 different blastocyst stages based on fresh and frozen-thawed mouse embryonic fibroblasts

MEF	No. of replicates	Blastocyst stages	No. of blastocysts	Percent attachment (n)	Percent successful consecutive passages (n)		
					P1 (n)	P2 (n)	P3 (n)
Fresh	38	Early	104	30.16 ±6.37^{b,x} (69)	23.50 ±5.27^{ab,x} (42)	18.16 ±4.87^{ab,xy} (22)	9.43 ±4.08^{a,xy} (7)
	38	Mid	231	47.92 ±6.17^{b,xy} (140)	42.17 ±6.14^{b,y} (92)	31.61 ±6.34^{ab,yz} (50)	16.32 ±4.80^{a,xy} (20)
	38	Expanded	340	76.05 ±4.24^{d,z} (261)	59.34 ±5.40^{c,z} (162)	44.34 ±6.05^{b,z} (94)	21.68 ±5.43^{a,y} (41)
	38	Hatching	337	58.96 ±5.81^{c,y} (213)	42.55 ±5.45^{b,y} (126)	28.77 ±5.29^{ab,yz} (57)	15.09 ±4.50^{a,xy} (19)
	38	Hatched	105	38.65 ±7.38^{c,x} (75)	23.33 ±5.03^{b,x} (36)	10.04 ±3.98^{ab,x} (9)	6.58 ±3.85^{a,x} (3)
Total	190		1117	50.35 ±2.93	38.18 ±2.61	26.59 ±2.53	13.82 ±2.06
Frozen-thawed	31	Early	145	46.47 ±6.48^{c,xy} (104)	32.69 ±5.88^{bc,xy} (53)	24.73 ±6.46^{ab,x} (21)	12.10 ±5.30^{a,x} (7)
	31	Mid	246	47.00 ±5.14^{b,xy} (130)	49.48 ±10.28^{b,y} (67)	24.99 ±5.99^{a,x} (30)	14.73 ±5.37^{a,x} (10)
	31	Expanded	308	58.16 ±5.12^{c,y} (187)	45.30 ±5.79^{bc,xy} (92)	31.11 ±6.41^{b,x} (43)	12.90 ±5.02^{a,x} (14)
	31	Hatching	334	53.67 ±4.35^{c,y} (197)	45.06 ±5.00^{c,xy} (96)	27.29 ±5.82^{b,x} (31)	8.06 ±3.85^{a,x} (6)
	31	Hatched	231	35.82 ±5.13^{c,x} (123)	26.38 ±5.24^{bc,x} (46)	15.00 ±5.66^{ab,x} (17)	2.69 ±1.91^{a,x} (4)
Total	155		1264	48.22 ±2.41	39.78 ±3.05	24.63 ±2.72	10.10 ±2.01

^{abc} Means with different superscripts in a row within a group were significantly different (P<0.05).
^{xyz} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.51 shows percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells on fresh and frozen-thawed mouse embyonic fibroblast at Passages 1 and 2. There were no significant differences ($P>0.05$) in percent successful consecutive passages on murine embryonic stem cell lines between Passage 1 (P1) and Passage 2 (P2) for fresh mouse embryonic fibroblast with values of $37.83 \pm 3.18\%$ versus $38.61 \pm 4.35\%$, $23.57 \pm 3.00\%$ versus $30.32 \pm 4.25\%$ and $13.13 \pm 2.85\%$ versus $14.67 \pm 2.97\%$ for Passages 1, 2 and 3, respectively. Whereas for Passsages 1 and 2 on frozen-thawed mouse embyonic fibroblast, there were significant differences ($P<0.05$) at Passage 3 (P3) with values of 5.40 ± 1.61 versus 18.64 ± 4.66, respectively.

Table 4.52 shows percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells derived from 5 different blastocyst stages on fresh and frozen-thawed mouse embryonic fibroblast at Passage 1 (P1) and Passage 2 (P2). The expanded blastocysts showed the highest percent attachment of blastocysts and consecutive passages for both Passages 1 and 2 in fresh mouse embryonic fibroblast with the respective values of $77.10 \pm 5.68\%$, $56.14 \pm 6.65\%$, $38.23 \pm 7.54\%$ and $19.44 \pm 7.35\%$ for murine embryonic stem cells lines at Passages 1, 2 and 3 (P1) and $74.74 \pm 6.56\%$, $63.29 \pm 9.00\%$, $51.88 \pm 9.74\%$ and $24.45 \pm 8.26\%$ for murine embryonic stem cell lines at Passages 1 and 2 only.

Table 4.51: Percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells (%, mean ±SEM) on fresh and frozen-thawed mouse embryonic fibroblast at Passage 1 (P1) and Passage 2 (P2)

MEF	No. passages MEF	No. of replicates	No. of blastocysts	Percent attachment (n)	Percent successful consecutive passages (n)		
					P1 (n)	P2 (n)	(n)
Fresh	P1	105	648	56.01 ±3.91^{d,y} (442)	37.83 ±3.18^{c,x} (248)	23.57 ±3.00^{b,x} (100)	13.13 ±2.85^{a,x} (31)
	P2	85	469	43.35 ±4.33^{c,x} (316)	38.61 ±4.35^{bc,x} (210)	30.32 ±4.25^{b,x} (132)	14.67 ±2.97^{a,x} (59)
Total		190	1117	50.35 ±2.93	38.18 ±2.61	26.59 ±2.53	13.82 ±2.06
Frozen-thawed	P1	100	794	50.10 ±3.10^{d,x} (493)	35.56 ±3.09^{c,x} (231)	21.64 ±3.16^{b,x} (89)	5.40 ±1.61^{a,x} (20)
	P2	55	470	44.82 ±3.80^{b,x} (248)	47.46 ±6.43^{b,x} (123)	30.05 ±5.01^{a,x} (53)	18.64 ±4.66^{a,y} (21)
Total		155	1264	48.22 ±2.41	39.78 ±3.05	24.63 ±2.72	10.10 ±2.01

^{abcd} Means with different superscripts in a row within a group were significantly different (P<0.05).
^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Table 4.52: Percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cells (%, mean ± SEM) derived from 5 different blastocyst stages on fresh and frozen-thawed mouse embryonic fibroblast at Passage 1 (P1) and Passage 2 (P2)

MEF	No. passages MEF	No. of replicates	Blastocyst stages	No. of blastocysts	Percent attachment (n)	Percent successful consecutive passages (n)		
						P1	P2	P3
Fresh	P1	21	Early	64	35.86 ± 9.37^{a,x} (42)	26.33 ± 6.98^{a,x} (26)	17.46 ± 5.83^{a,x} (12)	13.49 ± 6.86^{a,x} (4)
		21	Mid	129	50.28 ± 8.49^{c,xy} (72)	32.13 ± 6.42^{bc,xy} (37)	21.68 ± 7.36^{ab,xy} (14)	8.73 ± 5.35^{a,x} (3)
		21	Expanded	180	77.10 ± 5.68^{c,z} (141)	56.14 ± 6.65^{b,z} (80)	38.23 ± 7.54^{ab,y} (37)	19.44 ± 7.35^{a,x} (12)
		21	Hatching	220	66.12 ± 6.79^{c,yz} (144)	47.24 ± 5.96^{b,yz} (86)	27.63 ± 5.97^{a,xy} (32)	13.79 ± 5.67^{a,x} (10)
		21	Hatched	55	47.55 ± 10.49^{b,xy} (43)	25.05 ± 7.43^{a,x} (19)	11.51 ± 5.47^{a,x} (5)	9.52 ± 6.56^{a,x} (2)
		105	Total	648	55.48 ± 3.91	37.47 ± 3.17	23.34 ± 2.98	13.00 ± 2.83
	P2	17	Early	40	23.11 ± 8.27^{a,x} (27)	20.00 ± 8.18^{b,x} (16)	19.02 ± 8.37^{a,xy} (10)	4.41 ± 3.21^{a,x} (3)
		17	Mid	102	45.01 ± 9.22^{a,x} (68)	54.58 ± 10.68^{a,yz} (55)	43.89 ± 10.34^{a,yz} (36)	25.69 ± 8.06^{a,y} (17)
		17	Expanded	160	74.74 ± 6.56^{b,y} (120)	63.29 ± 9.00^{b,z} (82)	51.88 ± 9.74^{b,z} (57)	24.45 ± 8.26^{a,y} (29)
		17	Hatching	117	46.24 ± 9.74^{b,x} (69)	33.98 ± 9.67^{ab,xy} (40)	28.56 ± 9.33^{b,x} (25)	15.88 ± 7.13^{a,xy} (9)
		17	Hatched	50	27.65 ± 9.91^{b,x} (32)	21.20 ± 6.70^{ab,x} (17)	8.24 ± 5.96^{ab,x} (4)	2.94 ± 2.94^{a,x} (1)
		85	Total	1117	43.35 ± 4.33	38.61 ± 4.35	30.32 ± 4.25	14.67 ± 2.97

		20	Early	89	43.23 ±7.75^{c,x} (62)	27.38 ±6.28^{bc,xy} (34)	23.33 ±8.12^{b,x} (12)	2.50 ±2.50^{a,x} (1)
		20	Mid	149	49.94 ±7.28^{b,x} (86)	37.32 ±7.49^{b,xy} (39)	17.95 ±5.88^{a,x} (16)	7.00 ±3.85^{a,x} (4)
		20	Expanded	193	61.77 ±6.82^{b,x} (130)	46.13 ±7.84^{b,y} (64)	25.72 ±7.37^{a,x} (29)	10.00 ±5.53^{a,x} (9)
	P1	20	Hatching	202	53.24 ±5.77^{c,x} (121)	46.09 ±6.53^{c,y} (63)	26.30 ±7.29^{b,x} (19)	5.83 ±3.26^{a,x} (3)
		20	Hatched	161	42.32 ±6.63^{c,x} (94)	20.89 ±4.66^{b,x} (31)	14.92 ±6.86^{ab,x} (13)	1.67 ±1.67^{a,x} (3)
Frozen-thawed		100	Total	794	50.10 ±3.10	35.56 ±3.09	21.64 ±3.16	5.40 ±1.61
		11	Early	56	52.35 ±11.94^{a,y} (42)	42.35 ±11.90^{a,x} (19)	27.27 ±11.13^{a,x} (9)	29.55 ±12.98^{a,x} (6)
		11	Mid	97	41.65 ±6.02^{a,xy} (44)	71.59 ±24.99^{a,x} (28)	37.80 ±12.60^{a,x} (14)	28.79 ±12.73^{a,x} (6)
	P2	11	Expanded	115	51.60 ±7.38^{b,y} (57)	43.79 ±8.38^{ab,x} (28)	40.91 ±12.02^{ab,x} (14)	18.18 ±10.16^{a,x} (5)
		11	Hatching	132	54.46 ±6.68^{b,y} (76)	43.19 ±7.98^{b,x} (33)	29.09 ±10.13^{ab,x} (12)	12.12 ±9.29^{a,x} (3)
		11	Hatched	70	24.02 ±6.99^{b,x} (29)	36.36 ±11.89^{ab,x} (15)	15.15 ±10.41^{ab,x} (4)	4.55 ±4.55^{a,x} (1)
		55	Total	1264	44.82 ±3.80	47.46 ±6.43	30.05 ±5.01	18.64 ±4.66

^{abc} Means with different superscripts in a row within a group were significantly different (P<0.05).
^{xyz} Means with different superscripts in a column within a group were significantly different (P<0.05).

4.4 EFFECTS OF INNER CELL MASS ISOLATION TECHNIQUES, CULTURE MEDIUM AND MOUSE EMBRYONIC FIBROBLAST FREEZING ON PRODUCTION OF MURINE AND CAPRINE EMBRYONIC STEM CELL LINES USING MOUSE EMBRYONIC FIBROBLAST AS FEEDER CELL LAYER (EXPERIMENT 4)

The objectives of this experiment were a) to determine the effects of blastocyst isolation techniques, b) culture medium and c) fresh and frozen-thawed mouse embryonic fibroblasts on production of murine and caprine embryonic stem cell lines using mouse embryonic fibroblasts as feeder cell layer. In murine species, a total of 6831 female murine (ICR: 2879, CBA/ca: 2093, C57BL/6J: 1859) have been used in this experiment. The total number of murine and caprine blastocyst used in this project were 6831 and 169, respectively.

4.4.1 Effects of Inner Cell Mass Isolation Techniques, Culture Medium and Mouse Embryonic Fibroblast Freezing on Production of Murine Embryonic Stem Cell Lines

In Table 4.53 shows percent attachment of inner cell mass, primary inner cell mass outgrowth and successful consecutive passages of murine embryonic stem cell lines on 3 different inner cell mass isolation techniques, namely whole blastocyst culture, manual cut and laser dissection. A total of 6831 murine blastocysts were used in this experiment consisting of 2438, 2062 and 2331 for whole blastocyst culture, manual cut and laser dissection techniques, respectively. There were significant differences ($P<0.05$) in percent attachment of inner cell mass for 3 different inner cell mass isolation techniques with the values of $66.52 \pm 2.18\%$, $78.03 \pm 0.94\%$ and $71.06 \pm 1.11\%$ for whole blastocyst culture, manual cut and laser dissection techniques, respectively. Manual cut inner cell mass isolation technique gave the highest percent primary outgrowth of inner cell mass with the values of $78.35 \pm 1.24\%$, followed by laser

dissection (75.67±0.85%) and whole blastocyst culture (75.32±1.73%). There was significant decrease in percent successful consecutive passages from Passages1, 2 and 3 for 3 different inner cell mass isolation techniques. Figure 4.13 shows the 3 different inner cell mass isolation techniques with their ICM outgrowth.

Table 4.53: Percent attachment of inner cell mass, primary inner cell mass outgrowth and successful consecutive passages of murine embryonic stem cell lines (%, mean ±SEM) on 3 different inner cell mass isolation techniques

Isolation techniques	No. of females	No. of blastocysts	Percent ICM attachment (n)	Percent primary ICM outgrowth (n)	Percent successful consecutive passages (n)		
					P1 (n)	P2 (n)	P3 (n)
Whole blastocyst culture	241	2438	$66.52 \pm 2.18^{c,x}$ (1565)	$75.32 \pm 1.73^{d,xy}$ (1167)	$72.51 \pm 2.25^{cd,y}$ (841)	$41.62 \pm 3.23^{b,x}$ (380)	$25.77 \pm 3.34^{a,x}$ (131)
Manual cut	494	2062	$78.03 \pm 0.94^{d,z}$ (1624)	$78.35 \pm 1.24^{d,y}$ (1295)	$67.84 \pm 1.60^{c,y}$ (906)	$52.06 \pm 1.94^{b,y}$ (545)	$36.52 \pm 2.45^{a,y}$ (255)
Laser dissection	357	2331	$71.06 \pm 1.11^{d,y}$ (1690)	$75.67 \pm 0.85^{d,x}$ (1275)	$58.75 \pm 2.25^{c,x}$ (843)	$45.06 \pm 2.35^{b,x}$ (457)	$30.49 \pm 2.52^{a,xy}$ (194)
Total	1092	6831	72.97 ± 0.79	75.67 ± 0.85	65.70 ± 1.19	47.23 ± 1.39	31.96 ± 1.57

^{abcd} Means with different superscripts in a row within a group were significantly different ($P<0.05$).
^{xyz} Means with different superscripts in a column within a group were significantly different ($P<0.05$).

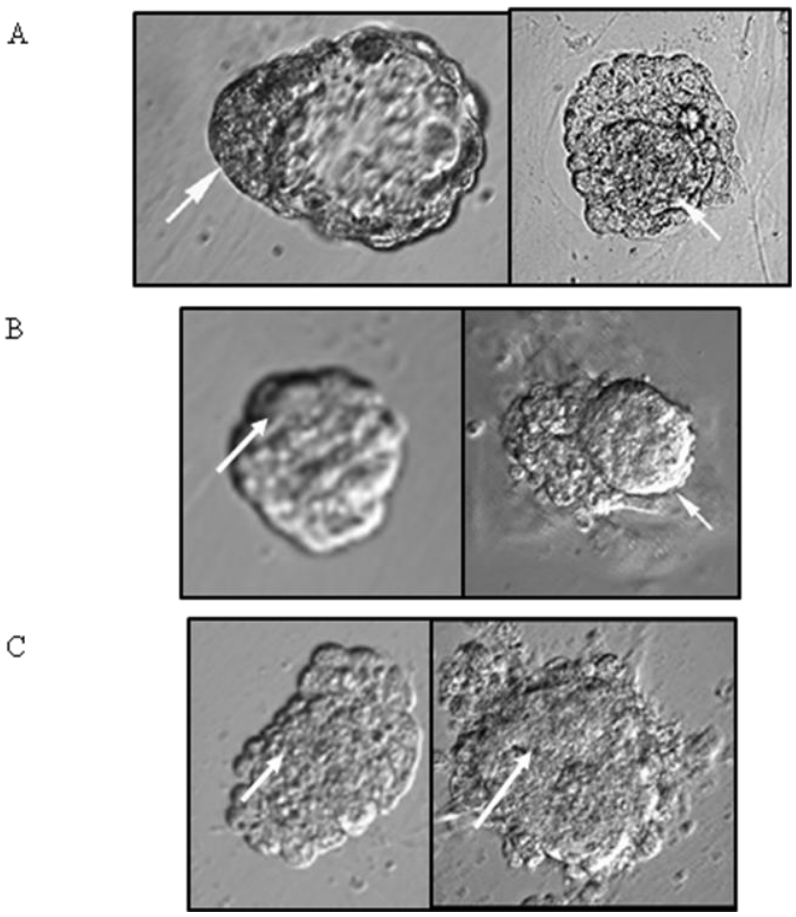

Figure 4.13: A) Whole blastocyst culture, B) manual cut ICM and C) laser dissection ICM isolation techniques with theirs ICM outgrowth shown by arrow.

Three different pure-strains of murine and 3 different inner cell mass isolation techniques are shown in Table 4.54 with special reference to their percent attachment of inner cell mass, primary inner cell mass outgrowth and successful consecutive passages of murine embryonic stem cell lines. There were significant differences ($P<0.05$) in percent successful consecutive passages in 3 different inner cell mass isolation techniques in ICR strain with the values of 75.34±3.34% versus 43.86±4.99% versus 22.86±4.31% (whole blastocyst culture); 72.36±1.46% versus 59.58±2.55% versus 39.36±3.37% (manual cut) and 70.58±1.74% versus 52.95±2.02% versus 38.77±2.81% (laser dissection) for Passages 1, 2 and 3, respectively. Furthermore, C57BL/6J showed the highest inner cell mass attachment, primary inner cell mass outgrowth and consecutive passages of murine embryonic stem cells lines rates followed by ICR and

CBA/ca by manual cut isolation techniques. Similar as previous table shown, there was significant decrease in percent successful consecutive passages from Passages 1, 2 and 3 for 3 different inner cell mass isolation techniques in 3 different pure-strains of murine.

In Table 4.55 shows percent attachment of inner cell mass, primary inner cell mass outgrowth and successful consecutive passages of murine embryonic stem cells on 2 different culture media, namely DMEM+10% FBS and αMEM+10% FBS and 3 different inner cell mass isolation techniques. In percent attachment of inner cell mass, manual cut technique gave the highest attachment rate compared to laser dissection followed by whole blastocyst with the values of 77.92±1.41%, 69.68±1.53% and 68.30±2.86% in DMEM+10% FBS culture medium, respectively. Manual cut always gave the highest percent successful consecutive passages of murine embryonic stem cell lines in both culture media. There was obviously significant decrease in percent successful consecutive passages from Passage 1 up to Passage 3 in both DMEM+10% FBS and MEM+10% FBS culture media.

Table 4.54: Percent attachment of inner cell mass, primary inner cell mass outgrowth and successful consecutive passages of murine embryonic stem cells (%, mean ±SEM) derived from 3 different pure-strains of murine and 3 different inner cell mass isolation techniques

Murine strains	ICM isolation techniques	No. of selected blastocysts	Percent ICM attachment (n)	Percent primary ICM outgrowth (n)	Percent successful consecutive passages (n)		
					P1 (n)	P2 (n)	P3 (n)
ICR	Whole blastocyst	971	$81.05 \pm 1.88^{c,y}$ (777)	$79.05 \pm 2.54^{c,x}$ (606)	$75.34 \pm 3.34^{c,x}$ (444)	$43.86 \pm 4.99^{b,x}$ (207)	$22.86 \pm 4.31^{a,x}$ (71)
	Manual cut	725	$80.60 \pm 1.37^{d,y}$ (583)	$78.80 \pm 1.21^{d,x}$ (463)	$72.36 \pm 1.46^{c,x}$ (340)	$59.58 \pm 2.55^{b,y}$ (212)	$39.36 \pm 3.37^{a,y}$ (96)
	Laser dissection	1183	$73.24 \pm 1.54^{c,x}$ (862)	$75.89 \pm 1.48^{c,x}$ (659)	$70.58 \pm 1.74^{c,x}$ (459)	$52.95 \pm 2.02^{b,y}$ (250)	$38.77 \pm 2.81^{a,y}$ (107)
Total		2879	77.82 ± 0.97	77.72 ± 1.00	72.50 ± 1.26	52.52 ± 1.88	34.47 ± 2.06
CBA/ca	Whole blastocyst	758	$55.83 \pm 2.81^{b,x}$ (429)	$74.22 \pm 2.30^{c,y}$ (315)	$74.21 \pm 3.15^{c,z}$ (237)	$43.69 \pm 2.61^{a,y}$ (109)	$34.74 \pm 6.16^{a,y}$ (38)
	Manual cut	722	$70.25 \pm 1.32^{d,z}$ (516)	$68.88 \pm 2.24^{d,y}$ (365)	$56.56 \pm 3.25^{c,y}$ (203)	$35.21 \pm 3.14^{b,y}$ (84)	$24.59 \pm 4.61^{a,y}$ (26)
	Laser dissection	613	$64.29 \pm 2.11^{d,y}$ (413)	$57.26 \pm 2.70^{d,x}$ (258)	$32.38 \pm 4.11^{b,x}$ (118)	$18.20 \pm 4.46^{b,x}$ (40)	$7.80 \pm 3.62^{a,x}$ (8)
Total		2093	65.72 ± 1.19	66.18 ± 1.59	52.12 ± 2.62	31.54 ± 2.34	21.14 ± 2.96
C57BL/6J	Whole blastocyst	709	$48.96 \pm 2.54^{bc,x}$ (359)	$69.17 \pm 3.81^{d,x}$ (246)	$65.30 \pm 4.99^{cd,x}$ (160)	$35.19 \pm 7.88^{ab,x}$ (64)	$22.46 \pm 8.02^{a,x}$ (22)
	Manual cut	615	$84.51 \pm 1.36^{d,z}$ (525)	$88.73 \pm 1.38^{d,y}$ (467)	$76.50 \pm 1.82^{c,y}$ (363)	$63.91 \pm 2.47^{b,y}$ (249)	$47.47 \pm 3.64^{a,y}$ (133)
	Laser dissection	535	$74.51 \pm 1.86^{c,y}$ (415)	$83.33 \pm 1.94^{d,y}$ (358)	$66.76 \pm 2.87^{bc,x}$ (266)	$59.91 \pm 2.71^{b,y}$ (167)	$40.42 \pm 5.16^{a,y}$ (79)
Total		1859	74.18 ± 1.72	83.08 ± 1.40	71.07 ± 1.68	56.91 ± 2.40	40.20 ± 3.02

[abcd] Means with different superscripts in a row within a group were significantly different ($P<0.05$).
[xyz] Means with different superscripts in a column within a group were significantly different ($P<0.05$).

Table 4.55: Percent attachment of inner cell mass, primary inner cell mass outgrowth and successful consecutive passages of murine embryonic stem cells (%, mean ±SEM) on 2 different culture media using 3 different inner cell mass isolation techniques

Culture medium	ICM isolation techniques	No. of blastocysts	Percent ICM attachment (n)	Percent primary ICM outgrowth (n)	Percent successful consecutive passages (n)		
					P1 (n)	P2 (n)	P3 (n)
DMEM+10% FBS	Whole blastocyst	1398	$68.30 \pm 2.86^{c,x}$ (894)	$74.81 \pm 2.47^{c,xy}$ (663)	$74.56 \pm 3.28^{c,z}$ (492)	$42.71 \pm 4.43^{b,x}$ (241)	$27.98 \pm 4.62^{a,x}$ (95)
	Manual cut	1127	$77.92 \pm 1.41^{d,y}$ (890)	$78.68 \pm 1.92^{d,y}$ (719)	$65.26 \pm 2.52^{c,y}$ (494)	$55.01 \pm 2.70^{b,y}$ (315)	$35.66 \pm 3.30^{a,x}$ (153)
	Laser dissection	1146	$69.68 \pm 1.53^{d,x}$ (823)	$68.91 \pm 2.36^{d,x}$ (605)	$55.66 \pm 3.67^{c,x}$ (404)	$42.57 \pm 3.76^{b,x}$ (220)	$28.57 \pm 3.76^{a,x}$ (88)
Total		3671	72.90 ± 1.10	74.59 ± 1.31	64.48 ± 1.87	47.93 ± 2.05	31.49 ± 2.19
αMEM+10% FBS	Whole blastocyst	1040	$64.23 \pm 3.37^{c,x}$ (671)	$75.98 \pm 2.41^{d,x}$ (504)	$69.86 \pm 2.93^{cd,y}$ (349)	$40.22 \pm 4.75^{b,x}$ (139)	$22.91 \pm 4.81^{a,x}$ (36)
	Manual cut	935	$78.16 \pm 1.23^{d,z}$ (734)	$77.98 \pm 1.52^{d,x}$ (576)	$70.76 \pm 1.83^{c,y}$ (412)	$49.61 \pm 2.70^{b,x}$ (230)	$37.49 \pm 3.69^{a,y}$ (102)
	Laser dissection	1185	$72.30 \pm 1.58^{d,y}$ (867)	$76.82 \pm 1.04^{d,x}$ (670)	$61.51 \pm 2.69^{c,x}$ (439)	$47.29 \pm 2.93^{b,x}$ (237)	$32.21 \pm 3.41^{a,xy}$ (106)
Total		3160	73.05 ± 1.12	76.82 ± 1.04	67.02 ± 1.45	46.79 ± 1.86	32.47 ± 2.26

^{abcd} Means with different superscripts in a row within a group were significantly different ($P<0.05$).
^{xyz} Means with different superscripts in a column within a group were significantly different ($P<0.05$).

In Table 4.56 shows the percent successful attachment of inner cell mass and consecutive passages of murine embryonic stem cell lines based on 3 different inner cell mass isolation techniques on fresh and frozen-thawed mouse embryonic fibroblasts. In fresh mouse embryonic fibroblast, there were no significant differences (P>0,05) for 3 different inner cell mass isolation techniques with the values of 79.05±2.54%, 78.80±1.21% and 75.89±1.48% for whole blastocyst culture, manual cut and laser dissection techniques, respectively. There were significantly different (P<0.05) between whole blastocyst culture and manual cut as well as laser dissection in frozen-thawed mouse embryonic fibroblast with the values of 52.39±1.95% versus 76.92±1.20% versus 74.51±1.86%, repsectively. There were no significant differences (P>0.05) at Passage 1 (P1) murine embryonic stem cell lines for both fresh and frozen-thawed mouse embryonic fibroblast for 3 different inner cell mass isolation techniques. However, there were significantly different (P<0.05) in inner cell mass attachment and primary inner cell mass outgrowth rates between whole blastocyst culture and manual cut as well as laser dissection in frozen-thawed mouse embryonic fibroblast whereby the laser dissection isolation techniques gave higher primary inner cell mass outgrowth rate (83.33±1.94%) followed by manual cut (78.16±1.70%) and whole blastocyst culture (71.69±2.24%) techniques in frozen-thawed mouse embryonic fibroblasts. Also, laser dissection technique gave the higher passages of murine embryonic stem cell lines at Passages 2 and 3 compared with manual cut and whole blastocyst culture techniques.

Figure 4.14 shows the development of murine embryonic stem cell from primary sub-cultured up to Passage 3 and embryonic stem cell colonies obtained. Differentiated murine embryonic stem cells with outgrowth of differentiated cells surrounding the inner cell mass is shown in Figure 4.15.

Table 4.56: Percent successful attachment of inner cell mass and consecutive passages of murine embryonic stem cell lines (%, mean ±SEM) based on 3 different inner cell mass isolation techniques on fresh and frozen-thawed mouse embryonic fibroblasts

MEF	ICM isolation techniques	No. of blastocysts	Percent ICM attachment (n)	Percent primary ICM outgrowth (n)	Percent successful consecutive passage (n)		
					P1 (n)	P2 (n)	P3 (n)
Fresh MEF	Whole blastocyst	971	81.05 ±11.10^{c,y} (777)	79.05 ±2.54^{c,x} (606)	75.34 ±3.34^{c,x} (444)	43.86 ±4.99^{b,x} (207)	22.86 ±4.31^{a,x} (71)
	Manual cut	725	80.60 ±1.37^{d,y} (583)	78.80 ±1.21^{d,x} (463)	72.36 ±1.46^{c,x} (340)	59.58 ±2.55^{b,y} (212)	39.36 ±3.37^{a,y} (96)
	Laser dissection	1183	73.24 ±1.54^{c,x} (862)	75.89 ±1.48^{c,x} (659)	70.58 ±1.74^{c,x} (459)	52.95 ±2.02^{b,y} (250)	38.77 ±2.81^{a,y} (107)
Total		2879	77.82 ±0.97	77.72 ±1.00	72.50 ±1.26	52.52 ±1.88	34.47 ±2.06
Frozen-thawed MEF	Whole blastocyst	1467	52.39 ±1.95^{a,x} (788)	71.69 ±2.24^{d,x} (561)	69.75 ±3.00^{d,x} (397)	39.44 ±4.15^{b,x} (173)	28.60 ±5.09^{a,x} (60)
	Manual cut	1337	76.92 ±1.20^{d,y} (1041)	78.16 ±1.70^{d,y} (832)	65.88 ±2.18^{c,x} (566)	48.77 ±2.49^{b,x} (333)	35.29 ±3.20^{a,x} (159)
	Laser dissection	1148	74.51 ±1.86^{c,y} (828)	83.33 ±1.94^{d,y} (616)	66.76 ±2.87^{bc,x} (384)	59.91 ±2.71^{b,y} (207)	40.42 ±5.16^{a,x} (87)
Total		3952	70.87 ±1.20	77.67 ±1.21	66.93 ±1.54	48.77 ±1.87	34.74 ±2.41

^{abcd} Means with different superscripts in a row within a group were significantly different (P<0.05).
^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

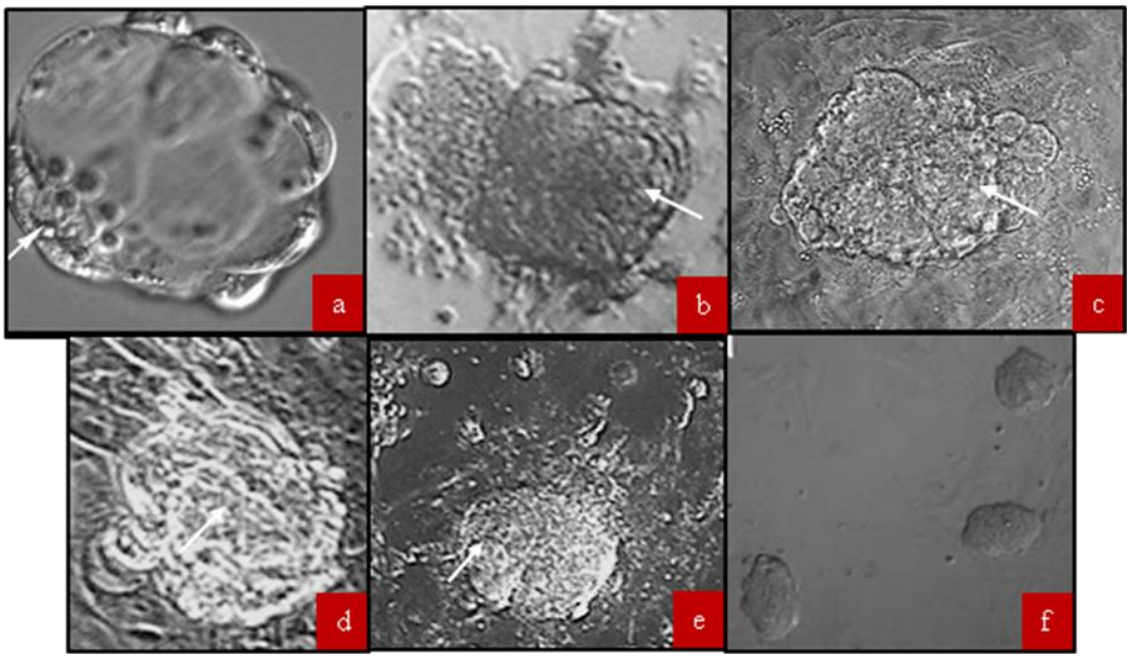

Figure 4.14: a) Blastocyst without the zona pellucida. b) Attachment and primary outgrowth of ICM at day 3. c) Primary ICM outgrowth was sub-cultured by 0.05% trypsin/EDTA (Passage 1). d) Passage 2 of murine embryonic stem cell was sub-cultured by manual dissociation before differentiation occurred. e) Undifferentiated murine embryonic stem cells at Passage 3 with the sharp and clear edge as well as dome-shape f) Embryonic stem cell colonies obtained. Arrow: inner mass (ICM).

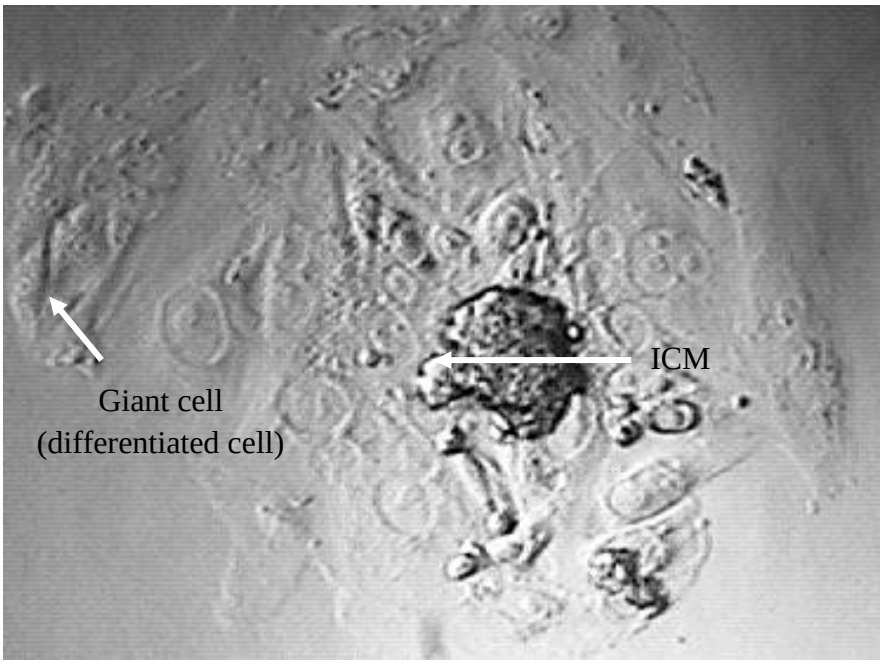

Figure 4.15: Differentiated murine embryonic stem cells with outgrowth of differentiated cells surrounding the inner cell mass.

4.4.2 Effects of Caprine Blastocyst Isolation Techniques, Culture Medium and Mouse Embryonic Fibroblasts Freezing on Production of Caprine Embryonic Stem Cell Lines

A total of 169 caprine blastocysts were used as a source of inner cell mass isolation for production of caprine embryonic stem cell lines. Percent attachment of caprine inner cell mass, primary inner cell mass outgrowth and successful consecutive passages caprine embryonic stem cell lines on whole blastocyst culture, manual cut and laser dissection techniques are shown in Table 4.57. There were no significant differences ($P>0.05$) in percent attachment of inner cell mass and primary inner cell mass outgrowth for 3 different inner cell mass isolation techniques with the values of $62.92 \pm 6.65\%$, $74.36 \pm 7.45\%$ and $74.00 \pm 7.25\%$ as well as $83.33 \pm 8.33\%$, $71.60 \pm 7.49\%$ and $80.44 \pm 7.68\%$, respectively. However, manual cut technique shows the highest percent attachment of inner cell mass, followed by laser dissection and whole blastocyst culture with the values of $74.36 \pm 7.45\%$, $74.00 \pm 7.25\%$ and $62.92 \pm 6.65\%$, respectively. In percent successful consecutive passages of caprine embryonic stem cell lines, manual cut technique always show the highest percent passages compared to laser dissection and whole blastocyst culture techniques. Morphology of primary outgrowth of caprine inner cell mass is shown in Figure 4.16.

Table 4.57: Percent attachment of inner cell mass, primary inner cell mass outgrowth and successful passages of caprine embryonic stem cell lines (%, mean ±SEM) on 3 different inner cell mass isolation techniques

Isolation techniques	No. of replicates	No. of blastocysts	Percent ICM attachment (n)	Percent primary ICM outgrowth (n)	Percent successful consecuitve passages (n)		
					P1 (n)	P2 (n)	P3 (n)
Whole blastocyst culture	8	26	$62.92 \pm 6.65^{bc,x}$ (16)	$83.33 \pm 8.33^{c,x}$ (13)	$39.58 \pm 12.96^{ab,x}$ (6)	$22.90 \pm 13.00^{ab,x}$ (3)	$12.50 \pm 12.50^{a,x}$ (1)
Manual cut	22	90	$74.36 \pm 7.45^{b,x}$ (79)	$71.60 \pm 7.49^{b,x}$ (68)	$71.28 \pm 9.00^{b,y}$ (59)	$46.44 \pm 9.19^{a,x}$ (43)	$35.04 \pm 9.05^{a,x}$ (25)
Laser dissection	15	53	$74.00 \pm 7.25^{cd,x}$ (42)	$80.44 \pm 7.68^{d,x}$ (34)	$43.89 \pm 7.39^{ab,xy}$ (19)	$30.00 \pm 6.10^{bc,x}$ (13)	$12.20 \pm 4.90^{a,x}$ (5)
Total	45	169	72.20 ± 4.50	76.63 ± 4.68	56.52 ± 5.85	45.30 ± 6.40	27.13 ± 5.91

^{abcd} Means with different superscripts in a row within a group were significantly different ($P<0.05$).
^{xy} Means with different superscripts in a column within a group were significantly different ($P<0.05$).

* Caprine blastocysts derived from both *in vivo* and *in vitro* sources of blastocyst.

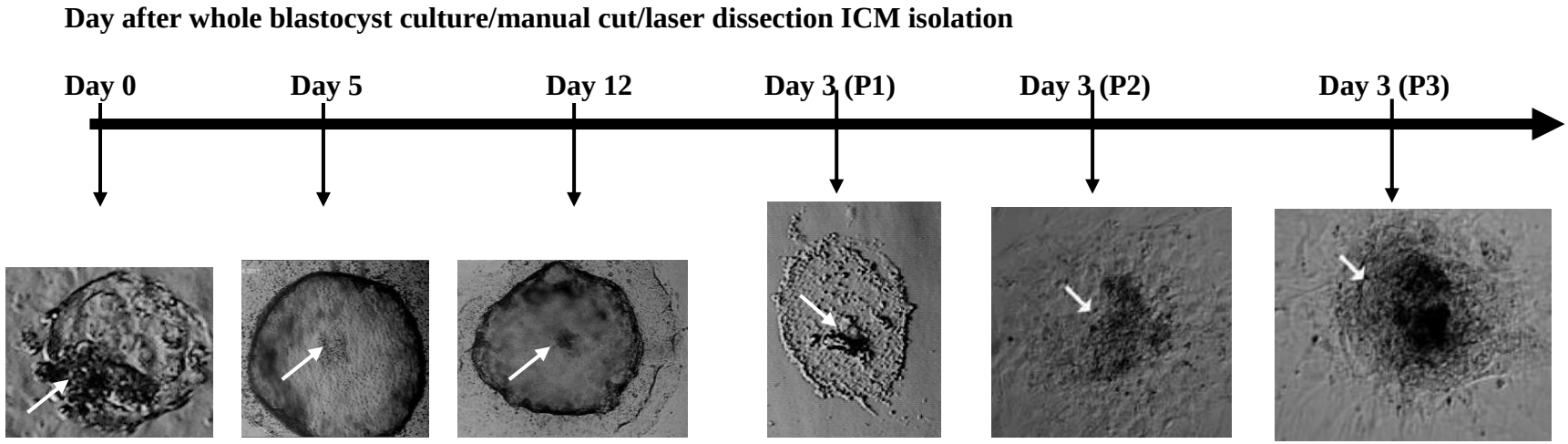

Figure 4.16: Morphology of primary outgrowth of caprine ICM development after (day 0 to day 12). On day 12, the primary ICM outgrowth was subcultured by 0.05% trypsin/EDTA but the later passages were performed by manual dissociation.

Table 4.58: Percent attachment of inner cell mass, primary inner cell mass outgrowth and successful consecutive passages of caprine embryonic stem cell lines (%, mean ±SEM) on 2 different culture media

Isolation techniques	No. of replicates	No. of blastocysts	Percent ICM attachment (n)	Percent primary ICM outgrowth (n)	Percent successful consecutive passages (n)		
					P1 (n)	P2 (n)	P3 (n)
DMEM+10% FBS	22	65	$79.02 \pm 7.01^{c,x}$ (55)	$82.12 \pm 6.57^{c,x}$ (48)	$67.65 \pm 7.86^{bc,x}$ (37)	$48.11 \pm 7.52^{ab,x}$ (23)	$32.58 \pm 9.14^{a,x}$ (12)
αMEM+10% FBS	23	104	$65.69 \pm 5.51^{bc,x}$ (82)	$71.39 \pm 6.60^{c,x}$ (67)	$45.87 \pm 8.18^{b,x}$ (47)	$42.61 \pm 10.39^{ab,x}$ (36)	$21.92 \pm 7.62^{a,x}$ (19)
Total	45	169	72.20 ±4.50	76.63 ±4.68	56.52 ±5.85	45.30 ±6.40	27.13 ±5.91

[abc] Means with different superscripts in a row within a group were significantly different (P<0.05).
[x] Means with same superscripts in a column within a group were significantly different (P>0.05).

Table 4.58 shows percent attachment of inner cell mass, primary inner cell mass outgrowth and successful consecutive passages of caprine embryonic stem cell lines on 2 different culture media, namely DMEM+10% FBS and αMEM+10% FBS. A total of 45 replicates were carried out which 22 replicates for DMEM+10% FBS and 23 replicates for αMEM+10% FBS. 169 total of caprine blastocyst were used to determine the quality of caprine embryonic stem cell lines in 2 different culture media. There were no significant differences (P>0.05) in percent attachment of inner cell mass, primary inner cell mass outgrowth, successful consecutive passages from Passage 1 up to Passage 3. However, DMEM+10% FBS culture medium gave higher percent attachment of inner cell mass, primary inner cell mass outgrowth, successful consecutive passages than αMEM+10% FBS culture medium.

In Tables 4.59, 65 and 104 number of caprine blastocyst were used for DMEM+10% FBS and αMEM+10% FBS in this experiment. Manual cut technique always show the highest percent attachment of blastocysts, primary outgrowth inner cell mass and successful consecutive passages caprine embryonic stem cell lines in both DMEM+10% FBS and αMEM+10% FBS culture media compared to whole blastocyst culture and laser dissection techniques. However, there were no significant differences (P>0.05) in percent attachment of inner cell mass, primary inner cell mass outgrowth, and consecutive passages caprine embryonic stem cell lines in both culture media.

Table 4.59: Percent attachment of inner cell mass, primary inner cell mass outgrowth and successful consecutive passages of caprine embryonic stem cells (%, mean ±SEM) on 2 different culture media using 3 different inner cell mass isolation techniques

Culture medium	No. of replicates	ICM isolation techniques	No. of blastocysts	Percent ICM attachment (n)	Percent primary ICM outgrowth (n)	Percent successful consecutive passages (n)		
						P1 (n)	P2 (n)	P3 (n)
DMEM+10%FBS	5	Whole blastocyst	25	60.67 ±10.98^{bc,x} (24)	93.33 ±6.67^{c,x} (22)	53.33 ±16.16^{b,x} (21)	30.00 ±20.00^{ab,x} (15)	0.00 ±0.00^{a,x} (11)
	10	Manual cut	17	87.50 ±10.03^{a,x} (10)	85.00 ±10.08^{a,x} (9)	88.00 ±9.98^{a,x} (5)	64.17 ±9.94^{a,x} (2)	66.67 ±12.91^{a,y} (0)
	7	Laser dissection	23	80.00 ±14.47^{c,x} (21)	70.00 ±13.97^{bc,x} (17)	48.81 ±12.66^{bc,x} (11)	38.10 ±101.0^{ab,x} (6)	7.14 ±7.14^{a,x} (1)
Total	22		65	79.02 ±7.01	82.12 ±6.57	67.65 ±7.86	48.11 ±7.52	32.58 ±9.14
αMEM+10%FBS	3	Whole blastocyst	65	66.67 ±0.00^{a,x} (55)	66.67 ±16.67^{a,x} (46)	16.67 ±16.67^{a,x} (38)	33.33 ±33.33^{a,x} (28)	33.33 ±33.33^{a,x} (14)
	12	Manual cut	9	63.40 ±10.00^{b,x} (6)	60.44 ±10.11^{b,x} (4)	57.35 ±13.29^{b,x} (1)	31.67 ±13.59^{ab,x} (1)	8.68 ±5.86^{a,x} (1)
	8	Laser dissection	30	68.75 ±5.62^{ab,x} (30)	89.58 ±7.00^{b,x} (17)	39.58 ±8.87^{a,x} (8)	62.50 ±18.30^{ab,x} (7)	37.50 ±15.67^{a,x} (4)
Total	23		104	65.69 ±5.51	71.39 ±6.60	45.87 ±8.18	42.61 ±10.39	21.92 ±7.62

^{abc} Means with different superscripts in a row within a group were significantly different (P<0.05).
^{x} Means with same superscripts in a column within a group were significantly different (P>0.05).

Table 4.60 shows the percent attachment of inner cell mass, primary inner cell mass outgrowth and successful consecutive passages of caprine embryonic stem cell lines on fresh and frozen-thawed mouse embryonic fibroblast. A total of 169 number of blastocysts were used in fresh (98) and frozen-thawed (71) mouse embryonic fibroblast in this experiment. There were no significant differences ($P>0.05$) in percent attachment of inner cell mass, primary inner cell mass outgrowth, successful consecutive passages of caprine embryonic stem cell lines for fresh and frozen-thawed mouse embryonic fibroblast. However, fresh mouse embryonic fibroblast gave higher percent attachment of inner cell mass, primary inner cell mass outgrowth and successful consecutive passages up to Passage 3 (P3) than frozen-thawed mouse embryonic fibroblast with the values of 79.53±6.38% versus 63.04±5.7%, 80.27±6.09% versus 72.10±7.31%, 63.53±7.99% versus 47.75±8.37%, 46.33±7.45% versus 44.00±11.20% and 28.67±8.31% versus 25.21±8.54%, respectively.

Table 4.60: Percent attachment of inner cell mass, primary inner cell mass outgrowth and successful consecutive passages of caprine embryonic stem cell lines (%, mean ±SEM) on fresh and frozen-thawed mouse embryonic fibroblasts

MEF	No. of replicates	No. of blastocysts	Percent ICM attachment (n)	Percent primary ICM outgrowth (n)	Percent successful consecutive passages (n)		
					P1 (n)	P2 (n)	P3 (n)
Fresh MEF	20	98	79.53 ±6.38^{c,x} (77)	80.27 ±6.09^{c,x} (64)	63.53 ±7.99^{bc,x} (46)	46.33 ±7.45^{ab,x} (35)	28.67 ±8.31^{a,x} (19)
Frozen-thawed MEF	25	71	63.04 ±5.76^{bc,x} (60)	72.10 ±7.31^{c,x} (51)	47.75 ±8.37^{abc,x} (38)	44.00 ±11.20^{ab,x} (24)	25.21 ±8.54^{a,x} (12)
Total	45	169	72.20 ±4.50	76.63 ±4.68	56.52 ±5.85	45.30 ±6.40	27.13 ±5.91

^{abc} Means with different superscripts in a row within a group were significantly different (P<0.05).
^{x} Means with same superscripts in a column within a group were significantly different (P>0.05).

A total of 45 replicates have been carried with 20 replicates for fresh mouse embryonic fibroblast and 25 replicates for frozen-thawed mouse embryonic fibroblasts in this experiment as shown in Table 4.60. Out of 9 (whole blastocyst culture), 59 (manual cut) and 30 (laser dissection) number of caprine blastocyst have been cultured on fresh mouse embryonic fibroblast (Table 4.61). There were no significant differences ($P>0.05$) in percent attachment of inner cell mass and primary caprine inner cell mass outgrowth on fresh mouse embryonic fibroblast for 3 different inner cell mass isolation with represented values of $66.67 \pm 0.00\%$ versus $56.76 \pm 11.89\%$ versus $68.75 \pm 5.62\%$ for percent attachment of caprine blatocysts and $65.67 \pm 16.67\%$ versus $56.36 \pm 12.74\%$ versus $59.58 \pm 7.00\%$ for primary outgrowth of caprine inner cell mass, respectively. There were also no significant differences ($P>0.05$) in percent attachment of inner cell mass, primary inner cell mass outgrowth and passage caprine embryonic stem cell lines up to Passage 2 on frozen-thawed mouse embryonic fibroblast cultured for 3 different inner cell mass isolation techniques.

Table 4.61: Percent successful attachment of inner cell mass, primary inner cell mass outgrowth and consecutive passages of caprine embryonic stem cells (%, mean ±SEM) derived from 3 different inner cell mass isolation techniques using 5 different blastocyst stages

MEF	No. of replicates	ICM isolation techniques	No. of blastocysts	Percent ICM attachment (n)	Percent primary ICM outgrowth (n)	Percent successful consecutive passages (n)		
						P1 (n)	P2 (n)	P3 (n)
Fresh MEF	3	Whole blastocyst	9	66.67 ±0.00^{a,x} (6)	65.67 ±16.67^{a,x} (4)	46.67 ±16.67^{a,x} (1)	33.33 ±33.33^{a,x} (1)	33.33 ±33.33^{a,x} (1)
	9	Manual cut	59	56.76 ±11.89^{b,x} (50)	56.36 ±12.74^{b,x} (43)	55.36 ±14.11^{b,y} (37)	31.11 ±15.67^{ab,x} (27)	11.57 ±7.66^{a,x} (14)
	8	Laser dissection	30	68.75 ±5.62^{ab,x} (21)	59.58 ±7.00^{b,x} (17)	39.58 ±8.87^{a,xy} (8)	62.50 ±18.30^{ab,x} (7)	37.50 ±15.67^{a,x} (4)
Total	20		98	79.53 ±6.38	80.27 ±6.09	63.53 ±7.99	46.33 ±7.45	28.67 ±8.31
Frozen-thawed MEF	5	Whole blastocyst	17	70.67 ±10.98^{c,x} (10)	63.33 ±6.67^{bc,x} (9)	53.33 ±16.16^{b,x} (5)	30.00 ±20.00^{ab,x} (2)	0.00 ±0.00^{a,x} (0)
	13	Manual cut	31	86.54 ±8.31^{b,x} (29)	80.77 ±8.62^{ab,x} (25)	75.38 ±12.02^{ab,x} (22)	57.05 ±10.67^{ab,x} (16)	51.28 ±12.72^{a,y} (11)
	7	Laser dissection	23	80.00 ±14.47^{c,x} (21)	70.00 ±13.97^{bc,x} (17)	48.81 ±12.66^{bc,x} (11)	38.10 ±10.10^{ab,x} (6)	7.14 ±7.14^{a,x} (1)
Total	25		71	63.04 ±5.76	72.10 ±7.31	47.75 ±8.37	44.00 ±11.20	25.21 ±8.54

^{abc} Means with different superscripts in a row within a group were significantly different (P<0.05).
^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

Comparison between *in vivo* and *in vitro* caprine blastocysts sources are shown in Table 4.62. There was highly significant differences ($P<0.05$) between *in vivo* and *in vitro* derived blastocyst sources in percent caprine embryonic stem cell lines Passages 1 and 3 with the values of $95.00 \pm 5.00\%$ versus $52.76 \pm 6.10\%$ and $79.20 \pm 12.50\%$ versus $20.83 \pm 5.53\%$, respectively. There were highly significant differences ($P<0.05$) between *in vivo*-derived blastocysts and *in vitro*-derived blastocysts whereby the percent attachment of inner cell mass, primary inner cell mass outgrowth and consecutive caprine embryonic stem cell lines passages were higher in the former with the values of $100.00 \pm 0.00\%$ versus $69.49 \pm 4.73\%$, $87.50 \pm 7.98\%$ versus $75.57 \pm 5.06\%$, $95.00 \pm 5.00\%$ versus $52.76 \pm 6.10\%$, $81.25 \pm 11.97\%$ versus $41.79 \pm 6.70\%$ and $79.20 \pm 12.50\%$ versus $20.83 \pm 5.53\%$, respectively.

Table 4.62: Percent successful attachment of inner cell mass and consecutive passages of caprine embryonic stem cells (%, mean ±SEM) derived from *in vivo* and *in vitro* sources of blastocyst

Treatment	No. of replicates	No. of blastocysts	Percent ICM attachment (n)	Percent primary ICM outgrowth (n)	Percent successful consecutive passages (n)		
					P1 (n)	P2 (n)	P3 (n)
In vivo	4	13	100.00 ±0.00^{a,x} (13)	87.50 ±7.98^{a,x} (11)	95.00 ±5.00^{a,y} (10)	81.25 ±11.97^{a,x} (8)	79.20 ±12.50^{a,y} (7)
*In vitro**	41	156	69.49 ±4.73^{c,x} (124)	75.57 ±5.06^{c,x} (104)	52.76 ±6.10^{b,x} (74)	41.79 ±6.70^{b,x} (51)	20.83 ±5.53^{a,x} (24)
Total	45	169	72.20 ±4.50	76.63 ±4.68	56.52 ±5.85	45.30 ±6.40	34.74 ±2.41

^{abc} Means with different superscripts in a row within a group were significantly different (P<0.05).
^{xy} Means with different superscripts in a column within a group were significantly different (P<0.05).

**In vitro* included somatic cell nuclear transfer and parthenogenesis treatments.

4.5 CONFIRMATION OF CAPRINE AND MURINE EMBRYONIC STEM CELLS BY IMMUNOFLUORESCENT STAINING PROTEIN MARKERS (EXPERIMENT 5)

4.5.1 Confirmation of Murine Embryonic Stem Cells by Specific Embryonic Stem Cell Markers

Embryonic stem cells protein markers (immunofluorescent staining) were used in determining the embryonic stem cells formation. Specific embryonic stem cell markers were found in murine embryonic stem cells such as Oct 4 (green) and SSEA 1(green) in this experiment. There were no colour shown for TRA-1-60 and TRA-1-81 embryonic stem cell markers in murine species (Figure 4.17). Therefore, it indicated the presence of embryonic stem cells in murine species. Alkaline phosphatase staining was carried out to determine the alkaline phosphatase activities that found in the murine embryonic stem cell lines. Purplish colour was shown in murine embryonic stem cells after underwent alkaline phosphatase staining. It confirmed the presence of true murine embryonic stem cells which showed alkaline phosphatase activities.

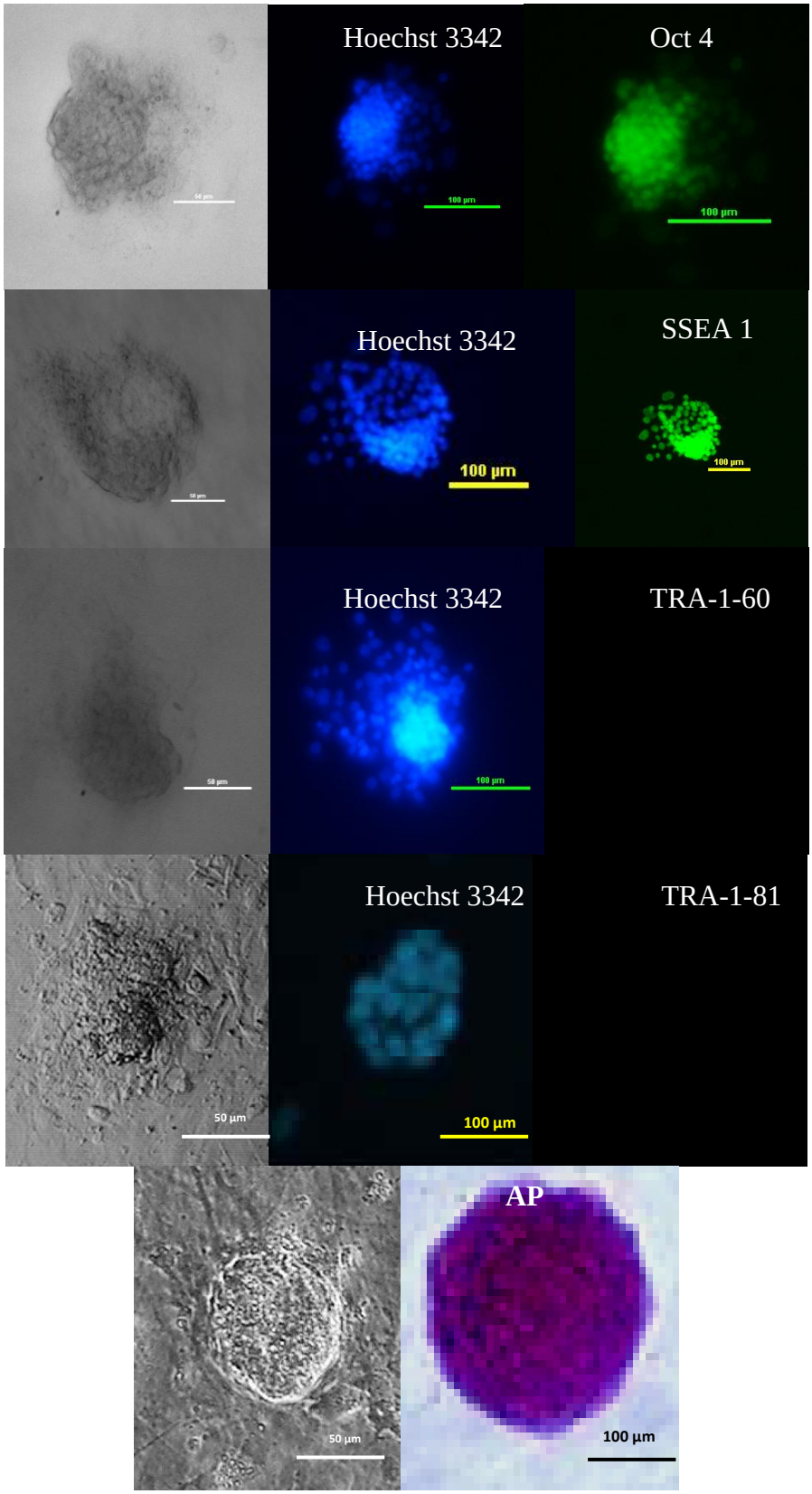
Hoechst 3342
Oct 4
100 µm
Hoechst 3342
SSEA 1
100 µm
100 µm
Hoechst 3342
TRA-1-60
100 µm
Hoechst 3342
TRA-1-81
50 µm
100 µm
AP
50 µm
100 µm

Figure 4.17: Murine embryonic stem cells were confirmed by the expression of murine specific embryonic stem cell markers (Oct 4, SSEA 1) and human embryonic stem cell specific markers (TRA-1-60 and TRA-1-81) as negative control. Transmission light images and Hoechst DNA staining were showed in the first and second column. The alkaline phosphatase activities were positive and showed in the bottom line of the picture.

4.5.2 Confirmation of Caprine Embryonic Stem Cells by Specific Embryonic Stem Cell Markers

Embryonic stem cell markers used in caprine embryonic stem cells in this experiment were Oct 4 (green) and SSEA 1(green) (Figure 4.18). It indicates the presence of embryonic stem cells in caprine species. Alkaline phosphatase staining was carried out to determine the alkaline phosphatase activities that found in the caprine embryonic stem cell lines. Purplish colour was shown in caprine embryonic stem cells after underwent alkaline phosphatase staining. It was confirmed that by alkaline phosphatase activities indicating the presence of caprine embryonic stem cells.

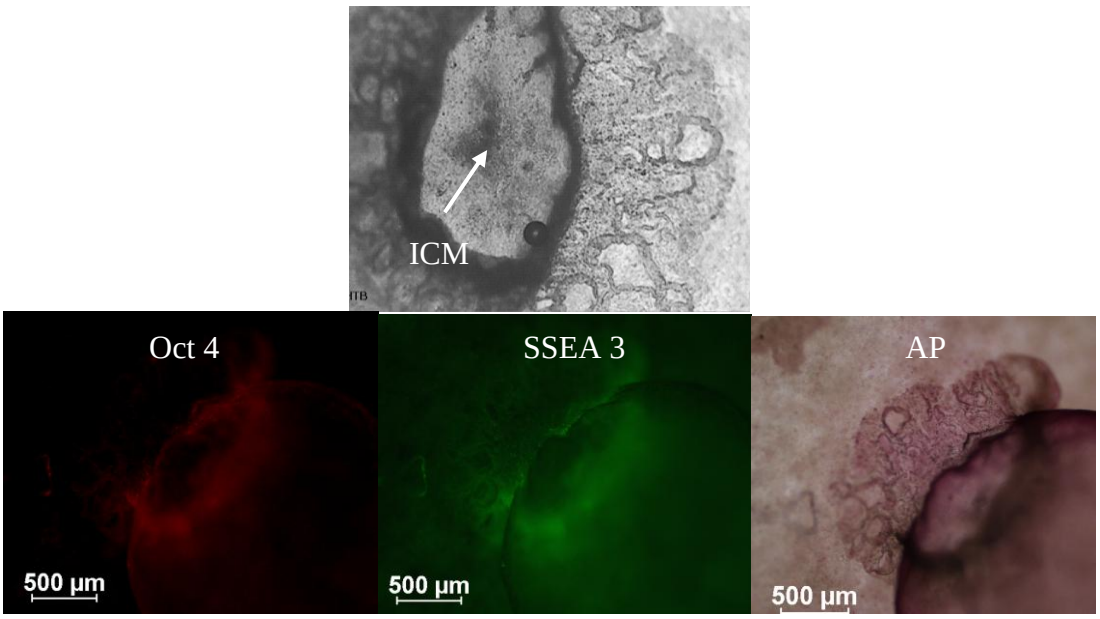

Figure 4.18: Caprine embryonic stem cells were confirmed by the expression of caprine specific embryonic stem cell markers that is Oct 4 (red) and SSEA3 (green). The alkaline phosphatase activities were shown in caprine embryonic stem cells which gave purplish colour for alkaline phosphatase staining.

Table 4.63: Summarised results in comparison between murine and caprine embryonic stem cells

Pluripotent markers	Murine embryonic stem cells	Caprine embryonic stem cells
AP	+	+
SSEA-1	+	-
SSEA-3	-	+
SSEA-4	-	-
TRA-1-60	-	-
TRA-1-81	-	-
Oct-4	+	+
Feeder cell	Need	Need
Cytokine factor control self-renew	LIF and some growth factors that work through GP130 receptor	LIF and some growth factors that work through GP130 receptor
Morphology of undifferentiated colony	Multi layers clump, dome shape, clear edge	Mono layer clump, loose, flat colony

Chapter 5

5.0 DISCUSSION

Chapter 5

5.0 DISCUSSION

5.1 ESTABLISHMENT OF MOUSE EMBRYONIC FIBROBLAST AS FEEDER CELLS FOR PRODUCTION OF MURINE EMBRYONIC STEM CELL LINES (EXPERIMENT 1)

In order to produce murine embryonic stem cells (mESC), source from a large number of blastocysts is very important. In this experiment, 3 sub-topics are discussed including a) effects of murine pure-strain on superovulation responses, b) effects of murine source on the percent cleavage for the production of blastocyst (such as *in vivo* oviduct or uterine flushing; *in vitro*-derived blastocyst through somatic cell nuclear transfer) and c) effects of 3 different pure-strain of murine for mouse embryonic fibroblast (MEF) cell lines on murine embryonic stem cell lines performance by looking into freezing of mouse embryonic fibroblast (P1 and P2), mouse embryonic fibroblast passages numbers (P0, P1, P2 and P3) and culture media (DMEM and αMEM).

5.1.1 Effects of Murine Pure-strain on Superovulation Responses

Superovulation was required to obtain a large number of blastocysts for embryonic stem cell (ESC) culture. Ideal strain of murine should be the one that produces a large amount of normal oocytes or embryos after superovulation which will successfully develop to the blastocysts stage. Sato *et al*. (2007) reported that 2 different strains of murine (ICR and BDF) were superovulated with large amount gonadotrophins to induce maturation of murine oocytes. The impact of exogenous stimulation with increasing amounts of gonadotrophins on the number and quality of oocytes per pre-embryos recovered from outbred BALB/c murine was investigated by Ozgunen *et al*. (2001). By

selecting the suitable strain of murine, embryonic stem cell lines can be produced more efficiently. A poor choice of strain will result in low efficiency and quality of embryonic stem cell lines derived, thus wasting resources. Superovulated mature females were found to be lower responders than were superovulated prepubescent murine (Ozgunen *et al*., 2001).

In present study, the performance of superovulation using pregnant mare's serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) treatments in 3 pure-strains murine (ICR, CBA/ca and C57BL/6J) gave the percentages of successful superovulation ranging from 65-77% ($P>0.05$). This is in agreement with the findings of Goh and Abdullah (2008). However, the success rates of superovulation of 3 strains were considered low when compared to previous findings in ABEL laboratory (Goh and Abdullah, 2008). Superovulation with hormones is widely used to increase the number of embryos per female, consequently reduces the number of murine used in experiments. However, the efficacy of exogenous stimulation varies with the strain, age, nutritional status and health, breeding-housing conditions of a female murine, and the dose of administered gonadotrophins (Licht *et al.*, 1979; Hogan *et al*., 1986). In present study, the possible factors that could be explained for the reduction in the superovulation response could be due to poor health condition and significant death of the murine that might be related to the unavoidable transfer of murine to a new animal house. Other factors could be the efficacy of new batch of gonadotrophins used that was often resulted in superovulation responses variation and the intraperitoneal gonadotrophin injection technique as well as unskillful embryo flushing by the personnel (Ainul Bahiyah *et al.*, 2010).

In addition, oestrous cycle of the murine during which the gonadotrophin injection is made may affect the success rate of superovulation. In present study, injections of gonadotrophins were done without taking into account the oestrous stage

of the murine. Some researchers reported no successful superovulation when pregnant mare's serum gonadotrophin was injected during the dioestrous stage of murine (Edwards and Fowler, 1960); while others have reported success but with lower number of embryos (Redina *et al.*, 1994). Both findings agree that the oestrous cycle stage of the murine has significant influence affecting the success rate of superovulation and suggest the optimum time of gonadotrophin injection is during the dioestrous stage.

Age of the murine also is one of the factors that determine the success of superovulation. As reported in the literature, the recommended age for superovulation usually falls between 3 and 5 weeks during the prepubescent stage to produce the highest number of oocytes (Hogan *et al*., 1986). At the other end of the spectrum, using older murine, especially above 10 weeks of age may produce lower amount of embryos.

Another factor affecting the performance of superovulation in murine is the strain or genetic background of the species. Ozgunen *et al*. (2001) strongly suggested that very high doses of hormone must be avoided in the prepubescent BALB/c murine in order to prevent increased numbers of degenerated or parthenogenetic oocytes. Therefore, these factors that influence the number and quality of superovulated oocytes must be optimised for any given strain of murine. For example, both CBA/ca and C57BL/6J murine were classified by Hogan *et al.* (1986) as high ovulators, while other strains such as A/J and BALB/cJ were considered low ovulators. This may due to the variations in the different murine strains have different natural gonadotrophin levels and number of ovarian luteinising hormone (LH) receptors available for stimulation (DeLeon *et al.*, 1990).

Although there were no significant differences ($P>0.05$) between number of 2-cell embryos and blastocyts per female, the number of 2-cell stage embryos and blastocyts embryos obtained per female were highest in CBA/ca, followed by ICR and C57BL/6J. This may be attributed to both the quality of the oocytes and also the quality

of sperm from the male studs of each murine strain. There were significant differences ($P<0.05$) in number of oocytes for ICR and CBA/ca with C57BL/6J. This may be due to the strains or genetic background whereby, for example, BALB/c females were reported to be a difficult strain to work with in superovulation (Hogan *et al*., 1986). The results of this study showed that C57BL/6J is less suitable as a source of blastocysts due to the lowest rate of superovulation compared to other strains and lowest number of 2-cell embryos as well as blastocysts obtained. CBA/ca may provide blastocysts of better quality as high number of blastocysts obtained.

The number superovulated oocytes varies depending on strain among inbred murine, however, sufficient numbers of oocytes and embryos for practical and research use could be easily obtained. Researchers often encounter difficulty obtaining sufficient numbers of oocytes and embryos from laboratory rats due to their limited responses to superovulatory treatment. Various methods, such as combination of pregnant mare's serum gonadotrophin-human chorionic gonadotrophin injection regime used in murine (Zarrow and Wilson, 1961; Wilson and Zarrow, 1962; Miller and Armstrong, 1981a, 1981b; Mukumoto *et al*., 1995; Corbin and McCabe, 2002; Sotomaru *et al*., 2005; Kagabu and Umezu, 2006), continuous infusion of the gonadotrophin (Armstrong and Opavsky, 1988) and anti-inhibin in antiserum treatment (Ishigame *et al*., 2004), have been shown to induce superovulation in rats. Among these methods, combination of pregnant mare's serum gonadotrophin-human chorionic gonadotrophin injection regime seems to be the most practical and economical method of inducing superovulation of cloned animals. Therefore, the combination of pregnant mare's serum gonadotrophin and human chorionic gonadotrophin was used in this study to induce superovulation, as recommended by Hogan *et al.* (1986).

5.1.2 Effect of Murine Sources on the Percent Cleavage for the Production of Blastocysts

The sources of murine blastocyst for murine embryonic stem cell lines production could be obtained from either *in vivo* or *in vitro*-derived blastocysts. In the present study, *in vivo*-derived blastocysts were obtained from *in vivo* oviduct after flushing embryos at 2-cell stage or *in vivo* uterine flushing to obtain blastocysts. As for *in vitro*-derived blastocysts were obtained via somatic cell nuclear transfer (SCNT). There were no significant differences ($P>0.05$) among the 3 different pure-strains of murine. However, the blastocyst rate obtained in ICR was the highest followed by CBA/ca and C57BL/6J. When comparing the rate of blastocysts formation in both *in vivo* oviduct and *in vivo* uterine flushing, C57BL/6J showed the lowest rate compared to other strains. One possible explanation is that the health of murine has been compromised, which led to non-response to gonadotrophin treatment and lower blastocysts formation. This may be due to the higher humidity in the animal house at the ISB Mini farm. Also, there were times whereby the pellet was out of stock due to failure of the supplier to deliver the pellet on time, thus causing minor starvation on murine. Therefore, to overcome this problem, pellet feed should be available at all time to ensure the murine are not under starvation.

Although the number of blastocysts obtained from *in vivo*-derived blastocysts were higher than *in vitro*-derived blastocysts, the C57BL/6J showed the lowest rate of blastocysts formation in *in vivo* oviduct or uterine flushing compare to other murine strains; on the contrary, C57BL/6J gave the highest hatched blastocyst rate formation through *in vitro*-derived blastocysts by somatic cell nuclear transfer techniques compared to other murine strains. This could be due to an increased incidence of rare imprinting disorders associated with assisted reproduction technologies (ARTs) such as somatic cell nuclear transfer (Sato *et al.*, 2007). In normal embryo development,

genomic imprinting, which describes the allele-specific expression of certain genes, accounts for the requirement of both maternal and paternal genomes (Ohlsson *et al*., 1998; Reik and Walter, 1998; Surani, 1998; Tilghman, 1999). In the murine, the sex-specific epigenetic modifications are imposed during gametogenesis and act as markers to distinguish the maternal and paternal alleles (Surani, 1998). Allele-specific DNA methylation has been observed in the vicinity of most imprinted genes in somatic cells (Reik and Walter, 1998; Paulsen and Ferguson-Smith, 2001) when cloning was carried out. Thus, *in vitro*-derived blastocysts rate in C57BL/6J is higher than *in vivo*-derived blastocysts which could be due to imprinting gene and DNA methylation mechanism occurred in the former. Besides that, superovulation has been shown to have a negative effect on the embryos, causing an increase in incidence of chromosome abnormality in blastocyst for different strains of murine (Fujimoto *et al.*, 1974). The ability of the donor cell to support development has been shown to be affected by the murine strains (Wakayama and Yanagimachi, 2001).

For *in vitro*-derived blastocysts via somatic cell nuclear transfer, the effects of pre-intracytoplasmic injection (pre-ICI) duration have been evaluated. There were highly significant differences ($P<0.05$) in pre-intracytoplasmic injection durations treatments at 90 minutes, whereby it gave the highest cleavage rate for all 3 different pure-strains murine compared to simultaneous injection as well as 30, 60 and 120 minutes pre- intracytoplasmic injection durations. Whereas, simultaneous injection right after enucleation showed the lowest cleavage rates in ICR, CBA/ca and C57BL/6J. According to Wakayama *et al*. (1998), enucleated oocytes were incubated in incubator before injection of donor nuclei to restore the oocyte viability due to the sensitivity of the oocytes and can easily lyse just after enucleation. Therefore, pre-intracytoplasmic injection duration for 90 minutes seemed to be more suitable for somatic cell nuclear

transfer in murine which was significantly higher compared to other pre-intracytoplasmic durations.

5.1.3 Effect of 3 Different Pure-strains of Murine for Mouse Embryonic Fibroblast Cell Lines on Murine Embryonic Stem Cell Lines Performance

Mouse embryonic fiborblasts have been used reliably as feeder cell layer for murine embryonic stem cells since the early 1980s when the first murine embryonic stem cell lines were being derived and cultivated. Mouse embryonic fiborblast cells are isolated from murine embryos at day 14 of gestation as described by Robertson (1987). Conventionally, mouse embryonic fibroblast serve as feeder cells in embroynic stem cell culture (Evans and Kaufman, 1981; Thomson *et al*., 1998; Smith, 2001; Turksen, 2002). The specific role of mouse embryonic fiborblast in prolonging embryonic stem cell culture is less clear. It is generally known that mouse embryonic fibroblast that provides a befitting environment for the interplay of signaling networks that regulate the fate of embryonic stem cells (Xu *et al*., 2001; Lim and Bodnar, 2002). In this experiment, we discussed the suitable strain of murine to be used to derive mouse embryonic fibroblast, based on their growth rate. The mouse embryonic fibroblasts showed similar growth rates regardless whether they were Passages 1 and 2, fresh or frozen-thawed, Dulbecco's Modified Eagle's Medium (DMEM) or Alpha Minimum Essential Medium Eagle (αMEM). This finding is very important as it shows that there were no differences in term of the quality of mouse embryonic fiborblasts. Thus, they all could be used for embryonic stem cell culture. However, the mouse embryonic fibroblast were not passaged to higher numbers which only up to Passage 2 because there will be a significant reduction in quality and increase in senescence at more passages number (C. Lorthongpanich, personal communication) which is not good for embryonic stem cell culture.

In the present study, there were no significant differences (P>0.05) in the growth rate of mouse embryonic fiborblast derived from ICR, CBA/ca and C57BL/6J. Therefore, 3 different pure-strains of murine also could be used as feeder cell layers for production of murine embryonic stem cell lines. However, CAB/ca gave the lowest growth rate for Passage 1 frozen-thawed mouse embryonic fiborblast compared to ICR and C57BL/6J. According to Kawase *et al*. (1994), different strains is a possible factor as it has been shown that the same cell type derived from different strains of murine has different degree of sensitivity to trypsinisation. Therefore, this finding suggested that mouse embryonic fiborblasts from the CBA/ca strain may be more sensitive to trypsinisation compared to ICR and C57BL/6J strains, and thus causing their lower growth rate. Thus, CBA/ca may be less suitable as a source to derived mouse embryonic fibroblasts.

For mouse embryonic fibroblast culture medium, the foetal bovine serum (FBS) is commonly used as an essential supplement to cell culture media. Foetal bovine serum is a cocktail of most of the factors required for cell attachment, growth, and proliferation *in vitro* (Gstraunthaler, 2003). There were no significant differences (P>0.0.5) between DMEM+10%FBS and αMEM+10%FBS culture media for fresh mouse embryonic fibroblast cells at Passage 0 and Passage 2 as well as frozen-thawed mouse embryonic fiborblast cells at Passages 1 and 2. Therefore, both DMEM+10%FBS and αMEM+10%FBS culture media could be used in culturing the mouse embryonic fibroblast cell lines. However, the DMEM+10% FBS culture medium gave higher growth rate than αMEM+10%FBS culture medium. This could be due to Dulbecco's Modified Eagle's Medium culture medium contains a 4-fold higher concentration of amino acids and vitamins, as well as additional iron (ferric nitrate) compared to Alpha Minimum Essential Medium Eagle culture medium. Dulbecco's Modified Eagle's Medium was developed in 1969 and is a modification of Basal

Medium Eagle (BME) that contains 1000 mg/L of glucose and was first reported for culturing murine embryonic stem cells. Therefore, apparently DMEM+10% FBS is more suitable culture medium for culturing mouse embryonic fibroblasts.

5.2 PRODUCTION OF CAPRINE BLASTOCYSTS AS A SOURCE OF INNER CELL MASS FOR THE ESTABLISHMENT OF CAPRINE EMBRYONIC STEM CELL LINES (EXPERIMENT 2)

The aims of this experiment were (a) to compare the efficiency and quality of embryonic stem cells growth from *in vitro* or *in vivo* derived caprine blastocysts, (b) to compare the squeezing and laser enucleation techniques in deriving the blasotcysts as source for caprine embryonic stem cells production, (c) to compare the grades of caprine oocytes in deriving of caprine blastocysts and (c) to compare the pre-intracytoplasmic injection durations for simultaneous, 30 and 60 minutes in production of high caprine blastocysts. In addition, bovine somatic cell nuclear transfer and parthenogenesis to produce blastocysts as a comparative study (control) to caprine species were included in this experiment.

5.2.1 Production of Bovine Blastocysts through Somatic Cell Nuclear Transfer and Parthenogenesis (Control)

Recent evolutions of somatic cloning by nuclear transfer are reported, especially in the bovine species where potential applications are underway for biomedicine in association with transgenesis, or for agriculture by improving livestock productivity. The overall efficiency of this biotechnology remains low in terms of viable offspring, but significant progress has been achieved on the different steps of the technique. In this experiment, bovine species was used as a comparative study (control) to caprine species due to lack of caprine sample as we need a lot of sample numbers to carry out fundamental

experiments for each treatment as well as getting more consistent and accurate results before it could be applied into caprine species. In other words, with the more sample number from the bovine species, it could be practiced more on the somatic cell nuclear transfer techniques such as squeezing and laser enucleation techniques, injection technique and modification of culture medium to be applied in the caprine species. Bovine species could be used as comparative preliminary studies and learning curve for our laboratory to improve the techniques and modified culture conditions and media in caprine species.

Somatic cell nuclear transfer in bovine species has been successfully applied by Cibelli *et al*. (1998). Since 1998 when the first calves derived from somatic cell nuclear transfer were born (Cibelli *et al*., 1998; Kato *et al*., 1998), many experiments of bovine somatic cell nuclear transfer have been performed (Thongphakdee *et al*., 2008; Yang *et al*., 2008). In nearly all of these experiments, oocytes used for cloning were obtained from the ovaries of slaughtered bovine so that genetic background of the oocytes was usually unknown.

One of the major steps involved in the nuclear transfer procedure is the removal of genetic material from the recipient oocyte (enucleation). It is clear that this procedure also removes important cytoplasmic components which may reduce cytoplast viability. In routine nuclear transfer procedures, one third or more of the ooplasm is frequently removed that would presumably result in a corresponding decrease in the total cell number of cloned blastocysts (S.Y. Goh, unpublished data). This reduced cell number may contribute to the decreased viability of cloned embryos (Zakhartchenko *et al*., 1997). Zakhartchenko *et al*. (1997) reported that the volume of enucleation is critical to the development of embryonic cell nuclear transfer. Peura *et al*. (1998) reported that embryos containing 150% of the original oocyte volume would improve the embryonic development. However, how much to be removed during nuclear transfer and its

removal mechanism are still not clear. Successful enucleation is important for nuclear transfer. A less harmful, more productive, fast, and reliable enucleation technology for somatic cell nuclear transfer would allow more efficient production of embryos for transfer. At present, various enucleation methods have been reported, which include blind enucleation (Kubota, 2000), Hoechst 33342 induced enucleation (Tsunoda *et al*., 1988), chemical enucleation (Russell *et al*., 2005) and Demecolcine induced oocyte enucleation (Ibanez *et al*., 2003; Li *et al*., 2007).

In this study, we compared 2 different approaches to enucleate oocytes, namely squeezing and laser enucleation techniques in order to obtain high production of bovine blastocysts. Embryos development at various stages were significantly higher in laser enucleation technique compared to squeezing enucleation technique for 2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst. Also, no cloned blastocyst was obtained by using squeezing techniques. According to Lee *et al*. (2008), in the squeezing method, the zona pellucida was partially dissected and a small amount of cytoplasm containing metaphase II (MII) chromosomes and the first polar body (PB) were pushed out, and this method took much longer duration compared to laser technique. For laser enucleation techniques, the used of laser in enucleation of porcine oocytes was shown to be easier to operate (V.T. Nguyen, personal communication). Although expensive, the laser technique gave the highest results in somatic cell nuclear transfer in bovine species in the present study.

The cleavage rate of bovine embryos based on oocytes grading regardless of enucleation techniques are shown to have the highest maturation (49.77±3.21%), enucleation (84.34±3.12%) and injection (86.73±3.03%) rates of Grade A followed by Grades B, C, D and E. Similarly, cleavage rates for each developmental stage decreased as the grades of oocytes decreased. The present results indicate that good quality oocytes surrounded by multi-layers of compact investment with a homogenous ooplasm

(Grade A, B) had significantly higher cleavage and development rates up to the hatched blastocyst stage compared to those of fair or poor quality oocytes (Grades C,D and E). This finding signifies the essential role of cumulus cells in promoting normal cytoplasmic maturation of oocyte necessary for fertilisation, cleavage and subsequent development of cloned embryos. It has been reported that cumulus cells are a sub-population of granulosa cells that surround the oocyte by providing nutrients and signals that regulate its growth and maturation (Eppig, 1991; Picton *et al*., 1998; Tanghe *et al*., 2002). Furthermore, oocyte quality is determined by various criteria such as the degree of expansion, number of cumulus cell layers and texture of the ooplasm, where all these criteria were found to have significant effects on the rates of maturation, fertilisation and cleavage *in vitro* (Tanghe *et al*., 2002). This could be observed in the present study, where the oocytes with the most cumulus cell layers with more than 5 layers (Grade A) had the highest cleavage rates up to hatched blastocysts. Whereas, embryos derived from oocytes with 3 to 4 layers of cumulus cells but with homogenous cytoplasm (Grade B) also developed up to hatched blastocyst similar to those with 5 layers of cumulus cells (Grade A). However, the percentage of cleaved embryos was significantly lower in the latter.

The present results also indicate that oocytes from Grade C were less competent than Grades A and B. This may be due to the fewer layers of cumulus cells may possible come from smaller or more atretic follicles than those with more layers (Martino *et al*., 1995). Thus, oocytes derived from smaller follicles may require a longer culture time for *in vitro* maturation (Qian *et al*., 2001). Oocytes that lacked cumulus cells (Grade D) had negatively affected cleavage rates. It has been shown in several species that removal of cumulus cells had negatively affected oocyte maturation and development (Zhang *et al*., 1995; Cecconi *et al*., 1996; Ka *et al*., 1997; Goud *et al*.,

1998). However, clarifications of the physiological mechanism that underline these observations require further studies.

It is noteworthy that, albeit a limited number in the present study, some embryos originating from Grades D and E were able to cleaved and develop to the blastocyst stage. This indicated that some oocytes lacking of cumulus cells and showing evidence of degeneration may retain developmental capacity.

There were no significant differences ($P>0.05$) between squeezing and laser enucleation techniques for Grades B, C, D and E, except Grade A where the laser percent enucleation was higher than squeezing enucleation. Also, there were significant differences ($P<0.05$) in percent injection between squeezing and laser enucleation for Grade A bovine oocytes. The possible reason for this phenomenon could not be explained in the present study and needs future research in the future.

It was clearly evident shown that laser enucleation technique gave the highest cleavage rate for all the grades compared to squeezing enucleation technique from 2-cell stage embryos up to hatched blastocysts. The probable reasons are laser enucleation technique is easier and less time consuming compared to squeezing technique, where the latter needs to pierce a slit or hole on zona pellucida by cutting needle and then pressed out the DNA of oocyte through the piercing hole. By pressing the oocytes, it might damage the chromosome of the enucleated oocyte and this would affect the oocytes development in future. Therefore, using laser enucleation technique was the preferred to prevent the damage of chromosome in the enucleated oocyte without pressing through the chromosome of the oocytes. Thus, the laser enucleation technique could be applied in caprine species to obtain higher blastocyst formation rate for embryonic stem cell lines production.

In the present study, we compared the embryo developmental rates between somatic cell nuclear transfer and parthenogenesis. There were significant differences

($P<0.05$) between parthenogenesis and somatic stem cell nuclear transfer, where the parthenogenesis showed higher cleavage rates than somatic cell nuclear transfer technique. Replacement of the somatic cell nuclear transfer with other alternatives is important for obtaining more sources of blastocyst for production of embryonic stem cells. The establishment of embryonic stem cells by parthenogenesis has not been reported, except for one case in primates (Cibelli *et al*., 2002). The parthenogenetic activation of oocytes is important in cloning research, as artificial activation of oocytes is an essential component of nucleus transfer protocols. Optimised activation protocols could enhance complete reprogramming of reconstructed embryos, which may increase success rates in cloning. Furthermore, parthenogenetic activation represents a valid tool to mimic the fertilisation Ca^{2+} transients and oscillation in nucleus transplantation experiments and to investigate the comparative roles of paternal and maternal genomes in controlling early embryonic development.

Parthenogenesis of bovine oocytes can be induced with an electrical pulse (Collas *et al*., 1993; Prochazka *et al*., 1993), ethanol (Nagai, 1987; Fukui and Sawai, 1992; Presicce and Yang, 1994), calcium ionophore A23187 (Ware *et al*., 1989; Liu *et al*., 1998), cycloheximide (CHX) (Presicce and Yang, 1994; Saeki *et al*., 1997), 1,4,5-inositol triphosphate (Ahn *et al*., 2001), ionomycin (Susko-Parrish *et al*, 1994; Winger *et al*., 1997), or strontium (Meo *et al*., 2004). Activation with ethanol and N-6 dimethylaminopurine (6-DMAP) combined could induce not only a transient inactivation of maturation promoting factor (MPF) and a single Ca^{2+} rise but also a persistent inhibition of maturation promoting factor (Susko-Parrish *et al*, 1994; Liu *et al*., 1998). Therefore, in the current study, activation with calcium ionophore (CaI) plus N-6 dimethylaminopurine resulted in higher cleavage and blastocyst developmental rates in bovine oocytes species. In bovine, depending on the activation method, percentages of oocyte activation varies from 29% to 52% (Suttner *et al*., 2000) and this

is similar to the results obtained in this study. The different methods of parthenogenetic activation used to date attempt to induce these reactions directly or indirectly. In fact, oocytes could be activated by agents that induce a rise in the intracellular concentration of calcium (Ca^{2+}), either by enabling the entrance of this cation from the external medium (Kaufman, 1983; Onodera and Tsunoda, 1989) or by the action of specific ionophores that liberate calcium from intracellular stores (Steinhardt *et al.*, 1974; Vincent and Johnson, 1992). Embryo reconstitution by nuclear transfer depends upon a number of important technical and biological variables such as oocyte quality, enucleation and cell transfer procedures, and oocyte activation. Ultimately, the nucleus of the donor somatic cell must render itself to be reprogrammed by the oocyte, which in turn must be capable to induce all the epigenetic changes to down-regulate somatic genes and up-regulate embryonic ones. Therefore, the cleavage rates of parthenogenesis were highly significant different ($P<0.05$) compared to somatic cell nuclear transfer.

5.2.2 Production of Caprine Blastocysts Through Somatic Cell Nuclear Transfer and Parthenogenesis (Control)

In this experiment, we discussed in details on the effects of a) oocyte source, b) oocyte quality, c) enucleation technique, d) pre-intracytoplasmic injection duration, e) parthenogenesis versus somatic cell nuclear transfer and f) pregnant mare's serum gonadotrophin and human chorionic gonadotrophin treatment on *in vivo* uterine flushing derived blastocysts on percent caprine blastocyst formation as a source for production of caprine embryonic stem cell lines.

Cleavage rates of caprine embryos through somatic cell nuclear transfer by 3 different sources of oocytes, namely laparoscopic ovum pick-up (LOPU), ovariectomy and abattoir were carried out in present study. There were significant differences ($P<0.05$) in maturation and enucleation rates for these 3 different sources. Even though

insignificance in embryo developmental, the embryo development at various stages was apparently higher in laparoscopic ovum pick-up, followed by ovariectomy and abattoir. However, ovariectomy showed the highest blastocysts and hatched blastocysts rates, followed by laparoscopic ovum pick-up and abattoir.

In this experiment, ovaries that were surgically ovariectomised consistently provided greater follicle surface exposure for oocyte retrieval that could be due to the ability of more clearly visualised follicles on the ovaries (Cognie, 1999). Slicing of ovary from ovariectomy or abattoir sources was performed as an alternative protocol due to unavoidable circumstances such as repeated low response of old donor caprine to hyperstimualtion after repeated oocyte retrieval cycles or physically abnormal ovary such as adhesion, polycystic ovary syndrome or degenerative ovary or small follicular diameter of caprine ovaries. In regards to small follicular (diameter between 2.5 to 3.0 mm), it was necessary to recover the oocytes by slicing the ovarian surface to yield high number of oocytes (Martino *et al*., 1994). Although caprine showing low reproductive capabilities were usually ovariectomised for ovarian slicing as most of them might be non-responsive to hormone stimulation, more oocytes will be obtained via ovariectomy and chances to develop and blastocyst formation is higher compared to laparoscopic ovum pick-up oocyte source.

In the present study, total number of oocytes retrieved was 641 and 487 from slicing and laparoscopic ovum pick-up techniques, respectively. It is generally agreed that the ovarian slicing method yields higher number of oocyte. This is agreement with Keskintepe (1994) who also obtained higher results of oocytes by surgically removing the ovaries of caprine donor. Martino *et al*. (1994) obtained more oocytes from ovarian mincing or slicing than follicular dissection or aspiration in caprine donor. The reasons for higher ooccyte yield may be attributed to the presence of some follicles embedded deeply within the ovarian cortex, which could only be released by slicing of the ovary.

Additionally, unlike laparoscopic ovum pick-up, the slicing protocol was done under visual observation regardless of ovarian follicle size; therefore, ensuring that more oocytes were retrieved.

However, collections of oocytes after exposure of the ovary by laparotomy or ovariectomy has been progressively replaced by laparoscopic techniques in small ruminants including caprine (Rahman, 2008). The low time consumption and repeatable cycle on the same animal showed that laparoscopic ovum pick-up is relatively a simple and efficient technique compared to slicing method (Baldassarre *et al*., 2002, Koeman *et al*., 2003). Since the embryo development at 2-, 4-, 8-cell and morula stages was apparently higher from laparoscopic ovum pick-up as shown in the present study, it was suggested that repeated laparoscopic ovum pick-up performed on common caprine genotypes in Malaysia either prepubertal or pubertal caprine after hyperstimulation treatment is a valuable tool for the synchronisation of follicular growth and retrieval of component oocytes. The impact of oocyte retrieval methods on the quantity and quality of oocytes retrieved is believed to be of major important. Laparoscopic ovum pick-up technique in combination with *in vitro*-produced of cloned-caprine embryos has the potential to improve the number of hatched blastocyst obtained. The laparoscopic ovum pick-up can be repeated several times without ovarian damage or decrease in the donors' fertility (McKelvey *et al*., 1986; Stangl *et al*., 1999; Alberio *et al*., 2002; Pierson *et al*., 2004). Additionally, compared with oocytes sourced from abattoir ovaries, laparoscopic ovum pick-up allows one to source the oocytes from live animals of known health status, which is very important for the production of therapeutic proteins for pharmaceutical applications. Laparoscopic ovum pick-up technique already allows field application in commercial programmes (Hasler *et al*., 1995). In addition, this method can be applied to prepubertal animals and thereby could contribute to a considerable shortening of the generation interval. However, *in vitro* developmental

competence of these oocytes from various qualitites obtained from different factors needs to be evaluated in further study.

Regardless the sources of oocyte retrieved, there were no significant differences ($P>0.05$) in percent cleavage at Grades A, B and C for 2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst. However, Grades A, B and C showed better cleavage rates than Grades D and E for all stages of embryo development. The data obtained in the present experiment indicated that Grade A oocytes are highly competent in terms of maturation and subsequent embryo development compared with Grades B and C. The Grade D oocytes (no layers of cumulus cells (denuded) with evenly granulated cytoplasm) and E (pale-coloured or heterogeneous cytoplasm) were least competent. The roles of cumulus cells in the acquisition of full developmental competence of the oocytes have been investigated earlier (Leibfried-Rutledge and First, 1976; Sato *et al*., 1977; Xu *et al*., 1986). Visual assessment of morphology of cumulus cells investment surrounding an oocyte remains the most important criteria for selection of developmentally competent oocytes during recovery (Shioya *et al*., 1988; Madison *et al*., 1992; Lonergan *et al*., 1994). It was suggested that earlier that expanded cumulus cells investment mature and good quality oocytes, while compact cumulus cells characterises immature oocytes (Veeck, 1988).

In regard to enucleation techniques, laser enucleation gave highest maturation, enucleation and injection rates for Grades B, D and E, except for Grades A and C in overall. Several enucleation methods have been published (Willadsen *et al*., 1986) and most rely on the removal of a karyoplast via micromanipulation in combination with a DNA fluorescent stain to confirm positive enucleation (Westhusin, 1992). This technique has improved enucleation and development as compared with enucleation by blind oocyte biopsy (Willadsen, 1986), but it has limitations associated with the time required for the production of large numbers of nuclear transfer embryos through

micromanipulation. However, enucleation is a major limiting step in the nuclear transfer procedure. The successful and efficient removal or the destruction of genetic material in the recipient oocyte is dependent upon the technique used. Staining by places the oocyte under stresses associated with exposure to Hoechst 33342 and ultra-violet irradiation (Smith, 1993), which may also affect development. In earlier reports, germinal vesicle enucleation was performed rather 'indirectly' by increasing the pressure inside a holding pipette to expel a germinal vesicle karyoplast (nucleus) through a slit made in the zona (Sun *et al*., 1991). The zona pellucida was penetrated by pressing a glass micro-needle tangentially into the perivitelline space against the holding pipette also known as squeezing enucleation technique.

Similar as previous findings in bovine species, we compared the squeezing and laser enucleation techniques in caprine oocytes. Same results were shown where most of the laser enucleation technique gave the highest maturation, enucleation and injection rates compared with squeezing enucleation techniques for all grades of oocytes, except Grade A. These most probably due to the laser technique that have been master the skills rather than using squeezing enucleation technique in the previous learning curve. There were obviously significant differences ($P<0.05$) of embryos development at the later stages cloned embryos (morula, blastocyst and hatched blastocyst) where the laser enucleation techniques gave significantly higher percent cleavage rates compared with squeezing enucleation technique as shown on Grade A. This could be due to maternal zygotic transition occurred where the onset of maternal zygotic transcription is species dependent such as murine 1 to 2-cell stage (Bolton *et al*., 1984); bovine, caprine and ovine 8 to 16 (Calarco and McClaren, 1976; Camous *et al*., 1986) and Xenopus approximately 4000 (Newport and Kirschner, 1982). Therefore, the caprine species at later stages were passed over the maternal zygotic transcription and reach the stability

on the cleavage rates in order to obtain good quality of blastocysts compared to early stage of embryos (2-, 4- and 8-cell).

In cloned embryos, reprogramming usually occurs aberrantly. Incomplete reprogramming may contribute to the low efficiency of cloning. Global epigenetic reprogramming of somatic nuclei is mainly in most preimplantation cloned embryos (Dean *et al*., 2001). Therefore, correct epigenetic reprogramming is necessary for successful and normal developmental of clones (Dean *et al*., 2001). The low success rate could be caused by an incomplete reprogramming of the epigenetic state of nuclei from differentiated donor cells. It is easy to understand that genes permanently switched off in the course of cell differentiation may be hard to reactivate efficiently. The incomplete reactivation of Oct 4 may account for some failures of nuclear transfer embryo development (Boiani *et al*., 2002; Bortvin *et al*., 2003). Most of cloning used an unfertilised and mature oocyte as recipient, reprogramming occurred within the brief interval between the transfer of the donor nucleus into the enucleated oocyte and here the start of zygotic transcription. Thus, pre-zygotic modifications (any modifications that have occurred before the mature oocyte stage) are expected to be less efficiently reprogrammed than post-zygotic modifications.

For pre-intracytoplasmic injection durations, it was vividly shown that for each embryo developmental stages from 2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst the cleavage rates were significantly higher ($P<0.05$) for 60 minutes followed by simultaneous injection and 30 minutes pre-intracytoplasmic injection durations. Similar as previous findings in murine species, the caprine enucleated oocytes need time to restore the oocyte viability due to sensitivity of the oocytes by incubated in incubator before injection of donor nuclear. Simultaneous injection right after enucleation could cause the lower oocyte viability as it could easily lyse just after enucleation. This is agreement with Wakayama *et al*. (1998) findings. Therefore, pre-

intracytoplasmic injection duration for 60 minutes seemed to be more suitable for somatic cell nuclear transfer in caprine speceis which was significantly higher compared to other pre-intracytoplasmic injection durations.

Although insignificance in comparison between parthenogenesis and somatic cell nuclear transfer in present experiment, the percent cleavage of somatic cell nuclear transfer were apparently higher than parthenogenesis for all the stages, except blastocyst. This could be explained in maternal zygotic transcription occurred in parthenogenesis start at 8- to 16-cell stages in caprine species (Calarco and McClaren, 1976; Camous *et al*., 1986).

Besides *in vitro*-derived caprine blastocysts as source for producing caprine embryonic stem cells, *in vivo*-derived caprine blastocyst also carried out in this experiment in order to obtain more number of blastocsyst to produce good quality of caprine embryonic stem cell lines. Two different hormones are used in superovulated to obtain more number of blastocysts, namely pregnant mare's serum gonadotrophin and follicle stimulating hormone. There were obviously significant differences in number of blastocyst obtained between pregnant mare's serum gonadotrophin and follicle-stimulating hormone treatments. By using the follicle stimulating hormone, it gave the highest number of caprine blastocysts (27) compared with pregnant mare's serum gonadotrophin (0). Superovulation in caprine is frequently restricted by the cost of gonadotropin or the handling requirements. In this situation pregnant mare's serum gonadotrophin has the advantage of a lower cost and single dose protocol, but the variability of response obtained restricts its use. Partial replacement of follicle-stimulating hormone with pregnant mare's serum gonadotrophin at the end of treatment also did not compromise the number of embryos collected. This variability is even more marked when the gonadotropin used to induce superovulation is pregnant mare's serum gonadotrophin (Dieleman, 1993), rather than follicle stimulating hormone. The same

observation has been made in caprine (Armstrong *et al*., 1983; Pendleton *et al*., 1992). However, pregnant mare's serum gonadotrophin provides important economical and labour advantages over follicle stimulating hormone (Alfuraji *et al*., 1993), which justifies its choice in some circumstances. Use of pregnant mare's serum gonadotrophin substantially reduces the cost of hormones and labour involved (Alfuraji *et al*., 1993), and the administration in a single dose may represent another advantage over follicle stimulating hormone, since the stress caused by repeated handling contributes to poor reproductive results in some breeds (Fenandez-Arias *et al*., 1997). However, these advantages are eclipsed by reported variable responses to pregnant mare's serum gonadotrophin, with a high number of unfertilised ova, low ovulation rate, high number of large follicles, and an apparent increase in the incidence of premature luteal regression (Armstrong *et al*., 1983, Pendleton *et al*., 1992). Present study showed the more ovulated number in follicle stimulating hormone (65) than pregnant mare's serum gonadotrophin (12). Our results also confirm how follicle stimulating hormone provides a more constant ovarian response in caprine variations in follicle stimulating hormone protocols suggest that dose of follicle stimulating hormone plays a determinant role in superovulatory response.

5.2.3 Comparison Between Caprine and Bovine Species on Production of Blastocysts Through Somatic Cell Nuclear Transfer and Parthenogenesis

Among domestic mammals, successful nuclear transfer has also been reported in caprine (Yong *et al*., 1991). In present results, there were significant differences ($P<0.05$) in maturation, enucleation and injection rates between caprine and bovine species. Also, percent maturation, enucleation and injection in caprine were apparently higher than bovine species for all the grades of oocyte. This might be due to the sources of oocyte retrieved where part of the caprine oocytes retrieved from laparoscopic ovum

pick-up which provide more high quality of oocytes compared with bovine species where the oocytes obtained only from abattoir source with unknown history or background.

There were obviously significant differences (P<0.0.5) between caprine and bovine in percent embryos development for 2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst where caprine species gave higher cleavage rates compared with bovine species. One of the most prominent developments, particularly in bovine embryo cloning, has been the use of *in vitro* matured oocytes to prepare large numbers of recipient cytoplasms (Yang *et al*., 1993). However, the introduction of blastomere nuclei into enucleated metaphase II oocytes results in poor development, probably due to damage induced by premature chromosome condensation caused by high levels of maturation promoting factor activity in such cytoplasts (Szollosi *et al*., 1988; Collas and Robl, 1991) as the genetic background of the bovine species from abattoir source is unknown. Therefore, the efficiency of the trials with clones is dependent on the heritability of the trait under study. The use of clones is more appropriate if the trait has a high degree of heritability and if a high technical expenditure is required per day and animal in the trial.

In the present experiment, there were significant differences (P<0.05) in percent enucleation and injection between caprine and bovine at Grades A and C. Thus, the quality and cost of recipient oocytes are important in the economics of cloning. The use of *in vitro* matured oocytes has a number of advantages as compared to *in vivo* derived. Firstly, the use of slaughterhouse derived material has an obvious cost advantage allowing the collection of large numbers of oocytes. However, more importantly the use of *in vitro* derived oocytes provides greater control over the maturation process allowing collection of oocytes at defined stages during maturation. This results in a

greater consistency of oocyte quality for animal production and further research of oocyte reprogramming activities.

There were significant differences ($P<0.05$) between caprine and bovine species in percent enucleation for squeezing and laser enucleation techniques where the percent enucleation were higher in the former compared to the latter in both enucleation techniques. Caprine species always showed higher percent cleavage than bovine species for both enucleation techniques. Caprine has emerged as an excellent model system for studies involving reproductive physiology, endocrinology, and virology (Marini *et al*., 2002). Although, many researchers used bovine species as model in somatic cell nuclear transfer research, the unknown genetic background of the bovine oocyte sources caused abnormalities in cloned embryos obtained. Therefore, the source and genetic background of oocytes are significant important to prevent abnormal embryos development. In comparing to caprine species, it is one of the most widely studied animal models of human influenza infection (Pearson and Gorham, 1998) and is also an ideal choice for modeling genetic lung diseases such as cystic fibrosis (CF) (Li and Engelhardt, 2003). In part, this is due to the remarkable similarity between caprine and human lung cell biology (Mercer *et al*., 1994; Choi *et al*., 2000; Wang *et al*., 2001). Moreover, the caprine has a 42 day gestation period and reaches sexual maturity in 4 to 5 months (Fox and Bell, 1998), making it one of the more rapidly reproducing species for animal modeling by somatic cell nuclear transfer. The successful production of viable caprine progeny following somatic cell nuclear transfer provides exciting new opportunities for basic research, investigating early embryogenesis and the propagation of endangered black-footed caprine and European minks. Given the relatively short total reproductive cycle of the caprine, with a gestation period of 42 days and 4 to 5 months to sexual maturity (Fox *et al*., 2002), the caprine represents an attractive animal model for genetic manipulation in conjunction with somatic cell nuclear transfer.

To be used as cytoplast recipients for somatic cell nuclear transfer, the genetic material must be removed from the oocytes. In general, this has been achieved by aspiration of the metaphase II spindle using a small glass pipette, so called enucleation (Li *et al*., 2004b). Not only does this technique require significant skill but the process also removes a variable portion of the oocyte cytoplasm which may affect subsequent development. To simplify the process and reduce the volume of cytoplasm removed various modifications to the enucleation technique have been developed. In most species, the metaphase plate of metaphase II oocytes is not visible by light microscopy due to the presence of cytoplasmic lipid. Enucleation has been achieved by so called "blind enucleation" using the first polar body as a marker for the location of the metaphase II plate. Generally, the first polar body and a small volume of cytoplasm (10 to15%) located below first polar body are removed by squeezing them out. The disadvantages of squeezing enucleation technique were in many cases the metaphase plate is not close to first polar body. The removal of cumulus cells prior to oocyte manipulation can further disrupt the relationship between the metaphase II spindle and first polar body. These factors could result in a proportion of the oocytes containing residual DNA following enucleation (Li *et al*., 2004c). In fact it has been reported that less than 50% of metaphase plates are located beneath first polar body in bovine (Nour and Takahashi., 1999). A second problem is that up to one-third of recipient cytoplasm just beneath the first polar body is generally squeezed out in order to improve enucleation efficiency. The removal of such a large proportion of the oocyte cytoplasm may result in the oocyte having a reduced capacity for epigenetic reprogramming of the transferred nucleus and subsequent development. Therefore, laser enucleation technique without disrupt the relationship between the metaphase II and first polar body and small volume of cytoplasm were removed were more advantageous compared with squeezing

enucleation technique. Thus, most of the cleavage values were significantly higher in laser enucleation technique for both species in the present study.

5.3 EFFECTS OF STRAINS, CULTURE MEDIUM AND MOUSE EMBRYONIC FIBROBLAST FREEZING ON PRODUCTION OF MURINE EMBRYONIC STEM CELL LINES USING WHOLE BLASTOCYST CULTURE TECHNIQUE (EXPERIMENT 3)

In the present experiment, we discussed about the effects of a) murine strains, b) culture medium and c) fresh and frozen-thawed mouse embryonic fibroblast on production of murine embryonic stem cell lines performance by using whole blastocyst culture technique. Also, 5 different stages of murine blastocyst have been studied, namely early-, mid-, expanded-, hatching- and hatched-blastocyst to produce good quality of murine embryonic stem cell lines.

5.3.1 Effects of Murine Strains on Production of Murine Embryonic Stem Cells Lines Using Whole Blastocyst Culture Technique

The effects of strain and blastocyst stage on the efficiency of deriving murine embryonic stem cell lines were discussed in this experiment. Different success rates of embryonic stem cells derived from different strains of murine were reported by Kawase *et al*. (1994) and Bryja *et al.* (2006). Our results have shown that there were significant differences between CBA/ca and ICR with C57BL/6J, whereby murine embryonic stem cells derived from CBA/ca strain had higher success rate of passaging up to Passage 3 compared with ICR and C57BL/6J. Although the difference is not significant, it suggests that CBA/ca strain may be more suitable for deriving murine embryonic stem cells.

Previously, it was found that blastocysts from C57BL/6J and CBA/ca have an efficiency of deriving embryonic stem cells of 58% and 66% (Schoonjans *et al.*, 2003) which was considerably higher than the findings of this study. However, it should be noted that in their study, only 12 blastocysts were used for each strain, whereas in this study 124 and 129 blastocysts from C57BL/6J and CBA/ca were used. No data was found on the performance of blastocysts derived from ICR strain. The overall low efficiency in deriving murine embryonic stem cells compared to other researchers may be attributed to the use of 35mm culture dishes instead of multi-well plates which provide greater contact between the blastocysts and feeder cells (Williams *et al.*, 1988; Bryja *et al.*, 2006). In general, only a few strains of murine have been used in the establishment of germ line competent embryonic stem cell lines (McWhir *et al*., 1996). Although refinements in conditions have resulted in improved efficiencies, very few lines have been established in inbred strains other than the 129 strain (Schoonjans *et al*., 2003; Cheng *et al*., 2004). In strains that are considered non-permissive, pluripotent cells within the inner cell mass may be more susceptible to differentiation signals. McWhir and co-workers selectively destroy the differentiating cells to obtain embryonic stem cell lines from the CBA/ca mouse strain from which no embryonic stem cell lines had previously been established.

Comparison among the blastocyst stages were shown in Table 4.56, whereby expanded blastocysts showed a significantly higher success rate of reaching Passage 3 (17.24±3.67%) compared to early (9.18±2.98%) and hatched blastocysts (8.08±2.94%). The low success rate of deriving embryonic stem cells from hatched blastocysts is in agreement with the findings of Movaassagh-Pour *et al.* (2003). No reports were found comparing the 5 different blastocyst stages. The factors behind the differences in efficiency are poorly understood. A possible explanation could be derived by looking into the events taking place during the development of blastocysts from early to hatched

stage. In early blastocysts, the inner cell mass and trophectoderm have not fully separated. Therefore, when cultured on the feeder cell layer, the consecutive outgrowth will have a high mixture of both inner cell mass and trophectoderm derived cells. The interaction among the cells possibly makes the embryonic stem cells more likely to differentiate. Moreover, hatched blastocysts have inner cell mass that were already beginning to differentiate into the epiblast. This could be supported by the fact that Oct 4 expression in blastocysts decreases sharply in at 4.5 days post coitum (Kehler *et al.*, 2004). Oct 4 expression is crucial in maintaining the the pluripotency and undifferentiated state in embryonic stem cells (Masui *et al.*, 2007). Thus, embryonic stem cells derived from them may have a higher tendency to differentiate.

By comparing each strain and blastocyst stage combination, expanded blastocyst stage always showed the highest percent successful attachment of blastocysts and consecutive passages up to Passage 3 throughout the 3 different pure-strains of murine compared to other blastocyst stages. Meanwhile, the success rate was very low in early and hatched blastocysts for all the murine strains in this finding. In summary, embryonic stem cells could be successfully derived from different strains of murine and blastocyst stages. Blastocysts from the CBA/ca strain, especially expanded blastocysts were the most efficient for deriving murine embryonic stem cell lines, whereas early and hatched blastocysts were not suitable for deriving murine embryonic stem cell lines.

5.3.2 Effect of Culture Media on Production of Murine Embryonic Stem Cell Lines Using Whole Blastocyst Culture Technique

In the present study, there were no significant differences in percent attachment of blastocysts and successful consecutive passages of murine embryonic stem cell lines for both DMEM+10%FBS and αMEM+10% FBS culture media. However, the DMEM+10% FBS culture medium showed higher percent successful attachment of blastocysts and consecutive passages of murine embryonic stem cell lines than αMEM+10% FBS culture medium. An important element affecting the efficiency of deriving embryonic stem cells is the composition of the culture medium. Most of the researchers used Dulbecco's Modified Eagle's Medium for culturing embryonic stem cells rather than Alpha Minimum Essential Medium Eagle. Dulbecco's Modified Eagle's Medium with 10 to 20% foetal bovine serum has been used since the early days of murine embryonic stem cell culture (Evans and Kaufman, 1981; Tesar, 2005). Lately, Knockout Serum Replacement (SR) is frequently used to substitute foetal bovine serum and have shown significantly better results (Wakayama *et al.*, 2007; Doungpunta *et al.*, 2009). Another chemical often added is leukaemia inhibitory factor, which helps to further maintain the embryonic stem cells in an undifferentiated state. Their addition has also increased efficiency (Schoonjans *et al.*, 2003). Some researchers' findings reported that one possible factor is the use of embryonic stem cells culture media conditioned with rabbit fibroblast cell lines which may have increased the amount of leukaemia inhibitory factor and other support factors in the culture medium, thus improving efficiency (Schoonjans *et al.*, 2003). Other modifications to the culture medium include pre-conditioning the culture medium on fibroblast (Schoonjans *et al.*, 2003) and addition of 6-bromoindirubin-3'-oxime (BIO) which activates the Wnt signalling pathway involved in the maintenance of pluripotency (Sato *et al.*, 2004; Doungpunta *et al.*, 2009). Similar findings also obtained for the combination of

different culture media (DMEM and αMEM) with the 5 different blastocyst stages, we found that most expanded blastocysts gave the highest percent attachment of blastocysts and successful consecutive passages of murine embryonic stem cell lines up to Passage 3 compared with other blastocyst stages for both culture media used.

5.3.3 Effect of Fresh and Frozen-thawed Mouse Embryonic Fibroblast on Production of Murine Embryonic Stem Cell Lines Using Whole Blastocyst Culture Technique

Furthermore, there were no significant differences ($P>0.05$) between fresh and frozen-thawed mouse embryonic fibroblast for percent successful attachment of blastocysts, consecutive passages of murine embryonic stem cell up to Passage 3. However, there was slightly decreasing in the growth rate of mouse embryonic fibroblasts after frozen-thawed. Nevertheless, as the difference in small, the reduction in quality of mouse embryonic fibroblast should be little as well, thus both can still be used as feeder cells. The present results showed that majority of expanded blastocysts gave the highest percent attachment of blastocysts and successful consecutive passages in both fresh and frozen-thawed mouse embryonic fibroblast. Freezing of mouse embryonic fibroblast when they have reached the confluent state in culture have been shown to result in higher cell viability after thawing (Buck *et al.*, 1981). The cell density during freezing also affects the viability rate (Freshney, 1994). Therefore, the thawing step significantly affects the viability and quality of cells, especially in regards to the thawing rate (Freshney, 1994). It has been reported that the centrifugation time and speed after thawing has an effect on cell viability (Cheng *et al.*, 2004). The rate of dilution (addition of culture medium to the cell suspension) also influences the viability.

In general, cryopreservation is the cooling of samples to low sub-zero temperatures for long term storage (Shaw and Jones, 2003). The reasons for the freezing of mouse embryonic fibroblast cells were the inevitable senescence of cells, possible

contamination after prolonged cultured, discard the need to maintain the cell lines in continuous culture and allow the cells to be distributed to other users (Freshney, 1994). Cells are typically cooled to a low temperature and finally stored in liquid nitrogen (LN_2), which has a temperature of -196℃ (Freshney, 1994). Despite the inevitable cellular damage, thawed and subsequently cultured cells exhibit rapid regeneration (Pereira and Marques, 2008). Thus, with the right methods, cells can be stored for later use and revived effectively. The period of equilibration usually completes once the temperature reaches -80℃, whereby the cells and then transferred to lower temperatures for storage (Shaw and Jones, 2003). During this period, water leaves the cells (dehydration), thus minimising intracellular ice crystallisation and reducing cryogenic damage from the highly concentrated solutes (Freshney, 1994). Low concentrations of cryoprotectant are used, thus minimising their chemical toxicity and osmotic shock (Pereira and Marques, 2008). Nevertheless, due to their low concentration, they are less potent to prevent ice crystallisation compared to techniques using higher cryoprotectant concentrations (Pereira and Marques, 2008).

In regard to the passages number of mouse embryonic fibroblast, we used only Passages 1 and 2 for both fresh and frozen-thawed mouse embryonic fibroblast in our study for culturing murine embryonic stem cell lines. There were no significant differences ($P>0.05$) in percent successful consecutive passages on murine embryonic stem cell lines between Passages 1 and 2 for fresh mouse embryonic fibroblast, whereas for Passsages 1 and 2 on frozen-thawed mouse embyonic fibroblast, there were significant differences ($P<0.05$) at Passage 3. Smilar as previous findings, the expanded blastocysts always showed the highest percent attachment of blastocysts and consecutive passages for both Passages 1 and 2 in fresh and frozen-thawed mouse embryonic fibroblasts. Puck *et al.* (1956) first described a method of increasing the growth rate of *in vitro* cell cultures through the use of feeder cells layer. While some

cell lines can grow efficiently on plates containing nutrient medium alone, others grow poorly without the use of feeder cells layer (Fisher and Puck, 1956). In general, cells survive and expand better layer (Degrassi *et al.,* 1993). In the early days, the use of feeder cells layer simply involved using inactivated cells of the same type of the primary cell culture (Puck *et al.,* 1956). These cells could no longer multiply effectively, but due to their metabolism remaining active, they continue to secrete growth factors necessary to support the primary cell culture. However, the use of the same cell type as feeder cells layer may not be applicable in every condition. Mature, differentiated cells such as neurons rely on other cells for growth factors (Pellitteri *et al.*, 2006).

Feeder cells provide support in a few ways. In an incomplete medium, feeder cell layer will provide certain nutrients not found in the medium (Fisher and Puck, 1956). Feeder cell layer also negate the effects of certain inhibitory substances found in the medium, such as those originating from the serum extract (Fisher and Puck, 1956). They can also form intercellular junctions such as tight junctions, adherens junctions and gap junctions with the cell culture, thus providing physical support to assist in the attachment of the cells (Ehmann *et al.,* 1998). Besides that, feeder cells layer also secrete factors that support the clonal growth and expansion of the cell culture, such as various growth factors called fibroblast growth factors (FGF) (Shimoaka *et al.*, 2002) and anti-apoptotic factors (Tseng *et al.,* 1996). Thus, whenever a cell type is to be considered as a suitable candidate for use as feeder cells layer, they should exhibit the above characteristics. But once the feeder cells layer underwent cryopreservation, it might be affected their original properties and thus could not performed well compared with fresh mouse embryonic fibroblasts. The present of dimethyl sulfoxide (DMSO) as cryoprotectant in cryopreservation the mouse embryonic fibroblasts cells might gave toxic to the feeder cells layer if in high concentration. Therefore, it would cause the

reduced growth rate as compared with fresh mouse embryonic fibroblasts. In a developing murine embryo, mouse embryonic fibroblasts function to assist in tissue morphogenesis, remodeling, repair, fibrogenesis and overall embryogenesis via connective tissue growth factors (Kennedy *et al.*, 2007). While in *in vitro* culture, it has been observed that growth rate of mouse embryonic fibroblasts was not exponential, but declines progressively within and after each passage, beginning from the initial passage (Karatza *et al.*, 1984). The reduction in growth potential is not due to the increase in the cell cycle duration, but due to reduction in the fraction of dividing cells.

5.4 EFFECTS OF INNER CELL MASS ISOLATION TECHNIQUES, CULTURE MEDIUM AND MOUSE EMBRYONIC FIBROBLAST FREEZING ON PRODUCTION OF MURINE AND CAPRINE EMBRYONIC STEM CELL LINES USING MOUSE EMBRYONIC FIBROBLAST AS FEEDER CELL LAYER (EXPERIMENT 4)

The objectives of this experiment were a) to determine the effects of inner cell mass isolation technique, b) culture medium and c) fresh and frozen-thawed mouse embryonic fibroblast on production of murine and caprine embryonic stem cell lines using mouse embryonic fibroblasts as feeder cell layer.

5.4.1 Effects of Inner Cell Mass Isolation Techniques, Murine Strains, Culture Medium and Mouse Embryonic Fibroblast Freezing on Production of Murine Embryonic Stem Cell Lines

Inner cell mass isolation technique is an important step to ensure the success of the embryonic stem cells establishment. In the present study, the whole blastocyst culture gave the lowest inner cell massattachment, primary inner cell mass outgrowth and successful passages up to Passage 3 rates compared to manual cut and laser dissection inner cell mass isolation techniques. This may be due to in intact embryos where

trophoblastic cells induce inner cell mass differentiation to be 3 embryonic germ layers by suppressing Oct 4 and Nanog expression level (Roberts, 2004). Therefore, completely removed trophoblastic cells provide more benefit to inner cell mass cells turn to be embryonic stem cells.

The inner cell mass could be separated and isolated from the trophectoderm by 5 ways, namely immunosurgery (Solter and Knowles, 1975), with mechanical processes (Bongso *et al*., 1994), with whole embryo culture of the blastocysts and partial embryo culture methods (Kim *et al*., 2005) or single blastomeres (Chung *et al*., 2006) and laser dissection (Turetsky *et al*., 2008). In the present study, we only compared among the whole blastocyst culture, manual cut and laser dissection inner cell mass isolation techniques. In the present experiment, C57BL/6J showed the highest inner cell mass attachment, primary inner cell mass outgrowth and consecutive passages of murine embryonic stem cells lines rates followed by ICR and CBA/ca by manual cut isolation techniques. Therefore, C57BL/6J is the more suitable strain to produce murine embryonic stem cell lines.

Manual cut always gave the highest percent successful consecutive passages of murine embryonic stem cell lines in both DMEM+10% FBS and αMEM+10% FBS culture media. This could be proved by mechanical isolation of the inner cell mass has previously been successfully used in the derivation of 2 cell lines, as reported by Mummery (2004) , Genbacev *et al*. (2005) and van de Stolpe *et al*. (2010). Manual cut inner cell mass isolation technique has been proven to improve results (C. Lorthongpanich, personal communication). However, the difficulty of the manual cut procedure limits the rate at which blastocysts can be dissected. Furthermore, some inner cell mass may become damaged due to the use of needles to pull the inner cell mass away from the overlying trophectoderm. The embryo tended to rotate away from the 30 G needle when it was cut towards one pole. The choice of fully expanded blastocysts

enabled trophoblast to be obtained with little risk of inclusion of inner cell mass. Ideally, more training and practice should be done to master manual cut inner cell mass isolation technique to allow comparisons in terms of efficiency.

In fresh mouse embryonic fibroblast, there were no significant differences ($P>0.05$) for 3 different inner cell mass isolation techniques. However, there were significant differences ($P<0.05$) in inner cell mass attachment and primary inner cell mass outgrowth rates between whole blastocyst culture and manual cut as well as laser dissection in frozen-thawed mouse embryonic fibroblast, whereby the laser dissection isolation techniques gave higher primary inner cell mass outgrowth rate followed by manual cut and whole blastocyst culture techniques in frozen-thawed mouse embryonic fibroblasts. Also, laser dissection technique gave the higher passages of murine embryonic stem cell lines at Passages 2 and 3 compared with manual cut and whole blastocyst culture techniques. Laser dissection method are used in the present study was to evaluate a new method of isolation inner cell mass and derivation of embryonic stem cell lines in a murine. Usually, laser technology is commonly used for assisted hatching with some applications. One of them consists of making the embryonic membrane weaker to make the exit of the future blastocyst easier and so favour the derivation (Antinori and Steirteghem, 2000). Until now, this new laser dissection method has been suggested to derive stem cell lines by Wang *et al*. (2005), although without presenting any conclusive results. A recent paper, Tanaka *et al*. (2006) reported preliminary results for a murine model using this method; subsequent culture of embryonic stem cells in a serum or cell-free culture system was achieved. To our knowledge, some notion of which of these methods are the most efficient for the isolation of inner cell mass is very important for future research in this field. Accordingly, we have compared the inner cell mass isolation method using laser dissection in a murine blastocyst with one of the most commonly used methods, the whole blastocyst culture (Kim *et al*., 2005). However,

with good quality blastocysts with a large and distinct inner cell mass, we used the laser drill (Hamilton, USA).

In our experiment, the murine blastocyst to be treated with laser shot was positioned at the center of the field of view under 40x magnification. The blastocyst position was moved when necessary. Blastocysts can be secured by 2 holding pipettes with the inner cell mass positioned at '9 o'clock' if desired (Tanaka *et al*., 2006). After focusing on the trophectoderm cells, the object had to be moved so that the part of the trophectoderm to be treated was located at the cross-hair position displayed on the monitor as the impact location of the laser focus. Thus this new mechanical method destroyed the trophectoderm cells by shooting the laser over them carefully without damaging the inner cell mass. The length of radiation time of the laser and the number of laser shots were controlled by the embryologist. Although some reported that the whole blastocyst culture method of the blastocysts is more effective than the laser method (70% versus 52.4%) (Polzin *et al*., 2010), taking into account that we have used the laser drill for the good quality blastocysts and that the concealment of the inner cell mass by the trophectoderm cells is the only disadvantage of the whole blastocyst culture method (Bongso *et al*., 1994). Therefore, we must continue to improve the laser dissection technique so that the trophectoderm cells are destroyed and do not interfere with inner cell mass (Tanaka *et al*., 2006). Curiously, the blastocysts treated with the laser at day 1 formed a pseudo-trophectoderm, with a few cells not destroyed by the laser because they were near the inner cell mass. Trophectoderm cells disappeared after repeated passages (Polzin *et al*., 2010).

5.4.2 Effects of Caprine Inner Cell Mass Isolation Techniques, Culture Medium and Mouse Embryonic Fibroblast Freezing on Production of Caprine Embryonic Stem Cell Lines

A total of 169 caprine blastocysts were used as a source of inner cell mass isolation for production of caprine embryonic stem cell lines. Isolation of inner cell mass is a very important step in the culture of embryonic stem cells. Although caprine embryonic stem cells could be produced from intact whole blastocyst culture, the efficiency was low as compared to manual cut and laser dissection techniques. When *in vitro* produced blastocysts were used, they found that intact or whole blastocyst culture was better than isolated inner cell mass culture as small or unclear inner cell mass were present *in vitro* produced blastocysts (Li *et al*., 2003a). Li *et al*. (2003a) reported that isolated inner cell mass is superior to intact blastocyst culture when *in vivo* produced blastocysts were used. More primary colonies were formed from inner cell mass cells than intact or whole blstocysts (Li *et al*., 2003b). It is probably that trophoblasts affects inner cell mass growth in the intact or whole blastocysts culture and induced inner cell mass cells differentiation in culture at the early stage. Therefore, manual cut technique shows the highest percent attachment of inner cell mass, followed by laser dissection and whole blastocyst culture. In percent successful consecutive passages of caprine embryonic stem cell lines, manual cut technique always show the highest percent passages compared to laser dissection and whole blastocyst culture techniques. The similar reasons were discovered, whereby completely removed trophoblastic cells provide more benefit to inner cell mass cells turn to be embryonic stem cells.

A total of 45 replicates were carried out in the present study, where 22 replicates for DMEM+10% FBS and 23 replicates for αMEM+10% FBS. 169 total of caprine blastocyst were used to determine the quality of caprine embryonic stem cell lines in 2 different culture media. There were no significant differences ($P>0.05$) in percent attachment of inner cell mass, primary inner cell mass outgrowth, successful

consecutive passages of caprine embryonic stem cell lines from Passage 1 up to Passage 3. However, DMEM+10% FBS culture medium gave higher percent attachment of inner cell mass, primary outgrowth of inner cell mass, successful consecutive passages than αMEM+10%FBS culture medium. This may be due to DMEM culture medium containing a 4-fold higher concentration of amino acids and vitamins, as well as additional iron (ferric nitrate) compared to Alpha Minimum Essential Medium Eagle culture medium. The Dulbecco's Modified Eagle's Medium was developed since 1969 and is a modification of Basal Medium Eagle (BME) that contains 1000 mg/L of glucose and was first reported for culturing murine embryonic stem cells. Therefore, DMEM+10% FBS is probably suitable culture medium for culturing caprine embryonic stem cells. Furthermore, most of the researchers used Dulbecco's Modified Eagle's Medium for culturing embryonic stem cells rather than Alpha Minimum Essential Medium Eagle, where Dulbecco's Modified Eagle's Medium with 10 to 20% foetal bovine serum has been used since the early days of murine embryonic stem cell culture (Evans and Kaufman, 1981; Tesar, 2005).

Manual cut technique always show the highest percent attachment of inner cell mass, primary inner cell mass outgrowth and successful consecutive passages caprine embryonic stem cell lines in both DMEM+10% FBS and αMEM+10% FBS culture media compared to whole blastocyst culture and laser dissection techniques. Culture conditions currently in use for caprine embryonic stem cells have been mainly developed from murine embryonic stem cell culture and it would appear that they are not very effective for maintaining caprine embryonic stem cells. Various components are usually supplemented to embryonic stem cell culture media. One of the components is the cytokine, such as leukaemia inhibitory factors (LIF), basic fibroblast growth factor (bFGF) and stem cell factor (SCF). Some researchers used only feeder cell layer and did not add any foreign cytokines in the embryonic stem cell medium (Chen *et al*.,

1991) while others added leukaemia inhibitory factors, basic fibroblast growth factor and stem cell factor to the embryonic stem cell medium and also used feeder cell layer (Piedrahita *et al*., 1990). Although leukaemia inhibitory factors can inhibit differentiation of embryonic stem cell, basic fibroblast growth factor can stimulate embryonic stem cells proliferation (Schmitt *et al*., 1991) and feeder cell layer is one of the key factors influencing embryonic stem cell culture (Piedrahita *et al*., 1990), we only add to our embryonic stem cell culture medium and used mouse embryonic fibroblast as feeder cell layer. Leukaemia inhibitory factor has been proved to leukemia inhibitory factors inhibit differentiation of embryonic stem cells (Niwa *et al*., 1998).

As discussed in previous findings in murine species, the trophectoderm cells will induce the differentiation of inner cell mass. Therefore, completely removal of trophectoderm cells from inner cell mass cells is very important in order to obtain high quality and efficacy of caprine embryonic stem cell line production. This has been proved that mechanical isolation of the inner cell mass has previously been successfully used in the derivation of 2 cell lines (Mummery, 2004; Genbacev *et al*., 2005; van de Stolpe *et al*., 2010). Our results have been improved by manual cut inner cell mass isolation technique in caprine species as described in the previous findings in murine species. In previously studied in other laboratories, inner cell mass cells were isolated from blastocysts by immunosurgery (Solter and Knowles, 1975; Wianny *et al*., 1997; Chen *et al*., 1999), but it is not always effective when antiserum is used during immunosurgery. In addition, making antiserum is laborious. In this study, an easy method to isolate inner cell mass cells from blastocysts was established. We found that it is very easy to isolate inner cell mass cells from blastocysts by manual cut as it is not costly and obtained healthier inner cell mass cells and better results of consecutive embryonic stem cell culture.

The present results showed that no significant differences (P>0.05) in percent attachment of inner cell mass, primary outgrowth of inner cell mass, successful consecutive passages of caprine embryonic stem cell lines for fresh and frozen-thawed mouse embryonic fibroblasts. However, fresh mouse embryonic fibroblast gave higher percent attachment of inner cell mass, primary inner cell mass outgrowth and successful consecutive passages of caprine embryonic stem cell lines up to Passage 3 than frozen-thawed mouse embryonic fibroblasts. It has been reported that mouse embryonic fibroblast secrete some kinds of cytokines (Smith *et al*., 1988), which may stimulate embryonic stem cells growth and inhibit their differentiation. Mouse embryonic fibroblast has been shown to produce the factors responsible for maintaining embryonic carcinoma cells in undifferentiated state by inhibiting their differentiation as reported by Martin and Evans (1975). In addition, mitomycin C treat time also affected the mouse embryonic fibroblast activity. If the time was more than 3 hours, the mouse embryonic fibroblast could not be used as a feeder cell layer as the cell's ability to adhere to the dishes was decreased significantly. Once the mouse embryonic underwent cryopreservation, the above properties of mouse embryonic fibroblast would get affected. Thus, the frozen-thawed mouse embryonic fibroblast would gave lower percent attachment of inner cell mass, primary outgrowth of inner cell mass and successful consecutive passages of caprine embryonic stem cell lines up to Passage 3 than fresh mouse embryonic fibroblasts.

There were highly significant differences (P<0.05) between *in vivo*- and *in vitro*-derived blastocyst sources in percent caprine embryonic stem cell lines in Passages 1 and 3. Correspondily, the percent attachment of inner cell mass, primary inner cell mass outgrowth and consecutive caprine embryonic stem cell lines passages were higher in the former. The present study indicates that culture inner cell mass from *in vivo*-derived caprine blastocysts is more effective than *in vitro*-derived blastocysts. When *in vitro*

produced blastocysts were used, they found that intact or whole blastocyst culture was better than isolated inner cell mass culture as small or unclear inner cell mass were present *in vitro* produced blastocysts (Li *et al*., 2003b). Li *et al*. (2003b) reported that isolated inner cell mass is superior to intact blastocyst culture when *in vivo* produced blastocysts were used.

5.5 CONFIRMATION OF CAPRINE AND MURINE EMBRYONIC STEM CELLS BY IMMUNOFLUORESCENT STAINING PROTEIN MARKERS (EXPERIMENT 5)

5.5.1 Confirmation of Murine Embryonic Stem Cells by Specific Embryonic Stem Cell Markers

In the present study, we used the specific embryonic stem cell markers such as Oct 4, SSEA 1, SSEA3, SSEA 4, TRA-1-60 and TRA-1-81 to confirm the presence of embryonic stem cell in murine species. Five widely adopted antibody markers (anti-Oct 4, anti-Sox 2, anti-SSEA 1, 3 and 4, anti-TRA-1-60 and anti-TRA-1-81) are the protein markers that usually found on embryonic stem cells. Several transcription factors, including Oct 3/4 (Nichols *et al*., 1998; Niwa *et al*., 2002), Sox 2 (Avilion *et al*., 2003) and Nanog (Chambers *et al*., 2003; Mitsui *et al*., 2003) function in the maintenance of pluripotency in both early embryos and embryonic stem cells. Oct 4 and Sox 2 are transcription factors highly expressed in undifferentiated embryonic stem cells and embryonic germinal carcinoma (Looijenga *et al*., 2003; Masui and Shinji, 2007; Zhao and Daley, 2008).

Oct 4 marker was found in murine embryonic stem cell in the present results, where it showed green colour after underwent embryonic stem cell markers staining. A classical marker of embryonic stem cells (cultured cells derived from the pluripotent

inner cell mass cells of pre-implantation embryos) and *in vivo* embryonic blastocyst cells is the transcription factor, Oct 4. Oct 4, exclusively expressed by embryonic and germ cells, plays a critical role in the establishment and/or maintenance of pluripotency (Donovan and Gearhart, 2001; Pesce *et al*., 1998a, b) The Oct 4 gene transcript is found in the totipotent and pluripotent stem cells of the early murine embryo and is down regulated when the cells differentiate (Scholer *et al*., 1990; Palmieri *et al*., 1994). Oct 4 is active in the inner cell mass cells and down regulated in the pre-trophectoderm cells, or cells of the outer layer of the blastocyst. The Oct 4 transcript levels are maintained in the epiblast, but expression soon becomes gradually restricted to cells of the germline as gastrulation proceeds (Pesce *et al*., 1998a; Yeom *et al*., 1996). Oct 4 is the first described protein which appears to be specific for the mammalian totipotent cycle (Yeom *et al*., 1996; Yoshimizu *et al*., 1999). The Oct 4 protein could either repress or activate transcription in particular target genes during embryonic development. These varied, initiating molecular events remain uncertain and complex, and deciphering these cell differentiation pathways is pivotal to the growth of stem cell technology (Ovitt and Scholer, 1998). The Oct 4 gene has been found only in mammals, and the human sequence is 87% identical to that of the murine (Ovitt and Scholer, 1998; Brehm *et al*, 1998).

The transcription factor, Oct 4 is regarded as a candidate master regulator for the initiation, maintenance, and differentiation of pluripotent cells (Nichols *et al*., 1998; Niwa and Smith, 2000; Boiani *et al*., 2002; Zhang *et al*., 2005). An understanding of this gene's expression will provide experimental evidence for clarification of target genes controlled by Oct 4 (Zhang *et al*., 2005). Because the quantitative expression of Oct 4 defines differentiation and self-renewal of embryonic stem cells (Niwa and Smith, 2000), the ability to regulate Oct 4 expression is a vehicle to understanding

tumorigenesis, increasing pluripotency of somatic cells, and modeling of the multiple layers of transcription and growth factors involved in embryology.

In murine, Oct 4 expression is crucial to the success of murine preimplantation development (Scholer *et al*., 1989a, 1990; Okamoto *et al*., 1990; Takeda *et al*., 1992; van Eijk *et al*., 1999). Maternal Oct 4 mRNA and protein are present in unfertilised murine oocytes (Palmieri *et al*., 1994) and in the nuclei of subsequent cleavage stages (Palmieri *et al*., 1994; Yoem *et al*., 1996; Kirchhof *et al*., 2000). During the first 2 cleavage stages, the levels of Oct 4 mRNA decrease to background and then steadily increase thereafter. After activation, in murine hatched blastocysts, Oct 4 mRNA is found in the inner cell mass which differentiates into epiblast and hypoblast (second extraembryonic lineage) (Pesce *et al*., 1998b); as the murine blastocyst forms, Oct 4 expression is downregulated, and with expansion, Oct 4 protein and mRNA are primarily found in the inner cell mass (Pesce *et al*., 1998a; Palmieri *et al*., 1994). As the inner cell mass differentiates into the epiblast (embryonic ectoderm) and the hypoblast (embryonic endoderm), and the hypoblast cells migrate along the inner surface of the trophectoderm, high levels of Oct 4 protein are found within these tissues (Pesce *et al*., 1998a; Scholer *et al*., 1990; Kirchhof *et al*., 2000). The pluripotent cells of the murine embryo and germ line, and embryonic stem cells in culture are characterised by expression of the homeobox gene, Oct 4 (Rathjen *et al*., 1999). Oct 4 was identified as a nucleic acid binding embryonic stem cell-enriched gene (Ramalho-Santos *et al*., 2002).

For nuclear transfer murine embryos, less than half of all somatic cell clones develop to the blastocyst stage, and of those, less than one-third develop beyond implantation. They died shortly after implantation with an inability to form embryonic tissues. An absence of Oct 4 results in preimplantation lethality before the murine oocyte cylinder forms (Nichols *et al*., 1998). The murine embryos deficient in Oct 4 developed to blastocysts that lacked an inner cell mass. Murine Oct 4 is expressed in the

murine inner cell mass but not in the pre-trophectodermal cells (Palmieri *et al*., 1994; van Eijk *et al*., 1999). Expression of the gene is uniform in all cells of the embryo through the morula stage, however, as the outer cells differentiate into trophectoderm cells, Oct 4 expression becomes restricted to the cells of the inner cell mass in the blastocyst (Scholer e*t al*., 1990; Yeom *et al*., 1996; Ovitt and Shocler, 1998).

Expression stage-specific embryonic antigen (SSEA) also appeared on murine embryonic stem cells. There are several types of stage-specific embryonic antigen that found in pluripotent of embryonic stem cells, namely SSEA 1, SSEA 3 and SSEA 4. In present study, only SSEA 1 was found in murine embryonic stem cells which gave green colour for embryonic stem cell markers staining. SSEA 1 is a globoseries carbohydrate antigen present on the surface of murine embryonic stem cells, but not human embryonic stem cells (Kohji *et al*., 2008). It was localised on the stem cells of differentiating solid teratocarcinomas and on the surface of core cells of solid embryoid bodies. At the embryo cylinder stage, the antigen is restricted to embryonic ectoderm and visceral endoderm. During subsequent development, SSEA 1 becomes localised to portions of the brain and primordial germ cells. In adult murine, the epithelium of the oviduct, the endometrium, and the epididymis are the cells most reactive with the monoclonal antibody to SSEA 1; although some areas of the brain and kidney tubules are weakly positive. SSEA 3 and SSEA 4 is a globoseries carbohydrate antigen present on the surface of human embryonic stem cell, but not murine embryonic stem cell (Solter and Knowles, 1978; Przyborski, 2001). Upon differentiation, murine embryonic stem cells are characterised by the loss of SSEA 1 expression and may be accompanied, in same instances, by the appearance of SSEA 3 and SSEA 4 (Solter *et al*., 1979).

Murine embryonic stem cells also express high levels of membrane alkaline phosphatase (AP) as found in the present experiment. Alkaline phosphatase is a stem cell membrane marker. Elevated expression of alkaline phosphatase is associated with

pluripotent status (Thomson *et al*., 1998). Alkaline phosphatase is an enzyme in the blood, intestines, liver, and bone cells and exists as membrane-bound isoforms of glycoproteins sharing a common protein structure but differing in carbohydrate content. These enzymes are most active at alkaline pH-hence the name (Thomson *et al*., 1998). Undifferentiated human embryonic stem cells, embryonic carcinoma cells and embryonic germ cells have been shown to express a very high level of the liver or bone or kidney isozyme of alkaline phosphatase (Thomson *et al*., 1995; Millan and Fishman, 1996; Shamblott *et al*., 1998; Draper *et al*., 2004). Alkaline phosphatase staining was carried out to determine the alkaline phosphatase activities that found in the murine embryonic stem cell lines. Purplish colour was shown in murine embryonic stem cells after underwent alkaline phosphatase staining. Expression levels of alkaline phosphatase decrease following stem cell differentiation. It was found that decreasing in alkaline phosphatase activities once the stem cell underwent differentiation. It confirmed the presence of true murine embryonic stem cells which showed alkaline phosphatase activities. There were no colour shown for TRA-1-60 and TRA-1-81 embryonic stem cell markers in murine species. We used human embryonic stem cell specific markers (TRA-1-60 and TRA-1-81) as negative control in the present experiment. TRA-1-60 and TRA-1-81 antigens are expressed on the surface of human teratocarcinoma stem cells human embryonic germinal carcinoma and human embryonic stem cells (Andrews *et al*., 1984), but no expression in murine embryonic stem cells.

In summary, Oct 4, SSEA 1 and alkaline phosphatase activities were found in murine embryonic stem cells which prove the presence of true and pluripotent embryonic stem cells in murine species.

5.5.2 Confirmation of Caprine Embryonic Stem Cells by Specific Embryonic Stem Cell Markers

Similar as murine species, caprine embryonic stem cells also confirmed the true of embryonic stem cells by using embryonic stem cell markers staining. In the present study, Oct 4 (red) and SSEA 3 (green)were expressed on caprine embryonic stem cells as well as alkaline phopsphatase staining gave positive results by showing purplish colour after alkaline phospatase staining.

Expression of Oct 4 was found caprine (Pawar *et al*., 2009). To our knowledge, Oct 4 is an octamer binding protein stimulates transcription and is found in primary germ cells (PGC), unfertilised oocytes and pluripotent embryonic stem cells; when the stem cells are induced to differentiate, the amount of the octamer binding factor decreases (Scholer *et al*., 1989a, b). It was suggested by Scholer and co-workers that the Oct 4 protein was not only present in oocytes but could be synthesised *de novo* by zygotic expression in the embryo (Scholer *et al*., 1989b). This mammalian transcription factor is exclusively expressed by embryonic and germ cells. Transcription factors are DNA binding proteins that could control the rate of transcription of certain genes. Understanding how transcription factors are expressed and function is a requirement for comprehending developmental processes. Oct 4 plays an important role during the early stage of murine embryogenesis and in the germline as an essential component of regulation for germline maintenance (Brehm *et al*., 1998).

Developmental lineage begins at the end of the cleavage stage of an embryo with compaction and leads to the development of the blastocyst. The trophectoderm, or outer layer of the blastocyst, generates the trophoblastic components of the placenta, while the inner cell mass cells develop into the pluripotent progenitors of all of the foetal cell types, including the germ cells and the non-trophoblastic extraembryonic tissues (Nichols *et al*., 1998). Understanding the molecular basis of these pluripotent

cells is critical to efforts to propagate stem cells. The explanation of molecular interaction that governs the trophoblast and inner cell mass development will provide an outline of complex tissue interaction in further development (Nichols *et al*., 1998). Oct 4 could either repress or activate transcription in particular target genes and is one of the best candidates for an embryonic regulatory factor. These varied embryonic initiating molecular events remain uncertain and complex, while Oct 4 has emerged as a critical transcription factor that establishes pluripotency (Ovitt and Scholer, 1998). Deciphering the cell differentiation pathways is pivotal to growth of stem cell technology.

Oct 4 action appears to go beyond a simple repression of trophoblastic lineage and activation of embryonic stem cells; it activates transcription of genes through cooperation of protein "partners." Thus, Oct 4 regulates expression of multiple genes via interactions with other transcription factors such as: Sry-related (Sex determining region on the Y), Sox 2 (Nichols *et al*., 1998; Niwa and Smith, 2000; Du *et al*., 2001), a stem-cell-restricted E1A-like protein (Ovitt and Scholer, 1998; Nichols *et al*., 1998; Niwa and Smith, 2000; Du *et al*., 2001; Scholer *et al*., 1991) and the transcription factor Rox1 (Niwa and Smith, 2000). Maintaining Oct 4 expression within a certain range appears to be crucial for stem-cell renewal, while any increase or decrease triggers differentiation to endoderm or mesoderm or trophectoderm, respectively (Niwa and Smith, 2000). Oct 4 is a master regulator of pluripotency that determines lineage commitment and illustrates the complexity of critical transcriptional regulators with the need for quantitative analyses (Niwa and Smith, 2000; Pesce *et al*., 1999).

For cloned embryos through somatic cell nuclear transfer, the Oct 4 temporal activation in somatic cell clones appeared to be normal, the maintenance of expression and cell type-specific Oct 4 regulation in the trophectoderm and inner cell mass were not. The abnormalities of Oct 4 expression in clones suggested that pluripotency was compromised. Activation of Oct 4 is not due to a general opening of chromatin after

nuclear transfer. Thus, gene regulation in later stage clones may involve transcription factors available in the oocyte cytoplasm or the chromatin remodeling might become dysfunctional (Boiani *et al*., 2002). Theoretically, totipotency can be restored to the nuclei of somatic cells by reprogramming the nucleus with the technique of nuclear transfer. A change in gene expression of the somatic cells must accompany this nuclear reprogramming. In addition, maintaining Oct 4 expression within a certain level appears to be critical for stem cell renewal.

Alkaline phosphatase is one of the markers to evaluate pluripotent cells and has been used to identify stem cells in culture for many years (Wobus *et al*., 1984; Talbot *et al*., 1993). Our results indicate that the caprine embryonic stem cells obtained in the present study are pluripotent, where it gave positive results for alkaline phosphatase staining. Alkaline phosphatase and Oct 4 expression has been used characteristically to identify pluripotent embryonic stem cells in many animal species such as buffalo (Verma *et al*., 2007), porcine (Li *et al*., 2003a), equine (Saito *et al*., 2002) and primate (Thomson and Marshall, 1998). Expression stage-specific embryonic antigen SSEA 3 only appeared on caprine embryonic stem cells (SSEA 1 is not found in caprine species as it is only found in murine embryonic stem cells).

In contrast, human embryonic stem cells and embryonic carcinoma cells typically express SSEA 3 and SSEA 4 but not SSEA 1, while their differentiation is characterised by down regulation of SSEA 3 and SSEA 4 and an up regulation of SSEA 1 (Andrews, 1984; Andrews *et al*., 1987; Fenderson *et al*., 1987). The differences of between murine and caprine embryonic stem cells were summarised in Table 4.74 based on expression of embryonic stem cells protein markers, morphoplogy and characteristics of embryonic stem cells. In summary, the caprine embryonic stem cells were successfully identified in the present study. These cells exhibit many important

features of pluripotent stem cells, including positive alkaline phosphatase activity, Oct 4 and SSEA 3 expressions.

5.6 GENERAL DISCUSSION

In order to obtain good quality and healthy embryonic stem cell lines, good sources of blastocyst obtained, suitability of feeder cell layer and culture medium are the main consideration to be look into in greater details. Before obtaining the blastocysts, superovulation of the female is conventionally necessary to produce large amount of oocytes and embryos to be used as a source for production of embryonic stem cells. In the present study, among the strains studied, CBA/ca gave the optimise superovulation response with regard to blastocyst obtained. Superovulation using gonadotrophins has the benefit to synchronise the oestrous cycle of many female donors, whereby it eases coordination of experiments as well as facilitates the use of immature female to obtain oocytes and collection of oocytes from less fertile animals (Pinkert and Martin, 2005). The general method in the traditional superovulation of murine is the injection of pregnant mare's serum gonadotrophin, followed by human chorionic gonadotrophin 48 hours apart, although the dose may vary among researchers (Ertzeid and Storeng, 1992; Van der Auwera and D'Hooghe, 2001). It was thought that successful superovulation in murine requires the synergistic effects of both pregnant mare's serum gonadotrophin and human chorionic gonadotrophin injection. Pregnant mare's serum gonadotrophin mimics the effects of follicle stimulating hormone produced by the female murine and functions to induce follicular growth and subsequently prepares them for ovulation (Christenson and Eleftheriou, 1972). More follicles were induced to mature as a result of increased amount of stimulating gonadotrophins, thus producing more embryos. On the other hand, human chorionic gonadotrophin also referred to as equine chorionic

gonadotrophin (eCG) (Spearow and Barkley, 1999) mimics the luteinising hormone (LH) to stimulate the ovulation of mature ova.

The efficiency of superovulation in females murine depends on several factors. The factors include the strains of murine, their physical condition such as age, weight and sexual maturity, the type, dose and timing of gonadotrophin administration (Hogan *et al.*, 1986) and external factors (Zarrow *et al.*, 1971; 1972). Differences in superovulatory response in different strains of murine have been observed (Hogan *et al.*, 1986; Byers *et al.*, 2006). Hogan *et al.* (1986) divides different strains of murine according to their superovulatory performance. High ovulators are C57BL/6J, 129/SvJ and CBA/CaJ strain while low ovulators are such as the A/J, BALB/CJ and DBA/2J strains (Hogan *et al*., 1986). The role of genetics in the variation in superovulatory response in murine is confirmed by Spearow and Barkley (1999). According to DeLeon *et al*. (1990), genetic variation among different strains of murine causes variation in the oestrous cycle in terms of the hormonal secretory patterns. Different strain differs in the timing of peak prolactin and progesterone secretion and the amount of prolactin that released is also different. In addition, murine with good reproductive performance have higher ovarian luteinising hormone receptors and lower prolactin concentration during the pro-oestrous stage compared to those with poor reproductive performance. Thus, this explains their better ovulatory response, as high concentration of prolactin will inhibit the induction of luteinising hormone receptors. The higher number of unoccupied ovarian luteinising hormone receptors allows better response to superovulatory treatment. Besides that, high concentration of prolactin would inhibit ovarian aromatase, responsible for oestrogen production (Tsai-Morris *et al.*, 1983). Lower oestrogen secretion in turn results in the irregular oestrous cycle and reduced fertility in murine (DeLeon *et al.*, 1990). Therefore, genetic differences in the various strains of murine result in different hormonal levels, which influence the number of

unoccupied ovarian luteinising hormone receptors available for superovulation, thus resulting in different superovulatory performance.

Individual murine exhibit varying degree of response to superovulation treatment depending on the individual physical condition. Sexually immature females of between 3 to 5 weeks old were the most receptive of pregnant mare's serum gonadotrophin treatment (Hogan *et al.*, 1986). This could be due to the high number of follicles available for stimulation. Unhealthy and underweight murine produces lower amount of embroys after superovulation (Hogan *et al.*, 1986). There was also some correlation between body weight and ovulation rate, whereby a healthy body weight positively influences the induction of luteinising hormone receptors, thus allowing better stimulation by gonadotrophins (Spearow and Bradford, 1983). The efficiency of superovulation in murine is also greatly dependent on the dose of gonadotrophins administered (Fowler and Edwards, 1957; Wilson and Zarrow, 1962). This also demonstrated in other rodent species (Mukumoto *et al.*, 1995). Researchers generally use the optimum dose of 5 to10 IU of pregnant mare's serum gonadotrophin for murine (Wilson and Zarrow, 1962; Ertzeid and Storeng, 2001; Van der Auwera and D'Hooghe, 2001). Excess doses of pregnant mare's serum gonadotrophin failed to induce superovulation in murine due to thickening of the zona granulosa of the follicles, which impede the release of the ova (Wilson and Zarrow, 1962). The dose of human chorionic gonadotrophin influences superovulation to a lesser extent. Ovulation will occur even if small amounts were administered. Excessive dose of human chorionic gonadotrophin did not affect the oocytes count (Wilson and Zarrow, 1962).

Superovulation is also influenced by the time of hormonal administration and subsequent time interval between the initial injection of pregnant mare's serum gonadotrophin and subsequent human chorionic gonadotrophin injection (Wilson and Zarrow, 1962). The optimal time for the administration of hormones is in turn

dependent on the local light-dark cycle, as it influences the endogenous luteinising hormone release in response to pregnant mare's serum gonadotrophin (Hogan *et al.*, 1986). Human chorionic gonadotrophin is best administered a few hours before endogenous luteinising hormone release. The time interval is also important, as sufficient time is required for maximum follicular stimulation, while mature follicles that are left for too long without release of oocyte will begin to degenerate. The optimum time interval reported by Wilson and Zarrow (1962) is between 30 to 50 hours apart. Others have found 42 to 48 hours to be optimal (Hogan *et al*., 1986). The widely used time interval is 40 hours (Fowler and Edwards, 1957; Wilson and Zarrow, 1962) and 48 hours (Van der Auwera and D'Hooghe, 2001). Superovulation in murine also facilitated by external factors, among them pheromones produced by male murine. In certain strain of murine such as the C57BL/6J, the presence of males causes a higher number of oocytes to be produced. The pheromones released by the male murine stimulate the endogenous release of follicles timulating and luteinising hormone, thus facilitating superovulation (Zarrow *et al.*, 1971; 1972).

From this study, it was determined that ICR is the more suitable strain to derive mouse embryonic fibroblasts. Thus, future studies involving murine embryonic stem cells coulb be carried out using ICR mouse embryonic fibroblast rather than a random or inferior strain as used in some previous studies (Brook and Gardner, 1997; Bryja *et al.*, 2006). Also, both fresh and frozen-thawed mouse embryonic fibroblast and Passages 1 and 2 could be used as feeder cell layer for embryonic stem cells. Besides that, both culture media (DMEM and αMEM) also could be used for cultured embryonic stem cells. However, Dulbecco's Modified Eagle's Medium is more suitable and produce high successful attachment of blastocyst, primary inner cell mass outgrowth and consecutive passages rate in embryonic stem cells.

Also, a significant finding in the present experiment is that expanded blastocysts from CBA/ca strain are better sources of embryonic stem cell line. The use of expanded blastocysts could be applied in future experiment involving embryonic stem cells to improve efficiency, rather than ignoring the blastocyst stage when deriving embryonic stem cell lines (Suemori and Nakatsuji, 1987; Kawase *et al.*, 1994; Brook and Gardner, 1997; Bryja *et al.*, 2006). It is important to increase the quality and efficiency of embryonic stem cells experiments because of its potential in regenerative medicine and also embryonic stem cells research requires the sacrifice of life in the form of murine and blastocysts, hence any form of unnecessary wasting should be minimised. This is crucial in embryonic stem cells research of other animals such as caprine which produces only a small amount of blastocysts even after superovulation, thus each and every blastocyst is very valuable and their use must be maximised. The findings in the effects of blastocyst stage on embryonic stem cells derivation is important as future studies involving embryonic stem cells could be enhanced by using the appropriate blastocyst stage. However, the exact mechanism causing this is still poorly understood, thus necessitating further research into factors such as possible differences in the expression of Oct 4 and Sox 2 among the blastocyst stages.

The inner cell mass isolation also is the crucial factor in determine the quality of murine embryonic stem cells production. Manual cut inner cell mass isolation technique is the most effective technique in producing high quality and undifferentiated murine embryonic stem cells. By total removing the trophectoderm cells from the inner cell mass cells, the murine embryonic stem cells could maintain in undifferentiated state and able to obtaine pure murine embryonic stem cell lines compared with whole blsatocyst culture and laser dissection techniques.

In summary, ICR is the suitable strain as feeder cell layer as well as CBA/ca is more suitable strain as a source of blastocyst for embryonic stem cells culture.

Meanwhile, the C57BL/6J strain is the least suitable. The present study also found that fresh and frozen-thawed mouse embryonic fibroblasts at Passages 1 and 2 have similar growth rates and both can be efficiently used for embryonic stem cell culture. Also, even though embryonic stem cells could be derived from any strain or blastocyst stage, expanded blastocysts from the CBA/ca strain gave the highest efficiency. This finding, coupled with the fact that CBA/ca strain gave the best performance in superovulation means that this strain is most suitable as a source for deriving murine embroynic stem cells. Early and hatched blastocysts are not suitable sources for deriving murine embryonic stem cells. The presence of true and pluripotent murine embryonic stem cells also confirmed by embryonic stem cells protein markers staining where it gave positive expression of Oct 4, SSEA 1 and alkaline phosphatase activities in our present experiment.

There were several constraints in the present experiment. Firstly, the murine should be maintain in the best possible condition and health to ensure high quality and quantity of embryos produced. For best performance in mating, a stud male should be isolated in a single cage 1 week prior to mating as the testosterone level will be lowered in the presence of other males, thus affecting the quantity and quality of sperm (Hogan *et al.*, 1986). To do this is barely possible due to insufficient amount of cages and space in the animal house. Also, to ensure best performance in superovulation, females should be on a high-fat diet so that they could be at the optimal weight once they hit sexual maturity, which is the time that they could mate and also have the best response to superovulation (Hogan *et al.*, 1986). Thus, to overcome these constrains, extra funds should be allocated to the maintenance of quality of murine especially in regard to space and feed.

The culture of embryonic stem cells is best done on multi-well plates as each blastocyst or inner cell mass should be separate from another during culture (Bryja *et*

al., 2006; Wakayama *et al.*, 2007). This will ensure that each embryonic stem cell line will contain the same genetic material originating from the same blastocyst and not mixed with embryonic stem cells from another blastocyst. The use of multi-well plates also provides a higher surface area for attachment (Williams *et al.*, 1988). The correct method of establishing an embryonic stem cell line is to firstly culture the blastocysts on separate wells on a 96-well plate, and once an outgrowth forms, they are transferred to a 48-well plate, and subsequently to larger wells according to the size of the colony (Bryja *et al.*, 2006). The use of different multi-well plates is much more costly compared to using 35 mm culture dishes. Therefore, this method could be used when there are extra research fundings. For now, the best way to improve efficiency is by improving the technique of the personnel.

The latest studies in embryonic stem cell used Knockout DMEM supplemented with serum replacment (SR) as the culture medium as it gives the best efficiency (Bryja *et al.*, 2006; Wakayama *et al.*, 2007). This medium selectively favours the proliferation of embryonic stem cells while inhibiting other cell types of the blastocyst (Bryja *et al.*, 2006). However, this medium is more costly. Thus, when there are enough research fundings, this medium can be used to replace the current use of DMEM supplemented with foetal bovine serum as the culture medium for embryonic stem cells.

In caprine species, laser enucleation technique seems to be more suitable compared to squeezing enucleation technique. However, the laser facility is costly and need practice to obtain optimal outcome in cloning. Also, pre-intracytoplasmic injection duration at 60 minutes seems to produce better cleavage rates in somatic cell nuclear transfer technique. These findings are believed to be first to be studied since there is no report regarding this in the literature. Furthermore, modification and method on preparation the *in vitro* maturation culture medium, where it supplemented with bFSH help in improve the maturation rates (Gupta, personnel communication).

General factors known to affect the production caprine embryonic stem cell lines were those such as types of feeder cell layer, embryonic stem cells culture medium, *in vivo*- or *in vitro*-derived blastocyst sources, inner cell mass isolation techniques and blastocyst stages used in producing caprine embryonic stem cell lines. In the present study, we used mouse embryonic fibroblast as feeder cell layer and mouse embryonic stem cell culture medium for culturing the caprine embryonic stem cells since it was working by culturing the murine embryonic stem cell lines. Although we manage to obtain caprine embryonic stem cells by applying the techniques and culture media from murine species, the caprine embryonic stem cells tends to differentiate when reach Passage 3. To our knowledge, different species required different growth factors such as cytokines to give nutrients to produce embryonic stem cell lines. This could be due to the type of feeder cell layer and embryonic stem cell culture medium used in this experiment. For future studies, we suggested that used goat foetal fibroblast as feeder cell layer as it may secrets some known and unkown factors which are closer to caprine species needed compared with mouse embryonic fibroblast (Pawar *et al*., 2009) as well as modified the murine embryonic stem cell culture medium by increased the leukaemia inhibitory factors concentration for obtaining higher and more undifferentiated caprine embryonic stem cell lines.

Also, sources of blastocyst derived are lysignificant important to obtain high efficacy of caprine embryonic stem cells production. In the present findings, we noticed that *in vivo*-derived blastocysts produce high percent of caprine inner cell mass outgrowth and successful consecutive passages of caprine embryonic stem cells up to Passage 3 compared with *in vitro*-derived blastocysts. The main reasons of these were *in vivo*-derived blatocysts are more stable which already developed as embryos in the body condition where it provides more suitable condition for embryos development, whereas the *in vitro*-derived blastocysts were culture *in vitro* which outside the body

would less stablised compared to *in vivo*-derived blastocysts. I*n vitro*-derived blastocysts such as embryos from nuclear transfer will produce less effective quality of embryos compared with *in vivo*-derived blastocysts. This is because the low frequency of successful development has been attributed to incomplete or inappropriate reprogramming of the transferred nuclear genome. Many studies have demonstrated that such reprogramming occurs by epigenetic mechanisms not involving alterations in DNA sequence, however, at present the molecular mechanisms underlying reprogramming are poorly defined. The first visible manifestation of the reprogramming of a somatic nucleus is the formation of a blastocyst, characterised by the differentiation of the trophectoderm and its separation with the inner cell mass. However, careful examination of these blastocysts reveals that they are, in fact, abnormal in several ways. For example, most caprine somatic cell nuclear transfer blastocysts exhibit an abnormal pattern of expression and methylation of imprinted genes, even those that are morphologically perfect (Mannen *et al*., 2003). The aberrant expression pattern of Oct 4 and related genes was also thought to account for the high mortality of somatic cell nuclear transfer embryos because of the inability to maintain pluripotent inner cell mass cells (Boiani *et al*., 2002; Bortvin *et al*., 2003). In blastocyst cell nuclei, an asymmetric methylation pattern begins to appear in fertilised embryos, with the extraembryonic lineages-trophectoderm, and later primitive endoderm-derived lineages being hypo-methylated compared to the epiblast (Santos *et al*., 2002; Monk *et al*., 1987). In nuclear transfer blastocysts, this process is apparently disrupted, with the trophectoderm being hyper-methylated (Dean *et al*., 2001; Bourc'his *et al*., 2001; Kang *et al*., 2002).

Nuclear transfer of murine embryonic stem cells (ntES) has always been relatively simple, provided the embryonic stem cell lines sources are available. Single murine embryonic stem cells survive well and predictably have high cloning efficiency, ranging up to 50%. This is not the case for caprine embryonic stem cells, which seem to

be exquisitely sensitive to dissociation and typically clone with efficiencies of less than 1%. Methods to improve cloning efficiencies are in active development, and include combinations of growth factors, co-cultures and optimising other medium components. The development of the reconstructed embryo is thought to be controlled by interactions between the recipient cytoplasm and the donor chromatin. The role of cytoplasm from the donor cell is undefined; however, our studies using nuclear donors with different volumes of cytoplasm provided evidence that a high karyoplast-cytoplast volume ratio may interfere with development of caprine nuclear transfer embryos (Zakhartchenko *et al*., 1997). Basic studies on nuclear transfer will continue to increase our understanding how genomic activation and cell cycle synchrony affect nuclear reprogramming and cloning efficiencies. Previous study shows that epigenetic reprogramming occurs aberrantly in most cloned embryos; incomplete reprogramming may contribute to the low efficiency of cloning. Global epigenetic reprogramming of somatic nuclei is aberrant in most preimplantation cloned embryos (Dean *et al*., 2001). Correct epigenetic reprogramming is necessary for successful and normal developmental of clones (Dean *et al*., 2001) The transplantation of somatic cell nuclei to enucleated oocytes has shown that genes can be reprogrammed to an embryonic pattern of expression, thereby indicating a reversal of their epigenetic state. In normal embryonic development, cells lose their pluripotent status and become restricted to a particular differentiation pathway by receiving signals from other cells. Once on the pathway to one kind of differentiation, cells do not redifferentiate in other directions and their commitment to differentiation is very stable. However, this does not mean that their nuclei have stably lost their earlier developmental plasticity. In addition, successful cloning involves efficient DNA replication, appropriate DNA methylation or demethylation, chromatin remodeling and X chromosome reactivation (Reik *et al*., 2001; Rideout *et al*., 2001; Shi *et al*., 2003), the low success rate could be caused by an incomplete reprogramming of

the epigenetic state of nuclei from differentiated donor cells. These considerations led us to explore further the nature of the constraints that limit the efficiency with which somatic cell nuclei can be reprogrammed by oocyte cytoplasm. It is easy to understand that genes permanently switched off in the course of cell differentiation may be hard to reactivate efficiently. An example of such a gene is Oct 4, whose product is required for embryonic development and the maintenance of stem cell status (Nichols *et al*., 1998). The incomplete reactivation of Oct 4 may account for some failures of nuclear transfer embryo development (Boiani *et al*., 2002; Bortvin *et al*., 2003).

Besides that, the inner cell mass isolation techniques also are crucial factor that affected the production of caprine embryonic stem cell lines where the manual cut technique produce more healthy and good quality of caprine embroyinc stem cell lines as similar to our previous findings in murine species. Totally removed the trophoblastic cells is very important to prevent the trophectoderm cells induced the differentiation of caprine inner cell mass. This is the new finding where no researchers have been reported by using this method before. Also, by looking into the caprine blastocyst stages as source for production caprine embryonic stem cells is very important. In the present study, we did not considering the blastocyst stages in caprine species due to the lack of caprine blastocysts source. More caprine sample is needed in order to obtain more consistent and accurate results for future study.

In summary, *in vivo*-derived caprine blastocysts, Dulbecco's Modified Eagle's Medium embryonic stem cell medium, fresh mouse embryonic fibroblast and manual cut technique provide the high efficacy and quality of caprine embryonic stem cell lines in our study. This could prove the pluripotent of caprine embryonic stem cells by confirmation with embryonic stem cells protein markers staining where it gave positive expression of Oct 4, SSEA 3 and alkaline phosphatase activities.

Several further studies could be carried out to investigate a few parameters that affect the efficiency and also as an extension to the current study. For murine species, it was previously reported that the murine strains used to derive mouse embryonic fibroblast feeder cell layer may have an effect on the efficiency of deriving embryonic stem cells (Suemori and Nakatsjui, 1987; Kawase *et al.*, 1994). In this study, the direct effect of mouse embryonic fibroblast from different strains on the embryonic stem cell culture was not investigated. Thus, further studies investigating the efficiency of mouse embryonic fibroblast derived from ICR, C57BL/6J, CBA/ca and other strains of murine in supporting the growth of embroynic stem cells could be carried out. This could lead to the finding of the mouse embryonic fibroblast and embroyinc stem cells strain combination that gives the best efficiency. Also, a study which compares the efficiency of embroyic stem cells derivation from different strains of murine in terms of expression of Oct 4 gene could also be carried out. Oct 4 is expressed in pluripotent embroyinc stem cells and the degree of expression determines the pluripotency and hence quality of the embryonic stem cell lines (Masui *et al.*, 2007). In the present study, efficiency is determined based on the success rate of reaching a higher passage and embryonic stem cells protein markers staining. Thus, gene expression and visualisation using fluorescence microscopy is a more accurate method. Similar experiments could also be carried out using Sox 2 expression.

An extension of the current experiment that could be done is a possible modification of the experiment that could be done to improve the results is by using microdissection of the inner cell mass of blastocyst or using mechanical isolation in murine and caprine species. Microdissesction of the inner cell mass has been proven to improve results (Wakayama *et al.*, 2007) while mechanical isolation has the same potential. Ideally, more training and practice should be done to master both the microdissection and mechanical isolation technique to allow comparisons in terms of

efficiency. A study which compares the efficiency of caprine embryonic stem cells derivation from different stages of blastocyst could be carried out. Using embryonic stem cell-like, it was shown that stage of blastocysts at inner cell mass isolation to be a significant factor in establishment of caprine embryonic stem-like cells colonies (Pawar *et al*., 2009). The authors showed that the inner cell mass isolated from hatched blastocyst enhanced primary colony formation (66.6%) as compared with 23.3% primary colony formation in inner cell mass obtained from early and expanded blastocysts. The mechanical isolation in caprine was found comparatively easier for hatched blastocyst than the early and expanded blastocyst (Pawar *et al*., 2009). This phenomenon could be studied in detail using caprine embryonic stem cell lines in the future. In addition, the further research is required to maintain and enhance the efficiency of embryonic stem cell culture in the caprine.

In order to confirm the pluripotency of the caprine embroyinc stem cells, differentiation of caprine embryonic stem cells could be carried out in future. To our knowledge, by culturing the caprine embryonic stem cells without feeder cell layer and leukaemia inhibitory factors, it would differentiate into specific type of cells such as muscle cell, heart cells and neuron cells by specific culture medium. By this way, we could prove the pluripotent characters in caprine embryonic stem cells. Furthermore, it could be applied into patient to cure some degenerative diseases such as cardiovascular, diabetes, Parkinson's and Alzhemier's diseases.

Chapter 6

6.0 CONCLUSIONS

Chapter 6

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This study presents the use of mouse embryonic fibroblasts (MEF) as feeder cells for following production of embryonic stem cell (ESC) lines in murine and caprine. The can be concluded from the findings of the present study:

1) For murine species

a) Superovulation was successfully carried out in ICR, CBA/ca and C57BL/6J strains of murine. There were no significant differences in terms of success rate. However, the CBA/ca (77.00±3.81%) strain showed the highest percent successful superovulation followed by ICR (75.34±3.66%) and C57BL/6J (65.71±4.12%).

b) Pre-intracytoplasmic injection durations treatments at 90 minutes gave the highest cleavage rate in 3 different pure-strains murine compared to simultaneous injection as well as 30, 60 and 120 minutes pre-intracytoplasmic injection durations.

c) Protocol for the derivation of mouse embryonic fibroblast cell lines and establishment of murine embryonic stem cell lines were successfully developed that may serve as the basic for further research in ABEL laboratory.

d) No murine strain differences in the growth rate of mouse embryonic fibroblasts. However, ICR is the suitable strain for use to derive mouse embryonic fibroblast cell lines used as feeder cell layer for murine embryonic stem cells whereby it gave highest growth rate of mouse embryonic fibroblast compared with CBA/ca and C57BL/6J.

e) Fresh and frozen-thawed mouse embryonic fibroblast as well as DMEM+10% FBS and αMEM+10% FBS culture media for mouse embryonic fibroblast at Passages 1 and 2 have similar growth rates. However, culture medium for DMEM+10% FBS

gave the highest growth rates (P1: 94.44±5.56%; P2: 97.22±2.78%) compared to αMEM+10% FBS culture medium (P1: 88.89±6.46%; P2: 91.67±6.06%).

f) There were no significant differences among the 3 different pure-strains of murine in establishment of murine embryonic stem cell lines. However, CBA/ca gave the highest percent attachment of blastocysts and successful consecutive passages murine embryonic stem cell lines compared with ICR and C57BL/6J. Thus, CBA/ca is the most suitable strain to produce high quality of murine embryonic stem cell lines.

g) Murine embryonic stem cell lines could be successfully derived from any strain or blastocyst stage. However, expanded blastocysts from the CBA/ca strain gave the highest efficiency, whereas early and hatched blastocysts are not suitable sources for deriving murine embryonic stem cell lines due to their poor efficiency.

h) Both DMEM+10% FBS and αMEM+10% FBS could be used for culture murine embryonic stem cells. However, DMEM+10% FBS culture medium is most suitable use for culture murine embryonic stem cells where it showed higher percent successful attachment of blastocysts and consecutive passages than αMEM+10% FBS culture medium.

i) Majority of expanded blastocysts gave the highest percent attachment of blastocysts and successful consecutive passages in both fresh and frozen-thawed mouse embryonic fibroblasts for Passages 1 and 2.

j) Murine embryonic stem cell lines could be successfully derived from any inner cell mass isolation techniques. However, manual cut inner cell mass isolation technique in C57BL/6J gave the highest percent primary outgrowth of inner cell mass with the values of 78.35±1.24%, followed by laser dissection (75.67±0.85%) and whole blastocyst culture (75.32±1.73%). Also, this technique in general gave higher percent inner cell mass attachment, primary outgrowth of inner cell mass and

consecutive passages of murine embroyinc stem cell lines for both DMEM+10% FBS and αMEM+10% FBS as well as fresh and frozen mouse embryonic fibroblast at Passages 1 and 2.

k) Oct 4 and SSEA 1 expression and alkaline phosphatase (AP) activites were found in murine embryonic stem cell lines.

2) For caprine species (bovine as comparative study)

a) Majority laser enucleation technique apparently gave higher maturation, enucleation, injection and cleavage rates in caprine and bovine species compared with squeezing enucleation technique.

b) Grade A, B and C oocytes showed the best performance in maturation, enucleation, injection and better cleavage rates than Grades D and E for all the stages in both caprine and bovine species.

c) Even though insignificance, the caprine embryo development at various stages was apparently higher in LOPU, followed by ovariectomy and abattoir. However, ovariectomy showed better results at blastocyst and hatched blastocysts compared to LOPU and abattoir.

d) Pre-intracytoplasmic injection durations at 60 minutes gave the highest cleavage rates compared with simultaneous injection and 30 minutes pre-intracytoplasmic injection durations in caprine species.

e) Caprine always showed higher percent maturation, enucleation, injection and cleavage rates for all the stages (2-cell to hatched blastocysts) as well as all the grades, except Grade A than bovine species.

f) Most of the cleavage values were significantly higher in laser enucleation technique for both species. However, caprine species always showed higher percent cleavage than bovine species for both enucleation techniques.

g) Average number of blastocysts obtained in FSH treatment showed higher than that of PMSG treatment in *in vivo* caprine uterine flushing.

h) Manual cut inner cell mass isolation technique shows the highest percent attachment of caprine inner cell mass, successful passages of caprine embryonic stem cell lines followed by laser dissection and whole blastocyst culture.

i) There were no significant differences in percent attachment of caprine inner cell mass, primary outgrowth of inner cell mass, successful consecutive passages caprine embryonic stem cell lines from Passage 1 up to Passage 3 for both DMEM+10% FBS and αMEM+10% FBS as well as fresh and frozen-thawed mouse embryonic fibroblast at Passages 1, 2 and 3. However, DMEM+10% FBS culture medium and fresh mouse embryonic fibroblast gave higher percent inner cell mass attachment, primary outgrowth of inner cell mass, successful consecutive passages caprine embryonic stem cell lines than αMEM+10% FBS culture medium and frozen-thawed mouse embryonic fibroblast.

j) *In vivo*-derived caprine blastocyst gave the highest percent inner cell mass attachment, primary outgrowth of inner cell mass and successful passages of caprine embryonic stem cell lines than *in vitro*-derived caprine blastocyst sources.

k) Expression of Oct 4 and SSEA 3 as well as alkaline phosphatase (AP) activities were found in caprine embryonic stem cell lines.

In summary, the results of this study show that murine and caprine embryonic stem cell lines could be established using mouse embryonic fibroblast as feeder layers. Murine strains, blastocyst stages and inner cell mass isolation techniques are factors that affect the production of embryonic stem cell lines. In murine, ICR is the best strain for deriving mouse embryonic fibroblast, expanded blastocyst from CBA/ca is the optimal

strain for deriving murine embryonic stem cell lines, while manual cut inner cell mass isolation technique is the most suitable technique in isolating inner cell masses. For caprine species, *in vivo*-derived blatocysts is the best source to produce high efficiency of caprine embryonic stem cell lines as well as manual cut inner cell mass isolation technique is the best technique in producing undifferentiated caprine embryonic stem cell lines.

3) Originality

There are several original findings obtained from caprine embryonic stem cell research. Firstly, mouse embryonic stem cells from 3 different strains of murine to be used as feeder cell layer for caprine embryonic stem cell culture. Second, laser enucleation technique in caprine species is a new approach to obtain good and high quality of blastocyst formation from cloned embryos to be used as source for producing caprine embryonic stem cell lines. Thirdly, manual cut and laser dissection techniques are the new techniques that can be used to isolated the inner cell mass from murine and caprine blastocysts to produce more healthy and undifferentiated murine and caprine embryonic stem cell lines. Lastly, blastocyst stages in murine species can be used to derive a good quality of embryonic stem cell lines.

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APPENDICES

APPENDICES

APPENDIX 1: LIST OF MATERIALS

Appendix Table 1.1: List of facilities and equipment

No.	Item/Product	Model/Catalogue no.	Manufacturer	Local agent
1.	Stereomicroscope	SZH10	Olympus	Matrix Optics (M) Sdn Bhd
2.	Compound microscope	CH-2	Olympus	-
3.	Inverted microscope	IMT-2	Olympus Optical Co., Japan	-
4.	Laminar flow	CMF series	ERLA, Malaysia	Trade Knight (M) Sdn Bhd
5.	Waterbath	870354	Memmert, Germany	Inter (M)
6.	Oven	40050-IP20	Memmert GmbH, Schwabach Germany	Inter (M)
7.	Autoclave	'Hiclave' Model HA-300M 2	Hirayama, Manufacturing Corp Tokyo, Japan	Ali Sdı
8.	CO_2 incubator	Model 3165	Forma Scientific Inc.	Ali Sdı
9.	Ultra-pure water system (Mili-Q UF Plus)	-	Millipore, Bedford, MA, USA	-
10.	Digital analytical balance	AB104	Mettlet, Toleda, Switzerland	-
11.	pH meter	HI 8417	HANNA Instrument, Singapore	-
12.	Osmometer	Vapro 5520	WESCOR Inc. USA	-
13.	Liquid nitrogen tank	-	-	MVE, USA
14.	Dissecting set	-	Aesculap, Germany	Chemolab Supplies
15.	Beaker (Pyrex)	-	-	-
16.	Micropipettor (10 µl, 100 µl, 1000 µl)	-	Eppendorf, Germany	-
17.	Flushing needle	30 G	B. Braun, Melsungen, Germany	
18.	Haemacytometer	-	-	-
19.	Centrifuge	D37520	Heraeus, Kendro Laboratory Products, Germany	-
20.	Digital camera	-	MicroLamba,	-

(Continued)

			Kuala Lumpur, Malayasia	
21.	Fluorescent microscope	Axiovert 135 M	Carl-Zeiss, Inc., Germany	-
22.	Flushing and aspiration system consists of the following: a) Aspriation system b) Flushing system c) Test tube heater	 K-MAR-5100 K-MAR-4000 K-FTH-1012	Cook Australia, Brisbance Technology Park, Eight Mile Plains, Queeland, Australia	-
23.	Flushing and aspiration pedals	6210-72535-OB	Hega Electric Limited, Suffolk, UK	-
24.	Impulse sealer	KF-300H	Khind, Taiwan	-
25.	Inverted microscope fitted with micromanipulators (for cloning)	IX71	Olympus Optical Co., Ltd, Tokyo, Japan	-
26.	La banana (hand-controlled pipette for handling oocytes or embryos)	-	Self-assembled	-
28.	Micropipette puller	P-97	SSutter Instrument CO., Novato, CA, USA	-
29.	Microforge	-	Technical Products International, St. Louis, MO, USA	-
30.	Microgrinder	EG-4	Narishige Co., Ltd., Tokyo, Japan	-
31.	Micropipette dispenser (2-20 µl, 10-100 µl, 200-1000 µl)	-	Eppendorf, Humburg, Germany	-
32.	Narishige hydraulic micromanipulators	ON3-99D	Narishige Co., Ltd., Tokyo, Japan	-
33.	Ovum pick-up needle (FAS Set C2)	-	Gynetics Medical Product, Hamont-Achel, Belgium	-
34.	Laparoscopic system consists of the following: a) Aesculap endoscopic camera system b) Autraumatic grasping forceps (pediatric grasper) c) CCD camera (David 3) d) Pediatric Storz	 PV431 PO951R PV430 PE 688A	Aesculap A.G. and Company, Tuttlingen, Germany	-

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	laparoscope (7.0 mm)			
	e) Electronic CO_2 gas insufflators unit	PG001		
	f) Light probe with fibre optic cable	OP913		
	g) Light system (Light source 300 W)	OP927		
	h) Small trocar with canula (5.5 mm) for grasper	EJ456		
	i) Trocar and canula (7.0 mm) for the pediatric Storz laparoscope	EJ457		
	j) Verrus needle attached to a plastic tubing	PG3		
34.	Refrigerator (Cooltech)	SR-21NME	Samsung Electronics, Seoul, Korea	-
35.	Spirit Burner (150 ml)	-	Shanghai Machinery Import and Export Company, Shanghai, China	-
36.	Surgical cradle (table)	-	Syarikat Copens Enterprise, Kuala Lumpur, Malaysia	-
37.	Laser machine	XYclone	Halmiton, USA	-
38.	Surgical tray	-	Syarikat Copens Enterprise, Kuala Lumpur, Malaysia	-
39.	Surgical trolley	-	Syarikat Copens Enterprise, Kuala Lumpur, Malaysia	-
40.	ThermoPlate (Stage warmer for cloning or inverted microscope)	HATS-U55R30	Tokai Hit Company Ltd., Tokyo, Japan	-
41.	Vortex mixer (Mixer UZUSIO)	VTX-3000L	LMS Co., Tokyo, Japan	-
42.	Water purification system (Milli-RO 10 Plus)	-	Millipore, Bedford, MA, USA	-

Appendix Table 1.2: List of labwares and disposables

No.	Item/Product	Model/Catalogue no.	Manufacturer	Local agent
1.	Hypodermic needle	18 G, 20 G, 21 G, 23 G, 25 G, 26 G, 30 G	Terumo Corporation, Tokyo, Japan	-
2.	Micropipette tips	Yellow and blue	-	Megalab Supplies
3.	Plastic culture dish	351008 (35 x 10 mm)	Becton Dickinson Labware, USA	FC- Bios Sdn Bhd
4.	Plastic culture dish	351007 (60 x 15 mm)	Becton Dickinson Labware, USA	Biomarketing (M) Sdn Bhd
5.	Tissue culture dish	35 mm	Nunc	Megalab Supplies
6.	Tissue culture dish	60 mm	Nunc	Megalab Supplies
7.	Aluminum foil	Diamond	Reynolds Consumer Product, Virginia, USA	Megalab Supplies
8.	Nylon	-	-	-
9.	Disposable glove	-	Cross Protection (M) Sdn Bhd	Megalab Supplies
10.	Autocalvable disposal bag	-	Megalab supplies, Subang Jaya, Selnagor, Malaysia	Megalab Supplies
11.	Beaker	50 ml, 100 ml, 250 ml, 500 ml	Pyrex®, Iwaki Glass, Japan	-
12.	Blades (Super Nacet)	-	Gillette, USA	-
13.	Borosilicate glass tubing	Microcaps®	Drummond Scienctific Company, Broomall, PA, USA	-
14.	CIDR applicator	-	Pharmacia and Upjohn Limited Company, Mt. Wellington, Auckland, New Zealand	-
15.	Conical centrifuge tube	15 ml, 50 ml; Falcon	Becton Dickinson Labware, Franklin Lakes, NJ, USA	Megalab supplies
16.	Cover slips	-	Hirschmann® Laborgerate, Germnay	-
17.	Disposable glass Pasteur pipettes	-	John Poulten Ltd., Essex, England	Megalab supplies
18.	Haematocrit capillary tube	75 mm	Hirschmann® Laborgerate, Germany	Megalab supplies
19.	Holding pipettes	-	Self-made	-

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20.	Injection needle	-	Self-made	-
21.	Cutting needle	-	Self-made	-
22.	Biopsy needle	-	Self-made	-
23.	Lens cleaning tissue	Kimswipe®EX-L	Kimberly-Clark, Roswell, GA, USA	-
24.	Measuring cyclinder	50 ml, 100 ml	Pyrex®. Iwaki Glass, Japan	-
25.	Microcentrifuge tubes	0.2 ml, 0.5 ml, 1.5 ml	Eppemdorf GmbH, Hambrug, Germany	-
26.	Millex®-GS syringe driven filter unit	0.22 µm	Millipore, Carringtwohill, Co. Cork, Ireland	-
27.	4-well dish	Nunc	Nunc, Roskilde, Denmark	-
28.	Parafilm	-	Pechiney Plastic Packaging, Chicago, IL, USA	-
29.	Schott bottle	50 ml, 100 ml, 250 ml, 500 ml, 1 litre	Duran, Germany	-
30.	Sterile gloves	-	Ansell International, Malaysia	-
31.	Syringes	1 ml, 5 ml, 10 ml, 20 ml, 50 ml	Terumo Corporation, Tokyo, Japan	-

Appendix Table 1.3: List of chemicals

No.	Item/Product	Chemical name/Content/ Description	Model/ Catalogue no.	Manufacturer	Local agent
1.	PMSG (Folligon)	1000 IU	-	Intervet International BV., Boxmeer, Holland	Lazuli Sdn Bhd
2	hCG (Chorulon)	5000 IU	-	Intervet International BV., Boxmeer, Holland	Lazuli Sdn Bhd
3.	Silicone oil	-	SI-380-00	Aldrich Chem. Co., USA	-
4.	PBS tablet	-	BR0014G	Oxoid Ltd, Hampshire, England	-
5.	DMSO	-	D-5879	Sigma-Aldrich Co., St. Louis, MO, USA	-
6.	BSA	-	A-9418	Sigma-Aldrich Co., St. Louis, MO, USA	-
7.	NaCl	-	S-5886	Sigma-Aldrich Co., St. Louis, MO, USA	-
8.	KCl	-	P-8014	Sigma-Aldrich Co., St. Louis, MO, USA	-
9.	KH_2PO_4	-	P-5655	Sigma-Aldrich Co., St. Louis, MO, USA	-
10.	$MgSO_4.7H_2O$	Anhydrous	M-8150	Sigma-Aldrich Co., St. Louis, MO, USA	-
11.	$NaHCO_3$	-	S-5761	Sigma-Aldrich Co., St. Louis, MO, USA	-
12.	$CaCl_2.2H_2O$	Dehydrate	C-7902	Sigma-Aldrich Co., St. Louis, MO, USA	-
13.	Glucose	Anhydrous	G-7021	Sigma-Aldrich Co., St. Louis, MO, USA	-
14.	Calcium lactate	-	2376	Sigma-Aldrich Co., St. Louis, MO, USA	-
15.	Lactic acid	-	L-4263	Sigma-Aldrich Co., St. Louis, MO, USA	-
16.	Natrium pyruvate	-	P-3662	Sigma-Aldrich Co., St. Louis, MO, USA	-
17.	L-glutamine	-	G8540	Sigma-Aldrich Co., St. Louis, MO, USA	-
18.	Taurine	Synthetic	T-7146	SIGMA Chem. Co., USA	-
19.	HEPES, sodium salt	-	H7006	Sigma-Aldrich Co., St. Louis, MO, USA	-
20.	Natrium EDTA	-	E-5143	Sigma-Aldrich Co., St. Louis, MO, USA	-
21.	Penicillin	-	-	Sigma-Aldrich Co., St. Louis, MO, USA	-
28.	Trypsin	-	-	-	-
22.	Streptomycin	-	S-9137	Sigma-Aldrich Co., St. Louis, MO, USA	-
23.	Phenol red	-	P-3532	Sigma-Aldrich Co., St. Louis, MO, USA	-
24.	Sucrose	-	S-1888	Sigma-Aldrich Co., St.	-

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				Louis, MO, USA	
25.	HCL	-	-	HmbG Chemicals, Germnay	-
26.	Alcohol	70%	-	Prepared from absolute ethanol	-
27.	DMEM	-	D5796 - 1L	Sigma-Aldrich Co., St. Louis, MO, USA	-
28.	Trypsin	-	-	-	-
29.	Pronase	-	-	-	-
30.	7X™ (non-toxic) detergent	-	-	FlowLab™, Sydney, Australia	-
31.	Absolute ethanol	100%	1322219	HmbG Chemicals, Germnay	-
32.	Hoechst 33342	-	B2261	Sigma-Aldrich Co., St. Louis, MO, USA	-
33.	Calcium ionophore	-	I0634	Sigma-Aldrich Co., St. Louis, MO, USA	-
34.	Cloprostenol (Estrumate®)	-	-	Schering-Plough Animal Health, Baulkham Hills, NSW, Australia	-
35.	Cysteamine	-	M9768	Sigma-Aldrich Co., St. Louis, MO, USA	-
36.	FSH	Ovagen™	Ofsh	ICPbio Limited, Auckland, New Zealand	-
37.	Gigasept®FF (disinfectant)	-	-	Schulke and Mary GmbH, Nonderstedt, Germnay	-
38.	Heparin, sodium salt	-	H0777	Sigma-Aldrich Co., St. Louis, MO, USA	-
39.	Hibiscrub cleanser	Hibiscrub	-	ZENECA Limited, Macclesfield, UK	-
40.	Hyaluronidase (bovine testes)	Type IV-S	H4272	Sigma-Aldrich Co., St. Louis, MO, USA	-
41.	HFl	-	1301030	HmbG Chemicals, Germnay	-
42.	Ketamine hydrochloride	Ketamil	L10077	Troy Laboratories Pty Ltd., NSW, Australia	-
43.	K-Y Jelly	-	-	Pharmedica Lb (Pty) Ltd., East London, South Africa	-
44.	Liquid nitrogen	-	-	Mox Gases Berhad, PJ, Selangor, Malaysia	-
45.	$MgCl_2.6H_2O$	-	M2393	Sigma-Aldrich Co., St. Louis, MO, USA	-
46.	Medium 199 (with Ealre's salt, L-glutamine and $NaHCO_3$)	-	M4530	Sigma-Aldrich Co., St. Louis, MO, USA	-
47.	Mineral oil	-	M8410	Sigma-Aldrich Co., St. Louis, MO, USA	-

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48.	Minimum Essential Media (MEM) non essential amino acid solution, 100x	-	M7145	Sigma-Aldrich Co., St. Louis, MO, USA	-
49.	Oestradiol-17β	-	E4389	Sigma-Aldrich Co., St. Louis, MO, USA	-
50.	Oestrus goat serum	-	-	Prepared ‘in-house’	-
51.	PVP medium	-	10890001	Medicult. Jyllinge, Denmark	-
52.	Pyruvic acid, sodium salt	-	P3662	Sigma-Aldrich Co., St. Louis, MO, USA	-
53.	Sodium DL-lactate (60% w/w syrup)	-	L4263	Sigma-Aldrich Co., St. Louis, MO, USA	-
54.	$NaH_2PO_4.H_2O$	-	S5011	Sigma-Aldrich Co., St. Louis, MO, USA	-
55.	Xylazine hydrochloride	Ilium Xylazine-20	L10600	Tryo Laboratories Pty Ltd., NSW, Australia	-
56.	Triton®X-100	-	93420	Fluka Chemie GmbH, Buchs, Germany	-
57.	Iodine solution	-	-	ICN Biomedicals, USA	-
58.	Mitomycin C	-	M4287	Sigma-Aldrich Co., St. Louis, MO, USA	-

APPENDIX 2: PUBLICATIONS, CONFERENCES AND WORKSHOP

A) JOURNALS

1) Goh, S.Y., R.B. Abdullah and W.E. Wan Khadijah. 2012. Characterization of caprine embryonic stem cell-like outgrowths, derived from the inner cell mass isolation. Small Ruminant Research. (In Press).
2) Goh, S.Y., R.B. Abdullah and W.E. Wan Khadijah. 2012. Effects of murine strain, blastocyst stage and inner cell mass isolation technique on the efficacy of murine embryonic stem cells. Malaysian Journal of Science. (In Press).
3) Goh, S.Y., R.B. Abdullah and W.E. Wan Khadijah. 2012. Production of cloned-caprine embryos through somatic cell nuclear transfer influenced by oocytes sources, oocyte grading, pre-intracytoplasmic injection durations and enucleation techniques. Journal of Animal Physiology and Animal Nutrition. (Submitted).

B) PROCEEDINGS

1) Goh, S.Y., R.B. Abdullah and W.E. Wan Khadijah. 2010. Comparison between bovine and caprine somatic stem cell nuclear transfer (SCNT) performance by using fresh cumulus cell through whole cell intracytoplasmic injection (WCICI) technique. Proceedings of the 7th Annual Conference of the Asian Reproductive Biotechnology Society (ARBS). p. 62.
2) Goh, S.Y., R.B. Abdullah and W.E. Wan Khadijah. 2010. Production of cloned-murine embryos through somatic stem cell nuclear transfer (SCNT) technique. Proceedings of the 31st Annual Conference of the Malaysian Society of Animal Production (MSAP). pp. 178-179.

C) CONFERENCES

1) The 7th Annual Conference of the Asian Reproductive Biotechnology Society (ARBS), 8-10th November 2010.
2) The 31st Annual Conference of the Malaysian Society of Animal Production (MSAP), 6-8th June 2010.

D) WORKSHOP

- 4th SUT Stem Cell Workshop " Stem Cell Innovation: A Milestone Beyond Life Science Roadblocks" at Suranaree University of Technology, Nakhon Ratchasima, Thailand, 26-30th July 2010.

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Characterization of caprine embryonic stem cell-like outgrowths derived from the inner cell mass isolation

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ABSTRACT

Embryonic stem (ES) cells have limitless potential in the field of biological sciences and regenerative medicine due to their pluripotency and ability for indefinite self-renewal. The aims of this study were: (i) to isolate goat ES cell lines from both *in vivo* and *in vitro* derived blastocysts and (ii) to characterize the growth characteristics and expression of markers indicative of pluripotency in goat ES cells. The goat inner cell mass (ICM) of *in vitro* and *in vivo* derived whole blastocysts was isolated by manually cutting or laser dissection, with *in vitro* cultured mouse embryonic fibroblasts (MEF's) as a feeder layer to obtain the ES cells. The *in vivo* derived blastocysts recorded a significant difference in producing goat ES cell lines at Passage 3, compared with *in vitro* derived blastocysts (91.7% vs. 20.8% respectively). The manually cut ICM (inner cell mass) isolation technique consistently recorded the highest success rate in goat ES cells for Passages 1 and 3, compared with the whole blastocyst culture (control) and those from the laser dissection technique (71.3% vs. 39.6% and 43.9%; 35.0% vs. 12.5% and 23.3% respectively). Alkaline phosphatase (AP), Oct-4 and SSEA-3 staining gave positive results in identifying the goat ES cells. In conclusion, goat ES cells could be produced by isolation of the ICM by using both manual and laser dissection of blastocysts, as characterized by AP, Oct-4 and SSEA-3 staining.

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1. Introduction

Embryonic stem (ES) cells are used as a therapeutic tool in humans. It is important to develop an animal model by using domestic animals which are immunologically and physiologically more closely related to humans than mice and rats (Gjorret and Maddox-Hyttel, 2005). ES cells can be used to study cell differentiation, embryonic development, gene regulation as well as be incorporated into biomedical research (Wheeler, 1994; Thomson and Odorico, 2000). ES cells were first successfully obtained in mice (Evans and Kaufman, 1981), sheep (Notarianni et al., 1991), primates (Thomson and Marshal, 1998), horses (Saito et al., 2002), pigs (Li et al., 2003), humans (Hovatta et al., 2003; Mitalipova et al., 2003), cattle (Yadav et al., 2005) and goats (Pawar et al., 2009). However, the methods of derivation, propagation and differentiation of ES cells for domestic animals have not been fully established.

Under appropriate culture conditions, isolated ICM (inner cell mass) will form ES cells and be maintained in an undifferentiated state in the culture. The ES cells subsequently have the potential to differentiate into various types of cells.

Proper characterization of ES cell lines were based on the expression of pluripotency markers such as Oct-4, SOX 2, Nanog, SSEA-1, 3 and 4. Oct-4 is a homeodomain transcription factor of the POU family. This protein is essentially involved in the self-renewal of the undifferentiated embryonic stem cells. Too much Oct-4 expression will induce differentiation of the cells (Niwa et al., 2000). SSEA-3 is a "globo-series" carbohydrate antigen present on both the glycolipid and glycopeptide cell surfaces. It is also found

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on the surface of human teratocarcinoma cells (EC), embryonic germ cells (EG) and ES cells (Takeda et al., 1992; Niwa et al., 2000).

Due to limited information on the ICM isolation and establishment of goat ES cell lines, the present study was designed to explore new techniques to isolate the ICM from goat blastocysts and to characterize the embryonic stem cell-like outgrowths.

2. Materials and methods

2.1. Reagents and media

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo, USA), unless otherwise stated. Plastic dishes were obtained from Nunc (Roskilde, Denmark). All media were obtained from Chemicon (Millipore, USA).

2.2. Feeder cells

Mouse embryonic fibroblasts (MEF's) were prepared from 13.5 to 14.0 d.p.c. mouse foetuses and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich Co., St. Louis, MO, USA), supplemented with 10% foetal bovine serum (FBS; Invitrogen, Singapore), 200 mM L-glutamine and 3× penicillin/streptomycin. The fetuses were washed in phosphate buffer saline without calcium and magnesium [PBS(−)]. Then, the head limbs and internal organs were removed and minced into small pieces and the minced tissue placed in a beaker. A volume of trypsin (0.25%) and EDTA (0.05%) was added into the beaker and stirred with a magnetic stirrer for 30 min. Subsequently, it was filtered and centrifuged twice at 3000 × *g* for 5 min. The supernatant was removed. The pellet were re-suspended with culture media and immediately seeded in a 60 mm tissue culture dish. The primary MEF's were cultured at 37 °C in a 5% CO_2 incubator, until the cells reached 80–90% confluency. At 80–90% confluency the MEF's were sub-cultured to expand and frozen for the feeder cell bank, for future use. In order to be used as feeder cells, the feeder layers were prepared by expanding the MEF cultures on gelatinized Petri dishes, before seeding the cells. Culture dishes were coated with 0.1% gelatin prior to the culturing of the MEF's. The source of MEF's was either freshly passaged or thawed from frozen stock. The MEF's were seeded at a rate of 3.0×10^5 viable cells per dish, to allow confluency to be reached within 2–3 days. The confluent MEF was filled with DMEM + 10% FBS, supplemented with 10 μg/ml mitomycin C and incubated for 3 h to inactivate the propagation of MEF's. The feeder cells were prepared 1 day before and incubated with an ES cell culture media at least 3 h before being used.

2.3. Flushing of embryos

Goat blastocysts were derived *in vitro* or *in vivo* (Fig. 1). For *in vivo* derived goat blastocyts, uterine flushings were carried out. The doe was superovulated with 160 mg of a follicle stimulating hormone (FSH) for 4 consecutive days. The goat donor being treated with a control internal drug release (CIDR) device inserted for 17 days. This was removed on Day 18. Estrous detection was carried out and the doe in estrus was mated with a fertile buck 3 times per day. This process was carried out for 2–3 days, until the end of estrus. The blastocyst recovery was performed on Day 7 through uterine flushing. All the collected blastocysts were cultured in a k-simplex optimization medium (KSOM), prior to ICM isolation.

2.4. In vitro embryo production

For *in vitro* derived blastocysts, goat blastocysts were derived by a somatic cell nuclear transfer (SCNT) to produce goat ES cell lines. Different goat oocytes retrieval techniques were used to produce blastocysts, namely laparoscopic ovum pick-up (LOPU), ovariectomy (via laparotomy) and ovary slicing from local abattoir material. Estrous synchronization was conducted to facilitate the time and date for retrieval of the oocytes for LOPU.

The goat ovaries were obtained from local abattoirs and transported to the laboratory within 2 h in a warm saline medium at 35–38 °C. In the laboratory, the ovaries were washed thoroughly with saline medium (37 °C) and the oocytes recovered by slicing. All retrieved oocytes were washed with the TL-hepes working solution, followed by placment in an *in vitro* maturation medium, prior to the oocytes being graded for the subsequent experiment. Cumulus oocyte complexes (COC's) were classified according to the cumulus cell characteristics and cytoplasm morphologies under a stereomicroscope and cultured in 50 μl drops of a maturation medium under mineral oil at 38.5 °C, in a 5% CO_2 incubator and humidified atmosphere. All the retrieved oocytes from the above sources were further processed by SCNT and parthenogenetic activation (PA) to obtain goat blastocysts as a source of the goat ES cell lines.

2.5. Isolation of inner cell mass (ICM's)

The zona pellucida of the blastocysts were removed using pronase (0.5%). ICM's were isolated by using manual cut (30 gauge needle), laser dissection or whole blastocyst cultures (control).

For the manual cut technique, a cut was performed between ICM and trophectoderm (TE), by using two 30 gauge needles. Then, the ICM was transferred carefully onto an inactivated feeder in a cell layer with a sufficient gap. A single dish contained 10–15 ICM's. The culture was observed on Day 2. Media were changed every other day.

For the laser isolation technique, the ICM isolation was carried out on the micromanipulator system where the holding pipette (left side) and biopsy needle (right side) were used in the manipulation of the blastocyst. The ICM was located at the 9 o'clock position and the laser shot between the ICM and the TE cells. The biopsy needle was sucked on the other side of the blastocyst to remove the zona pellucida and TE from the ICM. The isolated ICM was placed and cultured onto the inactivated feeder layer in a 5% CO_2 incubator, at 37 °C. After 5–12 days, the growing colonies were individually dissociated into clumps after treating with a mixture of trypsin (0.05%) and EDTA (0.01%). The resultant small clumps containing approximately 20–50 cells were transferred to a new well with a fresh feeder layer and medium. The new colonies were inspected daily and sub-cultured at an interval of 6–10 days, according to their size and growth rate. Culture medium was changed every other day.

2.6. Culture of outgrowth colonies (OC's) and passages

The primary goat ICM outgrowths were sub-cultured by using trypsinization or mechanical dissection procedures with trypsin (0.05%) and EDTA (0.01%). After 6 to 10 days, the primary ICM outgrowths with undifferentiated cells characterized by slightly dark-pigmented clumps of cells, were selected (Fig. 2). The ICM with a flattened shaped colony surrounded with primitive endoderm, homogeneous and a higher nuclear to cytoplasm ratio (bigger nucleus) was selected.

For trypsinization, the ICM outgrowths were lifted from the feeder cells and transferred to trypsin (0.05%) and EDTA (0.01%) for 3 min at 37 °C. Then, it was gently pipetted up and down several times to break the ICM outgrowth into small pieces, which contained 20 to 30 cells/piece and spread onto a new feeder layer. The cell colonies grew 4–6 days after trypsinization (Passage 1) and were subsequently sub-cultured.

For the mechanical dissection, a pulled Pasteur pipette connected with mouth piece was used to cut the colony into smaller pieces. The ICM outgrowths were detached and transferred to 30 μl trypsin (0.05%) and EDTA (0.01%); and gently disaggregated into smaller aggregates. The clumps that did not disaggregate into single cells were transferred into a 4-well dish and incubated at 37 °C in 5% CO_2. The cells with goat ES cell characteristics were checked daily and selected for sub-culture.

2.7. Alkaline phosphatase (AP), SSEA and Oct-4 staining

In order to confirm the pluripotency of the goat ES cells in the present study, AP staining was performed. The cell was fixed in paraformaldehyde (4%) for 30 min, after which the ES cells were washed with PBS(−), 5 times. After the AP was added into the substrate and incubated for 30 min in a dark environment, the ES cells were washed again with PBS(−) twice and PBS(−) was again added to the ES cells. The goat ES cells showed a purplish colour, while the differentiated cells were colourless after staining, as shown in Fig. 3.

The goat ES cells could also be confirmed by using an ES cell marker staining kit, from Chemicon (Millipore, USA and Canada). The ES cells were fixed in paraformaldehyde (4%) for 30 min. Then, the cells were washed with PBS(−) 5 times, followed by addition of a blocking solution [10% FBS + PBS(−)] and left for 2 h at room temperature (25 °C). The primary antibodies (Oct-4, SSEA-1, SSEA-3, SSEA-4) were prepared at the

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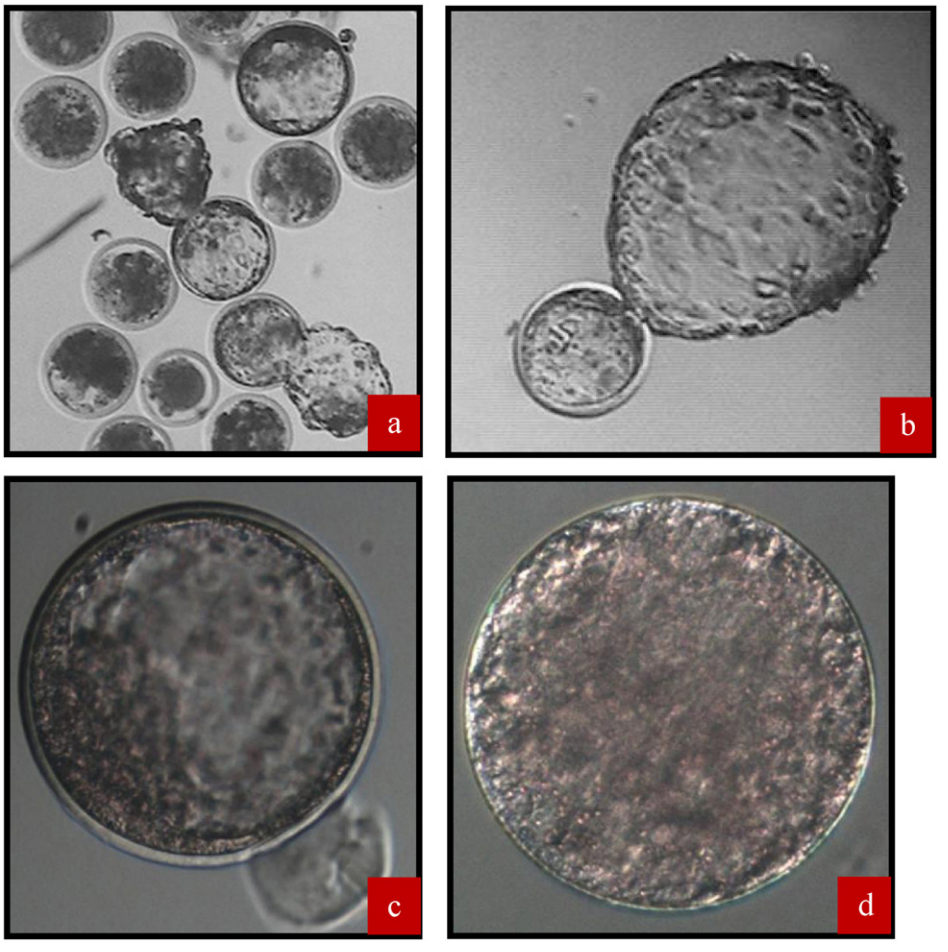

Fig. 1. (a and b) *In vitro*-derived caprine blastocysts from somatic cell nuclear SCNT. (c and d) *In vivo*-derived caprine blastocyst from uterine flushings.

optimal concentration in the ratio of 1:250. After 2 h of immersion in the blocking solution, the blocking solution was removed and the specific diluted individual primary antibody added. Then, the mixture was incubated overnight at 4 °C. After that, the primary antibody was removed and washed 5 times with PBS(−). After washing with PBS(−), a secondary antibody (diluted in ratio 1:1000) was added and incubated for 2 h in a dark environment. After a 2 h period, the secondary antibody was removed and washed 5 times with PBS(−). Lastly, Hoechst 33342 (5 μg/ml) was added to immerse the cells and incubated for 5 min in a dark environment. The cell staining was recorded. The cell staining being observed using a fluorescent microscope with the expression of glow exhibited from the secondary antibody and the nucleus (Hoechst 33342), as shown in Fig. 3.

2.8. Experimental design

Two experiments were conducted to develop an isolation method of homogenous embryonal outgrowths of the ES cells from the *in vivo or in vitro* derived goat blastocysts; and to characterize the growth

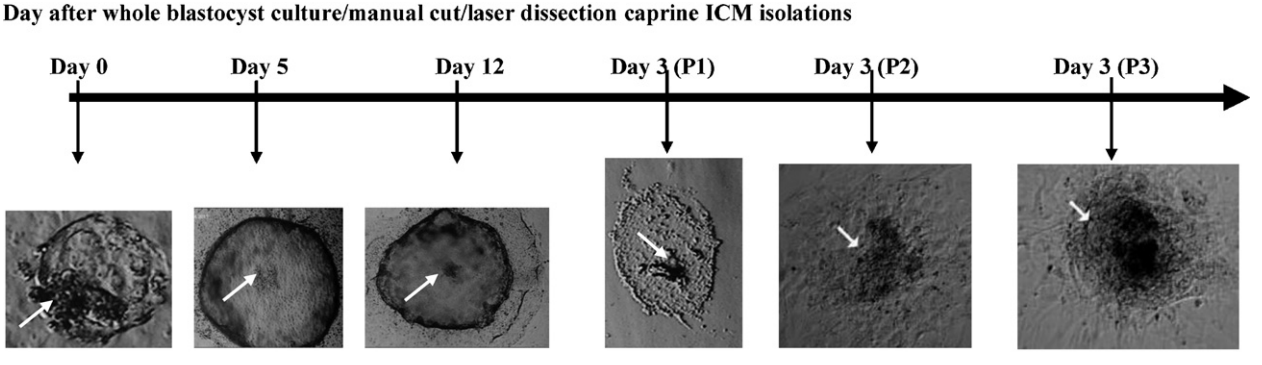

Fig. 2. Morphology of primary outgrowths of caprine ICM development after (Days 0–12). On Day 12, the primary ICM outgrowth was sub-cultured by 0.05% trypsin 0.01% EDTA but the later passages were performed by manual dissociation. Arrow: ICM outgrowths.

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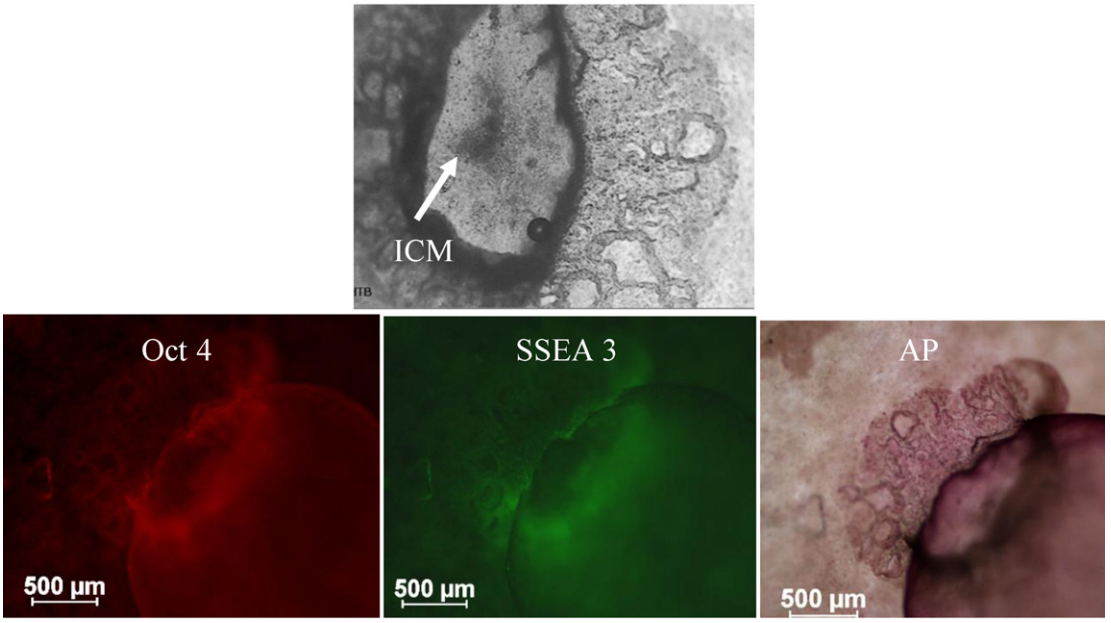

Fig. 3. Caprine embryonic stem cells confirmed by the expression of caprine specific embryonic stem cell markers Oct-4 (red) and SSEA-3 (green). The alkaline phosphatase activities in caprine embryonic stem cells gave a purplish colour for alkaline phosphatase (AP) staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

characteristics and expression of markers indicating the pluripotency in ES cells. Experiment 1 used a manual cut, laser dissection and a whole blastocyst culture (control) to isolate the ICM from both *in vivo* and *in vitro* derived goat blastocysts. Experiment 2 used AP staining to determine the AP activities in the ES cell lines. A purplish colour confirmed the pluripotency of the ES cells. Specific colours were shown for specific protein markers (Oct-4: red and SSEA-3: green) to indicate the pluripotency of ES cells.

2.9. Statistical analysis

The effects of the inner cell mass isolation techniques and blastocyst sources on the percentage of ICM attachment, primary ICM outgrowth and successful ES cell passages were analyzed using the Statistical Package for Social Science (SPSS 16.0 version). Means were obtained and analyzed by one-way analysis of variance (ANOVA). The differences between the means were determined by using the Duncan's Multiple Range Test (DMRT). The data were presented as mean ± standard error of the means (SEM).

3. Results

3.1. Experiment 1: embryo culture and goat ES cell derivation

A comparison between the *in vivo* and *in vitro* derived goat blastocysts as a source for ES cell production is set out in Table 1. A total of 13 and 156 blastocysts were derived *in vivo* and *in vitro*, respectively. There were significant differences between *in vivo* and *in vitro* derived blastocysts regarding the percentage successful cell lines in Passage 1 (95.0% vs. 52.8% respectively) and Passage 3 (91.7% vs. 20.8% respectively). Even though insignificant in most cases, the means of parameters *in vivo* were consistently higher than those *in vitro*.

3.2. Experiment 2: ICM isolation techniques to produce ES cell lines

The percentage attachment of goat ICM, primary ICM outgrowths and successful ES cell passage on the whole blastocyst culture, manually cut or by laser dissection techniques are summarized in Table 2. There were no significant differences ($P > 0.05$) in the percentage attachment of ICM and primary ICM outgrowths for the ICM isolation techniques, with values of 62.9%, 74.4% and 74.0%, as well as 83.3%, 71.6 and 80.4%, respectively. Except for Passage 2, manual cutting showed a consistently higher percentage of ICM attachment and primary ICM outgrowths in Passage 1 and Passage 3.

3.3. Experiment 3: characterization of ES cells: PA, Oct4 and SSEA-3 staining

The goat ICM outgrowths expressed positive AP activity after AP staining, which then exhibited a purplish colour. Specific ES cell markers were expressed in the goat ES cells as Oct-4 (red) and SSEA-3 (green) in the goat species. The expressions of ES cell protein markers are summarized in Table 3.

4. Discussion

Embryonic stem (ES) cells may be produced from the ICM of intact blastocysts (Evans and Kaufman, 1981; Robertson, 1987), by immunosurgery (Solter and Knowles, 1975; Martin, 1981), and by other methods to isolate pluripotent cells constituting the ICM (Brook and Gardner,

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Table 1
Mean (±SE) percentage attachment of blastocysts, primary ICM outgrowths and consecutive passages of goat ES-like cell lines.

Source	No. of replicates	No. of blastocysts	% Blastocyst attachments (*n*)	% Primary ICM outgrowths (*n*)	% Successful ES cell passages (*n*)		
					P1	P2	P3
In vivo	4	13	$100.0 \pm 0.0^{a,x}$ (13/13)	$87.5 \pm 8.0^{a,x}$ (11/13)	$95.0 \pm 5.0^{a,y}$ (10/11)	$81.3 \pm 12.0^{a,x}$ (8/11)	$79.2 \pm 12.5^{a,y}$ (7/11)
In vitro[*]	41	156	$69.5 \pm 4.7^{c,x}$ (124/156)	$75.6 \pm 5.1^{c,x}$ (104/124)	$52.8 \pm 6.1^{b,x}$ (74/104)	$41.8 \pm 6.7^{b,x}$ (51/104)	$20.8 \pm 5.5^{a,x}$ (24/104)
Total	45	169	72.2 ± 4.5	76.6 ± 4.7	56.5 ± 5.9	45.3 ± 6.4	34.7 ± 2.4

[abc] Means with different superscripts in a row within a group were significantly different ($P < 0.05$).
[xy] Means with different superscripts in a column within a group were significantly different ($P < 0.05$).
[*] *In vitro* included SCNT and PA treatments.

Table 2
Mean (±SE) percentage attachment of ICM, primary ICM outgrowths and consecutive passages of goat ES-like cell lines.

Isolation technique	No. of replicates	No. of blastocysts	% ICM attachments (n)	% Primary ICM outgrowths (*n*)	% Successful ES cell passages (*n*)		
					P1	P2	P3
Whole blastocyst culture (control)	8	26	$62.9 \pm 6.7^{bc,x}$ (16/26)	$83.3 \pm 8.3^{c,x}$ (13/16)	$39.6 \pm 13.0^{ab,x}$ (6/13)	$22.9 \pm 13.0^{ab,x}$ (3/13)	$12.5 \pm 12.5^{a,x}$ (1/13)
Manual cut	22	90	$74.4 \pm 7.5^{b,x}$ (79/90)	$71.6 \pm 7.5^{b,x}$ (68/79)	$71.3 \pm 9.0^{b,y}$ (59/68)	$46.4 \pm 9.2^{a,x}$ (43/68)	$35.0 \pm 9.1^{a,x}$ (25/68)
Laser dissection	15	53	$74.0 \pm 7.3^{cd,x}$ (42/53)	$80.4 \pm 7.7^{d,x}$ (34/42)	$44.0 \pm 7.4^{ab,xy}$ (19/34)	$30.0 \pm 6.1^{bc,x}$ (13/34)	$12.2 \pm 4.9^{a,x}$ (5/34)
Total	45	169	72.2 ± 4.5	76.6 ± 4.7	56.5 ± 5.9	45.3 ± 6.4	27.1 ± 5.9

[abcd] Means with different superscripts in a row within a group were significantly different ($P < 0.05$).
[xy] Means with different superscripts in a column within a group were significantly different ($P < 0.05$).

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Table 3
Summarization of ESC protein marker expressions on goat ES cells.

Pluripotent markers	Goat ES cells
AP	+
SSEA-1	–
SSEA-3	+
SSEA-4	–
TRA-1-60	–
TRA-1-81	–
Oct-4	+

1997) or single blastomeres (Sills et al., 2005; Chung et al., 2006).

The current culture conditions used for mammalian ES cells have been mainly developed for mouse ES cell culture, and it appears that these culture conditions were not very effective for maintaining ES cells in domestic animals. In the present study, ICM from goat blastocysts were successfully isolated by using whole blastocyst cultures, using manual cutting or laser dissection to produce ES cells, as characterized by AP, SSEA-3 and Oct-4 staining. The ICM's were cultured on a MEF feeder layer to produce the goat ES cells. The ICM from *in vivo* derived blastocysts gave a higher percentage of successful ES cell passages, compared to *in vitro* derived blastocysts. The pig ES cells obtained were reported to be lower for blastocysts, produced from *in vitro* produced blastocysts, compared to *in vivo* produced embryos (Vassiliev et al., 2010), and similar to those in the present study.

Attempts in the primary culture of the ICM of the feeder cell layer (MEF), in the presence of leukaemia inhibitory factors (LIF), have seldom been successful. The feeder layer was shown to be one of the key factors influencing ES cell culture (Piedrahita et al., 1990). Various components have been used as supplements in the ES cell culture media. One of the components being cytokine e.g. LIF, a basic fibroblast growth factor (bFGF) and the stem cell factor (SCF). Some researchers used feeder cell layers only, without adding any foreign cytokines to the ES cell medium (Chen et al., 1991). Others again added LIF, bFGF and SCF into the ES cell (Piedrahita et al., 1990). Although LIF could inhibit the differentiation of ES cells, bFGF may stimulate ES cell proliferation (Schmitt et al., 1991). LIF has been shown to inhibit the differentiation of ES cells (Niwa et al., 1998). In this study, LIF was added to the ES cell culture medium and MEF was used as the feeder cell layer. It has been reported that MEF's secrete some kind of cytokines (Smith et al., 1988), which may stimulate ES cell growth and inhibit their differentiation. MEF's had been shown to produce the factors responsible for maintaining embryonic carcinoma cells in an undifferentiated state, by inhibiting their differentiation (Martin and Evans, 1975). In addition, mitomycin C treatment time also affected the MEF activity. If the incubation period exceeded 3 h, the MEF could not be used as a feeder layer, due to the decrease in cell adhesion to the dishes.

ICM cells were isolated from the blastocysts by using an immuno-surgery technique (Solter and Knowles, 1975; Wianny et al., 1997; Chen et al., 1999). However, the retrieval of ICM cells was not always successful, as antiserum was used during immuno-surgery. It has been shown earlier that mechanical ICM isolation was successful in producing cat ES cells (Yu et al., 2008). Subsequently, Wolf et al. (2011) showed that cat ICM's could be obtained by using manual isolation, immunosurgery, immunosurgery with manual cleaning or whole blastocyst culture. Although all isolation methods resulted in ES-like cell outgrowth colonies (OC's), immunosurgery with manual cleaning yielded significantly higher rates of ICM attachment and subsequent ES-like morphology. An alternative method to isolate ICM cells from blastocysts was suggested and it was found that it was easier to isolate ICM cells from blastocysts by manually cutting (Wolf et al., 2011).

Alkaline phosphotase (AP) (Wobus et al., 1984; Talbot et al., 1993), Oct-4 (Pawar et al., 2009) and SSEA-3 (Yang et al., 2010) have been the most common markers used to evaluate pluripotent cells and to identify stem cells. As shown in Table 2, the ES cells obtained from ICM confirmed their pluripotency by using the mentioned staining, which was similar for the buffalo (Verma et al., 2007), pig (Li et al., 2003), horse (Saito et al., 2002) and primates (Thomson and Marshal, 1998). Expression stage-specific embryonic antigen SSEA-3 only appeared on goat ES cells.

5. Conclusions

In summary, the understanding of culture conditions such as by using MEF as a feeder layer in the presence of LIF in this study is important to develop an efficient derivation of ES cells in goats. In addition, appropriate isolation techniques of the ICM of blastocysts such as e.g. the whole culture of blastocysts, manually cut and laser dissection should be given due consideration in order to establish a routine and practical protocol for the production of ES cells. The goat ES cells were positively characterized by AP, Oct-4 and SSEA-3 staining.

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EFFECTS OF MURINE STRAIN, BLASTOCYST STAGE AND INNER CELL MASS ISOLATION TECHNIQUE ON THE EFFICACY OF MURINE EMBRYONIC STEM CELLS

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ABSTRACT The aim of this study was to compare the effects of murine strain, blastocyst stage and inner cell mass (ICM) isolation technique on the efficiency of deriving murine embryonic stem cell (mESC) lines. Foetal mouse embryonic fibroblasts (MEFs) were cultured to Passage 2, cryopreserved and thawed at each passage to be used as feeder layer for mESC culture. Five blastocyst stages from *in vivo* and *in vitro* produced blastocysts were cultured on the MEFs by using 3 different ICM isolation techniques. ICM outgrowths were disaggregated by trypsin/EDTA (0.05%) and manual dissociation, cultured on new inactivated MEFs in CO_2 (5%) incubator, 37ºC. The attachment, primary ICM outgrowth and successful consecutive passages rates up to P3 were compared among the murine strains, blastocyst stages and ICM isolation techniques. There were significant differences ($P<0.05$) in successful passage rate at P3 between CBA/ca with ICR and C57BL/6J (19.81% vs. 9.00% and 8.50%), respectively, also mESC at P1 for mid-, expanded- and hatching blastocyst stages versus early- and hatched blastocyst (45.35%, 52.79% and 43.01% vs. 27.88% and 24.53%), respectively. Manual cut ICM isolation technique consistently gave the highest attachment, primary ICM outgrowth and successful mESC P2 and P3 rates compared with whole blastocyst culture and laser dissection techniques (78.03% vs. 66.52% and 71.06%; 78.35% vs. 75.32% and 75.67%; 52.06% vs. 41.62% and 45.06%; 36.52% vs. 25.77% and 30.49%), respectively. In conclusions, the CBA/ca strain, expanded blastocyst stage and manual cut ICM isolation techniques showed the highest results obtained in production of mESC lines.

ABSTRAK Kajian ini adalah untuk membandingkan strain mencit, peringkat blastosis dan teknik-teknik mengasingkan jisim sel dalaman (ICM) terhadap kecekapan dalam memperolehi titisan sel batang embrionik mencit (mESC). Fibroblas embronik mencit (MEF) dikultur sehingga pasaj 2, dikrioawet dan dinyahsejukbeku pada setiap pasaj untuk digunakan sebagai lapisan sel pembantu bagi pengkulturan mESC. Lima peringkat blastosis diperolehi daripada *in vivo* dan *in vitro* telah dikultur atas MEF dengan 3 teknik pengasingan ICM. Pertumbuhan ICM telah dipisahkan dengan cara trypsin/EDTA (0.05%) dan penceraian secara manual, dikultur di atas MEF baru tak teraktif dalam inkubator CO_2 (5%) pada 37ºC Pelekapan, pertumbuhan ICM primer dan kadar pasaj turutan yang berjaya sehingga ke P3 telah dibanding antara strain mencit, peringkat blastosis dan teknik pengasingan ICM. Terdapat perbezaan signifikan ($P<0.05$) dalam kadar pasaj yang berjaya pada P3 antara CBA/ca dengan ICR dan C57BL/6J (19.81% vs. 9.00% dan 8.50%), masing-masing, juga mESC pada P1 bagi peringkat blastosis pertengahan, pengembangan dan penetasan berlawan dengan peringkat blastosis awal- dan menetas (45.35%, 52.79% dan 43.01% vs. 27.88% dan 24.53%), masing-masing. Teknik penceraian ICM secara pemotongan manual adalah dengan konsisten memberi kadar-kadar dalam pelekapan, pertumbuhan ICM primer serta P2 dan P3 bagi mESC yang berjaya yang paling tinggi berbanding dengan kultur seluruh blastosis dan teknik pembedahan laser (78.03% vs. 66.52% dan 71.06%; 78.35% vs. 75.32% dan 75.67%; 52.06% vs. 41.62% dan 45.06%; 36.52% vs. 25.77% dan 30.49%), masing-masing. Secara ringkasnya, strain CBA/ca, peringkat pengembagan blastosis dan teknik pemotongan secara manual bagi teknik pengasingan ICM menunjukkan hasilan optimal diperolehi dalam menghasilkan titisan-titisan mESC.

(**Keywords**: Blastocyst stage, ICM isolation technique, mESC, murine strain)

INTRODUCTION

Murine serves as a preferred human therapeutic model for embryonic stem cell (ESC) research as this species has close similarities in terms of molecular biology, physiology and developmental process. Since murine species has relatively short generation interval and prolific,

easy to handle, bred and managed as well as capable of reproducing in large numbers, it is suitable to establish a large number of both MEF stock and to obtain the blastocysts needed for stem cell culture. ESC could differentiate into any cell type in the body, including gametes [1, 2]. It is defined functionally as cells that have the capacity to self-renew and the ability to generate differentiated cells [3, 4]. Isolation and culture of embryo-derived cell lines have been reported in many mammals such as murine [2, 4], ovine [6, 7], hamster [8, 9], porcine [10, 11, 12, 13], mink [14], rabbit [15, 16], bovine [17, 18], including primates [19] and humans [20].

mESC isolated from both whole embryos [21] and isolated ICM [2, 22], have similar morphological and biochemical properties to cells from the early murine embryos [23]. mESC have generate chimeric murine showing pluripotency in nature [24] and cultured ICM on MEF in the presence of leukaemia inhibitory factor (LIF) [25]. Blastocysts are either plated intact of feeder layer [26, 27] where they hatch and attach to feeder cell layer or the ICM is isolated from blastocysts either by immunosurgery [27, 28], enzymatic digestion using trypsin [26, 27] or mechanical isolation [26].

Feeder cell is one of the important factors affecting ESC culture [29]. Feeder cell of various types have been used for ESC culturing; STO fibroblasts in murine [2], bovine foetal fibroblasts, bovine uterus epithelial cells, MEF, human lung fibroblasts for ESC culturing in bovines [28, 30, 31] and buffalo foetal fibroblasts in buffalo [26]. It has been reported that feeder layer could secrete some kinds of cytokines, such as LIF [32], which may stimulate ESC growth and inhibit their differentiation. Without a suitable culture medium or feeder layer, ESC would spontaneously differentiate. The original protocol for deriving mESC by Evans and Kaufman [2] is highly inefficient. Even though improvements have been made [33], an efficient protocol is still needed to be developed.

The production of blastocysts as s source for mESC production involves either natural mating or superovulation of murine females to produce a large amount of fertilisable eggs, followed by timed-mating. The desirable blastocysts could also be obtained directly via uterine flushing or indirectly by culturing early stages oviduct-flushed embryos up to blastocyst stage. Many regard the 129 strain as the most favourable and efficient strain [33]. Experiments using other strains showed varying success rates [34, 33]. It has been established that strain difference has an effect on the efficiency of establishing mESC lines [35]. The reason behind this is still poorly understood. Although the 129 and C57BL/6J strains are commonly used, relatively few researchers used the ICR or CBA/ca strain to develop ESC lines.

Most studies ignore the blastocyst stage as a factor when deriving ESC [35, 33]. Movassagh-Pour *et al*. [36] found that the efficient of deriving ESC from hatched blastocysts were low. This study indicates that blastocyst stage may be a crucial factor affecting ESCs derivation.

mESC could be obtained by culturing the whole blastocyst, manual cut or laser dissection technique on a suitable culture medium with MEF as feeder cell layer. Besides that, other methods of deriving ESC include microdissection of blastocysts [34], isolation of the ICM through micromanipulation and culturing of single blastomeres [37]. The cells could then be used for subsequent steps in research, or cryopreserved for future usage.

Culture of ICM of murine blastocysts was carried out for production of mESC lines and characterised with marker expressed as alkaline phosphatase, Oct 4, SSEA 1, SSES 3, TRA-1-60 and TRA-1-81.

In the present study, we evaluated the effects of murine strain (ICR, CBA/ca and C57BL/6J), blastocyst stage (early-, mid-, expanded-, hatching- and hatched blastocyst) and ICM isolation technique (whole blastocyst culture, manual cut, laser dissection) on efficiency of mESC culture.

MATERIALS AND METHODS

Preparation of feeder cell

The feeder cells (MEF) were prepared from 13.5-14.0 d.p.c. murine foetuses. The pups were processed by removing the head, tail, limbs and internal organs, then transferred to a fresh PBS(-) solution for washing and minced it by using a sterile blade in small amount of trypsin/EDTA (0.25%) solution. Mincing was done for a few

more minutes to further reduce the size of the pieces. The large pieces were filtered out through a sheet of sterilised nylon and then centrifuged at 2000 rpm for 5 min. After centrifugation, the supernatant was removed and the pellet was re-suspended in around 2 to 4 ml of 3x MEF culture media. The cells were then seeded into the culture dishes coated with 0.1% gelatine with culture medium DMEM (Invitrogen) supplemented with 10% foetal bovine serum (FBS; Hyclone), 200mM L-glutamine (Invitrogen) and 1x penicillin/streptomycin (PS) (Invitrogen). The MFF were inactivated with 5 µg/ml mitomycin C (Sigma) for 2-3 hr followed by a thorough wash before plating.

When the cells from the explants reached 80% confluence, they were harvested using trypsin/EDTA (0.25%) and then sub-cultured to P1 or P2. The MEF at P1 or P2 were cryopreserved using DMSO (20%) mixed in the tissue culture medium and stored in liquid nitrogen. The frozen cells were thawed and cultured up to 80% confluence to use ready used as feeder cells for mESC cultured.

Embryo collection

Three pure-strains of murine (ICR, CBA/ca and C57BL/6J) blastocysts were collected by *in vivo* oviduct (2-cell stage embryos) or uterine (blastocysts) flushing. Murine females (6-8 weeks old) were superovulated with an intraperitoneal (i.p.) injection of pregnant mare's serum gonadotrophin (PMSG; 10 IU) followed by an intraperitoneal injection of human chorionic gonadotrophin (hCG; 10 IU) at 46-48 hr later. Each female murine was then placed in a cage with a stud male and copulation plug was checked on the next morning (day 1 pregnancy).

For recovery 2-cell stage murine embryos, oviducts of superovulated murine females were flushed with Hepes Whitten's medium (HWM) using a flushing needle (32 G) connected to a syringe (1 ml). Collected embryos were washed 3 times in equilibrated Whitten's medium (WM) and cultured under mineral oil at 37.5ºC in CO_2 (5%) in humidified air for *in vitro* development until blastocyst stage prior to use for producing mESC. Murine blastocyst stage will be located in the uterus. By using a method described by Hogan *et al*. [38], blastocysts were flushed from the uterus between days 3.5-4.5 d.p.c.

Isolation of inner cell masses from blastocysts

Three different ICM isolation techniques were used in present study, namely whole blastocyst culture, mechanical dissection or manual cut (30 G needle) and laser dissection, and the cells consecutively cultured to obtain primary ICM outgrowths (**Figure 2**). In whole blastocyst culture, the efficiency of early-, mid-, expanded-, hatching- and hatched-blastocysts for deriving mESC were compared. The zona pellucida of each blastocyst stage was removed by using pronase (0.5%). Then, the whole blastocysts were plating on inactivated MEF in a humidified atmosphere of CO_2 (5%) in air at 37˚C for culturing. After 6-8 days later, the growing colonies were individually dissociated into clumps after treating with trypsin/EDTA (0.05%). The resultant small clumps containing approximately 20-50 cells were transferred to a new well with a fresh feeder cell and medium. The new colonies were inspected daily and sub-cultured at an interval of approximately 6-10 days according to their size and growth rate, and medium were changed on every other day.

For manual cut ICM isolation technique, the zona pellucida of blastocyst was removed similar as previously described. A single murine blastocyst was transferred onto a droplet of ESCs culture medium. A cut was performed between the ICM and trophectoderm (TE) on blastocyst by using 2 needles (30 G). The murine ICM was isolated after the cutting and transferred onto an inactivated feeder cell and finally was placed onto the feeder cell with sufficient gap in between them.

In laser dissection techniques, the zona pellucida of blastocyst was not removed. This technique was carried out on the micromanipulator system where the holding pipette (left side) and biopsy needle (right side) were used in manipulated blastocyst. The ICM was located at the 9 o'clock position. The blastocyst was held by holding pipette and the laser was shot along the way between the ICM cells and TE cells. After shooting, the biopsy needle was sucked on the other side of the blastocyst to remove the zona pellucida and TE from the ICM. The isolated murine ICM was cultured onto the inactivated feeder cells and the primary outgrowth of the culture was observed.

Isolation and passages of embryo-derived cell lines

The primary ICM outgrowths were sub-cultured using trypsinisation or mechanical dissection procedures by trypsin/EDTA (0.05%). After around 4-6 days of culture, the primary ICM outgrowths were picked and sub-cultured into new feeder cells. The ICM that were selected had characteristics included dome shape colony surrounded with primitive endoderm, homogeneous and higher nuclear to cytoplasm ratio (bigger nucleus). They had a slightly refracted and were located directly next to the outgrowths (Fig. 1). The suitable outgrowths were picked before differentiation occurred.

Alkaline phosphatase (AP) staining

For alkaline phosphatise (AP) staining, the mESC was fixed with paraformaldehyde (4%) for 30 min. After 30 min, they were washed with PBS(-) for 5 times. The AP was added to the substrate just to cover the cell and incubated for 15-30 min in dark environment. After 15-30 min, it was washed again with PBS(-) for 2 times. AP staining was carried out to determine the AP activities that found in mESC lines. The mESC were purplish and differentiated cells were colourless after staining (**Figure 4**).

Characterisation mESC by ESC protein markers

The mESC can be confirmed by immunofluorescent staining. They were fixed with paraformaldehyde (4%) for 30 min and the cell was washed with PBS(-) for 5 times and blocking solution (10% FBS+PBS(-)) was added and leave it for 2 hr at room temperature. The primary antibody (such as Oct 4, SSEA 1, SSEA 3, SSEA 4, TRA-1-80 and TRA-1-60) was prepared at the optimal concentration with the ratio (1:250). After 2 hr of blocking solution, the blocking solution was removed and the diluted primary antibody was added and incubated overnight at 4°C. After that, the primary antibody was removed out and washed 5 times with PBS(-). The washing step took 10-15 min/time. Next, secondary antibody (diluted in ratio 1:1000) was added just to cover the cell and incubated 2 hr in dark environment. After 2 hr, the secondary antibody was discarded out and washed again 5 times with PBS(-). Lastly, Hoechst 33342 (5 μg/ml) was added and incubated 5 min in dark condition. The staining cell was observed under fluorescent microscope.

Statistical analysis

Data were analysed by analysis of variance (ANOVA) and Duncan's multiple range tests (D-MRT), using the SPSS statistical software package version 16. A probability of $P<0.05$ was considered significant for all statistical tests. Values were presented as mean±SEM.

RESULTS AND DISCUSSION

A total of 864 and 212 murine were used *in vivo* oviduct flushing and in *vivo* uterine flushing with representative numbers of 297 and 121 (ICR), 278 and 37 (CBA/ca), 289 and 54 (C57BL/6J), respectively. After *in vitro* culture, a total of 6324 (84.36%) murine blastocysts were obtained from 7544 two-cell stage murine embryos through *in vivo* oviduct flushing. Out of 6324 blastocysts obtained, 2589 (86.39%), 1988 (84.26%) and 1747 (81.84%) blastocysts were obtained from the ICR, CBA/ca and C57BL/6J murine pure-strains, respectively.

The percent successful attachment of blastocysts and consecutive passages of mESC lines up to P3 derived from 3 pure-strains of murine (ICR, CBA/ca and C57BL/6J) are summarised in **Table 1** and **Figure 1**. There were no significant differences ($P>0.05$) among the 3 pure-strains of murine in percent successful consecutive passages mESC lines up to P3. However, CBA/ca gave the highest percent attachment of blastocysts and successful consecutive passages mESC lines with the values of 62.68% (attachment rate), 41.32% (P1), 31.00% (P2) and 19.81% (P3), respectively. ESC derived from different strains of murine generally have different success rates [35, 33]. Our results have shown that there were significant differences between CBA/ca and ICR with C57BL/6J, whereby mESC derived from CBA/ca strain have higher success rate of passaging up to P3 compared to ICR and C57BL/6J. Therefore, it is suggested that CBA/ca strain may be more suitable for deriving mESC.

Previously, it was found that blastocysts from C57BL/6J and CBA/ca have an efficiency of deriving ESC of 58% and 66% [39], which was considerably higher than the findings of this

study. One possible factor is the use of ESC medium conditioned with rabbit fibroblast cell line which may have increased the amount of LIF and other support factors in the culture medium, thus improving efficiency [39] in their studies. However, it is noteworthy to mention that only 12 blastocysts were used for each strain in their reports; whereas in the present study 758 and 709 blastocysts from CBA/ca and C57BL/6J were used. No data were found on the performance of blastocysts derived from ICR strain. The overall low efficiency in deriving mESC (11.62%) compared to other researchers may be attributed to the use of 35 mm culture dishes instead of multi-well plates which provide greater contact between the blastocysts and feeder cells [40, 33].

Percent successful attachment of blastocysts and consecutive passages of mESC lines up to P3 from 5 blastocyst stages (early-, mid-, expanded-, hatching- and hatched blastocyst) are summarised in **Table 2**. There were no significant differences ($P>0.05$) in percent attachment for early-, mid-, expanded-, hatching- and hatched blastocyst with the values of 65.17%, 48.08%, 68.78%, 57.01% and 37.45%, respectively. Although each blastocyst stage could passage up to P3 in producing mESC lines, expanded blastocyst stage showed the highest percent successful attachment of blastocysts and successful consecutive passages compared to other blastocyst stages with the values of 68.78% (attachment rate), 52.79% (P1), 37.55% (P2) and 17.24% (P3), respectively. The low success rate of deriving mESC from hatched blastocysts is in agreement with the findings of Movaassagh-Pour *et al*. [36]. Up to now, no reports were found comparing the 5 different blastocyst stages in murine species on the mESC culture. The reason for this phenomenon is not known as this time. A possible explanation could be the biochemical and morphological events taking place during the development of blastocysts from early to hatched stage. In early blastocysts, the ICM and TE have not fully separated. Thus, when cultured on the feeder cell, the consecutive outgrowth would have a high mixture of both ICM and TE derived cells. The interaction among the cells possibly makes the mESC more likely to differentiate. Meanwhile, hatched blastocysts have ICM that were already beginning to differentiate into the epiblast. This is supported by the fact that Oct4 expression in blastocysts decreases sharply at 4.5 d.p.c. [41]. Oct 4 expression is crucial in maintaining the pluripotency and undifferentiated state in ESC [42]. Thus, ESC derived from them may have a higher tendency to differentiate. Therefore, hatched blastocysts were not suitable for deriving mESC.

A significant finding in this experiment is that expanded blastocysts are better sources of mESC lines. The use of expanded blastocysts could be applied in future experiment involving ESC to improve efficiency, rather than ignoring the blastocyst stages when deriving ESC lines [33, 34, 35, 43].

A total of 6831 murine blastocysts were used in this experiment consisting of 2438, 2062 and 2331 for whole blastocyst culture, manual cut and laser dissection techniques, respectively. There were significant differences ($P<0.05$) in percent attachment of blastocysts for 3 different ICM isolation techniques with the values of 66.52%, 78.03% and 71.06% for whole blastocyst culture, manual cut and laser dissection techniques, respectively (**Table 3**; **Figure 2**). Manual cut ICM isolation technique gave the highest percent primary ICM outgrowth with the values of 78.35%, followed by laser dissection (75.67%) and whole blastocyst culture (75.32%). There was a significant decrease in percent successful consecutive passages from P1, P1, P2 and P3 for 3 different ICM isolation techniques.

ICM isolation technique is an important step to ensure the success of the ESC establishment. In present study, the whole blastocyst culture gave the lowest attachment, primary ICM outgrowth and successful passages up to P3 rates compared to manual cut and laser dissection ICM isolation techniques. This may be due to intact embryos, where trophoblastic cells induce ICM differentiation to be 3 embryonic germ layers by suppressing Oct 4 and Nanog expression level [44]. Therefore, completely removed trophoblastic cells provide more benefit to ICM cells turn to be ESC. The ICM could be separated and isolated from the TE by 5 ways, namely immunosurgery [45], with mechanical processes [46], with whole embryo culture of the blastocysts and partial embryo culture methods [47] or single blastomeres [48] and laser dissection [49].

Mechanical isolation of the ICM has previously been successfully used in the derivation of 2 cell lines, as reported by Genbacev *et al*. [50], Mummery [51] and van de Stolpe *et al*. [52].

Manual cut ICM isolation technique has been proven to improve results (Chanchao Lorthongpanich, personal communication). However, the difficulty of the manual cut procedure limits the rate at which blastocysts can be dissected. Furthermore, some ICM may become damaged due to the use of needles to pull the inner cell mass away from the overlying trophectoderm. The embryo tended to rotate away from the 30 G needle when it was cut towards one pole. The choice of well expanded blastocysts enabled trophoblast to be obtained with little risk of inclusion of ICM. Ideally, more training and practice should be done to master manual cut ICM isolation technique to allow comparisons in terms of efficiency.

Laser dissection method are used in the present study was to evaluate a new method of isolation ICM and derivation of ESC lines in a murine. Laser technology is commonly used for assisted hatching with some applications. One of them consists of making the embryonic membrane weaker to make the exit of the future blastocyst easier and so favour the derivation [53]. Until now, this new laser dissection method has been suggested to derive stem cell lines by Wang *et al*. [54], although without presenting any conclusive results. A recent paper, Tanaka *et al*. [55] reported preliminary results for a murine model using this method; subsequent culture of ESC in a serum/cell-free culture system was achieved. We have compared the ICM isolation method using laser dissection in a murine blastocyst with one of the most commonly used methods, the whole blastocyst culture [47]. However, with good quality blastocysts with a large and distinct ICM, we used the laser drill (Hamilton, USA). In our experiment, the murine blastocyst to be treated with laser shot was positioned at the centre of the field of view under 40x magnification. Blastocysts can be secured by 2 holding pipettes with the ICM positioned at '9 o'clock' if desired [55]. After focusing on the TE cells, the object had to be moved so that the part of the TE to be treated was located at the cross-hair position displayed on the monitor as the impact location of the laser focus. Thus, this new mechanical method destroyed the TE cells by shooting the laser over them carefully without damaging the ICM. Although some reported that the whole blastocyst culture method of the blastocysts is more effective than the laser method (70% versus 52.4%) [56], we have used the laser drill for the good quality blastocysts and that the concealment of the ICM by the TE cells is the only disadvantage of the whole blastocyst culture method [46]. Therefore, we must continue to improve the laser dissection technique so that the TE cells are destroyed and do not interfere with ICM [55].

ESC protein markers were used in determining the ESCs formation. Specific ESC markers were found in mESC such as Oct 4 (green) and SSEA 1(green) in this experiment (**Figure 4**). There were no colours shown for TRA-1-60 and TRA-1-81 ESC markers in murine species. Therefore, it indicates the presence of ESC in murine species. Also, purplish colour was shown in mESC after underwent AP staining. It could be confirmed that the presence of true mESC and pluripotent of mESC have been found. Five widely adopted antibody markers (anti-Oct 4, anti-Sox 2, anti-SSEA 1, 3 and 4, anti-TRA-1-60 and anti-TRA-1-81) are the protein markers that usually found on ESC. Transcription factors (such as Oct 3/4 [57, 58], Sox 2 [59] and Nanog [60, 61]) function to identify pluripotency in both early embryos and ESC. Oct 4 and Sox 2 are transcription factors that are highly expressed in undifferentiated ESC and EGC [62, 63, 64]. In our study, mESC express high levels of membrane alkaline phosphatase (AP) and Oct 4, a transcriptional factor critical to ICM and germline formation. Expression stage-specific embryonic antigen (SSEA 1) only appeared on mESC.

For future studies, ESC could be directed towards differentiation pathways by changing growth conditions, leading to development of specialised cells such as heart muscle cells, neurons or insulin-secreting cells, opening the way to the development of new therapies in human regeneration medicine.

Table 1: Percent successful attachment of blastocysts and consecutive passages of mESC (%, mean±SEM) up to P3 from 3 pure-strains of murine.

Strain	No. of females	No. of blastocysts	Percent attachment (n)	Percent successful consecutive passages (n)		
				P1 (n)	P2 (n)	P3 (n)
ICR	63	971	$56.74\pm3.18^{d,x}$ (n=777)	$35.20\pm2.61^{c,x}$ (n=443)	$23.77\pm2.48^{b,x}$ (n=207)	$9.00\pm1.65^{a,x}$ (n=71)
CBA/ca	34	758	$62.68\pm20.90^{b,x}$ (n=429)	$41.32\pm3.49^{ab,x}$ (n=237)	$31.00\pm3.56^{ab,x}$ (n=109)	$19.81\pm3.51^{a,y}$ (n=38)
C57BL/6J	39	709	$44.68\pm2.53^{c,x}$ (n=359)	$40.71\pm4.49^{c,x}$ (n=160)	$22.10\pm3.75^{a,x}$ (n=64)	$8.50\pm2.59^{a,x}$ (n=22)
Total	136	2438	55.18±5.55	38.15±1.93	25.18±1.80	11.62±1.39

^{xy}Mean values within a column within a group with different superscripts was not significantly different (P>0.05).
^{abcd}Mean values within a row within a group with different superscripts were significantly different (P<0.05).

Table 2: Percent successful attachment of blastocysts and consecutive passages of mESC lines up to P3 (mean±SEM) from 5 blastocyst stages.

Blastocyst stage	No. of females	No. of blastocysts	Percent attachment (n)	Percent successful consecutive passages (n)		
				P1	P2	P3
Early	82	256	$65.17\pm24.88^{b,x}$ (n=198)	$27.88\pm3.79^{ab,x}$ (n=105)	$20.28\pm3.70^{a,xy}$ (n=46)	$9.18\pm2.98^{a,x}$ (n=14)
Mid	115	488	$48.08\pm4.00^{c,x}$ (n=278)	$45.35\pm5.60^{c,y}$ (n=164)	$28.11\pm4.29^{b,yz}$ (n=81)	$15.16\pm3.47^{a,x}$ (n=30)
Expanded	129	666	$68.78\pm3.38^{d,x}$ (n=465)	$52.79\pm3.92^{c,y}$ (n=262)	$37.55\pm4.36^{b,z}$ (n=138)	$17.24\pm3.67^{a,x}$ (n=55)
Hatching	122	682	$57.01\pm3.65^{d,x}$ (n=418)	$43.01\pm3.67^{c,y}$ (n=224)	$28.02\pm3.81^{b,yz}$ (n=89)	$11.60\pm2.95^{a,x}$ (n=25)
Hatched	97	346	$37.45\pm4.58^{c,x}$ (n=206)	$24.53\pm3.53^{b,x}$ (n=85)	$11.92\pm3.26^{a,x}$ (n=26)	$8.08\pm2.94^{a,x}$ (n=7)
Total	545	2438	5.44±5.48	38.71±1.94	25.18±1.80	2.27±1.44

^{xyz}Mean values within a column within a group with different superscripts were significantly different (P<0.05).
^{abcd}Mean values within a row within a group with different superscripts were significantly different (P<0.05).

Table 3: Percent attachment of blastocysts, primary outgrowth inner cell mass and successful consecutive passages of mESC lines (%, mean±SEM) on 3 different inner cell mass isolation techniques

Isolation techniques	No. of females	No. of blastocysts	Percent attachment (n)	Percent primary outgrowth (n)	Percent successful consecutive passages (n)		
					P1 (n)	P2 (n)	P3 (n)
Whole blastocyst culture	241	2438	$66.52\pm2.18^{c,x}$ (n=1565)	$75.32\pm1.73^{d,xy}$ (n=1167)	$72.51\pm2.25^{cd,y}$ (n=841)	$41.62\pm3.23^{b,x}$ (n=380)	$25.77\pm3.34^{a,x}$ (n=131)
Manual cut	494	2062	$78.03\pm0.94^{d,z}$ (n=1624)	$78.35\pm1.24^{d,y}$ (n=1295)	$67.84\pm1.60^{c,y}$ (n=906)	$52.06\pm1.94^{b,y}$ (n=545)	$36.52\pm2.45^{a,y}$ (n=255)
Laser dissection	357	2331	$71.06\pm1.11^{d,y}$ (n=1690)	$75.67\pm0.85^{d,x}$ (n=1275)	$58.75\pm2.25^{c,x}$ (n=843)	$45.06\pm2.35^{b,x}$ (n=457)	$30.49\pm2.52^{a,xy}$ (n=194)
Total	1092	6831	72.97±0.79	75.67±0.85	65.70±1.19	47.23±1.39	31.96±1.57

[xyz] Mean values within a column within a group with different superscripts were significantly different ($P<0.05$).
[abcd] Mean values within a row within a group with different superscripts were significantly different ($P<0.05$).

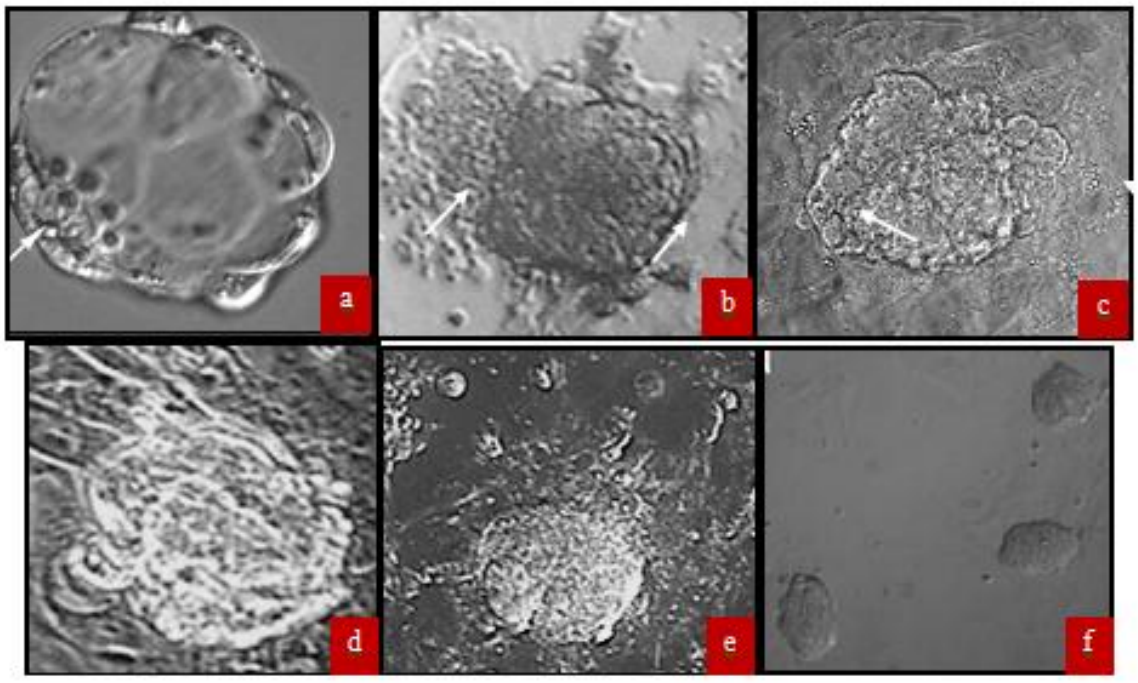

Figure 1: a) Blastocyst without the zona pellucida. b) Attachment and primary ICM outgrowth ICM at day3. c) Primary ICM outgrowth was sub-cultured by 0.05% trypsin/EDTA (P1). d) P2 of mESC was sub-cultured by manual dissociation before differentiation occurred. e) Undiffernetiated mESC at P3 with the sharp and clear edge as well as dome-shape. f) ESC colonies obtained. Arrow: ICM.

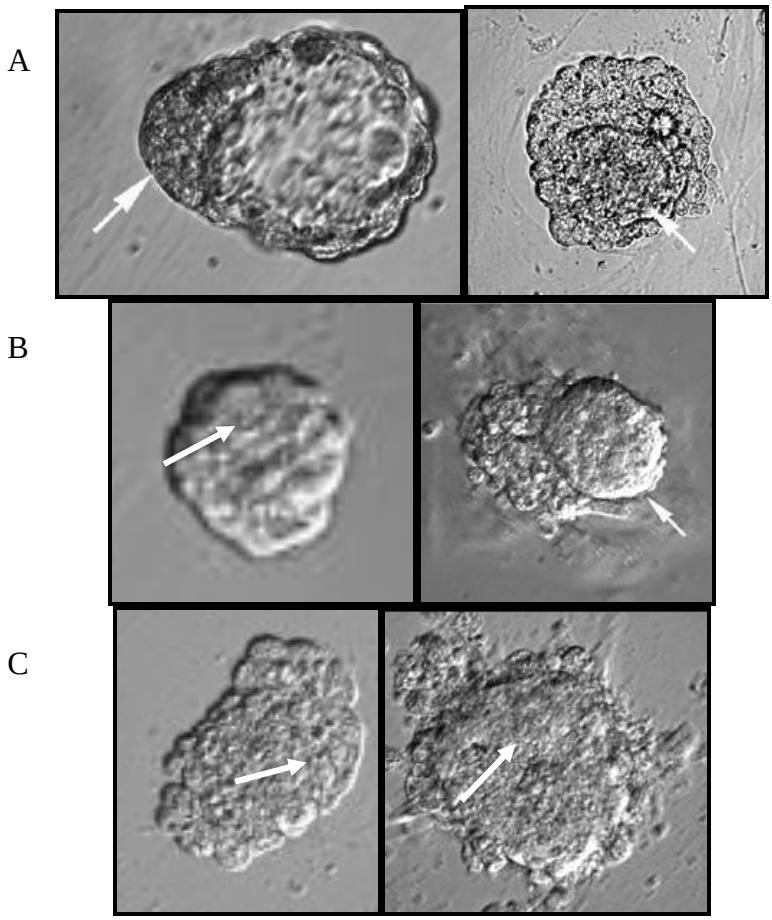

Figure 2: A) Whole blastocyst culture, B) manual cut ICM and C) laser dissection ICM isolation techniques with theirs ICM outgrowth shown by arrow.

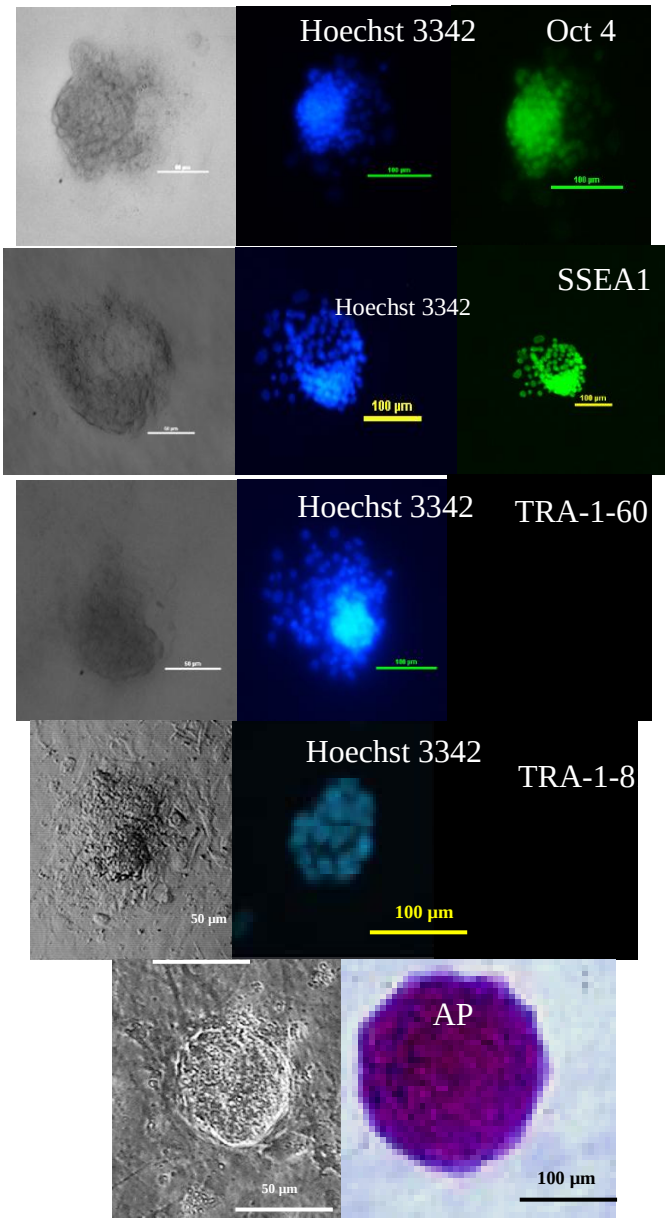

Figure 3: mESC were confirmed by the expression of murine specific ESC markers (Oct4, SSEA1) and human ESC specific markers (TRA-1-60 and TRA-1-81) as negative control. Transmission light images and Hoechst DNA staining were showed in the first and second column. The AP activities were positive and showed in the bottom line of the picture.

CONCLUSION

In conclusion, protocols for the derivation of MEF cell culture and establishment of mESC lines were successfully developed that may serve as the platform for future scientific research in ESC. Three murine strains could produce mESC lines. However, the CBA/ca strain shows satisfactory rate of consecutive passages mESC lines than ICR and C57BL/6J strains. With the establishment of ESC lines in murine by different stages of blastocyst and ICM isolation techniques, expanded blastocysts gave better growth rate of mESC lines then other stages. Manual cut gave highly significant successful rate in producing mESC lines compared the

other 2 techniques. mESC lines were confirmed where it was found that Oct 4 and SSEA 1 protein markers as well as gave positive results in AP staining.

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Production of cloned - caprine embryos through somatic cell nuclear transfer influenced by oocytes sources, oocyte grading, pre - intracytoplasmic injection durations and enucleation techniques

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Abstract: It has been successed since the first cloned mammals were generated from somatic cells using nuclear transfer, but the success rate for producing live offspring by cloning still remains low which less than 5%. The aims of this investigation were to compare the effects of a) oocytes sources, b) oocyte grading, c) pre - intracytoplasmic injection durations and d) enucleation techniques on somatic cell nuclear trasnfer performance. Source of caprine oocytes were obtained from laparoscopic ovum pick - up, ovariectomy and abattoir. The collected oocytes were in vitro matured and characterized by cumulus expansion as well as the extrusion of the first polar body. Cumulus cells were denuded off and oocytes that had extruded the first polar body as well as with evenly granulated cytoplasm were selected and grouped by Grades A, B, C, D and E for enucleation, which were placed into TCM 199 Hepes containing cytochalasin B (5.0 µg/mL). The first polar body and approximately 10 - 15% of the oocyte cytoplasm was squeezed out by 2 different techniques, namely laser or squeezing techniques. Fresh cumulus cells obtained from the matured oocytes could be used directly for nuclear transfer using whole cell intracytoplasmic injection technique. The reconstructed oocytes were activated by

calcium ionophore (5 min) and 6 - dimethylaminopurine for 4 h and cultured in vitro in KSOM at CO_2 (5%) incubator at 38.5^{o}C in humidified atmosphere for 8 - 9 days. The medium was changed every 2 days of in vitro culture. There were significant differences ($P < 0.05$) in maturation and enucleation rates for these 3 different sources with the values of 61.99 ± 3.12% and 66.16 ± 5.21% versus 51.23 ± 3.37% as well as 97.85 ± 1.23% and 98.32 ± 1.16% versus 90.27 ± 2.72% for laparoscopic ovum pick - up, ovariectomy and abattoir sources, respectively. Even though insignificance, the embryo development at 2 -, 4 -, 8 - cell and morula was higher in laparoscopic ovum pick - up, followed by ovariectomy and abattoir. However, ovariectomy showed the highest blastocysts and hatched blastocysts rates (24.52 ± 8.53% and 18.42 ± 6.79%), followed by laparoscopic ovum pick-up (13.33 ± 3.68% and 8.12 ± 2.29%) and abattoir (10.06 ± 5.83% and 5.01 ± 3.19%), respectively. There were significant differences ($P < 0.05$) in percent cleavage between Grades A, B and C with Grades D and E for all stages of embryo development. The 60 min pre – intracytoplasmic injection duration gave highly significant differences ($P < 0.05$) for all the cleavage rates followed by simultaneous injection and 30 min pre – intracytoplasmic injection duration. The results from this study showed that laser enucleation technique gives better cleavage rate compared to squeezing enucleation technique for all the stage embryos development. It is concluded that ovariectomy oocyte source, Grade A, B and C, 60 min pre – intracytoplasmic injection duration and laser enucleation were sucessfully develop in caprine species in our laboratory. It is hoped that the findings obtained from this research will provide the fundamental information for future studies and could be potentially applied to overcome issues in livestock production and wildlife conservation.

Key words: Oocyte sources, oocyte grading, enucleation techniques, pre - intracytoplasmic injection durations, caprine, somatic cell nuclear transfer

Introduction

The basic biology underlying nuclear transfer in cloning still remains unclear although it began more than 20 years ago. The birth of Dolly the sheep by Wilmut et al. (1), the first cloned mammal using somatic cell nuclear transfer (SCNT), success in cloning using adult somatic cells has been reported. The other mammalian species also reported including murine by Wakayama and Yanagimachi (2); Wakayama et al. (3); Wakayama et al. (4), bovine by Kato et al. (5); Galli et al. (6); Renard et al. (7); Wells et al. (8); Zakhaxtchenko et al. (9); Hill et al. (10); Kubota et al. (11), caprine by Baguisi et al. (12), porcine by Polejaeva et al. (13), and Bos gaurus (gaur) by Lanza et al. (14). A clone could be defined as a set of genetically identical animals was reported by Smith (15). Besides that, it also very useful in animal selection for the determination of the genetic traits or in research to reduce the number of experimental animals used as reported by Lamberson (16). The advantages of SCNT are valuable research animals may alleviate a limited availability of highly informative genotypes while to obtain optimum results by reducing the number of animals used. Today, cloning and transgenics are now available for producing phenotypes in large animals to study devastating disease and plays a relatively small role in research. Cloned animals could also replace more genetically dissimilar animals in the studies when the trait being investigated is highly heritable.

In the early 1990 s, Genzyme (17) was first attempt on cloning to produce therapeutic proteins in the milk of transgenic caprine. They inserted a genetic construct into the nucleus of embryo at one-cell stage by using microinjection technique. Furthermore, Yong and Yuqiang (18) from the Northwest University of Science and Technology for Agriculture and Forestry from China have successfully produced the first cloned caprine from ear fibroblast cells. The first success in cloning Asian Yellow Goat by nuclear transfer had recently been achieved in east China by Chen et al. (19). The improvement of the cloning technique may provide new approaches for conserving and saving endangered animal species. Successfu1 nuclear transfer has also been reported in caprine by Yong et al. (20). Cloning by nuclear transfer has great potential application in pharmaceutical protein production, xenotransplanation, and therapeutic cloning. The success rate of cloning is quite low due to many technical factors such as donor cell cycle, oocyte activation, reprogramming of donor nucleus and recipient cytoplast and chemical agents.

The cloned Dolly in 1997 showed us the way to physiologically differentiate already committed somatic cells as reported by Cibelli et al. (21). After Dolly, nuclear transfer enabled the cloning of murine, bovine, caprine and porcine where the era of livestock cloning had arrived. Cloning could be used as a great biochemical tool in providing invaluable advances in pharmacology and medicine. Thus, the objectives of this study were to investigate the effects of oocytes retrieval source, oocyte grading, pre – intracytoplasmic injection (pre – ICI) durations and enucleation techniques on caprine SCNT performance.

Materials and methods

Preparation of donor cells: The fresh caprine cumulus cells were obtained from in vitro matured oocytes harvested either from laparoscopic ovum pick - up, ovariectomy or abattoir ovaries. After 18 - 24 h of in vitro maturation, the cumulus cells were isolated by pipetting in hyaluronidase (0.2%) within 5 min at 37^{o}C. All fresh cumulus cells were collected and washed in holding medium (TL - hepes working solution) and centrifuged for 5 min. Cumulus cell pellet was formed and the supernatant was removed. TL - hepes working solution (100 µL) was added into the pellet and sucked up and down to make the fresh cumulus cells became a single cell (7 - 8 µm) and were ready for use as donor cells.

Oocyte retrieval and in vitro maturation: The caprine ovaries were obtained from LOPU (aspiration-flushing system), ovariectomy (ovaries were surgically removed) and abattoir [transported ovaries back to the laboratory in sterile NaCl (0.9%) supplemented with penicillin - G (60 µg/mL) and streptomycin (50 µg/mL) at 30 - 35^{o}C] sources. For ovariectomy and abattoir, the cumulus - oocyte complexes (COCs) were retrieved by checkerboard slicing the entire surface of the ovary inside the culture dish containing TL-hepes medium (holding medium) by using the razor blade. The retrieved oocytes group into Grades A, B, C, D and E based on the number layer of cumulus cells (Figure 1 a, b, c and d) and cultured in in vitro maturation (IVM) medium (TCM 199 supplemented with 10 µg/mL FSH, 1 µg/mL 17 - β estradiol, 100 µM/mL cysteamine, 0.2 mM sodium pyruvate, 10% FBS) overlaid with mineral oil in CO_2 incubator (5%) at 38.5^{o}C for 18 - 24 h.

Enucleation of matured caprine oocyte: After maturation, the COCs were denuded in hyaluronidase (0.2%) and subsequently washed 5 times in TL - hepes medium. All selected matured oocytes with the extrusion of first polar body were transferred to the microdroplet containing TL - hepes medium supplemented with cytochalasin B (5 µg/mL) for 10 min prior to enucleation. Two enucleation techniques, namely squeezing and laser enucleation technique were used. Briefly, a cut or small hole was made on the zona pellucida above the first polar body by using cutting needle or laser and 10 - 15% of the cytoplasm beneath the first polar body (containing metaphase II (MII) chromosomes) was gently squeezed out or pushed out, respectively. The enucleated oocytes were then washed 3 times in TL - hepes medium (without cytochalasin B) and simultaneous injection with donor cell or allowed to recover at 38.5^{o}C in KSOM medium in CO_2 incubator for 30 and 60 min (pre - incubated) prior to nuclear transfer.

Nuclear transfer: For whole cell intracytoplasmic injection, a single fresh cumulus cell (7 - 8 µm) was pipetted in and out few times in polyvinylpyrrolidone (PVP; 10%) medium using a blunt mouth injection needle (ID: 8 - 9 µm; OD: 9 - 10 µm) to break the plasma membrane prior to injection. Briefly, before injection, a small amount of cytoplasm of an oocyte was gently aspirated into the injection needle until a sudden flux of cytoplasm went into the needle observed. This was to confirm the breakage of the cytoplasm's membrane, thereby facilitating donor cell injection. A single cumulus cell was then gently deposited into the cytoplasm. All the injected oocytes were washed 3 times in TL - hepes medium, 3 times in KSOM medium and incubated in the final microdroplet of KSOM medium at least 60 min prior to activation.

Activation: All the injected caprine oocytes were subjected to activation with calcium ionophore (5 µM) for 5 min and followed with 6 - dimethylaminopurine (6 - DMAP; 1.9 µM) for 4 h at 38.5^{o}C under CO_2 (5%).

In vitro culture: Following activation, the reconstructed caprine oocytes were washed 5 times and then cultured in KSOM medium under CO_2 incubator (5%) at 38.5^{o}C. The developmental of cloned - caprine embryos were observed every 2 days and recorded. At the same time, the refreshment of culture medium was also made every 2 days culture.

Statistical analyses: All data were subjected to Analysis of Variance (ANOVA) followed by comparison of means using Duncan Multiple Range Test (DMRT). All statistical analysis was performed using SPSS (Statistical Package for Social Sciences) for windows Version 16.0. The data was presented as (mean ± SEM).

Results

Cleavage rates of caprine embryos through SCNT by 3 different sources of oocytes, namely LOPU, ovariectomy and abattoir were carried out in present study. There were significant differences ($P < 0.05$) in maturation and enucleation rates for these 3 different sources with the values of 61.99 ± 3.12% and 66.16 ± 5.21% versus 51.23 ± 3.37% as well as 97.85 ± 1.23% and 98.32 ± 1.16% versus 90.27 ± 2.72% for

laparoscopic ovum pick - up, ovariectomy and abattoir sources, respectively. Even though insignificance, the embryo development at 2 -, 4 -, 8 - cell and morula was higher in laparoscopic ovum pick - up, followed by ovariectomy and abattoir with values of 74.12 ± 5.30% versus 65.86 ± 7.87% versus 66.61 ± 6.64%; 63.40 ± 5.48% versus 56.83 ± 6.51% versus 51.46 ± 7.33%; 53.30 ± 5.84% versus 50.18 ± 9.05% versus 43.48 ± 8.14%; 48.56 ± 5.38% versus 44.68 ± 8.94% versus 26.28 ± 8.19%, respectively. However, ovariectomy showed the highest blastocyst and hatched blastocyst rates (24.52 ± 8.53% and 18.42 ± 6.79%), followed by laparoscopic ovum pick - up (13.33 ± 3.68% and 8.12 ± 2.29%) and abattoir (10.06 ± 5.83% and 5.01 ± 3.19%) respectively as shown in Table 1 (Figures 2 (a, b) and 3 (a, b)).

For caprine oocytes grading, there were no significant differences ($P > 0.05$) in percent cleavage at Grades A, B and C with values of 46.40 ± 5.26%, 42.89 ± 5.11%, 34.63 ± 4.91%; 31.06 ± 4.76%, 13.91 ± 3.56% and 6.88 ± 1.90%; 48.34 ± 5.08%, 40.43 ± 4.85%, 33.57 ± 4.70%; 29.79 ± 4.48% and 9.86 ± 2.95%, 5.99 ± 1.92%; 40.15 ± 5.17%, 32.78 ± 5.01%, 30.25 ± 5.00%, 26.62 ± 4.76%, 8.21 ± 2.80% and 5.38 ± 2.01% for 2 -, 4 -, 8 - cell, morula, blastocyst and hatched blastocyst, respectively. However, Grades A, B and C showed better cleavage rates than Grades D and E for all stages of embryo development (Table 2). For pre - ICI durations, it was vividly shown that for each embryo developmental stages from 2 -, 4 -, 8 - cell, morula, blastocyst and hatched blastocyst the cleavage rates were significantly higher ($P < 0.05$) for 60 minutes followed by simultaneous injection and 30 minutes pre - ICI durations (Table 3).

In regard to enucleation techniques, there were no significant differences ($P > 0.05$) in enucleation rates between squeezing and laser enucleation techniques. However, embryos development at various stages were significantly higher in laser

enucleation technique compared to squeezing enucleation technique with values of 82.07 ± 3.45% versus 58.15 ± 6.76%, 74.14 ± 3.62% versus 42.04 ± 6.53%, 70.93 ± 3.87% versus 25.73 ± 6.05%, 64.08 ± 3.91% versus 16.36 ± 5.22%; 25.33 ± 4.66% versus 0.00 ± 0.00% and 15.68 ± 2.99% versus 0.00 ± 0.00% for 2 -, 4 -, 8 - cell, morula, blastocyst and hatched blastocyst respectively (Table 4). For both enucleation techniques, the cleavage rates were decreasing as the stages of development progressing. No blastocysts were obtained for squeezing enucleation technique.

Discussion

Production of cloned-caprine embryos through SCNT is influence by many factors including oocytes retrieval source, oocyte grading, pre - ICI durations and enucleation techniques. The findings from this studies showed that ovariectomy showed the highest blastocyst and hatched blastocyst rates followed by laparoscopic ovum pick-up and abattoir. It could be due to the caprine ovaries that were surgically ovariectomized consistently provided greater follicle surface exposure for oocyte retrieval and the ability of more clearly visualized follicles on the ovaries as reported by Cognie (22). For small follicles, it was necessary to recover the oocytes by slicing the ovarian surface to obtain high number of oocytes as suggested by Martino et al. (23). Although caprine shows low reproductive capabilities are usually ovariectomized for ovarian slicing as most of them might be non - responsive to hormone stimulation during superovulation, more oocytes will be obtained via ovariectomy and chances to develop and blastocyst formation is higher compared to laparoscopic ovum pick - up oocyte source. It is generally agreed that the ovarian

slicing method gains higher number of oocyte. Martino et al. (23) obtained more oocytes from ovarian mincing or slicing than follicular dissection or aspiration. The reasons for higher oocyte obtain may be due to the presence of some follicles embedded deeply within the ovarian cortex, which could only be released by slicing of the ovary. Unlike laparoscopic ovum pick - up, the ovariectomy ovary by slicing protocol was done under visual observation regardless of ovarian follicle size; therefore, more oocytes were retrieved. Keskintepe et al. (24) who also obtained higher results of oocytes by surgically removing the ovaries of the does.

The data obtained in this study indicated that Grade A oocytes are highly competent in terms of maturation and subsequent embryo development compared with Grades B and C. The Grade D oocytes (no layers of cumulus cells with evenly granulated cytoplasm and E (pale - coloured or heterogeneous cytoplasm) were least competent. The roles of cumulus cells in the acquisition of full developmental competence of the oocytes have been investigated earlier by Leibfried-Rutledge and First (25), Sato et al. (26) and Xu et al. (27). It is important that visual assessment of morphology of cumulus cells that surrounding an oocyte remains the most important criterion for selection of developmentally competent oocytes during recovery as reported by Shioya et al. (28), Madison et al. (29) and Lonergan et al. (30). Veeck (31) suggested that earlier that expanded cumulus cells characterizes mature and good quality oocytes, while compact cumulus cells characterize immature oocytes.

The 60 min pre - ICI duration showed the highest cleavage rates followed by simultaneous injection and 30 min pre - ICI duration. This might be due to the enucleated caprine oocytes need time to restore the oocyte viability due to sensitivity of the oocytes after placing in CO_2 incubator before injection of donor nuclear. Simultaneous injection right after enucleation could cause the lower oocyte viability

as it could easily lyse just after enucleation. Therefore, pre - ICI duration for 60 minutes seemed to be more suitable for SCNT in caprine species which was significantly higher compared to other pre - ICI injection durations.

Our results demonstrate that using laser enucleation techniques have the ability to produce cloned - caprine hatched blastocyst stage. There is a scarce report on the caprine SCNT research using laser enucleation technique. Laser is a new and advance technique that has been used to enucleate recipient oocytes (cytoplast). In comparison with squeezing technique, laser enucleation technique is easier, less time consuming in enucleating the caprine oocytes. Low efficiency of cloned-caprine embryo development in the squeezing method might be due to the time consumption, where the squeezing method took much more longer duration compared to aspiration technique to remove the DNA (metaphase II) from caprine oocyte as reported by Lee et al. (32), whereas for laser enucleation techniques, the used of laser in enucleation of porcine oocytes was shown to be easier to operate. Although expensive, the laser enucleation technique gave the highest results in SCNT in caprine species in the present study.

In cloned embryos, reprogramming usually occurs. Low efficiency of cloning may be due to imcomplete reprogramming. Therefore, correct epigenetic reprogramming is necessary for successful and normal developmental of clones by Dean et al. (33). The incomplete reactivation of Oct - 4 may account for some failures of NT embryo development as reported by Boiani et al. (34) and Bortvin et al. (35). Reprogramming usually occurred within the brief interval between the transfer of the donor nucleus into the enucleated oocyte and here the start of zygotic transcription. Also, due to maternal zygotic transition occurred where the onset of maternal zygotic transcription is species dependent such as murine 1 to 2 - cell stage; bovine, caprine

and ovine 8 - 16 and Xenopus approximately 4000 as reported by Newport and Kirschner (36). Thus, a better understanding of the processes involved in nuclear reprogramming of in cloned embryos would enable to apply this knowledge to improving the overall efficiencies of NT.

In conclusions, cloned - caprine embryos were successfully produced in vitro from LOPU, ovariectomy and abattoir sources. The ovariectomy oocyte - retrieved seems to be more suitable source to produce cloned - caprine embryos through SCNT. Also, Grades A, B and C oocytes were more suitable to be used as karyoplast for SCNT compared with Grades D and E. The 60 min pre - ICI duration gave the highest cleavage rates in producing cloned - caprine embryos. The laser enucleation method shows higher cleavage rates up to hatched blastocyst than squeezing methods. With incorporation laser enucleation technology, the efficiency of SCNT will be enhanced for the production of cloned - caprine embryos.

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Table 1. Cleavage rates of caprine embryos (%, mean ± SEM) from in vitro culture through somatic cell nuclear transfer by 3 different sources of oocytes

Source of oocytes	No. of ovaries	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
						2 - cell (n)	4 - cell (n)	8 - cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
LOPU	91	487	61.99 ±3.12^{xy} (297)	97.85 ±1.23^{y} (292)	98.26 ±0.92^{x} (287)	74.12 ±5.30^{d,x} (227)	63.40 ±5.48^{cd,x} (207)	53.30 ±5.84^{bc,x} (184)	48.56 ±5.38^{b,x} (169)	13.33 ±3.68^{a,x} (66)	8.12 ±2.29^{a,xy} (43)
Ovariectomy	19	168	66.16 ±5.21^{y} (115)	98.32 ±1.16^{y} (111)	97.78 ±2.22^{x} (110)	65.86 ±7.87^{c,x} (75)	56.83 ±6.51^{c,x} (65)	50.18 ±9.05^{c,x} (54)	44.68 ±8.94^{bc,x} (45)	24.52 ±8.53^{ab,x} (29)	18.42 ±6.79^{a,y} (21)
Abattoir	99	641	51.23 ±3.37^{x} (355)	90.27 ±2.72^{x} (312)	94.61 ±2.25^{x} (289)	66.61 ±6.64^{e,x} (226)	51.46 ±7.33^{de,x} (190)	43.48 ±8.14^{cd,x} (166)	26.28 ±8.19^{bc,x} (136)	10.06 ±5.83^{ab,x} (75)	5.01 ±3.19^{a,x} (35)
Total	209	1296	59.76 ±2.28	95.99 ±1.10	97.28 ±0.86	71.23 ±3.83	59.59 ±3.99	50.43 ±4.32	42.45 ±4.20	13.85 ±2.93	8.57 ±1.86

^{abcde} Means with different superscripts in a row within a group were significantly different ($P < 0.05$).

^{xy} Means with different superscripts in a column within a group were significantly different ($P < 0.05$).

1 Table 2. Percent cleavage of caprine embryos (%, mean ± SEM) based on oocyte grading

Oocyte Grades	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
					2 - cell (n)	4 - cell (n)	8 - cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
A	366	51.73 ±4.83^{y} (253)	64.09 ±5.47^{y} (246)	63.53 ±5.45^{y} (240)	46.40 ±5.26^{d,y} (185)	42.89 ±5.11^{bc,y} (171)	34.63 ±4.91^{bc,y} (149)	31.06 ±4.76^{b,y} (133)	13.91 ±3.56^{a,y} (67)	6.88 ±1.90^{a,y} (39)
B	362	46.94 ±4.48^{y} (202)	63.22 ±5.43^{y} (190)	63.31 ±5.54^{y} (185)	48.34 ±5.08^{c,y} (144)	40.43 ±4.85^{bc,y} (129)	33.57 ±4.70^{b,y} (114)	29.79 ±4.48^{b,y} (99)	9.86 ±2.95^{a,y} (48)	5.99 ±1.92^{a,y} (28)
C	306	44.71 ±5.07^{y} (180)	54.73 ±5.67^{y} (166)	54.74 ±5.70^{y} (154)	40.15 ±5.17^{c,y} (116)	32.78 ±5.01^{bc,y} (101)	30.25 ±5.00^{bc,y} (87)	26.62 ±4.76^{b,y} (74)	8.21 ±2.80^{a,xy} (39)	5.38 ±2.01^{a,y} (21)
D	111	12.40 ±3.30^{x} (67)	15.14 ±4.06^{x} (60)	16.00 ±4.26^{x} (60)	12.33 ±3.46^{c,x} (47)	7.67 ±2.71^{bc,x} (38)	7.67 ±2.71^{bc,x} (38)	3.96 ±2.06^{ab,x} (28)	2.33 ±1.64^{ab,x} (14)	1.83 ±1.29^{a,xy} (11)
E	151	14.21 ±3.34^{x} (65)	19.67 ±4.47^{x} (53)	19.13 ±4.39^{x} (47)	9.44 ±3.00^{b,x} (26)	8.33 ±2.83^{b,x} (23)	6.67 ±2.63^{ab,x} (18)	5.33 ±2.15^{ab,x} (16)	1.33 ±0.94^{a,x} (2)	0.00 ±0.00^{a,x} (0)
Total	1296	34.00 ±2.09	43.37 ±2.51	43.34 ±2.52	31.33 ±2.18	26.42 ±2.05	22.56 ±1.95	19.35 ±1.82	7.13 ±1.16	3.65 ±0.69

2 ^{abcd} Means with different superscripts in a row within a group were significantly different ($P < 0.05$).

3

4 ^{xy} Means with different superscripts in a column within a group were significantly different ($P < 0.05$).

Table 3. Percent cleaved caprine embryos (%, mean ± SEM) based on different pre-intracytoplasmic injection (pre - ICI) durations

Pre-ICI durations	No. of ovaries	No. of oocytes	Percent cleavage (n)					
			2 - cell (n)	4 - cell (n)	8 - cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Simultaneous	68	322	61.17 ± 10.65^{d,x} (96/125)*	42.42 ± 8.77^{cd,x} (76/125)	37.12 ± 8.97^{c,x} (69/125)	29.39 ± 7.74^{bc,x} (59/125)	7.68 ± 5.93^{ab,xy} (30/125)	4.29 ± 3.41^{a,xy} (17/125)
30 min	31	194	45.39 ± 9.03^{c,x} (49/89)	33.82 ± 7.47^{bc,x} (36/89)	28.93 ± 7.11^{bc,x} (26/89)	24.83 ± 7.37^{b,x} (18/89)	0.45 ± 0.45^{a,x} (1/89)	0.00 ± 0.00^{a,x} (0/89)
60 min	110	780	83.33 ± 3.10^{d,y} (383/472)	74.42 ± 4.20^{cd,y} (350/472)	62.42 ± 5.47^{bc,y} (309/472)	53.10 ± 5.61^{b,y} (273/472)	20.49 ± 4.16^{a,y} (139/472)	12.95 ± 2.71^{a,y} (82/472)
Total	209	1296	71.23 ± 3.83	59.59 ± 3.99	50.43 ± 4.32	42.45 ± 4.20	13.85 ± 2.93	8.57 ± 1.86

*n = number of embryos (2 -, 4 -, 8 - cell, morula blastocyst and hatched blastocyst)/number of injected oocytes.

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

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

Table 4. Cleavage rates of caprine embryos (%, mean ± SEM) from in vitro culture through different enucleation techniques

Enucleation techniques	No. of ovaries	No. of oocytes	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)	Percent cleavage (n)					
						2 - cell (n)	4 - cell (n)	8 - cell (n)	Morula (n)	Blastocyst (n)	Hatched blastocyst (n)
Squeezing	89	516	51.74 ±3.19^{x} (509)	95.23 ±1.73^{x} (375)	94.71 ±1.76^{x} (320)	58.15 ±6.76^{d,x} (94)	42.04 ±6.53^{c,x} (71)	25.73 ±6.05^{b,x} (45)	16.36 ±5.22^{b,x} (16)	0.00 ±0.00^{a,x} (0)	0.00 ±0.00^{a,x} (0)
Laser	120	780	66.42 ±2.87^{y} (806)	96.61 ±1.42^{x} (699)	99.41 ±0.38^{y} (644)	82.07 ±3.45^{c,y} (412)	74.14 ±3.62^{bc,y} (327)	70.93 ±3.87^{b,y} (257)	64.08 ±3.91^{b,y} (195)	25.33 ±4.66^{a,y} (70)	15.68 ±2.99^{a,y} (33)
Total	209	1296	59.76 ±2.28	95.99 ±1.10	97.28 ±0.86	71.23 ±3.83	59.59 ±3.99	50.43 ±4.32	42.45 ±4.20	13.85 ±2.93	8.57 ±1.86

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

^{xy} Means with different superscripts in a column within a group were significantly different ($P < 0.05$).

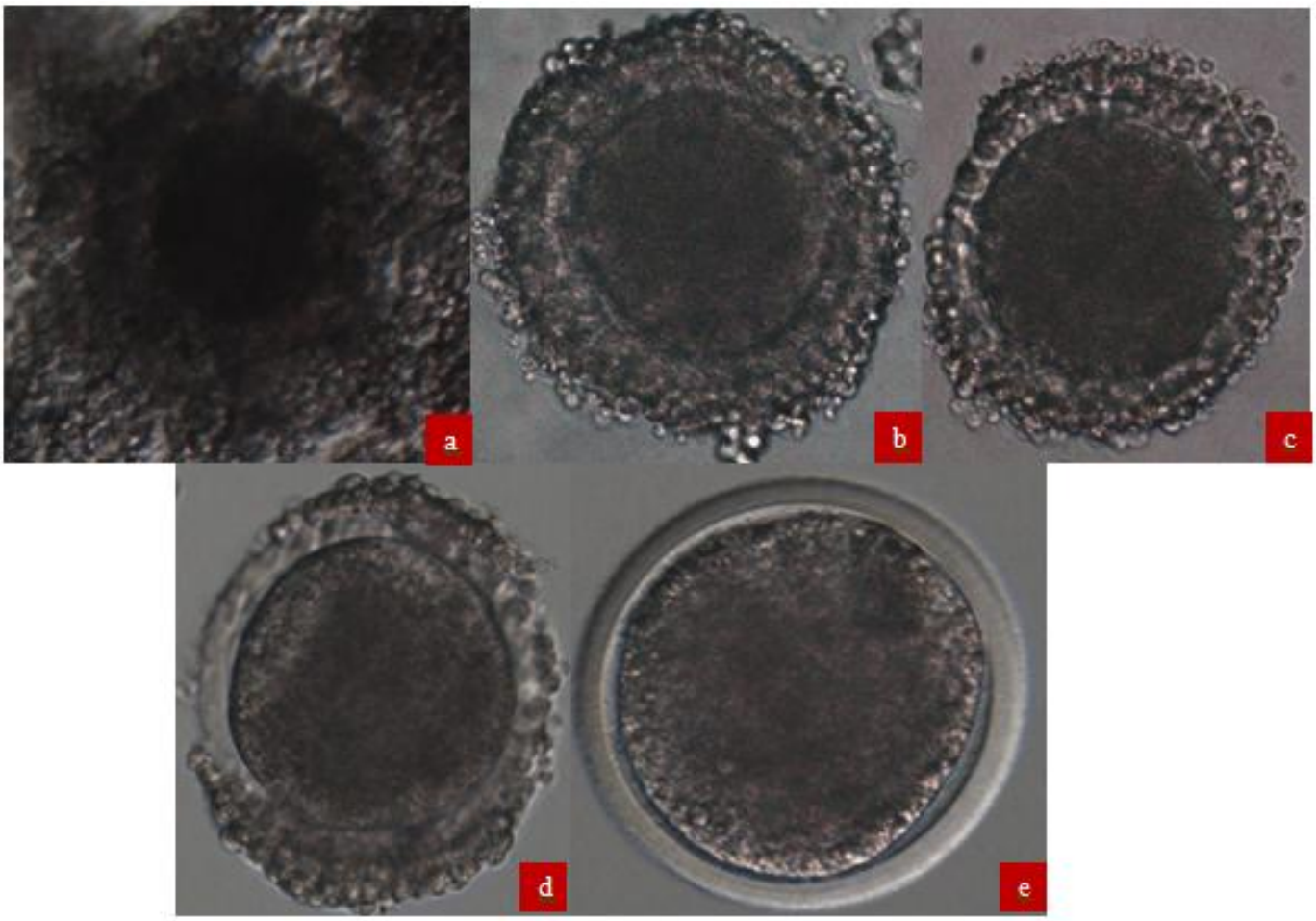

Figure 1. a) Grade A, b) Grade B, c) Grade C, d) Grade D and d) Grade E of caprine oocytes.

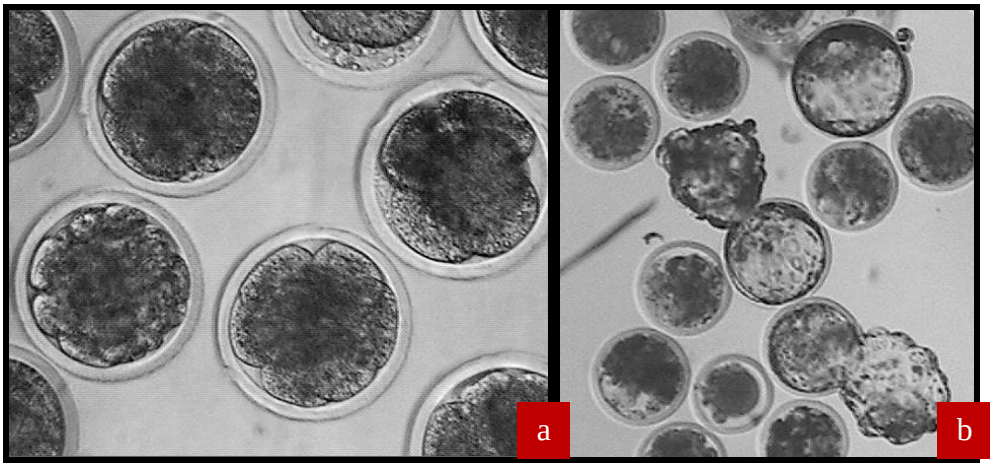

Figure 2. (a - b) Development of caprine - cloned embryos at day 3 (4 - to 8 - cells) and day 5 (blastocysts).

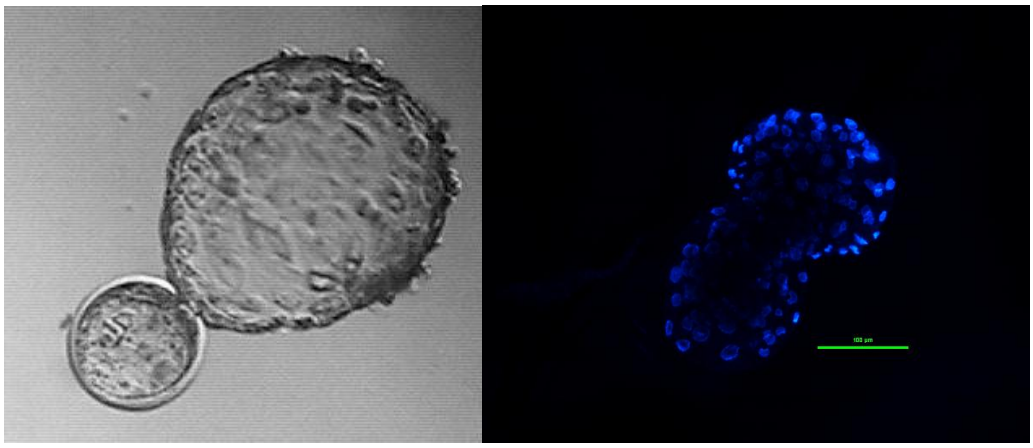

Figure 3. a) Hatching cloned caprine - blastocyst. b) hatching cloned - caprine blastocyst staining with Hoechst 3342 and observed under fluorescent microscope (20 × magnification).

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COMPARISON BETWEEN BOVINE AND CAPRINE SOMATIC STEM CELL NUCLEAR TRANSFER (SCNT) PERFORMANCE BY USING FRESH CUMULUS CELL THROUGH WHOLE CELL INTRACYTOPLASMIC INJECTION (WCICI) TECHNIQUE

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Although it has been 10 years since the first cloned mammals were generated from somatic cells using nuclear transfer (NT), the success rate for producing live offspring by cloning remains less than 5%. Cloning by somatic stem cell nuclear transfer (SCNT) involves the transfer of genetic material from a donor cell (karyoplast) to the cytoplasm of an oocyte or zygote from which the genetic material has been removed (cytoplast). The objectives of this study were to compare the different species oocytes and determine the effect of oocytes quality through the whole cell intracytoplasmic injection (WCICI) technique in ABEL laboratory. Goat oocytes were collected by laparoscopic ovum pick-up (LOPU) technique after superovulation with PMSG (1500 IU) and ovidrel (250 IU), whereas bovine ovaries were collected from abattoir and brought to the laboratory in normal saline at 30-35 ℃ within 4 h for slicing to obtain oocytes. Both collected oocytes were matured by *in vitro* and assessed by cumulus expansion and the extrusion of the first polar body. Cumulus cells were removed through use of finely drawn glass pipettes with 0.1% hyaluronidase. Oocytes that had extruded the first polar body and with evenly granulated cytoplasm which were grouped by Grades A, B, C, D and E were selected for enucleation which were placed into TCM 199 HEPES containing 5.0 μg/ml cytochalasin B. The first polar body and approximately 10% of the oocyte cytoplasm was squeezed out. Fresh cumulus cells obtained from the matured oocytes can be used directly for nuclear transfer, that is by transferring into enucleated oocytes using WICI technique. The reconstructed oocytes were activated by calcium ionophore (5 min) and 6-DMAP for 4 hours and cultured *in vitro* in KSOM. The cleaved embryos cell stages were observed and recorded daily. A total of 90 out of 192 (46.9%) matured oocytes were obtained from caprine while 271 out of 982 (27.6%) from bovine. There were no significant differences in maturation rate, successful enucleation and injection rates among caprine and bovine ($P>0.05$). Bovine showed the higher successful enucleation and injection rates (40.2±4.4; 37.4±4.3) than caprine (35.0±4.3; 35.1±4.3). The results from this study showed that bovine gives better cleavage rate at 2-cell (15.5±2.9) and 4-cell (11.3±2.5) but lower at 8-cell (5.5±1.9) and morula (0.7±0.7) compare to caprine. However, no significant differences were observed between caprine and bovine in regard to cleavage rate ($P>0.05$). Grade A (36.0±9.5; 43.4±8.1) oocyte give the highest cleavage rate followed by Grade B (18.0±7.0; 29.2±8.1), Grade C (8.0±4.7; 4.2±4.2), Grade D (0.0±0.0; 0.8±0.8) and Grade E (0.0±0.0; 0.0±0.0) for both caprine and bovine oocytes. Grade A (43.4±8.1) and B (29.2±8.1) bovine oocytes showed highest cleavage rate compare to caprine (36.0±9.5; 18.0±7.0) but Grade C (4.2±4.2) bovine oocytes is lower than Grade C (8.0±4.7) caprine oocytes. In conclusion, we successfully develop the WCICI technique in caprine and bovine species at ABEL laboratory. Improvement and standardization is still necessary for the more general use of this technique and its broader application to the study of livestock genetics.

COMPARISON BETWEEN BOVINE AND CAPRINE SOMATIC STEM CELL NUCLEAR TRANSFER (SCNT) PERFORMANCE BY USING FRESH CUMULUS CELL THROUGH WHOLE CELL INTRACYTOPLASMIC INJECTION (WCICI) TECHNIQUE

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INTRODUCTION

Although it has been 10 years since the first cloned mammals were generated from somatic cells using nuclear transfer (NT), the success rate for producing live offspring by cloning remains less than 5%. Cloning by somatic stem cell nuclear transfer (SCNT) involves the transfer of genetic material from a donor cell (karyoplast) to the cytoplasm of an oocyte or zygote from which the genetic material has been removed (cytoplast).

OBJECTIVES

a) To compare the **different species oocytes** between bovine and caprine through whole cell intracytoplasmic injection (WCICI) technique.
b) To determine the **effect of oocytes quality** through whole cell intracytoplasmic injection (WCICI) technique in ABEL laboratory.

MATERIALS AND METHODS

Goat oocytes were collected by laparoscopic oocyte pick-up (LOPU) technique after superovulation with PMSG (1500 IU) and ovidrel (250 IU), whereas bovine ovaries were collected from abattoir and do slicing to obtain oocytes. Both collected oocytes were matured by *in vitro* and assessed by cumulus expansion and the extrusion of the first polar body. Cumulus cells were removed with 0.1% hyaluronidase. Oocytes that had extruded first polar body and with evenly granulated cytoplasm which were grouped by Grades A, B, C, D and E were selected for enucleation which were placed into TCM 199 HEPES containing 5.0 µg/ml cytochalasin B. The first polar body and approximately 10% of the oocyte cytoplasm was squeezed out. Fresh cumulus cells were used for transferring into enucleated oocytes using WCICI technique. The reconstructed oocytes were activated by calcium ionophore (5 min) and 6-DMAP for 4 h and cultured *in vitro* in KSOM.

A) Oocyte Collection

Laparoscopic oocyte pick-up (LOPU)

Ovary slicing

B) Searching Oocytes Under Microscope

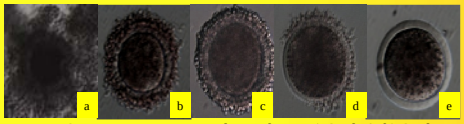

Figure 1: Ooocytes were categorized according to a) Grade A, b) Grade B, c) GradeC, d) Grade D and e) Grade E.

C) Enucleation and Injection of Donor Cell (SCNT)

Figure 2: Matured oocyte was obtained after IVM.

Figure 3: Matured oocyte was enucleated by using squeezing technique.

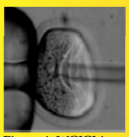

Figure 4: WCICI into enucleated oocyte.

D) Activation → E) *In vitro* culture (IVC)

Calcium ionophore (5 min) → 6-DMAP (4 h)

RESULTS

Table 1: Cleavage rate of caprine and bovine through WCICI technique

Species	No. of oocyte	Maturation rate	Successful enucleated rate	Successful injected rate	Cleavage rate			
					2-cell	4-cell	8-cell	Morula
Caprine	192	46.9	35.0	35.1	20.7± 4.4^{a,z}	18.0± 5.2^{a,yz}	9.7± 3.2^{a,xy}	4.0± 2.8^{a,x}
Bovine	982	27.6	40.2	37.4	25.6± 4.5^{a,z}	18.6± 3.9^{a,yz}	8.9± 3.0^{a,x}	1.8± 1.8 ^{a,xy}

^{a} Means with same superscript in a column were not significantly different (P>0.05).
^{xyz} Means with different superscript in a row were significantly different (P<0.05).

Table 2 (A-D): Effect of oocyte quality between caprine and bovine species through WCICI technique

A) 2-cell stage

Grades \ Species	Caprine (%)	Bovine (%)
A	36.0±9.5^{yz}	43.4±8.1^{z}
B	18.0±7.0^{xy}	29.2±8.1^{yz}
C	8.0±4.7^{x}	4.2±4.2^{x}
Total	**20.7±4.4**	**25.6±4.5**

^{xyz} Means with different superscript in a row were significantly different (P<0.05).
* Grade D and Grade E are not include because not cleavage.

B) 4-cell stage

Grades \ Species	Caprine	Bovine
A	28.0±8.9^{yz}	38.3±7.9^{z}
B	8.0±4.7^{x}	17.6±6.6^{xy}
C	4.0±2.8^{x}	0.0±0.0^{x}
Total	**18.0±5.2**	**18.6±3.9**

^{xyz} Means with different superscript in a row were significantly different (P<0.05).
* Grade D and Grade E are not include because not cleavage.

C) 8-cell stage

Grades \ Species	Caprine	Bovine
A	19.2±7.9^{xy}	22.7±8.0^{y}
B	8.0±4.7^{xy}	3.9±2.8^{x}
C	2.0±2.0^{x}	0.0±0.0^{x}
Total	**9.7±3.2**	**8.9±3.0**

^{xyz} Means with different superscript in a row were significantly different (P<0.05).
* Grade D and Grade E are not include because not cleavage.

D) Morula

Grades \ Species	Caprine	Bovine
A	4.0±4.0^{x}	3.6±3.6^{x}
B	4.0±4.0^{x}	0.0±0.0^{x}
Total	**4.0±2.8**	**1.8±1.8**

^{x} Means with same superscript in a row were not significantly different (P>0.05).
* Grade C, Grade D and Grade E are not include because not cleavage.

It is interesting to note that 46.9% (90/192) matured oocytes were obtained from caprine which was higher than bovine which was 27.6% (271/982). Bovine showed the higher successful enucleation and injection rates (40.2±4.4; 37.4±4.3) than caprine (35.0±4.3; 35.1±4.3). The results from this study showed that **bovine gives better cleavage rate at 2-cell (25.6±4.5) and 4-cell (18.6±3.9) but lower at 8-cell (8.9±3.0) and morula (1.8±1.8) compare to caprine.** However, no significant differences were observed between caprine and bovine in regard to cleavage rate (P>0.05).

Grade A (36.0±9.5; 43.4±8.1) oocytes gave the highest cleavage rate followed by Grade B (18.0±7.0; 29.2±8.1) and Grade C (8.0±4.7; 4.2±4.2) for both caprine and bovine oocytes. Grade A (43.4±8.1) and B (29.2±8.1) bovine oocytes showed highest cleavage rate compare to caprine (36.0±9.5; 18.0±7.0) but Grade C (4.2±4.2) bovine oocytes is lower than Grade C (8.0±4.7) caprine oocytes.

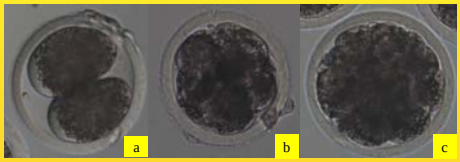

Figure 5: Development stage of cloned-bovine embryos. (a) 2-cell, (b) 8- cell and (c) morula.

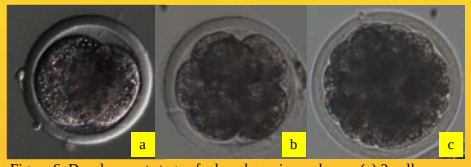

Figure 6: Development stage of cloned-caprine embryos. (a) 2-cell, (b) 8-cell and (c) morula.

DISCUSSION

The quality of the oocyte is the key factor determining the proportion of oocytes to develop or cleave to embryo (1). To simplify the manipulation procedure and increase the efficiency of SCNT, WCICI technique is used in obtained Asian yellow goat cloned embryos (2). WCICI technique is simple and efficient methods (3). However, one of the most important things to be concerned in WCICI for NT is that the plasma membrane of the donor cell may persist in the oocytes resulting in failure to release the nucleus. WCICI is also useful for the generation of ntESCs for preservation of valuable and infertile species.

CONCLUSIONS

We successfully develop the WCICI technique in caprine and bovine species at ABEL laboratory. Improvement and standardization is still necessary for the more general use of this technique and its broader application to the study of livestock genetics.

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ACKNOWLEDGEMENTS

The authors wish to thank ABEL members and staff of Mini Farm Institute of Biological Sciences, University of Malaya, for their advices and assistance throughout this project. This project was funded was PPP Research Grant PS287/2010A (UM).

PRODUCTION OF CLONED-MURINE EMBRYOS THROUGH SOMATIC STEM CELL NUCLEAR TRANSFER (SCNT) TECHNIQUE

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Although it has been 10 years since the first cloned mammals were generated from somatic cells using nuclear transfer (NT), the success rate for producing live offspring by cloning remains less than 5%. Cloning by somatic stem cell nuclear transfer (SCNT) involves the transfer of genetic material from a donor cell (karyoplast) to the cytoplasm of an oocyte or zygote from which the genetic material has been removed (cytoplast). The objectives of this study were to determine the effect of murine strains and the pre-intracytoplasmic injection (pre-ICI) duration of donor cells through the cloning procedure in ABEL laboratory.

The mature female mice from 3 different strains (ICR, CBA/ca and C57BL/6J) aged 6-8 weeks old were superovulated using pregnant mare serum gonadotrophin (PMSG) (10 IU) via intraperitoneal (i.p.) injections on Day-1 at 1800 hours and followed by injection of human chorionic gonadotrophin (hCG) (10 IU), 48 hours apart. After 13-15 hours post-hCG injections, the female mice were killed by cervical dislocation for oocyte recovery. The COCs were collected and denuded in 0.1% hyaluronidase to remove the cumulus cells. The matured oocytes with polar bodies were selected for enucleation to remove the spindle. Fresh cumulus cells (donor cells) were transferred into enucleated oocytes using whole cell intracytoplasmic injection (WCICI) technique with pre-intracytoplasmic injection (pre-ICI) duration of 30 min, 1 hr, 1 hr 30 min or 2 hr. After nuclear transfer, the reconstructed oocytes were activated by strontium chloride for 6 hr and cultured in *in vitro* culture medium (Whitten medium). The cleaved embryos cell stages were observed and recorded daily.

A total of 389 out of 658 (57.3±2.4) matured oocytes were obtained from mice. There were 358, 149 and 151 oocytes from the respective female of the superovulated ICR, CBA/ca and C57BL/6J strains. There were no significant differences in maturation rate among 3 different strains of mice where 60.0±3.5 (ICR), 59.3±4.4 (C57BL/6J) followed by 51.7±4.8 (CBA/ca) ($P<0.05$). C57BL/6J showed the highest successful enucleation and injection rates (67.6±6.4; 75.7±9.3), followed by CBA/ca (62.4±8.5; 65.0±9.1) and ICR (54.2±1.6; 59.7±5.6). There were no significant differences in successful enucleation and injection rates among the 3 different strains of mice ($P<0.05$). In this study, 1 hr 30 min (44.1±6.3) gave the optimised pre-ICI duration for nuclear transfer compared to 30 min (0.0%), 1 hr (6.7%) and 2 hr (25.0%). The results from this study showed that ICR gives better cleavage rate which were 34.2±3.9, 21.7±10.5, 3.2±3.2, 2.6±2.6 and 1.9±1.9 for 2-, 4-, 8-cell, morula and blastocyst, respectively, whereas C57BL/6J strain showed the lowest cleavage rates which were 6.3±4.4 (2-cell), 12.5±12.5 (4- and 8-cell) and 0.0% (morula and blastocyst) ($P<0.05$) by using the optimised pre-ICI injection duration (1 hr 30 min).

In conclusion, we successfully developed the standard somatic stem cell nuclear transfer (SCNT) protocol in murine species at ABEL laboratory. Improvement and standardisation is still necessary for the more general use of this technique and its broader application to the study of murine genetics.

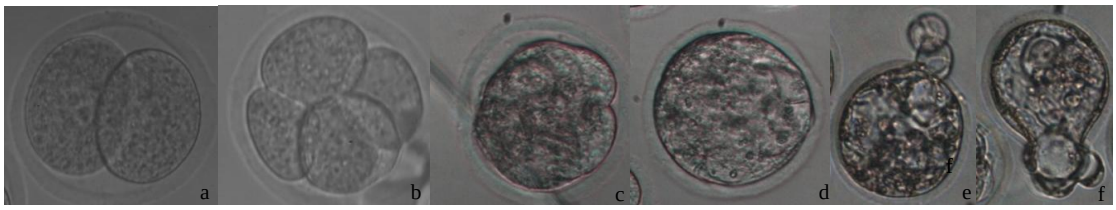

Figure 1: Cloned-murine embryos at (a) 2-cell, (b) 4-cell, (c) morula and (d-f) blastocyst stage.

The authors wish to thank ABEL members and staff of Institute of Biological Sciences (ISB) Animal House, University of Malaya (UM), for their advices and assistance throughout this project. This project was funded was PPP Research Grant PS197/2009A (UM).

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INTRODUCTION

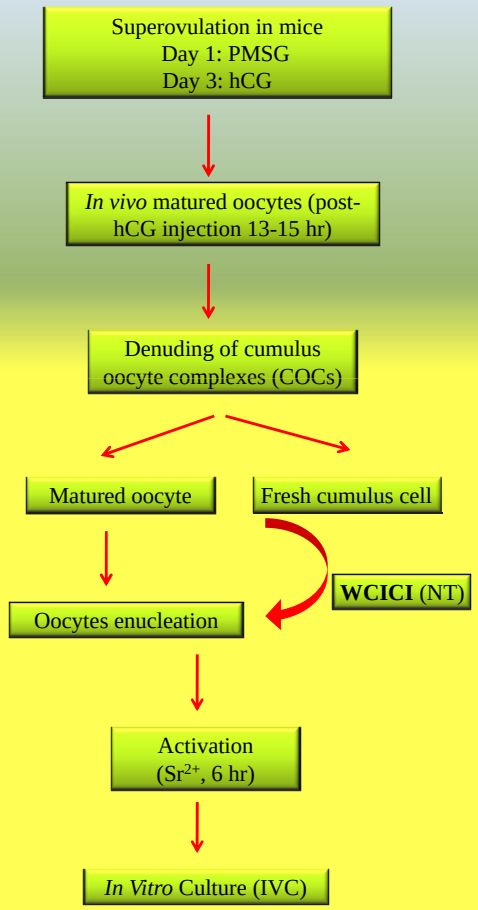
Advances in mammalian cloning is very rapid worldwide. However, there are many factors affecting the success of cloning using nuclear transfer. Mice is an excellent model animal to study cloning. However, there are different approaches of somatic stem cell nuclear transfer (SCNT) to produce cloned embryos which involves the whole cell intracytoplasmic injection (WCICI) and electrofusion techniques.

Objectives of this study:

a) To determine the effect of murine strains through the cloning procedure in ABEL laboratory.
b) To determine the pre-intracytoplasmic injection (pre-ICI) duration of donor cells through the cloning procedure in ABEL laboratory.

MATERIALS AND METHODS

Flow of Experiment



This cloning research involved the enucleation of oocytes from mice and injection of fresh donor cumulus cell into enucleated oocytes. The protocol is depicted in Figure 1 to 11.

Figure 1: Mouse was superovulated by intrapenitoneal injection.

Figure 2: Mouse was sacrificed by cervical dislocation.

Figure 3: Oviduct was collected by excision.

Figure 4: Oviduct with swollen part of ampulla.

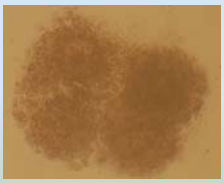


Figure 5: Cumulus oocyte complexes (COCs).

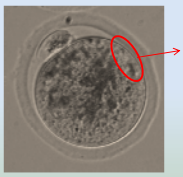


Figure 6: Matured murine oocytes with PB1.

Figure 7: Enucleated spindle of oocyte by squeezing technique.

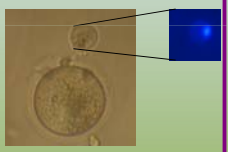


Figure 8: Enucleated oocyte was confirmed by Hoerchst 33324 staining.

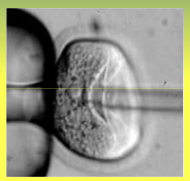


Figure 9: Injection of fresh donor cumulus cell (WCICI) into enucleated murine oocyte.

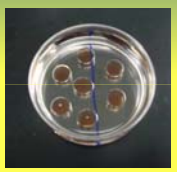


Figure 10: Nuclear transfer oocytes were activated with Sr^{2+}.

Figure 11: Nuclear transfer oocytes were cultured *in vitro*.

RESULTS

Table 1: Cleavage rate of murine by different pre-ICI duration of donor cells through SCNT technique

Pre-ICI duration	No. of replicate	Cleavage rate
30 min	5	0.0 ± 0.0^{a}
60 min	5	$6.7\pm6.7^{a,b}$
90 min	5	**44.1 ± 6.3^{c}**
120 min	5	25.0 ± 8.3^{c}
Total	**20**	**19.0±4.9**

[abc] Means with different superscript in a column were significantly different (P<0.05).

Table 2: Percentage of maturation, successful enucleated, successful injected and cleavage rate of murine through SCNT technique

Strains	No. of oocyte	Maturation rate	Successful enucleated rate	Successful injected rate	Cleavage rate				
					2-cell	4-cell	8-cell	Morula	Blastocyst
ICR	358	60.0 ± 3.5^{a}	54.2 ± 1.6^{a}	65.0 ± 5.6^{a}	**$34.2\pm3.9^{a,y}$**	$21.7\pm10.5^{a,y}$	$3.2\pm3.2^{a,x}$	$2.6\pm2.6^{a,x}$	$1.9\pm1.9^{a,x}$
CBA/ca	149	51.7 ± 4.8^{a}	62.4 ± 8.5^{a}	60.0 ± 9.1^{a}	$9.3\pm6.3^{a,x}$	$6.3\pm6.3^{a,x}$	$6.3\pm6.3^{a,x}$	$6.3\pm6.3^{a,x}$	$6.3\pm6.3^{a,x}$
C57BL/6J	151	59.3 ± 4.4^{a}	67.6 ± 6.4^{a}	75.7 ± 9.3^{a}	$6.3\pm4.4^{a,x}$	$12.5\pm12.5^{a,x}$	$12.5\pm12.5^{a,x}$	$0.0\pm0.0^{a,x}$	$0.0\pm0.0^{a,x}$
Total	**658**	**57.3±2.4**	**60.2±3.2**	**66.2±4.4**	**19.2±3.6**	**14.9±6.0**	**6.6±4.0**	**2.9±2.0**	**2.6±1.9**

[ab] Means with different superscript in a column were significantly different (P<0.05).
[xy] Means with different superscript in a row within a group were significantly different (P<0.05).

Figure 12: Development stages of cloned-murine embryos. (a) 2-cell, (b) 4-cell, (c) morula, (d) blastocyst and (e) hatching blastocyst.

DISCUSSION

Pre-ICI duration for 90 min seemed to be more suitable for somatic stem cell nuclear transfer in murine which was significantly higher compared to other pre-ICI duration. The chromosome-spindle complex, visible as a translucent region in mouse oocyte's cytoplasm (1). Enucleated oocytes were **incubated 90 min** in incubator before injection of donor nuclei to restore the oocyte viability due to the sensitivity of the oocytes and can easily lyse just after enucleation. Fresh donor cumulus cells were gently aspirated in and out of the injection pipette until their nuclei were largely devoid of visible cytoplasmic material (2). The ability of the donor cell to support development has been shown to be affected by the mouse strains (3). It is believed that **ICR strains gave highest cleavage rate** than CBA/ca and C57BL/6J. Finally, SCNT is useful for the generation of NT-ESCs for preservation of valuable and infertile strains.

CONCLUSIONS

We successfully produced cloned-murine embryos and applied WCICI technique in ABEL laboratory. Pre-ICI duration and strains of mice are important consideration in SCNT. Improvement and standardisation is still necessary for the more general use of this technique and its broader application to the study of murine genetics.

ACKNOWLEDGEMENTS

The authors wish to thank ABEL members and staff of Institute of Biological Sciences (ISB) Animal House, University of Malaya (UM), for their advices and assistance throughout this project. This project was funded was PPP Research Grant PS287/2010A (UM).

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7TH ASIAN REPRODUCTIVE BIOTECHNOLOGY SOCIETY (ARBS) CONFERENCE

2010

7th October 2010

Dear Sir/Madam

The 7th Asian Reproductive Biotechnology Society (ARBS) Conference 2010 Acceptance Letter

On behalf of the organising committee of the 7th ARBS Conference 2010, I would like to inform you that your abstract entitled **"Comparison Between Bovine And Caprine Somatic Stem Cell Nuclear Transfer (SCNT) Performance By Using Fresh Cumulus Cell Through Whole Cell Intracytoplasmic Injection (WCICI) Technique"** has been accepted for **POSTER PRESENTATION** in the 7th ARBS Conference 2010 that will be held in the Equatorial Hotel Kuala Lumpur from 8-10th November 2010.

Kindly follow the guidelines for the poster as attached in this letter. Please also visit the ARBS website at: http://www.cdb.riken.go.jp/arb/ for further details in relation to the accommodation, post-scientific conference tour, programme of the conference and etc.

I am looking forward to see you in this conference. Thank you.

Yours sincerely

Assoc Prof Dr Tunku Kamarulzaman
Head of Scientific Paper Committee
for the 7th ARBS Conference 2010

PROF. MADYA DR.TUNKU KAMARUL ZAMAN TUNKU ZAINOL ABIDIN
No. Pendaftaran Penuh MPM 35591
Pakar Perunding
PTj Surgeri Ortopedik
Pusat Perubatan Universiti Malaya

Asian Reproductive Biotechnology Society

The Seventh Annual Conference

Equatorial Hotel, Kuala Lumpur, Malaysia
November 8-12, 2010

Conference Program

November 7, 2010: Registration and Meeting of Organizing Committee

12:00 – 19:00 **Registration**

November 8, 2010

7:00 – 8:40 **Registration**

8:40 – 8:50 **Greeting and Opening Remarks by**
Prof. Dr. Ramli Bin Abdullah
Chairman of Local Organizing Committee of 2010 ARBS Conference

8:50 - 9:10 **Introduction of RIKEN-Center for Developmental Biology (CDB)**
Mr. Hazuki Hiraga, RIKEN Center for Developmental Biology, Kobe, Japan

Session I: **Frontier and New Achievement in Cloning Animal, Stem Cell and Regenerative Medicine**
Session Chairs: Eimei Sato & Ramli Bin Abdullah

9:10 – 9:50 **How to Improve the Nuclear Reprogramming Potential and its Limitations**
Dr. Teruhiko Wakayama
RIKEN Center for Developmental Biology,Kobe, Japan

9:50 – 10:30 **Mesenchymal Stem Cells: Research and Clinical Applications in Malaysia**
Dr. Tunku Kamarulzaman bin Tunku Zainol Abidin
The University of Malaya, Malaysia

10:30 – 10:40 **-- Break --**

Session I: (Continued) **Stem cells and Regenerative Medicine**
Session Chairs: Teruhiko Wakayama & Tunku Kamarulzaman bin Tunku Zainol Abidin

10:40 – 11:20 **Molecular Basis of Follicular Growth and Oocyte Maturation: Application in Animal Production and Medicine**
Dr. Eimei Sato
Tohoku University, Sendai, Japan

11:20 – 12:00 **Inactivation of Primordial Oocytes**
Dr. Takashi Miyano
Kobe University, Kobe, Japan

PERSATUAN PRODUKSI HAIWAN MALAYSIA
MALAYSIAN SOCIETY OF ANIMAL PRODUCTION

30 May 2010

Dear Sir/Madam

The 31st MSAP Annual Conference

On behalf of the organising committee of the 31st MSAP Annual Conference I would like to inform you that your paper has been accepted for POSTER PRESENTATION.

The list of posters with the poster numbers and the conference program are appended.

The posters should be prepared in A1 size, and should be displayed before 9 a.m. on 7 June 2010.

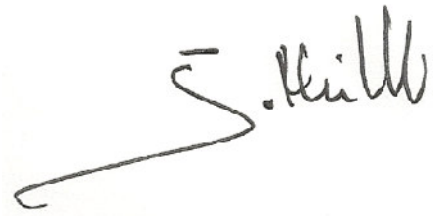
Please take note that the extended abstracts have been edited by the scientific committee; and they may have made changes to the title of your extended abstract.

The Malaysian Society of Animal Production will be giving awards for the Best Student Presenter / Best Young Presenter. If you are a registered student at one of the local universities OR are below 35 years of age, you are eligible for this award. If you fall into any one of these categories, please provide the secretariat with the following information:
Student Presenter – name, university, name of supervisor
Best Young Presenter – name, date of birth

Should you have any questions pertaining to your presentation or the award, you may contact me (03-89466896) or the secretariat. If you require any further information with regards to the conference, please contact the secretariat.

Thank you.

Yours sincerely



ASSOC. PROF. DR. JOTHI M. PANANDAM
Chairman, Scientific Committee
31st MSAP Annual Conference

31ST MSAP ANNUAL CONFERENCE 2010
PROGRAMME

6 June 2010 (Sunday)	
1030 – 1230	***Registration***
1400 – 1700	***FIELD TRIP***
7 June 2010 (Monday)	
0800 – 0900	***Registration***
0900 – 0930	**OPENING CEREMONY**
0930 – 1015	*Keynote Lecture* **Issues And Challenges in Livestock Industry in Malaysia** Dr. Idris Kadir Department of Veterinary Services Malaysia
1015 – 1030	*TEA BREAK*
1030 – 1100	***POSTER SESSION***
Session 1:	Chairman: Prof. Dr. Zainal Aznam Mohd Jelan
1100 – 1130	*Plenary 1* **Feed Act 2009** Mr. Syed Hussein Syed Abdullah Department of Veterinary Services Malaysia
1130 – 1200	*Plenary 2* **Utilization of Agro-Industrial By-Products for Animal Feed** Prof. Dr. Abd. Razak Alimon Universiti Putra Malaysia, Malaysia
1200 – 1215	**Effects of Palm Oil Sludge as an Energy Source in Concentrate on Nutrient Utilization in Thai Native Cattle** Vuttichai Seephueak
1215 – 1230	**Alterations in the Fatty Acid Profile of the Liver in Goats Fed Oil Palm (Elaeis guineensis) Frond Supplemented Diets** Mohamed Ali Rajion
1230 – 1245	**Effect of Enzyme Supplementation on Performance of Broilers Fed Diets Containing Corn Dried Distiller's Grains With Solubles (DDGS)** Tang Siew Ching
1245 – 1300	**Changes in Growth Performance and Intestinal Microflora of Broiler Chickens Fed With Metabolite and Acidifier** Rosyidah Mohd Radzi
1300 – 1400	LUNCH

Session 2:	*Chairman:* Prof. Dr. Mohamed Ali Rajion
1400 – 1430	*Plenary 3* **Present Roughage Status in the Lower Southern Provinces of Thailand** Dr. Chaiyawan Wattanachant Prince of Songkhla University, Thailand
1430 – 1500	*Plenary 4* **Availability and Utilisation of Oil Palm By-Products and Waste as Bali Cattle Feed in Riau Province** Dr. Tantan Rustandi Wiradarya Indonesia
1500 – 1515	**Effects of Molasses and Rice Bran as Additives on Guinea Grass Silages** Aida Bt Zakaria
1515 – 1530	**Effect of *Weissella paramesenteroides* on Ensiled Guinea Grass (*Panicum maximum*)** Mahdi Pasebani
1530 – 1545	**Adoption of Complete Feed Technology on Peranakan Etawah Goat Farming in Banyumas Region, Indonesia** Novie Andri Setianto
1545 – 1600	**Effects of Non Antibiotic Feed Additives on Tibial Dyschondroplasia Incidence and Tibia Characteristics of Broilers Fed Low Calcium Diets** Mohamad Houshmand
1600 – 1615	**The Potential of Herbs to Control Helminth in Goats** Mohd Saufi bin Bastami
1615 - 1645	TEA BREAK
1630 - 1730	**MSAP AGM**
8 June 2010 (Tuesday)	
Session 3:	*Chairman:* Dr. Johari Jiken Abdullah
0830 – 0900	*Plenary 5* **Development and Utilization of Forages for Improving Ruminant Farming in Malaysia** Prof. Dr. Zainal Aznam Mohd Jelan Universiti Putra Malaysia, Malaysia
0900 – 0930	*Plenary 6* **Aquaculture Feed** Dr. Che Roos Saad Universiti Putra Malaysia, Malaysia
0930 – 0945	**Weaning Induced Gene Expression Changes in the Hypothalamus of Postpartum Beef Cows** Ainu Husna Binti M.S Suhaimi
0945 – 1000	**Association of Natural Resistance-Associated Macrophage Protein 1 with Response to *Salmonella enteritidis* in Malaysian Native Chickens** Reza Tohidi

1000 – 1015	**Lack of Variability in Mitochondrial DNA Cytochrome B Region in Three Cattle Breeds** Yow Weng Kit
1015 – 1030	TEA BREAK
1030 – 1100	***POSTER SESSION***
Session 4:	*Chairman:* Assoc. Prof. Dr. Halimatun Yaakub
1100 – 1130	*Plenary 7* **Enhancing the Development of Malaysian Livestock Industry: Government Initiatives Under the New Economic Model** Dr. Abas Mazni Othman Agro-Biotechnology Institute Malaysia, Malaysia
1130 – 1145	**Effect of Multiple Ejaculations on Semen Quality in Dorper Sheep Rams** Jasmi Bin Yahya
1145 – 1200	**Ovulation and Embryo Recovery Responses of Dorper Ewes to Day Zero Superovulation Treatment** Abdul Rashid Baba
1200 – 1215	**1,3-Butanediol in Vitrification Solution Enhances Nuclear Maturationo of Vitrified Immature Bovine Oocytes** Hadi Hajarian
1215 – 1230	**Effect of Ghrelin on *In Vitro* Maturation and Subsequent Embryo Development of Immature Bovine Oocytes** Mojtaba Dashtizad
1230 – 1245	**Efficacy of Cloned Caprine Embryos Production Using Intraspecies- Versus Interspecies SCNT Approach** Kwong Phek Jin
1245 – 1300	**Changes in Foetal Heart Size and Echogenicity as Indicator for Gestational Age Estimation in Pregnant Jermasia Does** Mohd Nizam A.R.
1300 – 1400	LUNCH
Session 5:	*Chairman:* Prof. Dr. Abd. Razak Alimon
1400 – 1430	*Plenary 8* **The Water Buffalo in Sabah and Sarawak** Dr Johari Jiken Abdullah Malaysia Agriculture Research and Development Institute, Malaysia
1430 – 1445	**Development Strategy for Increasing Goat Productivity and Population** Akhmad Sodiq
1445 – 1500	**The Potential of an Intensive Swiftlet Production System in Malaysia** Kamarudin, M.I.

1500 – 1515	**Halal Principles and Sustainable Agriculture: Consumers Perspective Study in Malaysia** Zainal Mohamed
1515 – 1530	**The Impact of Food Safety Information on Meat Demand in Peninsular Malaysia** Abdullahi Farah Ahmed
1530 – 1545	**Comparative Advantage and Competitiveness of Meat and Meat Preparation Sub-Sectors** Mansor Ismail
1545 – 1600	**Carcass and Meat Production of Improved Boer Goats** Juni Sumarmono
1600 – 1615	**Effects of Transportation and Stocking Density During Transportation on Pre-Slaughter Weight and Meat Quality of Boer Goats** Saeid Nikbin
1615 – 1700	***CLOSING CEREMONY***

The 4th SUT Stem Cell Workshop
Stem Cell Innovation:
A Milestone beyond Life Science Roadblocks

Organized by Embryo Technology and Stem Cell Research Center,
School of Biotechnology, Suranaree University of Technology

Venue: Suranaree University of Technology, Nakhon Ratchasima, Thailand
Lecture session: Suranaree Room, Surasammanakarn
Hands-on workshop session: Embryo Technology and Stem Cell Research Center, Equipment 1 (F1) Bldg.

Date
Lecture session: 24-25 July 2010
Hands-on workshop session: 26-30 July 2010

Registration Fee
Lecture session: 1000 Baht includes printed handouts together with banquets and refreshments (120 seats available)
Hands-on workshop session (20 seats available)**:** 14,000 Baht

Application Deadline: 20 June 2010

Lecture Programme: Suranaree Room, Surasammanakarn
24 July 2010

8.00-8.30	**Registration**
8.30-8.45	**Opening remarks**
8.45-9.30	**Embryonic Stem Cells: Past, Present and Future** ***Prof. Davor Solter, Ph.D.,*** *Mammalian Development Laboratory, Institute of Medical Biology, A*STAR, Singapore*
9.30-10.15	**The Biology of Human Embryonic Stem Cells** ***Prof. Peter W. Andrews, D.Phil.****, Centre for Stem Cell Biology, The University of Sheffield, UK*
10.15-10.45	**Refreshment**
10.45-11.15	**TGFb Family Signaling in the Regulation of Human ESC Self-Renewal and Early Stem Cell Fate** ***Dr. Stuart Avery, Ph.D.,*** *Mammalian Development Laboratory, Institute of Medical Biology, A*STAR, Singapore*
11.15-11.45	**The Role of Elabela in Human ESCs** ***Dr. Katie Avery, Ph.D.,*** *Mammalian Development Laboratory, Institute of Medical Biology, A*STAR, Singapore*
11.45-13.00	**Luncheon talk** **"Usefulness of Xeno-Free System in Stem Cells Banking and Therapy**
13.00-13.45	**Reprogramming as a Biological Process** ***Prof. Barbara Knowles, Ph.D.,*** *Mammalian Development Laboratory, Institute of Medical Biology, A*STAR, Singapore*
13.45-14.15	**Nuclear Reprogramming in One Cell Stage Embryo** ***Dr. Chanchao Lorthongpanich, Ph.D.,*** *Mammalian Development Laboratory, Institute of Medical Biology, A*STAR, Singapore*

14.15-15.00 **To Be, or Not to Be, Monoclonal**
Dr. Bruno Revesade, Ph.D., *Human Embryology Laboratory, Institute of Medical Biology, A*STAR, Singapore*

15.00-15.30 **Refreshment**

15.30-16.00 **Buffalo Embryonic Stem Cells and their Differentiation Potential**
Prof. Yindee Kitiyanant, DVM, *Department of Anatomy, Faculty of Science, Mahidol University, Thailand*

16.00-17.00 **Short communications (15 minutes each) will be selected from abstracts for poster presentation.**

18.00-20.30 **Welcome dinner**

25 July 2010

8.30-9.00 **Building Up the Network for International Stem Cell Research: the Lessons from the ISCI and the ESTOOLS**
Prof. Peter W. Andrews, D.Phil., *Centre for Stem Cell Biology, The University of Sheffield, UK*

9.00-9.30 **Asia Pacific Stem Cell Network and Thai Society for Stem Cell Research**
Prof. Surapol Issaragrisil, M.D., *Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand*

9.30-10.00 **Shedding New Life on Stem Cells Using SR-FTIR Microspectroscopy**
Dr. Kanjna Thumanu, Ph.D., *Synchrotron Light Research Institute (Public Organization), Thailand*

10.00-12.00 **Poster presentation together with refreshment**

12.00-13.15 **Luncheon talk**
Induce Pluripotent Stem Cells: Is it Really New Hope for Patients?

13.15-14.00 **The Birth of iPSC Mice from Tetraploid Complementation**
Prof. Qi Zhou, Ph.D., *State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China*

14.00-14.30 **Epigenetic Therapy in Cancer: a Perspective from Human Pluripotent and Nullipotent Stem Cells**
Mr. Patompon Wongtrakoongate, PhD candidate, *Centre for Stem Cell Biology, The University of Sheffield, UK*

14.30-15.00 **Neural Differentiation of Human Embryonic Stem Cells: a Tool to Reveal Neurodevelopment**
Dr. Parinya Noisa, Ph.D., *Embryo Technology and Stem Cell Research Center, School of Biotechnology, Suranaree University of Technology, Thailand*

15.00-15.15 **Refreshment**

15.15-15.45 **Survival and Differentiation of hESC-derived Otic Neural Progenitor Cells (ONPs) Transplanted into the Gerbil Cochlea**
Dr. Nopporn Jongkamonwiwat, Ph.D., *Faculty of Health Science, Srinakharinwirot University, Thailand*

15.45-16.15 **Nuclear Transfer for Producing Human ESCs**
Asst. Prof. Rangsun Parnpai, Ph.D., *Embryo Technology and Stem Cell Research Center, School of Biotechnology, Suranaree University of*

Technology, Thailand

16.15-16.30 **Poster presentation prize awarding**

16.30-17.00 **Final discussion by all speakers and closing remarks**

Hands-on Workshop Programme: Embryo Technology and Stem Cell Research Center, Equipment 1 (F1) Bldg.

26-30 July 2010

Group I: Embryonic Stem Cells (Available for 10 participants)

1. Establishment of mouse ES cells
    - 1.1. Preparation of mouse spleenocytes for raising antibody
    - 1.2. Primary mouse embryonic fibroblast preparation
    - 1.3. Feeder cell preparation
    - 1.4. Collection of mouse embryos at blastocyst stage
    - 1.5. Isolation of inner cell mass cell by immunosurgery
    - 1.6. Culture and passage of mouse ES cells
    - 1.7. Characterization of mouse ES cells
    - 1.8. Freezing of mouse ES cells
2. Culture and characterization of human ES cells
3. Differentiation of human ES cells into neural progenitor cells

Group II: Adult Stem Cells (Available for 10 participants)

1. Isolation, culture and characterization of rat bone marrow derived mesenchymal stem cells (MSCs).
2. Isolation, culture and characterization of rat adipose tissue derived MSCs.
3. Isolation, culture and characterization of rat amniotic epithelial cells.
4. Differentiation of rBM-MSCs into pre-adipocytes, osteoblasts and chondrocytes.