PRODUCTION OF CLONED-CAPRINE EMBRYOS *IN VITRO* OBTAINED FROM INTERSPECIES NUCLEAR TRANSFER USING BOVINE CYTOPLAST AND CAPRINE KARYOPLAST

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ABSTRACT

Production of cloned-caprine embryos using the intraspecies somatic cell nuclear transfer is limited by low source of caprine oocytes as the recipient cytoplast in Malaysia. Therefore, using the bovine oocytes as recipient cytoplast in interspecies somatic cell nuclear transfer is an alternative approach to produce large number of cloned-caprine embryos and subsequently offspring at a rapid rate. This study was aimed to produce cloned-caprine embryos *in vitro* by specifically evaluating the interspecies nuclear transfer technique such as enucleation methods, nuclear transfer methods and *in vitro* culture systems. Briefly, the bovine ovaries were collected from local abattoir and transported to the laboratory within 2 to 3 hours in saline solution (0.9% NaCl). Bovine oocytes were recovered by checkerboard slicing the entire surface of the ovary inside the culture dish by using the razor blade. Oocytes with several compact layers of cumulus cells were selected and cultured in *in vitro* maturation (IVM) medium for 20 to 22 hours. After maturation, cumulus oocyte complexes (COC) were denuded in hyaluronidase (0.1%) to remove the cumulus cells. The matured oocytes with extrusion of first polar body were selected for enucleation to remove the spindle. Caprine-foetal fibroblast cells (donor karyoplasts) were harvested and transferred into enucleated bovine oocytes. The reconstructed oocytes were activated and the reconstructed couplets were cultured in KSOM medium for *in vitro* embryos development in CO_2 (5%) incubator at 38.5^{o}C in humidified atmosphere for 8 days. The medium was changed every 2 days of *in vitro* culture. Samples of embryos from each stage were stained with Hoechst 33342 to examine the number of nuclei of the embryos. The data were presented as mean±SEM and were analysed using one-way ANOVA. The significant differences among treatments were further analysed by DMRT and $P<0.05$ was considered significant.

In Experiment 1, two enucleation methods were compared, *i.e.* squeezing and aspiration. There was no significant difference in the percentages successfully enucleated oocytes (P>0.05) for both enucleation methods (squeezing vs. aspiration: 88.01±3.00% vs. 91.68±1.92%, respectively). In terms of manipulation efficiency, the duration needed to complete the enucleation together with injection, the speed for aspiration with sub-zonal injection was significantly faster (P<0.05) than squeezing with sub-zonal injection (41.18±2.77 and 83.82±3.16 minutes, respectively). In *in vitro* embryo development, the percent of cloned-caprine embryos from 2 cell stage up to blastocyst stage using squeezing (2 cell: 60.18±2.43%, 4 cell: 53.80±2.84%, 8 cell: 37.71±3.30%, morula: 24.45±2.71% and blastocyst: 12.08±2.95%) and aspiration (2 cell: 61.55±4.20%, 4 cell: 49.86±3.87%, 8 cell: 39.22±4.26%, morula: 21.07±3.94% and blastocyst: 10.93±1.87%) methods did not differ significantly (P>0.05).

In Experiment 2, two nuclear transfer methods were compared, *i.e.* sub-zonal injection with electro-fusion and intracytoplasm injection. There were no significant differences (P>0.05) in the injection and reconstruction rates for both nuclear transfer methods. The percent cloned-caprine embryos obtained from interspecies SCNT at 2- and 8 cell using SUZI and ICI methods did not differ significantly (P>0.05). However, the percentages of cloned-caprine embryos at 4 cell, morula and blastocyst derived from SUZI method were significantly higher (P<0.05) compared to the ICI method (53.80±2.84% vs. 38.60±2.25%, 24.45±2.71% vs. 16.05±1.43% and 12.08±2.95% vs. 4.51±1.45%, respectively).

In Experiment 3, three different *in vitro* culture system were compared, *i.e.* Group 1 (KSOM A throughout duration of the culture), observation of the embryos were recorded on days 3, 5, 7 and 8 without changing the medium; Group 2 (KSOM A on days 1-3, changed with KSOM A on days 3 and 5), the embryos were observed and recorded on days 3, 5, 7 and 8; and Group 3 (KSOM A on days 1-3, changed with

KSOM B on days 3 and 5), the embryos were observed and recorded on days 3, 5, 7 and 8. Group 3 (2 cell: 60.18±2.43%, 4 cell: 53.80±2.84%, 8 cell: 37.71±3.30%, morula: 24.45±2.71% and blastocyst: 12.07±2.95%) showed significantly higher ($P<0.05$) in the *in vitro* development competence from 2 cell up to blastocyst stages compared to Groups 1 (2 cell: 49.01±2.02%, 4 cell: 36.92±3.02%, 8 cell: 26.46±1.74%, morula: 8.42±0.47% and blastocyst: 0.00±0.00%) and 2 (2 cell: 49.85±3.27%, 4 cell: 39.68±2.72%, 8 cell: 29.34±1.87%, morula: 10.22±1.49% and blastocyst: 0.00±0.00%).

In Experiment 4, an attempt to produce pregnancy after oviduct embryo transfer of interspecies SCNT embryos and using ultrasound scanning for pregnancy diagnosis were carried out. A total of 63 cloned-caprine embryos were obtained from interspecies SCNT experiment. However, only 55 embryos of Grades 1 and 2 (4- and 8 cell stages) were chosen and transferred into 9 recipients with at least 1 CL. Unfortunately, after ultrasound scanning on day 30 of gestation age after embryo transfer, there was no pregnancy observed in the recipient goats after embryo transfer experiment.

It can concluded from the present study that caprine embryos could be successfully produced through interspecies SCNT using caprine foetal fibroblast cell as donor karyoplast and bovine oocyte as recipient cytoplast under local setting in Malaysia. It is believe this is the first report of producing cloned-caprine embryos with satisfactory blastocyst rate in interspecies SCNT using KSOM supplementation additional of glucose. With this encouraging findings and future refined research, using caprine-bovine in interspecies SCNT to produce caprine embryos and offspring may offer a new approach to increase genetically superior goat population in Malaysia at a rapid rate to meet the goat meat and milk demand for the industry.

ABSTRAK

Penghasilan klon embrio kaprin dengan menggunakan pemindahan nukleus sel somatik intraspesies di Malaysia adalah terhad kerana sumber oosit kaprin yang rendah sebagai sitoplas penerima. Oleh yang demikian, menggunakan oosit bovin sebagai sitoplas penerima dalam pemindahan nukleus sel somatik interspesies adalah suatu pendekatan alternatif untuk menghasilkan klon embrio kaprin dalam bilangan yang banyak dan seterusnya menghasilkan zuriat pada kadar yang cepat. Kajian ini bertujuan untuk menghasilkan klon embrio kaprin secara *in vitro* dengan penilaian khusus terhadap teknik pemindahan nukleus interspesies seperti kaedah enukleasi, kaedah pemindahan nukleus dan sistem pengkulturan secara *in vitro*. Secara ringkasnya, ovari bovin dikumpul daripada rumah sembelihan tempatan dan diangkut ke makmal dalam tempoh 2 ke 3 jam dalam larutan salin (0.9% NaCl). Oosit bovin diperolehi secara penghirisan *ckeckerboard* terhadap keseluruhan permukaan ovari di dalam piring kultur dengan mengguna bilahan pisau cukur. Oosit yang mempunyai beberapa lapisan manpat sel kumulus dipilih dan dikultur di dalam medium kematangan *in vitro* selama 20 hingga 22 jam. Selepas menjadi matang, kumulus oosit kompleks (COC) telah ditanggalkan lapisan selnya dengan hialuronidase (0.1%) untuk membuangkan sel kumulus. Oosit matang yang jasad kutub pertamanya dinyah keluar telah dipilih untuk enukleasi bagi membuang spindelnya. Sel fibroblas fetus kaprin (karioplas penderma) dikutip dan dipindahkan ke dalam oosit bovin yang telah dienukleasi. Oosit yang telah direkonstruksi diaktifkan dan kuplet yang direkonstruksi telah dikultur di dalam medium KSOM untuk perkembangan embrio secara *in vitro* dalam inkubator CO_2 (5%) pada suhu 38.5^{o}C dalam suasana atmosfera lembap selama 8 hari. Medium dalam kultur *in vitro* telah ditukar setiap 2 hari. Sampel embrio daripada setiap peringkat telah diwarna dengan pewarna Hoechst 33342 untuk mengenalpasti bilangan nukleus embrio.

Data dikemukakan sebagai min±SEM dan dianalisis dengan menggunkan ANOVA sehala. Perbezaan yang signifikan di antara perlakuan telah dianalisis selanjutnya dengan menggunakan DMRT dan $P<0.05$ dianggap sebagai signifikan.

Dalam Eksperimen 1, dua kaedah enukleasi telah dibandingkan, iaitu picitan dan aspirasi. Tiada perbezaan signifikan dalam peratusan oosit telah berjaya dienukleasi ($P>0.05$) bagi kedua-dua kaedah enukleasi (picitan vs. aspirasi: 88.01±3.00% vs. 91.68±1.92%, masing-masing). Dari segi kecekapan manipulasi, tempoh yang diperlukan untuk menamatkan enukleasi bersama-sama dengan suntikan, kelajuan untuk aspirasi dengan suntikan sub-zona adalah lebih capat secara signifikan ($P<0.05$) berbanding picitan dengan suntikan sub-zona (41.18±2.77 dan 83.82±3.16 minit, masing-masing). Di dalam perkembangan embrio *in vitro*, peratusan klon embrio kaprin dari peringkat 2 sel sehingga peringkat blastosis menggunakan kaedah picitan (2 sel: 60.18±2.43%, 4 sel: 53.80±2.84%, 8 sel: 37.71±3.30%, morula: 24.45±2.71% and blastosis: 12.08±2.95%) dan aspirasi (2 sel: 61.55±4.20%, 4 sel: 49.86±3.87%, 8 sel: 39.22±4.26%, morula: 21.07±3.94% and blastosis: 10.93±1.87%) secara signifikannya tidak berbeza ($P>0.05$).

Dalam Eksperimen 2, dua kaedah pemindahan nukleus telah dibandingkan, iaitu suntikan sub-zona dengan elektro-fusi dan suntikan secara intrasitoplasmik. Tiada perbezaan signifikan ($P>0.05$) dalam kadar suntikan dan rekonstruksi bagi kedua-dua kaedah pemindahan nukleus. Peratusan klon embrio kaprin yang diperolehi daripada interspesies SCNT pada 2- dan 8 sel dengan menggunakan kaedah SUZI dan ICI tidak berbeza dengan signifikan ($P>0.05$). Walau bagaimanapun, peratusan klon embrio kaprin pada 4 sel, morula dan blastosis diperolehi daripada kaedah SUZI adalah lebih tinggi secara signifikan ($P<0.05$) berbanding dengan kaedah ICI (53.80±2.84% vs. 38.60±2.25%, 24.45±2.71% vs. 16.05±1.43% dan 12.08±2.95% vs. 4.51±1.45%, masing-masing).

Dalam Eksperimen 3, tiga sistem kultur secara *in vitro* yang berbeza telah dibandingkan, iaitu Kumpulan 1 (KSOM A sepanjang tempoh kultur), pemerhatian ke atas embrio direkodkan pada hari 3, 5, 7 dan 8 tanpa menukar medium; Kumpulan 2 (KSOM A pada hari 1-3, seterusnya ditukar dengan KSOM A yang baru pada hari 3 dan 5), embrio telah diperhatikan dan direkodkan pada hari 3, 5, 7 dan 8; dan Kumpulan 3 (KSOM A pada hari 1-3, seterusnya ditukar dengan KSOM B pada hari 3 dan 5), embrio diperhatikan dan direkodkan pada hari 3, 5, 7 dan 8. Kumpulan 3 menunjukkan lebih tinggi secara signifikan ($P<0.05$) di dalam *in vitro* kekompetenan perkembangan daripada peringkat 2 sel sehingga blastosis berbanding kepada Kumpulan 1 (2 sel: 49.01±2.02%, 4 sel: 36.92±3.02%, 8 sel: 26.46±1.74%, morula: 8.42±0.47% and blastosis: 0.00±0.00%) dan 2 (2 sel: 49.85±3.27%, 4 sel: 39.68±2.72%, 8 sel: 29.34±1.87%, morula: 10.22±1.49% and blastosis: 0.00±0.00%).

Dalam Eksperimen 4, suatu usaha untuk menghasilkan kebuntingan selepas pemindahan embrio secara oviduktus embrio dengan menggunakan embrio interspesies SCNT dan dengan menggunakan *ultrasound scanning* untuk mendiagnosis kebuntingan telah dijalankan. Sejumlah 63 klon embrio kaprin telah diperolehi daripada eksperimen interspesies SCNT. Walau bagaimanapun, hanya 55 embrio terdiri daripada Gred 1 dan 2 (peringkat 4-8 sel) telah dipilih dan dipindahkan ke dalam 9 penerima yang mempunyai sekurang-kurangnya 1 CL. Malangnya, selepas *ultrasound scanning* pada hari ke-30 usia kebuntingan selepas pemindahan embrio, tiada kebuntingan diperhatikan dalam kambing-kambing penerima setelah eksperimen pemidahan embrio.

Boleh disimpulkan daripada kajian ini bahawa embrio kaprin boleh dihasilkan dengan jayanya melalui interspesies SCNT menggunakan sel fibroblas fetus kaprin sebagai karioplas penderma dan oosit bovin sebagai sitoplas penerima di bawah suasana tempatan di Malaysia. Adalah dipercayai bahawa ini adalah laporan pertama yang menunjukan penghasilkan klon embrio kaprin dengan kadar blastosis yang memuaskan

dalam interspesies SCNT dengan menggunakan tambahan suplementasi glukosa. Dengan penemuan yang memberangsang ini dan penyelidikan yang lebih terperinci pada masa hadapan, penggunaan kaprin-bovin dalam interspesies SCNT untuk menghasilkan embrio dan zuriat kambing boleh menawarkan satu pendekatan baru untuk meningkatkan populasi kambing yang baik dari segi genetiknya di Malaysia pada kadar yang cepat untuk memenuhi permintaan daging dan susu kambing bagi industri.

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Sincerely,

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LIST OF PUBLICATIONS AND PRESENTATIONS

Publications

Soh, H.H., W.E. Wan Khadijah and R.B. Abdullah. 2012. Sub-zonal versus intracytoplasmic injection produces a higher rate of cloned caprine-bovine interspecies blastocyst. Small Ruminant Research. 105: 231-236. (ISI publication)

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Conferences (Poster Presentations)

Soh, H.H., Y.B. Qing, C.S. Xu, S. Lee, R.B. Abdullah, H.J. Wei and W.E. Wan Khadijah. 2010. Production of cloned-porcine embryos through somatic cell nuclear transfer (SCNT). Proceedings of 7th Annual Conference of the Asian Reproductive Biotechnology Society, November 8-12, Kuala Lumpur, Malaysia.

Soh, H.H., W.E. Wan Khadijah and R.B. Abdullah. 2011. Production of cloned-goat embryos through interspecies somatic cell nuclear transfer (iSCNT) using two different nuclear transfer methods. Proceedings of 32nd Annual Conference of the Malaysian Society of Animal Production, June 6-8, Tawau, Sabah.

Student Exchange Programme(Between University of Malaya and YunNan Agricultural University)

The experiment on porcine intraspecies SCNT was performed in YunNan Agricultural University (May – August 2010), China, on an agreement of "Memorandum on Academic Exchange between University of Malaya and YunNan Agricultural University, 2010-2015".

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%	percentage
^{o}C	degree Celcius
β	beta
mHz	megahertz
µl	microlitre
ml	millilitre
µm	micrometer
mm	millimeter
cm	centimeter
µsec	microsecond
min	minute
hr	hour
mg	milligramme
g	gramme
mM	millimolar
M	molar
mOsm	milliosmole
O_2	oxygen
CO_2	carbon dioxide
pH	hydrogen potential
kV/cm	kilovolt/centimeter
w/v	weight/volume
v/v	volume/volume
α-MEM	α-Minimum Essential Medium

6-DMAP	6-dimethylaminopurine
ABEL	Animal Biotechnology-Embryo Laboratory
AI	artificial insemination
AI/TI	anaphase I/ telophase I
ANOVA	analysis of variance
ARTs	advances reproductive technologies/ assisted reproductive technologies
BME	basal medium eagle
BSA-FV	bovine serum albumin-fraction V
Ca^{2+}	Calcium ion
$CaCl_2$	calcium chloride
$CaCl_2.2H_2O$	calcium chloride dihydrate
CaI	calcium ionophore
CB	cytochalasin B
CHX	cycloheximide
CIDR	controlled intravaginal drug release device
CL	corpus luteum
COC	cumulus oocyte complexes
CR1	Charles Rosenkrans 1
CSF	cytostatic factor
CZB	Chatot, Ziomek, Bavister medium
DC	direct current
DMRT	Duncan's Multiple Range Tests
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EMiL	Embryo Micromanipulation Laboratory

ESC	embryonic stem cell
ET	embryo transfer
e.g.	for example
et al.	et alii (and others)
FBS	foetal bovine serum
FSH	follicle stimulating hormone
G	gauge (needle size)
HDAC	histone deacetylases
HDACi	histone deacetylase inhibitor
ICI	intracytoplasmic injection
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
ID	inner diameter
IPPP	Institute of Research Management and Monitoring
IU	international unit
IVC	*in vitro* culture
IVF	*in vitro* fertilisation
IVM	*in vitro* maturation
IVP	*in vitro* production
i.e.	that is
KCl	potassium chloride
KH_2PO_4	potassium phosphate monobasic
KSOM	Potasium Simplex Optimisation Medium
LED	lymphoid enhancer factor
LOPU	laparoscopic oocyte pick-up

LOS	large offspring syndrome
M II	metaphase II
MAP	mitogen-activated protein
MEM	minimum essential medium
$MgCl_2.6H_2O$	magnesium chloride hexahydrate
$Mg(CH_3COO)_2$	magnesium chloride tetrahydrate
$MgSO_4$	magnesium sulphate
MPF	maturation promoting factor
mSOFaa	modified synthetic oviduct fluid with amino acids
mtDNA	mitochondrial DNA
NaTuRe	Nuclear Transfer and Reprogramming Laboratory
Na_2HPO_4	sodium pyrophosphate
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
$NaH_2PO_4.H_2O$	sodium phosphate monobasic monohydrate
Na lactate	sodium lactate
NEBD	nuclear envelope breakdown
OD	outer diameter
P-S	penicillin-streptomycin
PB-1	first polar body
PB-2	second polar body
PBS	phosphate buffered saline
PMSG	pregnant mare’s serum gonadotrophin
PVP	polyvinylpyrrolidone
RO	reverse osmosis

SCNT	somatic cell nuclear transfer
SEM	standard error of means
SOM	simplex optimisation medium
SUZI	sub-zonal injection
TCM-199	tissue culture medium-199
TSA	tricostatin A
UV	ultraviolet
volt	voltage
vs.	versus
WCICI	whole cell intracytoplasmic injection
WM	Whitten's medium
Xa	active X chromosome
Xi	inactive X chromosome
Xist	inactive specific transcript X chromosome
ZGA	zygotic genome activation

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Chapter 1

1.0 INTRODUCTION

1.1 BACKGROUND

Researchers and authorities have identified that improper breeding programme, poor management and nutrition, lack of advanced technologies, non-commercialisation and non-economical practices are predominant factors that interrupt the growth of goat (also known as caprine) industry in Malaysia. Due to the low productivity of goat production and increasing demand of goat meat each year in Malaysia, the economic value and market potential of goat meat are on the increase each year and consequently the Malaysian government gives a high priority to make agriculture as the third engine of economic growth besides manufacturing and services sectors. In Malaysia, approximately 95% of goats are imported from New Zealand, Australia, Brazil and some other countries to meet the local demand. This is because the present population of goat in Malaysia is less than 500,000 heads (Department of Veterinary Services, 2010). However, there is approximately 10 million goats are required to be slaughtered per year for the local consumption of Malaysian populations (per capita consumption: 0.5 kg per person per year). The high demand for goat meat and milk is also due to the country's population growth and increase in the standard of living of the Malaysians. Furthermore, goat meat could be eaten by all the different ethnic groups of Malaysia as well as goat meat (3%) is lower in fat content compared to other red meats such as beef (21%), pork (17%) and lamb (16%) (USDA Handbook, No. 8, 1989).

The application of reproductive technologies in goat is still low compared to other species such as cattle, sheep and pig. However, the current trend of goat commercialisation, they are vigorous efforts worldwide to incorporate the developed, advanced reproductive technologies (ARTs) such as artificial insemination (AI), *in vitro*

fertilisation (IVF), intracytoplasmic sperm injection (ICSI), oocytes and embryo cryopreservation and embryo transfer into the modern farm management practices. Other newer techniques such as somatic cell nuclear transfer (SCNT) and stem cell research are currently being actively research in various laboratories across the globe. These techniques, once established in the near future, would be an important cornerstone of technological development for the goat industries as well as for the applications for the treatment of human degenerative diseases.

In our laboratory at the University of Malaya, Malaysia, we have been actively conducting research in various goat reproductive technologies for the past 3 decades. One main issue of goat research in Malaysia is the lack of source of ovaries to obtain the oocytes for various reproductive techniques. This is due to the low goat population to be slaughtered in the country; and those slaughtered are mainly the male goats that are imported. Consequently, we have been constantly facing problems of obtaining sufficient number of oocytes for the various reproductive techniques such as *in vitro* fertilisation, intracytoplasmic sperm injection, oocytes and embryo cryopreservation and embryo transfer.

Recently, our laboratory has developed somatic cell nuclear transfer in goat. Even though we obtained encouraging results of producing cloned-caprine embryos using intraspecies somatic cell nuclear transfer (intraspecies SCNT), similar problem as that of other techniques in which the insufficient source of caprine oocytes hampered the progress of cloning and other related research activities. One of the reproductive techniques that we attempt to carry out in our laboratory is interspecies somatic cell nuclear transfer (interspecies SCNT) in goat. Dominko *et al.* (1999) reported the first interspecies SCNT experiments on cow-cattle, sheep-cattle, pig-cattle, monkey-cattle and rat-cattle with the blastocyst percentages 17.3, 13.9, 14.3, 16.6 and 0%, respectively, shortly after the cloning of a lamb, Dolly, from somatic cell nuclear transfer (Wilmut *et*

al., 1997). They suggested that the cell division and formation of an embryonic blastocoele cavity occurred at the time characteristic of embryos from the species of the donor nuclei; however, from their study there was no pregnancies reported after transferred of interspecies SCNT cloned embryos into surrogate animals.

Since then, interspecies SCNT becomes a new approach of several researchers to obtain embryos as well as offspring through interspecies SCNT by employing various model systems. However, most of the successful interspecies SCNT were done by combining closely related species (Beyhan *et al*., 2007). The first pregnancy obtained through interspecies SCNT was reported by White *et al*. (1999) using domestic sheep as recipient oocyte and argali sheep as donor cell (*Ovis aries/Ovis musimon*), however, the pregnancies had been lost by 59 days of gestation. Interspecies SCNT offspring such as gaur (*Bos grunensis*) (Lanza *et al*., 2000) and mouflon (*Ovis orientalis musimon*) (Loi *et al*., 2001) have been obtained. Sansinena *et al*. (2005) also reported with established two pregnancies of banteng-cattle cloned embryos, however, no live offspring was obtained. Besides that, some reported experiments on interspecies SCNT but no offspring obtained such as panda-rabbit (Chen *et al*., 2002), macaca-rabbit (Yang *et al*., 2003), water buffalo-cattle (Kitiyanant *et al*., 2001), takin-cattle (Li *et al*., 2006), however, have achieved success in blastocyst development.

1.2 STATEMENT OF PROBLEMS

To date, a lot of research progress has been made in interspecies somatic cell nuclear transfer in livestock animals including goats. However, there are still many issues need to be solved before this technique could be used routinely in livestock production. Interspecies somatic cell nuclear transfer is a new approach to produce cloned embryos. However, many technical problems need to be overcome since it involves 2 different unrelated species. Even though the percentage in getting cleavage rate is high after

nuclear transfer and subsequent culture, it is very hard to obtain blastocyst (<10%) or even harder to obtain cloned offspring.

Nuclear transfer is a complex protocol, which involved many steps and each step might affects the overall efficiency. The protocols involved are such as, the preparation of somatic cells, the preparation of enucleated oocytes, the injection or fusion of somatic cell nuclei into the enucleated oocyte, the reconstruction of the somatic chromosomes, oocyte activation and culture medium methods to produce a diploid cloned embryo. Over the years, researchers have shown their great effort to improve or solve the technical problem in getting cloned embryo as well as live and health offspring. However, there is still having unpredictability of the biological variation in recipient oocytes (cytoplasts) and donor cells (karyoplasts) which is difficult to control.

One obvious problem can be seen throughout the years is the low efficiency, with low proportions of embryos developing to be implanted, survive pregnancy and birth live cloned offspring (Wilmut *et al*., 1997; Spikings *et al*., 2006). However, up to now, the causes of this extremely high failure rate reported is unclear. Song *et al*. (2008) suggested that the mtDNA (mitochondria DNA) distribution with the progression of interspecies SCNT embryo development might hold the promise for future improvement of nuclear transfer efficiency.

Nevertheless, one of the most important criteria that accompany the success rate of interspecies SCNT outcome is the compatibility between recipient cytoplasm and donor nucleus. This is because a suitable recipient cytoplasm can support the donor nuclei to be reprogrammed. Dominko *et al.* (1999) reported that bovine cytoplasm has the ability to support the introduced differentiated nucleus regardless of chromosome number, species or age of the donor fibroblast cell. Due to the low population of goat in Malaysia, reproductive technologies experiments could not be carried out properly as expected; therefore correspondingly, one of the preferred solutions is by using

interspecies SCNT technique to use the bovine cytoplasm as recipient and caprine somatic cell as donor since bovine ovaries are relatively easily obtained from the abattoir compared to the caprine ovaries source.

Below are some of the pertinent questions needed to be answered related to interspecies somatic cell nuclear transfer in goat:

a) Is the caprine somatic nuclei can be reprogrammed in bovine cytoplasm in interspecies SCNT?
b) Is the caprine foetal fibroblast cell more compatible with bovine cytoplasm if compared to other donor karyoplast sources such as ear fibroblast cell and cumulus cell?
c) Is interspecies somatic cell nuclear transfer can develop into blastocyst *in vitro* and subsequently produce viable offspring after embryo transfer?
d) What is the most suitable enucleation (aspiration or squeezing) and nuclear transfer techniques (ICI or SUZI) in interspecies SCNT in order to produce large number of cloned embryos and subsequently offspring.
e) Are different culture systems (culture medium) will affect the development of cloned embryos *in vitro*?
f) Why cloning attempts give low percentage of viable offspring?

1.3 JUSTIFICATION OF THE STUDY

In Malaysia, we are facing the problems in getting caprine ovaries from abattoir in order to carry out reproductive experiments due to low number of goats slaughtered at the abattoir; however, one of the alternative ways to obtain the caprine oocyte source is via laparoscopic oocyte pick-up (LOPU) in the laboratory. But the cost of the LOPU is much higher than ovaries sample collection from abattoir. Therefore, to overcome this problem, we proposed to choose the bovine oocyte from abattoir as the recipient cytoplasm, at the same time, we established the caprine foetal fibroblast somatic cell line and use as the donor nucleus with the aim to produce interspecies cloned-caprine embryos as well as obtain pregnancy after embryo transfer of cloned-caprine embryo.

Our laboratory has been successfully obtained the both intra- and interspecies cloned-caprine embryos using caprine ear fibroblast cell as the donor karyoplast by a senior researcher, Kwong Phek Jin since year 2008 (Abdullah *et al*., 2011). She is the one who started the cloning in our laboratory, even in Malaysia. However, the percentage of getting the interspecies cloned blastocyst is still low. Hence, the present study is to optimise the technique on interspecies SCNT by using caprine foetal fibroblast as donor karyoplast to improve the production of cloned-caprine blastocyst and subsequently to produce pregnancy through embryo transfer.

This research is a new approach in the production of cloned-caprine embryos using bovine oocyte as recipient cytoplasm. Bovine oocyte has been proven to serve as a universal recipient cytoplasm to produce interspecies SCNT cloned embryos and offspring. From the studies, researchers have demonstrated that the bovine cytoplast is capable of reprogramming somatic cell nuclei from other species which include the horse (Hinrichs *et al*., 2000; Reggio *et al*., 2000; Li *et al*., 2002a), pig (Yoon *et al*., 2001), tiger (Hwang *et al*., 2001), buffalo (Kitiyanant *et al*., 2001; Saikhun *et al*., 2002) and gaur (Lanza *et al*., 2000).

Besides that, comparison of different enucleation and nuclear transfer techniques were carried out to evaluate the success of cloned-caprine embryos development in interspecies SCNT. In previous studies, Chen *et al*. (2007) has reported that the procedures of enucleation and donor cell injection are two of the important key factors that affect somatic cell nuclear transfer. At present, there were a few enucleation techniques had been reported, which are piezo-drive (Wakayama *et al*., 1998), squeezing (Wilmut *et al*., 1997) and aspiration (Polejaeva *et al*., 2000). While, for nuclear transfer techniques are such as sub-zonal injection (SUZI; Wilmut *et al*., 1997), intracytoplasmic injection (ISI; Wakayama *et al*., 1998) and whole cell intracytoplasmic injection (WCICI; Lee *et al*., 2003). However, there is less report on the effect of techniques on the development of cloned embryos in interspecies SCNT. Therefore, in the present study, the combination of squeezing with sub-zonal injection (SUZI) (with fusion), squeezing with ICI (without fusion) and aspiration with sub-zonal injection (SUZI) (with fusion) were carried out to optimise both of the enucleation and nuclear transfer techniques in our laboratory.

Embryo culture system also plays an important factor for all types of *in vitro* production (IVP) experiments. Campbell *et al*. (2007) suggested that the *in vitro* culture of SCNT embryos is one of the most important steps affecting the preimplantation development, pregnancy and the number of offspring generated. This is because *in vitro* produced mammalian embryos generally differ from their *in vivo* counterparts, due to the sub-optimal conditions of the *in vitro* culture systems. Therefore, in order to improve the efficiency of *in vitro* embryo production systems, the formulation of culture media have to be optimised to mimic the *in vivo* environment of the female reproductive tract (Summers and Biggers, 2003). In the present study, effects of different *in vitro* culture media with special focus to low and high glucose concentration KSOM medium on the interspecies cloned-caprine embryos were compared.

1.4 APPLICATION OF INTERSPECIES SCNT CLONING

A key reason behind the usefulness of cloning is that by producing near-identical genetic copies of an organism, outcomes of producing genetically superior animal including goat are faster and more predictable than in previous reproductive techniques. This is essential to ensure profitability and sustainability of goat production and commercialisation in Malaysia.

The first and main application of interspecies SCNT cloning is an exciting possibility for species with limited availability of oocytes and recipients for embryo transfer. In both intra- and interspecies nuclear transfer, it is necessary to prepare large quantities of high-quality of recipient oocytes (cytoplasts) to optimise the cloning techniques, in order to produce large number of cloned embryos as well as offspring. However, it is very difficult to obtain large quantities of caprine oocytes in Malaysia. Therefore, interspecies SCNT is a new approach to solve the oocytes limitation problems in Malaysia as well as other countries for different species.

By using interspecies SCNT cloning technique, it can allow genetic conservation of local breeds, for example Katjang goat, with unique tolerance for regional diseases or local climates. It can in principle be used to create an infinite number of clones of the very best farm animals and enhance the genetic improvement in a short time that is by shortening the generation interval. According to statistics (Department of Veterinary Services, 2010), Malaysian goat population is decreasing annually due to improper breeding, poor management and nutrition, non-commercialisation and non-economical practices. In order to decisively and effectively overcome this situation of low population and threatening from breed extinction to ensure preservation of genetic diversity, cloning would be the best alternative to be integrated systematically into goat breeding programmes and management practices.

Thirdly, interspecies SCNT may also play a very important role in country economic development such as Malaysia by rapidly increasing animal productivity through improvement in economic genetic traits such as meat and milk production. In other words, a rapid production of identical genetic modified elite individuals with desirable traits, such as in Katjang goat, for milk containing extra nutrients or meat more consistent in taste and quality could be achieved by using interspecies SCNT.

Fourthly, extending interspecies SCNT techniques to other applications, conservation and propagation of rare livestock breeds as well as endangered species and poorly reproduced zoo wild animals could be implemented and achieved. Consequently, by using somatic cells in interspecies cloning may allow the sustenance of general genetic diversity of a species. Besides that, it would also remain to be shown that clones do consistently deliver the expected commercial performance and are healthy and that the technology can be applied without compromising animal welfare. Compared with other reproductive technologies, such as IVF and ICSI, interspecies SCNT, could give a faster rate to produce large number of high quality identical gene animals.

Nevertheless, the most important prospective application of interspecies SCNT lies in its potential is to generate a preimplantation embryo to be used as a source of embryonic stem cells (ESC) in human therapeutic approach. We believe that interspecies SCNT may able to reprogramme the human somatic cells without many of the significant ethical challenges surrounding the use of human oocytes (Beyhan *et al*., 2007). Chen *et al*. (2003) reported that the somatic nuclei from a human patient could be reprogrammed by nuclear transfer into a rabbit oocyte to generate nuclear transfer ESC. The cells and tissues derived from this pathway would have nuclear DNA identical to the patient's and, therefore, would likely not be subject to immune rejection (Boiani *et al*., 2003; Colleoni *et al*., 2005; Beyhan *et al*., 2007). It is believe that the ability to produce ESC from interspecies SCNT embryos could facilitate the creation of

new cellular models of human diseases (for example, Parkinson's disease, Alzheimer's disease and other degenerative tissue diseases) and could significantly advance our understanding of basic nuclear-cytoplasmic interactions between a somatic cell and an oocyte from different species.

1.5 OBJECTIVES OF THE STUDY

This study was carried out with the aim to obtain cloned-caprine embryos *in vitro* from interspecies nuclear transfer with the following specific objectives:

a) To produce cloned-caprine embryos *in vitro* through interspecies somatic cell nuclear transfer.
b) To compare the effect of enucleation and nuclear transfer methods on interspecies SCNT cloned-caprine embryos developmental competency.
c) To compare the effect of culture medium changing on interspecies SCNT cloned-caprine embryos developmental competency.
d) To obtain the pregnancy on interspecies cloned-caprine embryo after embryo transfer at early stage through oviduct embryo transfer technique.

Chapter 2

2.0 REVIEW OF LITERATURE

2.1 BACKGROUND

The last century has seen a dramatic reduction of animal species, including large mammals, mainly caused by human-related activities (Loi *et al*., 2011). The obvious consequence of such a phenomenon is the progressive contraction in biodiversity world-wide. Paradoxically, this problem does not involve wild species only, but also domestic ones, as often local or typical breeds are being replaced by fewer, more productive genotypes. According to the Food and Agriculture Organisation of the United Nations (FAO, 2007), a total of 1491 breeds world-wide (or 20%) are classified as being either critically endangered, critical-maintained, endangered or endangered-maintained (Figure 2.1). Although in mammalian livestock the proportion of breeds classified at risk is lower than average (16%) in absolute terms, their number is very high (881 breeds) (FAO, 2007).

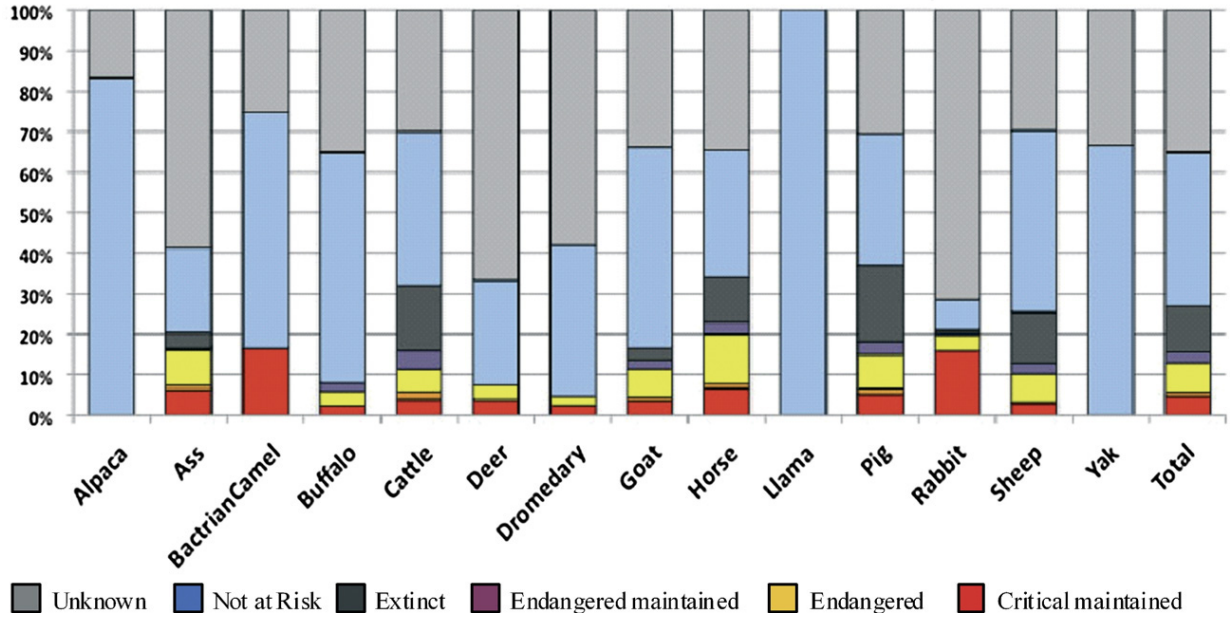

Figure 2.1: Status of endangered domestic breeds according to a FAO survey 2007 (adapted from Loi *et al.,* 2011).

For that reason, reproductive biologist and zoologist have started to consider reproductive technologies as a tool to expand small animal populations which are already composed of a critical number of individuals (Holt *et al*., 2004; Andrabi and Maxwell, 2007). Essentially, the target of such reproductive technology should be the enhancement of the reproductive performance of a threatened population (Loi *et al*., 2011).

To keep up with this alarming situation, it has been suggested that genetic banks, preferably in the form of cell lines, should be established from animal species threatened with extinction (Ryder *et al*., 2000). The idea is to re-establish or expand the threatened population by using these cells for somatic cell nuclear transfer (SCNT) (Wilmut *et al*., 1997; Holt *et al*., 2004). However, even though the potential of SCNT tool for conservation has already been demonstrated (Lanza *et al*., 2000; Loi *et al*., 2001, 2007), the efficiency of the nuclear transfer remains low in terms of offspring outcome. In addition, the high embryonic loss and perinatal mortality observed with the SCNT procedure requires that large number of oocytes must be available for the numerous attempts needed to establish pregnancies and produce live offspring (Colman, 2000; Hill *et al*., 1999). In exotic or endangered species, the lack of oocytes precludes the use of traditional SCNT procedure and an approach such as interspecies somatic cell nuclear transfer (interspecies SCNT) may be the only alternatively to produce embryos and offspring (Sansinena *et al*., 2005).

In Malaysia, the yearly increasing demand of goat meat consumption and its dairy products worldwide, served as the moving gear in goat breeding industries to multiply the goat population via the application of assisted reproductive technologies (ARTs) besides sustaining the conventional breeding programme. Among the ARTs that are applied in goat farming industry, reproductive cloning technologies in production of cloned-caprine embryos are foreseen to facilitate the effort of mass goat production in

just a short time frame (Abdullah *et al*., 2011). However, production of cloned-caprine embryos using the traditional SCNT is limited by low source of caprine oocytes as the recipient cytoplasts in Malaysia. Therefore, by using the bovine oocytes as recipient cytoplasts in interspecies SCNT is the only alternative approach to produce large number of cloned-caprine embryos and subsequently offspring at a rapid rate.

The first attempt of caprine interspecies SCNT was reported by Song *et al*. (2008) using the enucleated bovine oocyte as recipient cytoplast resulted in *in vitro* development of 7.9% blastocysts. Ma *et al.* (2008) obtained 7.4% of blastocysts using goat-sheep. Recently, our research group has successfully obtained 5 to 10% of the interspecies SCNT cloned-caprine blastocysts using bovine as recipient cytoplast (Kwong *et al.,* 2011; Soh *et al.,* 2011). However, there is still lack of information on the interspecies SCNT in goat; therefore, the optimal and practical interspecies SCNT procedures integrated with other technologies and appropriate management system are needed in the future, especially to increase goat population at a rapid rate for viable, profitable and sustainable industry.

Table 2.1: Timeline of significant findings of intra- and interspecies SCNT in caprine

Year	Author	Intra-/interspecies SCNT	Significant event/ finding
1999	Baguisi *et al*.	Intraspecies SCNT	First report on goat SCNT with obtaining of 3 healthy identical female offspring using foetal somatic cells as donor karyoplast.
2001	Keefer *et al*.	Intraspecies SCNT	Both *in vitro* transfected and non-transfected caprine foetal fibroblasts could direct full term development following nuclear transfer.

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Year	Author	Intra-/interspecies SCNT	Significant event/ finding
2001	Reggio *et al*.	Intraspecies SCNT	First report of cloned goats produced from nuclear transfer using cytoplast derived from abattoir ovaries.
2001	Zou *et al*.	Intraspecies SCNT	The survival rate of cloned-caprine embryos, obtained by injection, was higher than that derived from fusion (62.7 and 45.9%, respectively).
2002	Guo *et al*.	Intraspecies SCNT	The results of microsatellite DNA analyses indicated that the 2 kids were from the same donor fibroblast cell line derived from an adult caprine ear skin.
2002	Keefer *et al*.	Intraspecies SCNT	No significant differences in the rates of pregnancy and nuclear transfer efficiency between ranulose cells and foetal fibroblast cells.
2002	Zou *et al*.	Intraspecies SCNT	A foreign gene, such as the neo-resistant gene, could be introduced into caprine foetal fibroblast cells, and that the resulting transgenic cells were capable of being cloned to produce 100% transgenic animals.
2003	Das *et al*.	Intraspecies SCNT	Reported that 300 V resulted in better electrofusion, and cytochalasin B blocked synchronised cells and fast growing skin fibroblast cells of caprine could be used for nuclear transfer.
2003	Ohkoshi *et al*.	Intraspecies SCNT	Caprine nuclear transfer using anterior pituitary cells in an *in vitro* culture system had the developmental potential to produce offspring after embryo transfer.
2004	Zhang *et al*.	Intraspecies SCNT	Donor cell cycle at stage G0/G1 might be efficient ways to improve the developmental competence of reconstituted caprine embryos than stage G2/M.

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Year	Author	Intra-/interspecies SCNT	Significant event/ finding
2005	Melican *et al*.	Intraspecies SCNT	More offspring were produced utilising karyoplast cultured in low serum versus cycling cells grown to confluence to synchronise G0/G1 stage cells. In addition, more live offspring were produced using donor cells harvested by partial compared with complete trypsinisation.
2006	Lan *et al*.	Intraspecies SCNT	The fusibility and *in vitro* developmental potential of embryos reconstructed from foetal fibroblasts at passages 20 to 25 were significantly lower than those of embryos reconstructed from foetal fibroblasts at passages 3 to 5, and the cloning efficiency of the long term cultured cells was low (0.5%).
2006	Shen *et al*.	Intraspecies SCNT	SCNT is a viable technique for goat cloning and that increase electrical field strength for both fusion and activation of reconstructed embryos appeared to be beneficial for the development of cloned embryos.
2007	Chen *et al*.	Intraspecies SCNT	The method of telophase II enucleation combined with whole cell intracytoplasmic injection (WCICI) could properly reprogramme the somatic cells, and WCICI could provide an efficient and less labour-intensive protocol in Asian yellow goat cloning.
2008	Daniel *et al*.	Intraspecies SCNT	The difference in membrane surface properties between cumulus and fibroblast cell may contribute to the higher fusion rate obtained in cumulus cells for cloned-caprine embryo production.
2008	Ma *et al*.	Interspecies SCNT	Authors suggested that caprine foetal fibroblast derived mitochondria were degraded for the depression of bioenergetic functions, and then selectively eliminated during the embryogenesis of sheep (cytoplast)-goat (karyoplast) cloned embryos.

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Year	Author	Intra-/interspecies SCNT	Significant event/ finding
2008	Song *et al*.	Interspecies SCNT	Successfully obtained 7.9% cloned-caprine blastocyst using bovine as recipient cytoplast.
2008	Tao *et al*.	Intraspecies and Interspecies SCNT	Both interspecies and intraspecies reconstructed cloned-caprine embryos have similar development changes in the zona ellucid, rough endoplasmic reticulum, Golgi apparatus and nucleolus when compared with *in vivo*-produces embryos.
2008	Wang *et al*.	Intraspecies SCNT	Successfully developed an effective method to screen transgenic donor cells and improve the production efficiency of transgenic embryos.
2009b	Tao *et al*.	Intraspecies SCNT	Optimised nuclear transfer protocol and proper hCG treatment led to the successful birth of a cloned goat.
2009	Yuan *et al*.	Intraspecies SCNT	Live goats were generated by SCNT from caprine mammary gland epithelial cells using long term cultured cell lines (25 to 27 passages).
2010	Akshey *et al*.	Intraspecies SCNT	The foetal fibroblast cell was a suitable candidate as nuclear donor, and the flat surface culture system was suitable for zona-free blastocyst development by the hand-made cloning technique in the goat.
2010	Dalman *et al*.	Intraspecies SCNT	The use of full confluency was suitable for cell cycle synchronisation because it arrested cells at the G0/G1 phase and also induced less apoptosis in comparison with the serum starvation group.
2011	Abdullah *et al*.	Intraspecies and Interspecies SCNT	Cloned-caprine embryos could be produced *in vitro* via both intraspecies and interspecies SCNT approaches in which the efficacy of interspecies SCNT approach was comparable to that of intraspecies SCNT approach.

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Year	Author	Intra-/interspecies SCNT	Significant event/ finding
2011	Akshey *et al*.	Intraspecies SCNT	First report of effect of treatment of donor cells with rescovitine and different activation methods on handmade cloned embryo production in goat.
2011	Tang *et al*.	Intraspecies SCNT	mSOF medium supplemented with 10% FBS could better support the development of cloned caprine embryos, and the blastocysts cultured in this medium could develop to term and gave birth to a healthy kid at term.

In interspecies SCNT, a donor cell (karyoplast) is transplanted into a recipient enucleated oocyte (cytoplast) of a different species/family/order/class. The resulting embryo is then transplanted into the uterus of a suitable foster mother for development to term (Loi *et al*., 2011). Up to now, there are more than 40 articles have been published in which oocytes and somatic cells from a number of species have been used to generate embryos via interspecies nuclear transfer (Table 2.2). Most of the successfully live offspring were obtained by combining closely related species, such as gaur-cattle (Lanza *et al*., 2000), argali sheep-domestic sheep (White *et al*., 1999), river buffalo-swamp buffalo (Yang *et al*., 2010) and wild cat-domestic cat (Gomez *et al*., 2004).

In some of the reported research, however, genetic distance between donor and recipient species spanned taxonomic classes, such as chicken-rabbit (Liu *et al*., 2004) and panda-rabbit (Chen *et al*., 2002). The majority of these experiments have failed to produce viable embryos. A common limitation in making comparisons between these interspecies SCNT reports is that the definition of experimental endpoints and criteria for successful reprogramming was often ill-defined, except to those resulting in live

offspring (Beyhan *et al*., 2007). Nevertheless, the potential impact of a successful interspecies SCNT scheme is sufficiently attractive to maintain ample scientific interest in this subject (Beyhan *et al*., 2007).

Table 2.2: List of interspecies SCNT experiments in mammalian species reported to date (adapted and modified from Loi *et al*., 2011)

Taxonomic relationship	Year	Author	Cell donor species	Types of donor cell	Recipient cell species	Significant results
Intersubspecies	2004	Gomez *et al.*	African wild cat (*Felis sylvestris lybica*)	Skin fibroblast cells	Domestic cat (*Felis sylvestris catus*)	Offspring
	2010	Yang *et al.*	River buffalo *(Bubalus bubalis bubalis)*	Ear skin fibroblast cells	Swamp buffalo *(Bubalus bubalis carabensis)*	Offspring
Interspecies	1999	White *et al.*	Argala *(Ovis ammon)*	Skin fibroblast cells	Sheep *(Ovis aries)*	50 day foetuses
	2000	Lanza *et al.*	Gaur *(Bos gaurus)*	Skin fibroblast cells	Cattle *(Bos taurus)*	Offspring
	2001	Loi *et al.*	European Mouflon *(Ovis orientalis musimon)*	Granulosa cells	Sheep *(Ovis aries)*	Offspring
	2005	Murakami *et al.*	Yak *(Bos grunniens)*	Cumulus cells	Cattle *(Bos taurus)*	Blastocysts: 10.9%
	2005	Sansinena *et al.*	Banteng *(Bos javanicus)*	Ear skin fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 15.0-28.0%
	2006	Li *et al.*	Yak *(Bos grunniens)*	Ear skin fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 28.0%
	2008	Gomez *et al.*	Sand cat *(Felis margarita)*	NA (abstract)	Domestic cat *(Felis sylvestris catus)*	Offspring

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Taxonomic relationship	Year	Author	Cell donor species	Types of donor cell	Recipient cell species	Significant results
Interfamily	1999	Dominko *et al.*	Sheep *(Ovis aries)*	Ear skin fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 18.0%
	2001	Kitiyanant *et al.*	Buffalo *(Bubalus bubalus)*	Foetal fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 33.0%
	2004	Matshikiza *et al.*	African buffalo (*Syncerus caffer)*	Ear skin fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 3.5%
	2004	Matshikiza *et al.*	Eland (*Taurotragus oryx)*	Ear skin fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 2.0%
	2005	Lu *et al.*	Buffalo *(Bubalus bubalus)*	Ear skin fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 4.5%
	2005	Lu *et al.*	Cattle *(Bos taurus)*	Ear skin fibroblast cells	Buffalo *(Bubalus bubalus)*	Blastocysts: 3.0%
	2006	Li *et al.*	Takin *(Burdocas taxicolor)*	Ear skin fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 5.0%
	2006	Yin *et al.*	Leopard cat *(Pronailurus bengalensis)*	Ear skin fibroblast cells	Domestic cat *(Felis sylvestris catus)*	Blastocysts: 7.8%
	2008	Hua *et al*.	Sheep *(Ovis aries)*	Ear skin fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 24.0%

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Taxonomic relationship	Year	Author	Cell donor species	Types of donor cell	Recipient cell species	Significant results
	2008	Song *et al.*	Goat *(Capra hircus)*	Foetal fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 7.9%
	2008	Ma *et al.*	Goat *(Capra hircus)*	Foetal fibroblast cells	Sheep *(Ovis aries)*	Blastocysts: 7.4%
	2008	Tao *et al.*	Goat (*Capra hircus*)	Ear skin fibroblast cells	Cattle *(Bos taurus)*	16 cell: NA
	2010	Thongphakdee *et al.*	Marbled cat *(Pardofelis marmorata)*	Oviduct fibroblast cells	Domestic cat *(Felis sylvestris catus)*	Blastocysts: 5.0%
Interorder	1999	Dominko *et al.*	Pig *(Sus scrofa)*	Ear skin fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 6.0%
	1999	Dominko *et al.*	Rhesus monkey *(Macaca mulatta)*	Ear skin fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 16.6%
	2002	Chen *et al.*	Giant Panda *(Ailuropoda melanoleuca)*	Abdominal muscle cells	Rabbit *(Oryctolagus cuninculus)*	Blastocysts: 18.5%
	2003	Arat *et al.*	Mouse *(Mus musculus)*	Mouse embryonic fibroblast cells	Cattle *(Bos taurus)*	8 cell: 6.2%

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Taxonomic relationship	Year	Author	Cell donor species	Types of donor cell	Recipient cell species	Significant results
	2003	Chen *et al.*	Human *(Homo sapiens recens)*	NA (abstract)	Rabbit *(Oryctolagus cuninculus)*	Blastocysts: 10.4-13.5%
	2003	Wen *et al.*	Domestic cat *(Felis sylvestris catus)*	NA (abstract)	Rabbit *(Oryctolagus cuninculus)*	Blastocysts: 6.9%
	2004	Ikumi *et al.*	Antarctic mink whale *(Balaenoptera bonearensis)*	NA (abstract)	Pig *(Sus scrofa)*	2 to 4 cell: NA
					Cattle *(Bos taurus)*	2 to 4 cell: NA
	2005	Jiang *et al.*	Ibex *(Capra ibex)*	Ear skin fibroblast cells	Rabbit *(Oryctolagus cuninculus)*	Blastocysts: 19.5%
	2006	Zhou and Guo	Camel *(Camelus bactrianus)*	Ear skin fibroblast cells	Sheep *(Ovis aries)*	Blastocysts: 0%
	2006	Zhao *et al.*	Tibetan antelope *(Pantholops hodgsonii)*	Ear skin fibroblast cells	Rabbit *(Oryctolagus cuniculus)*	Blastocysts: 1.4-8.7%
	2006	Zhao *et al.*	Camel *(Camelus bactrianus)*	Ear skin fibroblast cells	Rabbit *(Oryctolagus cuniculus)*	Blastocyst: 0-7.5%

(continued)

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Taxonomic relationship	Year	Author	Cell donor species	Types of donor cell	Recipient cell species	Significant results
	2007	Hashem *et al.*	Siberian tiger *(Panthera tigris altaica)*	Skin fibroblast cells	Pig *(Sus scrofa)*	Blastocysts: 0.7%
	2007	Uhm *et al.*	Pig *(Sus scrofa)*	Skin fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 3.9%
	2007	Uhm *et al.*	Cattle *(Bos taurus)*	Skin fibroblast cells	Pig *(Sus scrofa)*	Blastocysts: 5.5%
	2008	Lorthongpanich *et al.*	Crab-eating monkey *(Maccaca fascicularis)*	Skin fibroblast cells	Cattle *(Bos taurus)*	16 cell: NA
	2009	Sha *et al.*	Human *(Homo sapiens recens)*	Human neural stem cells	Goat *(Capra hircus)*	Blastocyst: 10.7%
	2009	Song *et al.*	Rhesus monkey *(Macaca mulatta)*	Ear skin fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 0%
	2009	Sugimura *et al.*	Dog *(Canis domesticus)*	Tail tip fibroblast cells	Pig *(Sus scrofa)*	Blastocysts: 4.3-24.0%
	2009a	Tao *et al.*	Red panda *(Ailurus fulgens)*	Ear skin fibroblast cells	Rabbit *(Oryctolagus cuninculus)*	Blastocysts: 23.0%

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Taxonomic relationship	Year	Author	Cell donor species	Types of donor cell	Recipient cell species	Significant results
	2010	Lagutina *et al.*	Cattle *(Bos taurus)*	NA (abstract)	Pig *(Sus scrofa)*	4 cell: NA
	2010	Lagutina *et al.*	Pig *(Sus scrofa)*	NA (abstract)	Cattle *(Bos taurus)*	16-25 cell
Interclass	2004	Kim *et al.*	Fowl (chicken) *(Gallus gallus domesticus)*	Chicken embryonic fibroblast cells	Cattle *(Bos taurus)*	Blastocysts: 3.0%
	2004	Liu *et al.*	Fowl (chicken) *(Gallus gallus domesticus)*	Chicken blastodermal	Rabbit *(Oryctolagus cuniculus)*	Blastocyst: 9.7%

NA: Not available (in the abstract)

2.2 FACTORS AFFECTING INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER EFFICIENCY

Basically, there are 2 important main factors to the success of the application of interspecies nuclear transfer technology in any given species: (1) compatibility of recipient cytoplast and donor karyoplast between 2 different species (such as mitochondrial/genomic DNA compatibility, embryonic genome activation of the donor nucleus by the recipient oocyte and availability of suitable foster mothers for interspecies SCNT embryos), and (2) technical problems that affect the overall efficiency of the nuclear transfer procedure (such as selection of recipient cytoplast, selection of donor cells, stages of donor cell cycle, donor cell line passages, enucleation, nuclear transfer, fusion, activation and *in vitro* culture system).

2.2.1 Mitochondrial/Genomic DNA Compatibility

To date, most of the successful interspecies SCNT experiment to produce live offspring occurred when the recipient oocyte (cytoplast) and donor cell (karyoplast) sources used were derived from closely related species. This low efficiency of interspecies SCNT may be due to the incompatibility in mitochondrial physiology between the donor karyoplast nucleus and recipient cytoplast mitochondrial (Beyhan *et al.,* 2007).

Mitochondrial DNA (mtDNA) encodes some of the subunits of the electron transfer chain which is responsible for ATP production. Factors required for mtDNA replication, transcription and translation are encoded by nuclear DNA and, therefore, a coordinated mt/genomic DNA cross-talk is essential for normal development (Beyhan *et al.,* 2007). During mammalian gametogenesis, fertilisation and embryogenesis, mitochondria have an unusual morphology and pattern of transmission from one generation to another (Beyhan *et al.,* 2007). This genetic bottle-neck is thought to

ensure mitochondrial homoplasmy (defined as having mitochondria derived from a single source: in this case, the oocyte), which is important to the maintenance of proper mitochondrial function (Beyhan *et al.,* 2007).

It has been suggested that the typical pattern of maternal inheritance observed in many mammalian species does not apply in intraspecies SCNT by using mouse blastomere for nuclear transfer, and its varying degrees of heteroplasmy were observed in most of the resulting embryos, foetuses and live offspring (Hiendleder *et al.,* 2003). With few exceptions, neutral segregation of mtDNA in most bovine intraspecies SCNT, embryos and foetuses appear to be the dominant pattern of inheritance in which the amount of donor cell mtDNA is not more than the original amount contributed during reconstruction of the embryos (*i.e.* the donor cell mitochondria are not selectively replicated over the recipient mitochondria) (Hiendleder *et al.,* 2003).

Co-evolution of nuclear and mitochondrial genomes and the transfer of genetic information from mitochondrial to the nucleus have resulted in a very specific and unique complementation of mitochondrial and nuclear function within an individual species (Beyhan *et al.,* 2007). This specific interaction has also been proposed to contribute to the speciation process (Herrmann *et al.,* 2003). Therefore, it is important to understand that the compatibility of nuclear and mitochondrial genomes may be of paramount importance to interspecies SCNT experiments.

Nagao *et al.* (1998) reported that the type of mtDNA was also shown to affect the developmental ability of preimplantation congenic mouse embryos. In their study, the mtDNA-congenic strain was established from back-crossing *Mus musculus* female mice to B6 male mice for 20 generations, and it was shown that the percentage of embryos reaching the blastocyst stage was reduced from 94 to 35%, depending on their mtDNA composition. It was concluded that since in early embryonic development

mitochondria contribute to the energy production in preimplantation embryos, their developmental ability may be influenced by the type of maternally derived mtDNA and possible incompatibilities among nuclear and mitochondrial genomes could impair early embryonic development.

During SCNT, a relatively small number of the donor cell mitochondria are inserted into the reconstructed embryo, thus, resulting in mtDNA heteroplasmy (Beyhan *et al.,* 2007). SCNT studies on murine, ovine and bovine embryos indicate a high degree of variability in mitochondrial distribution, with some animals displaying complete homoplasmy (Evans *et al.,* 1999; Meirelles *et al.,* 2001; Hua *et al.,* 2008) and others displaying heteroplasmy to varying degrees (Han *et al.,* 2003; Hiendleder *et al.,* 2003; Takeda *et al.,* 2003; Inoue *et al.,* 2004). In heteroplasmic animals, the level of contribution from donor cell mitochondria is highly variable between subjects and within tissues of the same subject. It has been reported that the level of heteroplasmy increases when interspecies SCNT is performed (St John *et al.,* 2004). Although healthy, live offspring have been obtained by both intra- and interspecies SCNT, possible negative effects of heteroplasmy introduced in these animals may be responsible, in part, for many of the failures of interspecies SCNT. For example, incompatibility of mitochondrial and nuclear genomes could impair mitochondrial function, leading to suboptimal respiration (St John *et al.,* 2004).

To date, nuclear transfer studies addressing mitochondrial transmission have limited their scope to the detection of mtDNA and have provided no information about indicators of mitochondrial function. Two recent studies have investigated the amount of mtDNA, ATP production and gene expression, that is in bovine intraspecies SCNT embryos with different haplotypes (Jiao *et al.,* 2007) and mtRNA expression in sheep-goat interspecies SCNT embryos (Ma *et al.,* 2008). The results of these studies

suggested that the haplotype of recipient oocyte affects the ATP output and developmental competence of embryos and that of the donor cell's mitochondria are selectively eliminated in interspecies SCNT embryos during preimplantation development. Although metabolic pathways are well conserved among mammals, the proper activity of respiratory chain complexes (*i.e.* involving nuclei-mitochondrial compatibility) has never been directly studied in interspecies SCNT embryos.

2.2.2 Embryonic Genome Activation

During oogenesis, the early development of an oocyte in mammals is controlled by protein and mRNAs stored in the oocyte. After fertilisation, these factors direct the early cleavage divisions of an embryo and are gradually depleted. Depletion of these molecules seems to coincide with the activation of the embryonic genome (Beyhan *et al.,* 2007). The new genome established starts to become transcriptionally active at different stages in pre-implantation embryos, according to the species {e.g. mouse (2 cell) (Schultz, 1986; Telford *et al.,* 1990); pig (4 cell) (Prather *et al.,* 1989); human and rabbit (8 cell) (Braude *et al.,* 1979; Telford *et al.,* 1990); sheep, cattle and goat (16 cell) (Camous *et al.,* 1984; Bavister, 1988)}. The transcription is also often associated with a so-called critical or developmental block stage during *in vitro* culture of preimplantation embryos (Telford *et al.,* 1990). The *in vitro* block has been shown to coincide with the time of transition from maternal to embryo genomic control. Embryos appear to be particularly sensitive to culture conditions during this critical phase of their development (Chatot *et al.,* 1989; Telford *et al.,* 1990).

Transcription can start as early as the one cell stage and can gradually increase until the embryonic genome gains control. Latham (2005) reported that the expression of developmental genes is very critical for embryos to develop properly; the process of

embryonic genome activation plays a major role by providing control over spatial and temporal patterns of gene expression during preimplantation development. According to Hu *et al.* (2010), maternally expressed the transcription factors, whereby these factors accumulated and was stored in the oocyte cytoplasm, subsequently triggered the embryonic genome activation. However, in a preliminary study on interspecies experiments (cattle-pig and sheep-pig), it has been showed that in all cases the transplanted genome failed to be activated and transcribed in the host cytoplasm, leading to embryonic arrest (Fulka *et al.,* 2008). This finding has recently been confirmed by Song *et al.* (2009) and Lagutina *et al.* (2010). For an interspecies SCNT embryo to develop successfully into a blastocyst and beyond, it needs to coordinate both the donor and recipient components of embryonic genome activation (Beyhan *et al.,* 2007).

Indeed, the success of hybrid nuclear transfer (interspecies SCNT) embryo development is a function of the genetic/evolutionary distance between donor cell and recipient cytoplasm. In the case of intra-order SCNT the differences in the gene(s) products that trigger the first burst of transcription contained in the oocyte probably fail to bind the corresponding sequences of the introduced nucleus, leading to early embryonic arrest (Loi *et al.,* 2011). In the interspecies SCNT experiment, rabbit oocyte always served as the most “flexible” recipient oocyte, which is apparently able to promote embryonic genome activation across family/species/class boundaries.

Therefore, it is interesting to understand the embryonic genome activation occurs in the interspecies SCNT. Once the full sequences of the principal gene(s) triggering embryonic genome activation are identified, the gene(s)/gene protein(s) could be delivered by microinjection along with the foreign nuclei, or to promote the maternal to zygotic transition in interspecies SCNT embryos (Loi *et al.,* 2011).

2.2.3 Availability of Suitable Foster Mothers for Interspecies SCNT Embryos

Embryo transfer is the final step of embryos produced from all kinds of assisted reproductive technologies. This is because all the embryos need to undergo the embryo transfer procedure and subsequently produce an offspring. It is a very important and crucial step. In the threatened animals, the availability of females to be used as foster mothers within the endangered population is certainly limited. Loi *et al.* (2011) suggested that in intraspecies embryo transfer, the genetic background of the foster mother and the embryos may be a road-block, even more serious than the genomic/mitochondrial DNA compatibility or the embryonic genome activation described above. Therefore, the removal of species-specific boundaries for embryo transfer is a fundamental requirement for the successful multiplication of endangered genotypes through cloning, or even with other assisted reproductive technologies. Very little has been done with inter-intra specific embryo transfer in large animals, and much of this work has focused particularly on equine and bovine interspecies embryo transfer (Allen and Rowson, 1972; Dresser, 1986; Kraemer, 1982; Summers *et al.,* 1987; Hammer *et al.,* 2001).

Preimplantation development is characterised by a series of cleavages taking place in the oviduct, followed by the first differentiation event leading to 2 defined cell lineages: the inner cell mass (ICM), giving rise to the foetus proper, and the trophoblast (trophectoderm), which forms the extraembryonic tissue that finally becomes the definitive placenta (Loi *et al.,* 2011). The appearance of these 2 cell lineages breaks the symmetry typical of cleavage stage embryos and confers to the blastocyst an embryonic and ad-embryonic pole, probably established earlier in development (Plusa *et al.,* 2006).

In the case of endangered animals, the choice of trophoblast (placental) donor must be made according to the phylogenetic distance (the placental donor is also very

likely the donor of the host oocytes) and the pregnancy length, which must be close between the 2 species. A preliminary trial, where ICM was exchanged between sheep trophoblastic vesicles, has been successfully carried out (Loi *et al.,* 2007). It has been reported that the early loss of pregnancy is frequently associated with functional deficiencies occurring at the onset of placentation as evidenced in sheep and cattle by the lack of placentome development and abnormal vascularisation of extraembryonic tissues. These placental abnormalities could lead to severe nutrient deficiencies and induce growth retardation, ultimately causing preimplantation loss (Sinclair *et al.,* 1999; Wells *et al.,* 1999; Yang *et al.,* 2010). It is likely in the interspecies cloned embryos that incomplete reprogramming of the differentiated nucleus was a major constraint to the *in vivo* developmental potential of these embryos. Besides that, as the interspecies embryos were transferred to recipients, the immune system of surrogate mothers might exclude the embryos so that placenta could not form normally or formed incompletely after implantation (Jian-Quan *et al.,* 2007).

2.2.4 Selection of Recipient Oocyte (Cytoplast)

Interspecies somatic cell nuclear transfer (interspecies SCNT) involving the transfer of nucleus/nuclei from one species into the oocytes of another species. This provides a useful tool for studying interactions between the recipient cytoplasm and donor nucleus karyoplast in terms of nuclear reprogramming (Li *et al*., 2007). Therefore, the recipient oocyte plays an important role in interspecies SCNT, since the dominant distribution of mtDNA is from recipient oocytes (Steinborn *et al.,* 2002; Takeda *et al.,* 2003). However, one of the problems of interspecies nuclear transfer is the unavailability of species-specific competent recipient cytoplasm or oocyte (Zhao *et al.,* 2006).

Oocytes used for interspecies nuclear transfer should be easy to obtain, able to dedifferentiate the somatic cell nuclei of other species, and support development of the reconstructed embryo. Therefore, the types (species) of recipient oocytes is very important in order for the donor nuclei from another species to be reprogrammed. To date, most of the interspecies SCNT experiments used are either cattle, rabbit or sheep as the recipient cytoplasts. One of the first attempts on interspecies nuclear transfer using enucleated bovine oocytes as the recipient cytoplast was reported by Dominko *et al.* (1999). They suggested that bovine oocyte has the potential to serve as a universal recipient in interspecies SCNT. Monkey, sheep, pig and rat somatic cells were used as donor karyoplasts, resulting in various degrees of early *in vitro* development; however no pregnancies were reported. Several studies have demonstrated that the bovine cytoplast is capable of reprogramming somatic cell nuclei (donor karyoplast) from other species including horse (Hinrichs *et al.,* 2000; Reggio *et al.,* 2000; Li *et al.,* 2002a), pig (Yoon *et al.,* 2001), tiger (Hwang *et al.,* 2001), Korea cattle (Roh and Yoon, 2001), human (Cibelli *et al.,* 2001), buffalo (Kitiyanant *et al.,* 2001; Saikhun *et al.,* 2002), soala (Bui *et al.,* 2002), bongo (Lee *et al.,* 2002a), mouse (Koo *et al.,* 2003), black bear (Ty *et al.,* 2003), eland (Damiani *et al.,* 2003) and goat (Song *et al.,* 2008; Abdullah *et al.,* 2011; Kwong *et al.,* 2011; Soh *et al.,* 2011).

Sheep oocytes have also been used as recipients for interspecies nuclear transfer. In previous research, 2 pregnancies were established after interspecies nuclear transfer using domestic sheep oocyte (*Ovis aries*) as the recipient cytoplast and an exotic argali (*Ovis ammon*) as the donor karyoplast, but both pregnancies were lost by day 59 of gestation (White *et al.,* 1999). In addition, another study using domestic sheep (*Ovis aries*) oocytes as recipients and the mural granulose cells of a dead mouflon (*Ovis orientalis musimon*) as donor nuclei resulted in 1 live offspring (Loi *et al.,* 2001).

However, as reviewed by Beyhan *et al.* (2007), some of the research groups proposed that using rabbit oocytes could result interspecies SCNT embryos developed to the blastocyst stage with remarkably high efficiency, but, depending on the donor species, such as cat (Wen *et al.,* 2005; Thongphakdee *et al.,* 2006), ibex (Jiang *et al.,* 2005), panda (Chen *et al.,* 2002), camel (Zhao *et al.,* 2006), antelope (Zhao *et al.,* 2006), macaque (Yang *et al.,* 2003) and human (Chen *et al.,* 2003). Due to their small size, short reproductive life span, and easy manipulation and inducement of ovulation, rabbits have been one of them most popular animal models used for scientific research. Besides that, the rabbit oocyte has also been proven to be an ideal model for many types of studies due to its large size, elasticity and easy handling as well as the oocyte cytoplasm is capable of dedifferentiating somatic cell nuclei from other species (Zhao *et al.,* 2006). Therefore, rabbit oocyte as cytoplast is one of the ideal models for interspecies nuclear transfer.

2.2.5 Enucleation

Enucleation is a critical step in nuclear transfer. This is because before oocytes could be used as cytoplast recipients for interspecies SCNT, the genetic material must be removed from the oocytes. Enucleation is a very important factor that might influence the developmental ability of interspecies SCNT embryos and it is the most time-consuming procedure in interspecies SCNT (Peura *et al.,* 1998; Kawakami *et al.,* 2003; Savard *et al.,* 2004; Lee *et al.,* 2008).

2.2.5.1 Enucleation at different stages of oocyte

In most species (e.g. pig, cattle and goat), the metaphase plate of MII oocytes is not visible by light microscopy due to the presence of cytoplasmic lipid. To our knowledge,

a matured oocyte at MII, the MII plate in the cytoplasm of an oocyte is parallel beneath under the first polar body (PB-1). Therefore, enucleation has been achieved by so called "blind enucleation" using the first polar body as a marker for the location of the MII plate, and generally the first polar body together with a small volume of cytoplasm (10%) located below PB-1 are removed (Campbell *et al.,* 2007). To this, some of the researchers prefer to determine the position of the chromosomes by observing under ultraviolet (UV) light after staining oocytes with a DNA-specific dye (e.g. Hoechst-33342) during enucleation step. However, this method, although it increases enucleation accuracy, is harmful to embryo development (Liu *et al.,* 2000).

An alternative to the enucleation of MII oocytes is the enucleation of activated oocytes at telophase of the second meiotic division (TII). Because second polar body (PB-2) is expelled shortly after activation, second polar body remains closer to the nuclear materials as it has not had sufficient time to migrate. Therefore, mechanical aspiration or squeezing of the extruding second polar body and surrounding cytoplasm following activation is an effective and reliable enucleation method without the need for visualisation of the DNA by exposure to UV light. Bordignon and Smith (1998) and Mohamed Noor and Takahashi (1999) reported that enucleation rate in bovine oocytes was significantly higher at TII stage than that at MII stage (98.0% vs. 59.0%, 91.5% vs. 59.9%, respectively). This statement was similar reported by Chen *et al.* (2007). Also, they suggested that less cytoplasm was removed in TII enucleation than in MII enucleation, and subsequently, there is less disturbance of the micro-environment of the recipient oocyte for coordination with the donor cells.

Pre-treatment of mouse oocytes with sucrose (3%) was effective in aiding the visualisation of the metaphase spindle and chromosomes with standard light microscopy (Wang *et al.,* 2001) and a sucrose (0.3 M) treatment was found to be effective in

facilitating the localisation of the bovine chromosomes (Liu *et al.,* 2002). Furthermore, cloned piglets were born after chemically assisted enucleation of the oocytes (Yin *et al.,* 2002). In their study, a brief treatment of the MII porcine oocytes in demecolcine and sucrose resulted in a membrane protrusion that contained the condensed chromosomes, which were easily visualised and aspirated into a pipette without the need of staining. Although the mechanisms of action of demecolcine are not clear, the appearance of the protrusion might be related to the condensation of maternal chromosomes (Yin *et al.,* 2002).

2.2.5.2 Type of enucleation methods

There are various methods of enucleation that have been developed and reported from time to time, in order to increase and improve the efficiency of removing the polar body and MII chromosomes. These methods are such as squeezing, aspiration, piezo-drill electric pipette, and xyclone laser system. Squeezing is the most common enucleation methods, whereby a slit is made in the zona pellucida with a microneedle and then first polar body together with a small amount of cytoplasm containing MII chromosomes are removed by squeezing oocytes with pipette (Wilmut *et al.,* 1997). Enucleation by the aspiration method is done using a micropipette that has been bevelled and spiked to aid in the penetration of the zona pellucida (Mohamed Nour and Takahashi, 1999). Lee *et al.* (2008) demonstrated that the squeezing method resulted in a higher proportion of degenerated oocytes than the aspiration method (14% vs. 5%), however, the blastocyts formation was improved in oocytes enucleated by the aspiration method (5% vs. 9%).

An alternative method, which was first developed in mouse oocytes by Wakayama *et al.* (1998), the zona pellucida of an oocyte was ‘drilled’ by applying several piezo pulses to the tip of an enucleation pipette. The MII chromosome-spindle

complex, distinguished as a translucent spot in the ooplasm, was drawn into the pipette with a small amount of accompanying ooplasm and pulled gently away from the oocyte until a stretched cytoplasmic bridge was pinched off (Wakayama *et al.,* 1998). This method has shown a very successful production of cloned live offspring in mouse.

Recently, there was a new enucleation methods developed by Campbell *et al.* (2007), namely, xyclone laser system (Hamilton-Thorne, USA) to assist enucleation or to induce enucleation. By using the laser to breach the zona pellucida prior to aspiration of the oocyte spindle at AI/TI, MII or TII the enucleation process is simplified requiring less technical expertise and the production of more basic micromanipulation tools.

2.2.6 Nuclear Transfer (Reconstruction)

Several nuclear transfer procedures are currently used to produce cloned animals, including sub-zonal injection and subsequently electro-fusion (SUZI), assisted with piezo-actuated microinjection, intracytoplasmic injection (ICI) and whole cell intracytoplasmic injection (WCICI).

For SUZI method, it involves placing a donor cell in the perivitelline space of an enucleated recipient oocyte and fusing the donor and recipient cells with electrical pulses. It has been successfully applied to generate cloned cattle (Wells *et al.,* 1999) and goats (Baguisi *et al.,* 1999) as well as pigs (Polejaeva *et al.,* 2000).

Piezo-driven enucleation and reconstruction has been successfully improved the nuclear transfer outcome in the species such as mouse, cow and horse (Wakayama *et al.,* 1998; Choi *et al.,* 2002b; Galli *et al.,* 2002). This is due to the piezo drill generating mechanical pulses that travel longitudinally along the microinjection pipette and vibrate the pipette tip, drilling through the zona pellucida and the oolemma are facilitated without producing any net forward movement of the pipette. This is especially useful in

species with fragile oocytes or hard zona pellucida, such as the mouse and the horse (Choi *et al.,* 2002b). Because the pipette used for piezo-driven injection (7 to 10 μm) is smaller than those used in traditional nuclear transfer (20 to 25 μm), it also allows for the direct injection of donor cells or nucleus/nuclei into the cytoplasm without the need for electrofusion (Wakayama *et al.,* 1998; Lee *et al.,* 2003).

In the intracytoplasmic injection, which donor cells were pipetted in and out few times using a narrow microinjection pipette (8 to 10 μm) to isolate the donor nuclei and injected into enucleated oocytes (Doa *et al.,* 2002). This method was modified from piezo-driven enucleation method, while no electrical current was needed to remove the plasma membrane of a donor cell. This method is more practical and save money because no piezo-driven machine is needed.

Recently, Lee *et al.* (2003) suggested that the whole cell (e.g. foetal fibroblast and cumulus cells) intracytoplasmic injection procedure is less labour intensive, requires no special micromanipulation equipment and is as efficient in the generation of cloned pigs producing a relatively high blastocyst rate (37%). This is a new technique involving direct injection of a whole cell into an enucleated oocyte, bypassing both the fusion and nucleus isolation processes.

2.2.7 Selection of Donor Cells

The potential of various donor cell types to be used as the nucleus donor, such as embryonic cells (pre-blastocyst blastomere; Campbell *et al.,* 1993), fibroblasts (skin fibroblast and foetal fibroblast; Kato *et al.,* 1998), mammary gland cells (Wilmut *et al.,* 1997), cumulus granulosa cells (Wakayama *et al.,* 1998), oviduct cells (Kato *et al.,* 2000), leukocytes (Galli *et al.,* 1999), mural granulosa cells (Wells *et al.,* 1999), embryonic stem cells (inner cell mass; Eggan *et al.,* 2001) and liver cells (Brem and

Kuhholzer, 2002) have been reported for production of cloned animals. However, it is still unclear which type(s) of donor cell is(are) the most suitable for nuclear transfer into enucleated oocytes, especially in interspecies nuclear transfer which is currently lacking. However, closely related species always gives a better result in the production of interspecies cloned embryos as well as live offspring.

When the efficiency of various cell types from adult, newborn and foetal male and female donor fibroblast cells was compared in SCNT experiment, the percentage of blastocysts produced from each cell type was found not to be significantly different (Kato *et al.,* 2000). Similar results were obtained using various cell types derived from mice of different strains, sexes and ages (Wakayama and Yanagimachi, 2001).

Initially, all cloned animals derived from adult somatic cells were produced using cells from the female reproductive system, such as mammary gland (Wilmut *et al.,* 1997), oviduct (Kato *et al.,* 1998), cumulus and mural granulosa cells (Wakayama *et al.,* 1998; Wells *et al.,* 1999; Kato *et al.,* 2000) raised the question of whether male individuals could be cloned. Therefore, Wakayama and Yanagimachi (1999) reported the first male mice were cloned from male tail skin cells. Besides that, no significant differences were found in the developmental rates of embryos reconstituted with male or female nuclei in cattle (Kato *et al.,* 2000) and mouse (Wakayama and Yanagimachi, 2001).

Foetal fibroblast cells are believed to have less genetic damage and more proliferative ability (as measured by cell doublings) compared to the adult somatic cells, therefore, they have been the cell type of choice as donor karyoplasts (Hill *et al.,* 2000b). No significant differences were found in bovine embryo developmental rates to the blastocyst stage with adult, newborn or foetal cell nuclei; however, abortion in later stages of pregnancy was higher for cloned foetuses derived from adult cells (Kato *et al.,*

2000). Similarly, no differences among embryos derived from foetal and adult bovine fibroblasts with regard to fusion, cleavage and blastocyst formation were detected (Niemann *et al.,* 2002). However, more foetal losses after transfer into recipients were noted with embryos reconstructed with adult bovine donor cells (Hill *et al.,* 2000b; Niemann *et al.,* 2002).

The efficacy of different types of adult somatic cells as donor karyoplast is still controversial. To date, adult cells such as cumulus cells and ear fibroblasts are the most commonly used for nuclear transfer, as they are easy to obtain and result in no injuries to animals (Yang *et al.,* 2010). Yang *et al.* (2010) reported that in buffalo intraspecies SCNT, the fusion rate of couplets derived from ear fibroblasts was significantly lower than that from cumulus cells (76.8% vs. 82.5%), while there was no differences in cleavage rate and blastocyst rate between the two groups. Similar report on intraspecies SCNT in buffalo, Shah *et al.* (2009) suggested that cumulus cells were better than ear fibroblast, while Srirattana *et al.* (2010) reported that cumulus cells were similar to ear fibroblasts. In the intraspecies SCNT of sheep, with regard to the days 7 to 8 of blastocyst formation, the ratios of blastocyst formation were similar and not statistically different between the donor cell types derived from cumulus cells, male and female fibroblast cells (19.5, 17.4 and 15.2%, respectively) (Hosseini *et al.,* 2008). However, in human-rabbit interspecies SCNT experiments, Ji *et al.* (2007) demonstrated that the cumulus cell embryos showed significantly higher development rates than the ossocartilaginous cell and skin fibroblast cell.

Sugimura and Sato (2011) reported that the rate of blastocyst formation and the total number of cells at the blastocyst stage were significantly higher for embryos derived from dewclaw cells than for those derived from tail-tip cells in the production of interspecies cloned dog-pig embryos. Although this finding could not be definitively

explained, it indicates that the types of donor cells used influence the preimplantation competence of mouse SCNT embryos to develop to the blastocyst stage (Wakayama and Yanagimachi, 2001). Therefore, Sugimura and Sato (2011) suggested that the dewclaws of animals may contain relatively undifferentiated somatic cells, and that these cells may be able to develop into blastocysts and to improve cell proliferation at the blastocyst stage in each of the interspecies SCNT embryos derived from tail-tip cells.

2.2.8 Stages of Donor Cell Cycle

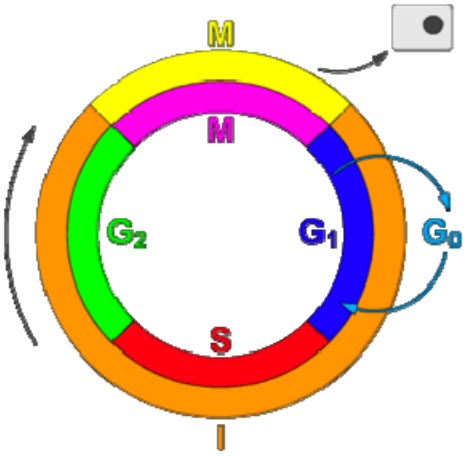

Figure 2.2: Stages of cell cycle.

Table 2.3: The description for each of the somatic cell stages

Stages	Phase	Abbreviation	Description
Quiescent/senescent	Gap 0	G0	A resting phase where the cell has left the cycle and has stopped dividing.
Interphase	Gap 1	G1	Cells increase in size in Gap 1. The G1checkpoint control mechanism ensures that everything is ready for DNA synthesis.
	Synthesis	S	DNA replication occurs during this phase.
	Gap 2		During the gap between DNA synthesis and mitosis, the cell will continue to grow. The G2 check-point control mechanism ensures that everything is ready to enter the M (mitosis) phase and divide.
Cell division	Mitosis	M	Cell growth stops at this stage and cellular energy is focused on the orderly division into 2 daughter cells. A check-point in the middle of mitosis (Metaphase check-point) ensures that the cell is ready to complete cell division.

(Adapted from: //en.wikipedia.org/wiki/Cell_cycle)

Donor cell cycle was a major contributing factor to the success of the somatic cell nuclear transfer (Campbell *et al.,* 1996b). According to Wilmut *et al.* (1997), the use of G0 cells could be beneficial for the success of nuclear transfer procedures. In the scientific literature, G0 and G1 cells are often grouped together as G0/G1, although these phases are quite distinct. G0 cells exit the normal cell division cycle and enter a quiescent state, whereas, G1 is a transient stage between M-phase and S-phase in proliferating cells (Sansinena *et al.,* 2005).

There are different methods for synchronising cells in different cell cycle phase. Methods of arresting cells in the G0 phase of the cycle include: (1) serum deprivation (Kues *et al.,* 2000; Gomez *et al.,* 2003), (2) contact inhibition (Boquest *et al.,* 1999; Kasinathan *et al.,* 2001; Mitalipov *et al.,* 1999; Gomez *et al.,* 2003) and (3) reversible cycle inhibitors such as rescovitine (Gibbons *et al.,* 2002; Gomez *et al.,* 2003). Synchronising cells at the G1 phase include: (1) culturing cells to early confluence (Cibelli *et al.,* 1998), (2) the "shake off" method, which is performed by physically shaking or vortexing a sub-confluent population cell culture to obtain newly divided couplets of cells which cytoplasmic bridges at the beginning of G1 phase (Kasinathan *et al.,* 2001) and (3) by serum deprivation to force the cells to enter in G0 phase and then stimulating re-enter in G1 phase by culturing cells for 10 hours in medium containing serum (Memili *et al.,* 2004).

The terms 'serum-starved' and 'quiescent' interchangeably should be avoided when referring to *in vitro* cell cultures for nuclear transfer, as quiescence is a state that cells can enter spontaneously while serum-starvation can cause a state of stress with potentially more profound implications within the cells (Wells *et al.,* 2003). Quiescent cells presumably arrested in G0 phase of the cell cycle have commonly been used to produce cloned animals (Campbell *et al.,* 1996a; Wilmut *et al.,* 1997; Baguisi *et al.,*

1999; Kues *et al.,* 2000; Reggio *et al.,* 2001; Gibbons *et al.,* 2002; Wells *et al.,* 2003; Yu *et al.,* 2003) and the specific method used to arrest donor cells can markedly affect foetal survival to term and neonatal survival (Gibbons *et al.,* 2002). Studies reported that the proliferating cells have also been successfully used for nuclear transfer (Cibelli *et al.,* 1998), although the exact stage of the donor cell cycle was not yet verified. Subsequently, several studies from bovine nuclear transfer laboratories have demonstrated that both quiescent and proliferating somatic donor cells can be fully reprogrammed after nuclear transfer and result in viable offspring (Cibelli *et al.,* 1998; Kasinathan *et al.,* 2001; Wells *et al.,* 2003).

When the oocyte becomes arrested at metaphase-II, MPF activity remains high; then following fertilisation or chemical activation, MPF activity rapidly declines. During the cell cycle, chromosomal DNA is replicated only once. The mechanisms by which a cell coordinates this event and prevents re-replication still remain unclear, although maintenance of an intact nuclear envelope appears to be of utmost importance (Blow and Laskey, 1988). The development of reconstructed embryos following nuclear transfer appears to be dependent upon a variety of factors and cell cycle synchrony appears to be a critical aspect. The donor nucleus must be in G1 or G0 when transferred to fresh oocytes with high levels of MPF to condense normally and maintain correct ploidy of subsequent embryos at the end of the first cell cycle (Campbell *et al.,* 1996a). The high levels of MPF in the mature, metaphase-II oocyte cause nuclear envelope breakdown (NEBD) and chromosome condensation of the transferred nucleus, irrespective of the cell cycle stage of the donor cell.

Subsequently, the exposure of the chromosomes to the licensing factors in the oocyte cytoplasm leads to the replication of DNA following the decay of MPF activity and the reformation of the nuclear membrane (Campbell *et al.,* 1993, 1996a; Dinnyés *et*

al., 2002). In the metaphase-II cytoplast, donor chromatin immediately condenses, in conjunction with the breakdown of the nuclear envelope (Collas and Robl, 1991). Spindle microtubules then form in association with condensed chromatin (Pinto-Correia *et al.,* 1993). When the embryo karyoplast is activated, causing a decrease in MPF, a nucleus forms and acquires the morphology of a large pronucleus (Collas and Robl, 1991). If DNA synthesis has commenced prior to NEBD, then DNA replication will take place, resulting in abnormal ploidy and a defective embryo. Furthermore, the progression of DNA synthesis in the donor nucleus is not compatible with normal chromatin condensation, which also results in a defective embryo. Early studies in bovine nuclear transfer embryos reconstructed by transfer donor karyoplast into metaphase-II cytoplasts showed that all nuclei underwent NEBD and DNA synthesis after reformation of the nuclear envelope, regardless of their cell cycle stage (Campbell *et al.,* 1993). Thus, it was hypothesised that only G1 nuclei (donor karyoplasts) should be used when transferring to metaphase-II cytoplasts.

2.2.9 Number of Cell Passage

Genetic damage may occur during the *in vitro* culture of donor cells prior to nuclear transfer. Cells cultured for prolonged periods of time are known to increase in their levels of aneuploidy over time (Freshney, 2000). Besides that, long-term cultured somatic cells undergo cellular senescence and have numerous mutations or allelic loss of gene accumulated through many rounds of cell divisions, which are known to cause improper genetic reprogramming after SCNT and subsequent abnormal development of the embryos (Walker *et al.,* 1996; Aladjem *et al.,* 1998; Cibelli *et al.,* 1998; Schmidt-Kastner *et al.,* 1998; De Sousa *et al.,* 1999; Kuhholzer *et al.,* 2000). Therefore, it is suggested that the use of long-term cultured senescent cells may decrease cloning

efficiency, which may be a limiting factor in the application of SCNT in animals for gene targeting. For these reasons, fresh or short-term cultured (<10 sub-passages) donor cells have been the cell type of choice for the production of cloned embryos.

Zakhartchenko *et al.* (1999) used late passage immortalised epithelial cells to clone cattle, but failed to obtain blastocyst. Roh *et al.* (2000) demonstrated that nuclei from both early (8-16) and late (17-32) passages were capable of supporting *in vitro* development of bovine cloned embryos, with reduced rates of blastocyst formation when late passage cells were used. In contract, one study reported higher developmental rates to the blastocyst stage for embryos reconstituted with adult somatic cells that had been sub-passaged 10 to 15 times compared with that of embryos reconstituted from cells with a lower number of sub-passages (Kubota *et al.,* 2000). In addition, cloned calves were obtained from embryos reconstituted with high sub-passage cells, with all cloned foetuses derived from low sub-passage donor cells aborting during pregnancy (Kubota *et al.,* 2000). Similar results were obtained using enhanced green fluorescent protein gene transfected and nontransfected bovine granulosa cell donor cells where *in vitro* developmental rates of cloned embryos derived from cells at passage 15 were higher than those for embryos derived from cells at lower sub-passages (Arat *et al.,* 2001). Results within and among various research laboratories are often conflicting due to procedural effect, oocyte variability, inherent differences among donor cell lines, age of donor animals or to effects of *in vitro* culture conditions.

Furthermore, some reports have shown that donor cells with high cell passage numbers result in lower fusion rates and lower blastocyst development rates in the reconstructed embryos (Wilmut *et al.,* 1997; Kato *et al.,* 1998; Hill *et al.,* 2000a; Roh *et al.,* 2000; Bhuiyan *et al.,* 2004). The possible explanation is that long-term *in vitro*

culture alters the donor cell metabolism and unbalanced regulation of imprinted genes may be induced, thus affecting nuclear transfer remodelling (Walker *et al.,* 1996).

2.2.10 Fusion

Over the years, the use of electrical stimuli for membrane fusion of the enucleated oocytes and transferred somatic cells has been the method of choice (Betthauser *et al.,* 2000; Kubota *et al.,* 2000; Du *et al.,* 2002; Daniel *et al.,* 2008). However, the overall cloning efficiency has remained low (Cibelli *et al.,* 1998; Wells *et al.,* 1998; Kuhholzer *et al.,* 2000). This may due to the low fusion efficiencies currently being achieved between the somatic donor cells and the recipient oocyte, following somatic nuclear transfer (Cibelli *et al.,* 1998; Kubota *et al.,* 2000).

Daniel *et al.* (2008) reported that the optimum fusion rate of 55.4% was achieved when 2.0-2.5 kV/cm DC current was applied compared to 24.0% when using 1.0-1.5 kV/cm DC current in the cumulus donor cell group in goat intraspecies SCNT. Furthermore, in their study, the higher DC current (3 kV/cm and above) resulted in a very low fusion rate (16.7%). Dimitor (1993) suggested that the low fusion rate observed with low electric strength may be the low geometric probability of membrane fusion due to a low pore concentration in the membranes. In contrast, high electric strength may result in heat generation and turbulent flows, which would decrease the fusion efficiency (Dimitor, 1993).

It is also interesting to understand that different types of donor cells may give different fusion rate. This statement was approved by Daniel *et al.* (2008), using the same electric strength, 2.0–2.5 kV/cm, the fusion rate in cumulus cells was significantly higher than the fibroblast cell line fused into the recipient oocytes in the goat intraspecies SCNT (55.4% vs. 31.9%). West and Baker (1987) demonstrated that the

nuclear transfer with cultured cumulus cells as nuclear donors recorded a high fusion rate (59%), when compared to that of skin fibroblasts (33%). It was concluded that the difference in membrane surface properties between the cumulus and skin fibroblast cells may contribute to the higher fusion rate of the cumulus cell, compared to the fibroblast cell. It is postulated that the cumulus cells and oocyte possess the similar membrane surface properties, in terms of the types of glycoproteins present (Reik *et al.,* 2001).

One of the early attempts of nuclear transfer in the horse reported fusion rates of 81% for quiescent and 52% for proliferating adult equine fibroblasts (Reggio *et al.,* 2000). More recently, greater fusion rates were reported when combining electrofusion with Sendai virus (82%) compared with the electrofusion technique alone (57%) (Li *et al.,* 2002b). Although the fusion efficiencies reported by different laboratories for the horse are comparable to those previously reported for the cattle (Cibelli *et al.,* 1998; Campbell, 1999; Colman, 2000), this step is still a source of variability in the outcome of the nuclear transfer combination with activation using equine sperm cytosolic factor which is used for nuclear transfer, reporting a cleavage rate as high as 51% (Choi *et al.,* 2002b).

2.2.11 Activation

During normal fertilisation, the sperm entry triggers a series of intracellular short-lived calcium (Ca^{2+}) oscillations which is critical to oocyte activation. Calcium is released in a pulsatile manner from internal stores, including the endoplasmic reticulum and mitochondria (Yanagimachi, 1994), and this elevation in intracellular calcium could persist for several hours (Carroll and Swann, 1992; Kline and Kline, 1992; Miyazaki *et al.,* 1993). These calcium oscillations are responsible for the cascade of events that follow, including the cortical granule reaction (Miyazaki *et al.,* 1990), zona pellucida

reaction (Yanagimachi, 1994) and the escape from the metaphase-II arrest (Whitaker and Irvine, 1984). However, it is different in nuclear transfer, where the lack of sperm-induced fertilisation steps requires the use of artificial activation in order to trigger nuclear reprogramming and further embryonic development (Wells *et al.,* 1999). Different artificial protocols have been developed to activate mammalian oocytes by simulating the biochemical and physiological events that normally occur during sperm-oocyte interaction.

Maturation promoting factor (MPF) and mitogen-activated protein (MAP) kinase are the most likely targets of calcium-stimulated events, because inactivation of these kinases is a prerequisite to the resumption and completion of meiosis, subsequent pronuclear formation and DNA synthesis (Collas *et al.,* 1993; Verlhac *et al.,* 1994; Moos *et al.,* 1996). Maturation promoting factor was first described as a complex of two subunits: a catalytic subunit (p34cdc2) and a regulatory subunit (cyclin B). p34cdc2 is a protein kinase regulated by changes in its phosphorylation state and by its association with cyclin. Throughout the cell cycle, the level of p34cdc2 remains constant but the level of cyclin varies. Maturation promoting factor peaks at metaphase in association with nuclear envelope breakdown, chromatin condensation, reorganisation of the cytoskeleton and the formation of the mitotic spindle (Doree and Galas, 1994; Moos *et al.,* 1996). Maturation promoting factor inactivation, which is necessary for the cell to exit the metaphase-II arrest, involves cyclin proteolysis by the proteosome system (Glotzer *et al.,* 1991).

In vertebrates, mature oocytes are arrested at metaphase-II of the meiotic division, with elevated MPF activity maintained by a cytostatic factor (CSF), which prevents the ubiquitin-dependent degradation of cyclin B and thus, inactivation of MPF. Intracellular calcium oscillations triggered by sperm down-regulate CSF activity and

allow for the degradation of cyclin. Proteolytic degradation of cyclin B and subsequent MPF inactivation releases oocytes from metaphase arrest and allows the beginning or resumption of mitotic cycles (Lorca *et al.,* 1993).

In nuclear transfer procedures, enucleated oocytes fused with a diploid donor cell must be artificially activated to continue development, since somatic cell nuclei could not initiate activation (Campbell, 1999). Different artificial activation treatments attempt to mimic sperm-triggered events and induce parthenogenetic development in metaphase-II oocytes. Successful activation has been achieved by a range of treatments including electrical stimulation, as well as chemicals such as strontium in mouse, ionomycin, calcium ionophore in cattle and sheep (Campbell *et al.,* 2007). In addition, there are many factors that might influence the efficient of activation, such as the concentration of chemical agents, duration between fusion and activation, activation media, strength of electric stimulation, post-treatments such as cytochalasin B or D, cycloheximide (CHX) or 6-dimethylaminopurine (6-DMAP) (Campbell *et al.,* 2007).

One of the most widely used activation protocols for reconstructed oocyte is the combination of ionomycin or calcium ionophore with 6-DMAP or CHX (Loi *et al.,* 1998; Wells *et al.,* 1999; Akagi *et al.,* 2003). The role of ionomycin is to mobilise intracellular calcium stores to induce only a single calcium release rather than a repetitive series as occurs naturally (Hoth and Penner, 1992). Subsequently, the calcium oscillations suppressing activity of the maturation promoting factor, followed by administration of chemicals such as 6-DMAP (a serine protease inhibitor) or CHX (a protein synthesis inhibitor) to suppress or prevent reformation of MPF activity (Szollosi *et al.,* 1993; Verlhac *et al.,* 1993; Susko-Parrish *et al.,* 1994; Yang *et al.,* 1994). In one previous study on interspecies SCNT in buffalo-cattle, the results showed a significantly higher percentage of blastocyst development in the nuclear transfer activated by calcium

ionophore and 6-DMAP when compared with 6-DMAP alone (33% vs. 17%) (Kitiyanant *et al.,* 2001). Bhak *et al.* (2006) reported that in bovine intraspecies SCNT, the cloned embryos obtained from 6-DMAP or CHX activation treatment were not significantly different in term of blastocyst development, total cell number and proportions of chromosome abnormalities, although CHX treatment is more desirable than 6-DMAP.

In the mouse, strontium has been demonstrated to be used to induce calcium oscillation produces a pattern similar to the oscillation by sperm, and repeated oscillation seems to be beneficial for later embryo development (Bos-Mikich *et al.,* 1997; Vitullo and Ozil, 1992). Wakayama *et al.* (1998) reported that the additional of cytochalasin B (5 g/ml) to the activation medium may prevent polar body extrusion, however, the concentration of both chemicals and time exposure to strontium in addition to post-activation conditions are needed to optimise in order to produce efficient embryonic development. Besides that, Yamazaki *et al.* (2005) showed that ionomycin combined with strontium can be used in the activation of intraspecies SCNT bovine embryos as in mouse and increased both *in vitro* development to blastocyst and *in vivo* development to term.

Electrical stimulation is a widely used method for activation during nuclear transfer procedures in sheep (Campbell *et al.,* 1996b; Wilmut *et al.,* 1997)) and pigs (Betthauser *et al.,* 2000; Onishi *et al.,* 2000; Polejaeva *et al.,* 2000). Few studied reported that the application of multiple pulses of lower field strength is beneficial for embryo development to blastocyst in porcine SCNT (Park and Kuroda, 2001; De Sousa *et al.,* 2002; Zhu *et al.,* 2002). Additionally, an elevated calcium concentration in fusion/activation medium also enhanced embryo development to blastocyst (Cheong *et al.,* 2002).

It is important also to know about the timing of activation of reconstructed oocytes in nuclear transfer. Generally, the timing of activation can be divided into 3 types: (1) pre-activation (prior fusion), (2) immediate activation (at the time of or immediately following fusion), and (3) delayed activation (at a specified time after fusion). It was reported that the prolonged exposure of donor cell nuclei to recipient oocyte cytoplasm may be beneficial in intraspecies SCNT embryos (Betthauser *et al.,* 2000; Onishi *et al.,* 2000; Im *et al.,* 2006). Wrenzycki *et al.* (2001b) demonstrated that the pattern of transcription in bovine blastocyst produced by intraspecies SCNT was affected by exposure time (Wrenzycki *et al.,* 2001a). In cattle, activation within 2.5 hours after fusion has shown to be improved in the rate of blastocyst development and nuclear morphology (Akagi *et al.,* 2003; Choi *et al.,* 2004; Aston *et al.,* 2006).

2.3 *IN VITRO* EMBRYO CULTURE

In vitro produced preimplantation embryos are sensitive to environmental conditions that could affect embryo morphology, gene expression, embryonic growth and developmental potential both pre- and post-natally (Summers and Biggers, 2003; Fleming *et al.,* 2004). Therefore, it appears that *in vitro* culture of cloned embryos before embryo transfer is one of the important steps in achieving pregnancy and delivery (Choi *et al.,* 2002a). Embryos are susceptible to a wide range of stressors *in vitro* (Lane, 2001; Lane and Gardner, 2005), including inappropriate medium formulation, medium supplementation, problem in the culture system, technical issues, or lack of appropriate quality control and quality assurance (Gardner, 2004). These stress factors could trigger the response mechanisms designed in the embryo that might affect the homeostatic balance. Short-term responses observed include changes in morphology (Pollard and Leibo, 1994; Abe *et al.,* 1999; Boni *et al.,* 1999; Crosier *et al.,*

2000, 2001; Abe and Hoshi, 2003), cell proliferation and apoptosis (Knijin *et al.,* 2002; Gjorret *et al.,* 2003), metabolism (Khurana and Niemann, 2000; Thompson, 2000; Leese, 2002; Houghton and Leese, 2004), transcriptome (Wrenzyeki *et al.,* 1999, 2001a, 2005; Fabian *et al.,* 2005; Corcoran *et al.,* 2006; Sagirkaya *et al.,* 2006) and proteame (Katz-Jaffe *et al.,* 2005).

Although several culture systems have been developed for the *in vitro* fertilised embryos obtained from IVF and ICSI, it is still unclear whether those are suitable for supporting preimplantation development from SCNT embryos (Choi *et al.,* 2002b). Thus, it was considered that optimisation of culture conditions would be needed for success of the interspecies SCNT technique (Dominko *et al.,* 1999; Sugimura and Sato, 2011). Therefore, the types of culture medium used and the culture conditions are essential to be optimised in order to improve the efficiency of development in *in vitro* culture for both intraspecies and interspecies SCNT embryos.

An optimal *in vitro* culture medium is one of the most important key factors to the success of interspecies SCNT because supplementation of appropriate nutrients, energy sources and growth factors are critical for the development of interspecies SCNT embryos (Lorthongpanich *et al.,* 2008). It has been suggested that the energy metabolism of interspecies nuclear transfer is differs from that allogeneic SCNT embryos because of disrupted cellular processes associated with mitochondrial function (Mastromonaco *et al.,* 2007).

The need for species-specific embryo culture medium is a widely accepted concept that has already been demonstrated in different species, such as North Carolina State University-23 (NCSU-23) medium for pig embryos (Lee *et al.,* 2003), Chatot, Ziomek, Bavister medium (CZB) (Wakayama and Yanagimachi, 1999), potassium simplex optimized medium (KSOM) (Nagy *et al.,* 2003; Wakayama *et al.,* 2005) and

Whitten medium (WM) (Goh *et al.,* 2010) for mouse, modified synthetic oviduct fluid with amino acids (mSOFaa) (Gardner, 1994; Melican *et al.,* 2005) goat embryos, Charles Rosenkrans 1 (CR1) (Doa *et al.,* 2002) for cattle embryos. The main chemicals that supplemented in these medum are such as essential and non-essential amino acids, growth factors, glucose, glutamine, gluthathione, taurine, cysteamine, β-mercaptoethanol and EDTA.

Amino acids, especially the non-essential amino acids, were suggested to be the most significant component affecting ruminant embryonic development *in vitro* (Thompson, 2000), which are important regulators of early embryonic development (Rosenkrans and First, 1994; Liu and Foote, 1995; Steeves and Gardner, 1999; Rezaei and Chian, 2005). Growth factors are importance to improve the development of an embryo to the blastocyst stage (Lonergan *et al.,* 1996; Palma *et al.,* 1997; Palasz *et al.,* 2000) and implantation rate (Block and Hansen, 1997). Gluthathione, taurine, cycteamine and β-mercaptoethanol are act as antioxidants. Glucose, especially during the late stage of embryos (morula and blastocyst), which meets the increased energy demand for blastulation, differentiation, and growth (Donnay *et al.,* 1999; Thompson and Peterson, 2000; Houghton and Leese, 2004; Lopes *et al.,* 2007; Harvey, 2007). EDTA is important in the intracellular chelation during early development of an embryo (Lane and Gardner, 1997).

It is not clear whether the culture medium is dependent on the donor karyoplast or the recipient cytoplast for interspecies nuclear transfer embryos (Zhao *et al.,* 2006). In the studies reported by Zhao *et al.* (2006) on interspecies SCNT using rabbit oocytes as recipient cytoplast and camel and Tibetan antelope somatic cells as donor karyoplasts, they indicated that M199+10% FCS culture medium was suitable for interspecies nuclear transfer embryos. Similar reports have also been shown that M199+10% FCS

supported *in vitro* development of panda-rabbit, cat-rabbit and chicken-rabbit embryos to the blastocyst stage (Chen *et al.,* 2002; Wen *et al.,* 2003; Liu *et al.,* 2004). Yang *et al.* (2010) demonstrated that using the similar culture medium, TCM-199+10% FBC, in the interspecies SCNT of buffalo-cattle was able to produce *in vitro* blastocyst (5.9%).

Another interspecies SCNT study was reported by Dominko *et al.* (1999), using CR1aa medium as the *in vitro* culture medium for reconstructed oocytes in monkey-cattle, pig-cattle and sheep-cattle. In their study, the culture medium successfully supported the interspecies nuclear transfer embryos up to blastocysts (16.6%, 14.3% and 13.9%, respectively). Similar medium was used and reported by Li *et al.* (2007) on yak-cattle with the blastocyst formation (30-34%). Hua *et al.* (2008) demonstrated that in their studies using mSOF medium in sheep-cattle interspecies SCNT was able to obtain the *in vitro* blastocyst (24.6%).

Recently, studied have suggested that the use of chromatin remodeling agents (Tricostatin A, TSA) could improve the efficiency of SCNT experiment. TSA is a histone-deacetylase inhibitor (HDACi), which enhances the pool of acetylated histones (Yoshida *et al.,* 1990) and DNA demethylation (Hattori *et al.,* 2004). Kishigami *et al.* (2006) suggested that the regulation of histone deacetylases (HDAC) is a clue for efficient reprogramming. They indicated that TSA-treatment after SCNT in mice could dramatically improve the practical application of current cloning techniques. However, further study should be carried out and focus on how TSA enhances the reprogramming in terms of DNA methylation and histone modifications in order to elucidate the mechanism of reprogramming (Kishigami *et al.,* 2006).

Serum and BSA are complex undefined mixtures containing hormones, growth factors, vitamins and numerous other factors. Serum is known to be detrimental to embryonic and foetal development (Campbell *et al.,* 2007). However, serum has been

used as a component of co-culture medium and addition of serum into the culture medium improves the kinetics of embryo development (Lazzari *et al.,* 2002; Lequarre *et al.,* 2003), cell number and the number of blastocysts reaching the blastocyst stage (Holm *et al.,* 2002; Lazzari *et al.,* 2002). A higher developmental rate to the blastocyst stage was generally obtained from medium supplemented with serum (Pinyopummintr and Bavister, 1994). However, it has been demonstrated that serum has a biphasic effect on embryonic development. The presence of serum in the medium may affect the speed of embryonic development, morphology, ultrastructure, metabolism and the gene expression profiles (Thompson *et al.,* 2007). This is often associated with the large offspring syndrome (LOS) (Thompson *et al.,* 1995; Sinclair *et al.,* 1999). Therefore, almost all media used for embryo culture generally contain bovine serum albumin (BSA), instead of serum as a source of protein to improve embryonic development, blastocyst formation and the hatching rates (Thompson, 2000; George *et al.,* 2008).

During *in vitro* culture of embryos, ammonia is generated from the spontaneous degradation of amino acids during culture and amino acid metabolism (Gardner, 1994). Production of ammonia could affect the development of *in vitro* culture embryos, therefore, strategy of replacing medium with fresh every 2nd day might optimise development rate (Gardner, 1994). The importance of removing ammonia is significant, as it is a primary candidate for the induction of "foetal oversize syndrome" induced during embryos culture (McEvoy *et al.,* 1997).

During the 1980s, co-culture, especially with primary cultures of oviduct epithelial cells, emerged as the most effective method for overcoming the so-called 8- to 16 cell development block in mammalian embryos (Gandolfi and Moor, 1987). Co-culture system generally increases the developmental rates and produced good quality of blastocysts and high pregnancy initiation rates (Massip *et al.,* 1996; Rizos *et al.,*

2001). Izquierdo *et al.* (1999) suggested that development with oviduct cells was superior to that in cumulus cells or in medium alone. Bavister (1988) reported that the oviduct tissue supports early development *in vitro* are not well known but it seems likely that these cells produce material beneficial to the young embryos, remove substances with negative effects from the medium and/or reduce the oxygen concentration, or both.

In addition, oxygen concentration in the embryo culture system has been considered to affect rates of development (Machaty *et al.,* 1998; Olson and Seidel Jr, 2000). Although most groups that successfully produce porcine nuclear transfer piglets used an atmosphere of 5% CO_2 in air to culture embryos (Betthauser *et al.,* 2000; Onishi *et al.,* 2000; Lai *et al.,* 2002), oxygen tension in the reproductive tract is lower than that in air (Fischer and Bavister, 1993). This study examined the hypothesis that reducing oxygen concentration would improve the development of preimplantation porcine nuclear transfer embryos in various culture media. Similar report has also demonstrated in the production of full-term goat following nuclear transfer (Keefer *et al.,* 2001). In their studies, low oxygen (5%) is needed to culture the reconstructed oocytes up to embryo transfer. Besides that, cattle somatic cell nuclear transfer embryos cultured under low oxygen concentrations had a higher development to the blastocyst stage than those cultured in a high oxygen concentration (Im *et al.,* 2000). In addition, a higher oxygen concentration in genital tract can produce the formation of reactive oxygen species (ROS) during embryo culture. ROS are known to have deleterious effects on cells, including DNA damage, lipid peroxidation and oxidative modification of proteins (Johnson and Masr-Esfahani, 1994). Therefore, embryos cultured under low oxygen concentration (5% O_2) had higher total cell number and lower apoptotic cell number (Yuan *et al.,* 2003).

2.4 OTHER FACTORS AFFECTING INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER EFFICIENCY

2.4.1 Nuclear Reprogramming

Nuclear reprogramming, the conversion of the epigenome of a differentiated cell to one that is similar to the undifferentiated embryonic state, could be facilitated by several methods, such as nuclear transfer, cell fusion, use of embryonic stem cell extracts, and more recently, by the introduction of exogenous transcription factors. Amongst these various strategies, somatic cell nuclear transfer is, by far, the most effective method of nuclear reprogramming. Nuclear reprogramming is a general term which describes the resetting of the cell memory established during cell commitment and differentiation. At the moment, a complete reprogramming compatible with development to term is achieved in a minority of the cloned embryos, which a large proportion of them show epigenetic deregulation and abnormal gene expression at pre- and post-implantation stages, and even after birth (Ogura *et al.,* 2002; Tsunoda and Kato, 2002; Tamashiro *et al.,* 2003; Latham, 2005; Kremenskoy *et al.,* 2006; Loi *et al.,* 2006; Morgan *et al.,* 2006; Chae *et al.,* 2009; Sawai, 2009; Suzuki *et al.,* 2009; Xing *et al.,* 2009).

2.4.2 Epigenetic Modification

In nuclear transfer, the percentage to produce live births is always ranging between 1 to 5%, and most of the results are found to be failure during gestational or neonatal death. Many of those that survive to term succumb to a variety of abnormalities that are likely due to inappropriate epigenetic reprogramming. Cloned embryos derived from donors, such as embryonic stem cells, that may require little or no reprogramming of early developmental genes developed substantially better beyond implantation than nuclear transfer clones derived from somatic cells. Survival of cloned animals to birth and

beyond, despite substantial transcriptional deregulations, is consistent with mammalian development being rather tolerant to epigenetic abnormalities, with lethality resulting only beyond a threshold of faulty gene reprogramming encompassing multiple loci. These epigenetic modifications include DNA methylation, genomic imprinting and X-chromosome inactivation (Rideout *et al.,* 2001). Epigenetic changes affect the local structure, composition and remodeling of chromatin, which in turn defines and maintains the accessibility and transcriptional competence of the nucleosomal DNA template (Wolffe and Matzke, 1999).

2.4.3 DNA Methylation

DNA methylation in mammals occurs predominantly at CpG dinucleotides and it involves in a number of key genome functions (Takahashi, 2004). These include roles in imprinted genes, X-linked genes in females, germline-specific and tissue-specific genes. Therefore, DNA methylation plays a critical role in the determination of cell fate and cell type specific gene expression (Shi *et al.,* 2003).

DNA methylation takes place in 2 steps: the first during germ line development, when DNA methylation imprints are erased (Hajkova *et al.,* 2002; Lee *et al.,* 2002a), and the second during fertilisation and preimplantation development (Rougier *et al.,* 1998; Oswald *et al.,* 2000; Santos *et al.,* 2002). During fertilisation, the paternal genome is formed and undergoes major transformations within the cytoplasm of the oocyte, including remodeling of sperm chromatin by replacement of protamines with histones followed by genome-wide demethylation (Perreault, 1992). Example in mouse, the active demethylation of the paternal genome was shown to occur a few hours after fertilisation and before the first cell division (Mayer *et al.,* 2000; Santos *et al.,* 2002), whereas, the genome-wide demethylation of maternal genome occurs gradually

(Rougier *et al.,* 1998; Santos *et al.,* 2002). Genome-wide *de novo* methylation (remethylation) occurs at the blastocyst stage in mouse (in cattle is at 8- to 16 cell stage), preferentially in the inner cell mass (Dean *et al.,* 2001b). In contrast, the male pronuclear demethylation does not occur in sheep embryos (Beaujean *et al.,* 2004).

In somatic cell nuclear transfer, the donor cell genome is compelled to bypass gametogenesis (Takahashi, 2004). The successful cloning of mammals by the transfer of embryonic or somatic cell nucleus into an enucleated oocyte demonstrated that the genetic and epigenetic programs could be reversed in order to achieve totipotency (Gurdon and Colman, 1999; Shi *et al.,* 2003). However, up to which extent of epigenetic reprogramming is conserved in cloned embryo development is still unknown (Young and Fairburn, 2000). Besides that, the inefficient reprogramming of DNA methylation patterns may be partly responsible for the low birth rates and developmental abnormalities that often result from nuclear transfer (Young and Fairburn, 2000). Dean *et al.* (2001a) reported that the initial demethylation appeared to be conserved in 1 cell cloned bovine embryos. However, further demethylation is observed at the 2 cell stage, and a precocious *de novo* methylation is found in many at the 4- to 8 cell stage. In contrast, Bourc'his *et al.* (2001) reported that in their studies, they failed to find any demethylation in cloned bovine embryos. The demethylation/remethylation waves generally appear to coincide with the activation and transcription of the embryonic genome (Sansinena, 2005). In mammals, it is well known that the onset of ge25 nomic activation differs between species, occurring at the 2 cell stage in mice, 4 cell stage in pigs, and the 8- to 16 cell stage in cows and goat (Bavister, 1988; Campbell, 1999).

2.4.4 Genomic Imprinting

Genomic imprinting is a mammalian specific epigenetic modification of the genome. Assessment of the integrity of the imprinting memory in somatic cell cloned animals is important not only for understanding of the "reprogramming" process during cloning by nuclear transfer, but also for the applications of this technique for therapeutic cloning in the future (Kohda *et al*., 2006).

By the mechanisms of genomic imprinting, specific genes are allowed to express dependent on their parental origin (Surani *et al.,* 1984). During early development of an embryo, the functional differences are present between maternal and paternal genome. In the paternal genome, it exerts its effects in the extraembryonic tissues, whereas the maternal genome will have influence in the development of the embryo (McGrath and Solter, 1983; Surani *et al.,* 1984). In mammals, imprinting genes are particularly implicated in the regulation of foetal growth, development and function of placenta (Barlow, 1995). Both DNA methylation and chromatin packaging have been implicated as imprinting marks. However, little is known how they are established and maintained (Shi *et al.,* 2003). It has been proposed that when a somatic nucleus is introduced into an enucleated oocyte, the methylation of imprinted genes needs to be protected from genome-wide demethylation so that imprints are maintained intact in the cloned organism. In this case, reprogramming could interfere with proper maintenance of imprints and this could explain, at least in part, the low efficiency of animal cloning (Shi *et al.,* 2003).

2.4.5 Inactive X Chromosome

During late DNA replication and epigenetic chromatin modifications, it involves inactive X chromosome (Xi) in mammals. Shi *et al.* (2003) reported that the CpG

islands of many X-linked genes are methylated on Xi but not on the active chromosome (Xa). Dosage compensation for X-linked genes between males and females is achieved by transcriptional inactivation of one of the two X chromosomes (Lyon, 1961). An untranslated RNA, encoded by the Xist (inactive specific transcript X chromosome) gene that is expressed only from Xi, is necessary for the initiation of inactivation (Penny *et al.,* 1996). Continuous presence of Xist RNA acts together with DNA methylation and histone hypoacetylation to accomplish high fidelity inheritance of the inactive form (Park and Kuroda, 2001).

In the female nucleus, one X chromosome is inactivated because it expresses the Xist RNA. The other X chromosome escapes silencing and remains active. The Xi occurs by epigenetic lineage where the paternal chromosome is preferentially shut down. On the other hand, in somatic cell nuclear transfer the recipient oocyte receives one Xi and one Xa from the donor cell. One of the studies in bovine cloned embryos, observation of aberrant patterns of X-chromosome inactivation is reported (Xue *et al.,* 2002). When comparing between 2 types of donor cells, adult somatic cells and foetal cells, the former types of donor cells was significantly higher in the Xist transcripts than the latter types of donor cells (Wrenzycki *et al.,* 2002). Another study showed aberrant patterns of X-linked genes, as well as, hypomethylation of Xist in the organs of deceased cloned calves (Xue *et al.,* 2002). Therefore, due to the process of X inactivation is faithfully recapitulated in cloned female embryos, but the few surviving foetuses were studied (Eggan *et al*., 2000).

Chapter 3

3.0 MATERIALS AND METHODS

3.1 INTRODUCTION

The main objectives of this study were to produce viable caprine embryos through interspecies somatic cell nuclear transfer (interspecies SCNT) technique (donor karyoplast: caprine; recipient oocyte: bovine) as well as an attempt to produce pregnancy on cloned-caprine embryos via early cell stage embryo transfer. Samples of bovine ovaries were collected once or twice weekly from local abattoirs, that is, a) Abattoir Complex, Department of Veterinary Services, Shah Alam, Selangor Darul Ehsan and b) Department of Veterinary Services, Senawang, Seremban, Negeri Sembilan Darul Khusus. The recipient does for embryo transfer were sourced from the ISB Mini Farm, University of Malaya. All the experiments in this study involving works on *in vitro* maturation (IVM), interspecies somatic cell nuclear transfer (interspecies SCNT), *in vitro* culture (IVC), staining of embryos and embryo transfer were carried out at the Nuclear Transfer and Reprogramming Laboratory (NaTuRe), Institute of Research Management and Monitoring (IPPP), University of Malaya. All the media and reagents were prepared in the Embryo Micromanipulation Laboratory (EMiL), Institute of Biological Sciences, Faculty of Science, University of Malaya. This study was conducted from May 2009 to June 2011.

3.2 EXPERIMENTAL ANIMALS

A total number of 2,805 oocytes from 269 abattoir ovaries were used throughout this study. Briefly, the ovaries were collected from local abattoir and transported to the laboratory within 1 to 2 hours (Shah Alam) or 3 to 4 hours (Senawang) by placing the ovaries in a thermos flask containing NaCl (0.9%) supplemented with penicillin-G and

streptomycin as the collecting medium and maintained the temperature between 30 to 35^{o}C. The breed, origin and health status of the bovine were unknown. Besides these, the ovaries were collected regardless of the phases of oestrous cycle, ages and pregnancy.

A total number of 12 does were used as recipient in embryo transfer throughout this study. The recipient does comprising of Boer crossbred and local mixed breed goats with the age ranging from 24 to 36 months old. All recipient does were fed with Napier grass and commercial pellets twice daily, with *ad libitum* access to water. The does were well managed in the ISB Mini Farm, University of Malaya. All recipient does used were maintained under good conditions and with animal welfare guidelines.

3.3 MATERIALS

Materials used in the present study which included various equipment; chemicals, reagents and media as well as lab-wares and disposables are briefly described in the following sections:

3.3.1 Equipment

The details of each equipment used in the present study with model number, manufacturer's and supplier's name are listed in Appendix Table 1.1. The commonly used equipment include autoclave, centrifuge, CO_2 incubator, fluorescent microscope, inverted microscope with micromanipulation, electrofusion machine, laminar air flow work station, liquid nitrogen tank, microforge, micropipette grinder, micropipette puller, ultrapure water purification water system, osmometer, pH meter, oven, stage warmer, stereomicroscope, surgical set and water bath.

3.3.2 Chemicals, Reagents and Media

Analytical grade laboratory chemicals and reagents were used in the preparation of all solutions and media in the present study. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. from USA. A detailed list of the chemicals, reagents and media with catalogue number, manufacturer's and supplier's name is described in Appendix Table 1.2.

3.3.3 Lab-wares and Disposables

A list of lab-wares and disposables with manufacturer's name used in the study is tabulated in Appendix Table 1.3.

3.4 METHODS

3.4.1 General Maintenance of Research Laboratory

It is very important to ensure that all the equipment used were in good condition; lab-wares and disposables used were properly cleaned and sterile in all types of experiments that carried out in the study. This is because both recipient oocyte and donor cell are very sensitive especially culture in *in vitro*, therefore, maintains the sterility environment is necessary in the laboratory in order to prevent any contamination from happening.

This study involved the use of CO_2 incubator for *in vitro* culture experiments with 5% of CO_2 in humidified atmosphere to maintain the correct physiological pH (7.2 to 7.4) and a temperature of 38.5^{o}C. The CO_2 incubator has to be cleaned once a month, which involves wiping the inside wall, doors and racks with ethanol (70%) soaked sterile gauze or towels. The tray and the RO water contained in it which to provide humidity should be sterile and changed with every cleaning regime. The CO_2 incubator must be monitored regularly and the LED display of temperature checked with

independent thermometer readings. Repeated opening and closing of the CO_2 incubator should be kept to the minimum because it might affect the stability of the oocyte or embryo culture environment.

All used glassware and non-disposable items in the present study included glass bottles, beakers, volumetric flasks, measuring cylinders, conical flasks, magnetic stirrer and conical tubes. Cleaning solution (7x®-PF) was used as a detergent for glassware washing and was filled in a squirt bottle. The used glassware was rinsed in water to wash away traces of medium; the label was immediately removed and soaked in soap bath of 7x®-PF overnight. The glassware was washed with diluted cleaning solution (7x®-PF) using a brush or sponge and immediately rinsed 5 times with water followed by 5 times with RO water. After rinsing was completed, the cap of glassware was placed loosely and covered snugly with a layer of aluminium foil. A piece of autoclave tape was placed on the foil. Alternatively, non-disposable items were washed with diluted cleaning solution (7x®-PF) and rinsed 5 times with water followed by 5 times with RO water. After washing, all non-disposable items were placed in an autoclave bag and sealed; a piece of autoclave tape was placed on the seal bag. All items were allowed to be autoclaved for 20 to 25 minutes at 120°C. After autoclaving was done, the glassware cap was tightened a little bit prevent any contaminants from entering and together with non-disposable items were transferred into the oven to dry before being transferred in a clean and close glassware cabinet or appropriate storage cabinets. Also, the cap was not tightened completely until the glassware had cooled to prevent a vacuum forming in the glassware.

Before starting any experiment, the inside surfaces of the laminar air flow work station, microscope stages and other equipment such as micropipettes were wiped with ethanol (70%). The residual traces of ethanol were allowed to evaporate for at least 20 minutes before commencing work. Any spillage was wiped immediately with dry tissue

and if necessary, with ethanol (70%). When work finished, the inside surfaces of the laminar flow work station and all the equipment were wiped again with ethanol (70%). Also, the water bath was cleaned and water was changed frequently.

3.4.2 Preparation of Stock Solutions and Media

All stock solutions and media used throughout this study were prepared under laminar air flow work station to maintain the sterile environment. Purified Milli-Q water was the base water source for use to prepare most of the solutions and media. All powder forms of chemicals were weighed using a digital analytical balance while the chemicals in liquid form were measured using either sterile disposable plastic pipette or sterile disposable micropipette tips.

Generally, the media for oocytes and embryos culture were prepared into two forms, which namely stock solution and working solution. Due to different culture media required accurate and involved different chemicals for it culture purpose, therefore, is it convenient to prepare in a series stock solutions for each medium and it can be kept for longer time, for example, 1 to 2 months. However, when needed for experiment, working solutions were prepared freshly from stock solutions and it can be kept for only 1 to 2 weeks. While, the media used for somatic cell line culture were prepared when needed and which would be able to be kept for up to 1 to 2 months. All solutions and media prepared were filter-sterilised using syringe filter (0.22 μm pore size), aliquot in microcentrifuge tubes or Scott bottles, finally, stored in the refrigerator (2 to 8^{o}C) or freezer (-20^{o}C) as appropriate.

3.4.2.1 Preparation of ovary collection medium

Ovary collection medium was used for washing and collecting ovaries from abattoir. The ovary collection medium consisted of NaCl (9 mg/ml) supplemented with

penicillin-G (0.06 mg/ml) and streptomycin (0.05 mg/ml). The chemicals were weighed and dissolved in Milli-Q water (1 litre) by stirring gently. After preparation, the medium was kept at room temperature (25^{o}C) with a shelf life of 3 months and each time aliquot 400 ml to a beaker (500 ml) to warm up in the water bath prior to use (Table 3.1).

Table 3.1: Composition of ovary collection medium with a shelf life of 3 months (stored at room temperature, 25^{o}C)

Chemical component	Catalogue number	Concentration	Quantity/litre
NaCl	S5886	0.9 (w/v)	9 g
Penicillin-G	P7794	0.06 mg/ml	0.06 g
Streptomycin	S1277	0.05 mg/ml	0.05 g
Milli-Q water	-	-	1 litre

3.4.2.2 Preparation of *in vitro* maturation (IVM) medium

In vitro maturation (IVM) medium was prepared freshly and equilibrated at least 3 hours in the CO_2 incubator (5%) prior to oocyte recovery (Table 3.2). The IVM medium was consisted of TCM-199 as a base medium supplemented with FSH, oestradiol-17β, sodium pyruvate, foetal bovine serum (FBS), cystein and gentamicin.

Table 3.2: Preparation of stock solutions for IVM medium

Stock solution	Catalogue number	Concentration	Method of preparation (storage duration)
FSH	Folltropin-V®	5 mg/ml	Folltropin-V® (5 mg) was weighed and dissolved in Milli-Q water (1 ml) in a microcentrifuge tube, labelled on the cover, sealed with parafilm, wrapped in aluminium foil and stored (4^{o}C). (6 months)

(continued)

(continued)

Gentamicin	Sigma, G3632	50 mg/ml	Gentamicin (1 g) was weighed and dissolved in DPBS (20 ml), aliquot (1 ml) in microcentrifuge tube, labelled on the cover, sealed with parafilm, wrapped in aluminium foil and stored (4^oC). (6 months)
Oestradiol-17β	Sigma, E8875	1 mg/ml	Oestradiol-17β (1 mg) was weighed and dissolved in ethanol (1 ml) in microcentrifuge tube, labelled on the cover, sealed with parafilm, wrapped in aluminium foil and stored (4^oC). (6 months)

To prepare the IVM medium, TCM-199 (8.9 ml) was measured using a sterile disposable micropipette tips (1 ml) and dispensed into a sterile conical tube (15 ml). Using a digital balance, cystein (0.9 mg) were weighed and dissolved in the TCM-199 solution. Following, sodium pyruvate (2.2 mg) was weighed and dissolved in 1 ml of TCM-199 solution in a microcentrifuge tube. From the mixture, using a sterile disposable micropipette tips (100 µl), TCM-pyruvate (100 µl) was measured and dispensed into the TCM-199 solution containing cysteamine. Then, FSH (10 µl), gentamicin (5 µl) and FBS (1 ml) were added to the TCM-199 solution. The resulting IVM medium was mixed well and filter-sterilised by using syringe filter (0.22 µm pore size). Oestradiol-17β (9.5 µl) was added last after the medium was filtered. The final IVM medium was sealed with parafilm, wrapped in aluminium foil and stored in the refrigerator (4^oC) with a shelf life of 1 to 2 weeks (Table 3.3).

Table 3.3: Composition of IVM medium with a shelf life of 1 to 2 weeks (stored at 4^{o}C)

Chemical component	Catalogue number	Concentration	Quantity/10 ml
TCM-199	M4530	-	8.9 ml
cystein	M9768	0.09 mg/ml	0.9 mg
TCM-pyruvate	P4562	2.2 mg/ml	100 µl
FSH	Folltropin-V	5 mg/ml	10 µl
Gentamicin	G3632	50 mg/ml	5 µl
FBS	10270	10% (v/v)	1 ml
Oestradiol-17β	E8875	1 mg/ml	9.5 µl

3.4.2.3 Preparation of cloning manipulation solutions

The media were used to manipulate the oocytes for interspecies SCNT. The media involved were TL-Hepes medium, hyaluronidase solution, oocytes waiting medium (TCM-199 with 10% FBS), cytochalasin B (CB) solution, polyvinylpyrrolidone (PVP) solution and fusion medium.

3.4.2.3(a) Preparation of TL-Hepes medium

TL-Hepes medium consisted of Hepes to maintain pH of the medium at 7.2 to 7.4, and it was used for oocytes collection, oocytes and embryos washing and manipulation work outside the CO_2 incubator.

(i) Preparation of TL-Hepes stock solution

Typically, TL-Hepes stock solution (500 ml) was prepared each time (Table 3.4). All the chemicals, except for sodium lactate, bovine serum albumin-fraction V (BSA-FV), gentamicin stock solution and sodium pyruvate, were weighed as shown in Table 3.4, using a digital balance and dispensed into a sterile conical flask (500 ml). Milli-Q water was then added by using a measuring cylinder (100 ml) to make the final volume of 500 ml solution. The chemicals were dissolved slowly by stirring gently using a magnetic stirrer on a stirrer machine and at the same time, sodium lactate was slowly added to the solution with the used of sterile disposable micropipette tips. The resulting TL-Hepes stock solution was filter-sterilised by using syringe filter (0.22 μm pore size) and stored in the refrigerator (4^oC) with a shelf life of 3 months.

Table 3.4: Composition of TL-Hepes stock solution with a shelf life of 3 months (stored at 4°C)

Chemical component	Catalogue number	Concentration	Quantity/500 ml
NaCl	S5886	114 mM	3.330 g
KCl	P5405	3.2 mM	0.120 g
$NaHCO_3$	S5761	2.0 mM	0.084 g
$NaH_2PO_4.H_2O$	S9638	0.4 mM	0.028 g
$CaCl_2.2H_2O$	C3881	2.0 mM	0.150 g
$MgCl_2.6H_2O$	M2393	0.5 mM	0.050 g
Hepes: $C_8H_{17}N_2O_4SNa$	H3784	10 mM	0.600 g
Hepes: $C_8H_{18}N_2O_4S$	H6147	10 mM	0.600 g
Penicillin G	PEN-NA	100 IU/ml	0.0325 g
Phenol red powder	P3532	-	0.0025 g
Na Lactate (60% syrup)	L7900	10 mM	0.93 ml
Milli-Q water	-	-	499.07 ml

(ii) Preparation of TL-Hepes working solution

Typically, TL-Hepes working solution (50 ml) was prepared each time (Table 3.5). Using a sterile disposable plastic pipette (10 ml, Falcon), TL-Hepes stock solution (50 ml) were measured and dispensed into a sterile conical tube (50 ml). Using a sterile disposable micropipette tips, gentamicin stock solution (25 µl) was measured and dispensed into the solution. Using a digital balance, BSA-FV and sodium pyruvate were weighed and dissolved in the solution. The resulting TL-Hepes working solution was filter-sterilised again by using syringe filter (0.22 µm pore size) prior to use. The working solution was kept in the refrigerator (4°C) with a shelf life of 1 to 2 weeks.

Table 3.5: Composition of TL-Hepes working solution with a shelf life of 1 to 2 weeks (stored at 4°C)

Chemical component	Catalogue number	Quantity/50 ml
TL-Hepes stock solution	-	49.95 ml
Gentamicin stock solution	-	25 µl
Sodium pyruvate	P4562	1.1 mg
BSA-FV	A7030	50 mg

3.4.2.3(b) Preparation of hyaluronidase solution

Hyaluronidase solution was used for removing the cumulus cell from COC after maturation. As indicated by the manufacturer, a type IV-S hyaluronidase from bovine testes was used to prepare hyaluronidase solution. Hyaluronidase solution (750-1500 IU/mg) was prepared by dissolving hyaluronidase powder (1 mg) in TL-Hepes working solution (1 ml). The prepared solution was mixed properly, filter-sterilised by using syringe filter (0.22 μm pore size), aliquot (100 μl each) in microcentrifuge tubes, labelled on the cover, sealed with parafilm, wrapped in aluminium foil and stored in the freezer (-20^{o}C) with a shelf life of 6 months. On the day of experiment, 1 tube of hyluronidase solution was withdrawn from the refrigerator, warm up on a stage warmer and use to denude the COC (Table 3.6).

Table 3.6: Composition of hyaluronidase solution with a shelf life of 6 months (stored at -20^{o}C)

Chemical component	Catalogue number	Concentration	Quantity/10 ml
TL-Hepes working solution	-	-	10 ml
Hyaluronidase	H4272	0.01 g/ml	0.1 g

3.4.2.3(c) Preparation of oocytes waiting medium (TCM-199 with 10% FBS)

Oocytes waiting medium consisted of TCM-199 supplemented with FBS (10%) and was used to incubate the oocytes in CO_2 incubator (5%) while waiting for manipulation work. Typically, using a sterile disposable micropipette tips, TCM-199 medium (4.5 ml) and FBS (0.5 ml) were measured and dispensed into a sterile conical tube (15 ml). The prepared solution was mixed properly, filter-sterilised by using syringe filter (0.22 µm pore size), aliquot (500 µl each) in microcentrifuge tubes, labelled on the cover, sealed with parafilm, wrapped in aluminium foil and stored in the refrigerator (4^{o}C) with a shelf life of 3 month. On the day of experiment, 1 tube of oocytes waiting medium was withdrawn from the refrigerator, prepared in droplet form and incubated for at least 3 hours prior to use (Table 3.7).

Table 3.7: Composition of oocytes waiting solution with a shelf life of 3 months (stored at 4^{o}C)

Chemical component	Catalogue number	Concentration	Quantity/5 ml
TCM-199	Medium 199 (1X) 11150	-	4.5 ml
FBS	Gibco 10270	10% (v/v)	0.5 ml

3.4.2.3(d) Preparation of cytochalasin B solution

Cytochalasin B solution was used to depolymerise microfilaments of the matured oocytes (M II) and facilitated the enucleation process. Enucleation was accomplished by removing the first polar body (PB-1) and the metaphase II plate together with a small amount of surrounding cytoplasm.

(i) Preparation of cytochalasin B stock solution

Cytochalasin B stock solution was prepared by dissolving cytochalasin B powder (1 mg) in DMSO (1 ml). The prepared solution was then aliquot (10 µl each) in microcentrifuge tubes, labelled on the cover, sealed with parafilm, wrapped in aluminium foil and stored in the freezer (-20^{o}C) with a shelf life of 6 months (Table 3.8).

Table 3.8: Composition of cytochalasin B stock solution with a shelf life of 6 months (stored at -20^{o}C)

Chemical component	Catalogue number	Concentration	Quantity/ml
DMSO	D5879	-	1 ml
Cytochalasin B	C6762	1 mg/ml	0.001 g

(ii) Preparation of cytochalasin B working solution

On the day of experiment, 1 tube of cytochalasin B stock solution (10 µl) was withdrawn from the refrigerator and diluted with TL-Hepes working solution (990 µl) to make a final volume of 1 ml (Table 3.9).

Table 3.9: Composition of cytochalasin B working solution with a shelf life of 1 week (stored at 4^{o}C)

Composition	Quantity/ml
Cytochalasin B stock solution	10 µl
TL-Hepes working solution	990 µl

3.4.2.3(e) Preparation of PVP solution

PVP solution was used for the donor cell (donor karyoplast) when doing nuclear transfer in intracytoplasmic injection (ICI). During nuclear transfer, a donor cell was drawn in and out by the injection pipette (8 to 9 μm) in PVP medium (100 mg/ml) until the plasma membrane of the donor cell was disrupted prior inject into enucleated oocyte. PVP solution was prepared by dissolving PVP powder (0.1 g) in TL-Hepes working solution (1 ml). The prepared solution was mixed properly (it take approximately 1 day for PVP powder to dissolve fully in TL-Hepes working solution) by placing in the refrigerator at 4^{o}C overnight, filter-sterilised by using syringe filter (0.22 μm pore size), into a microcentrifuge tubes (1.5 ml), labelled on the cover, sealed with parafilm, wrapped in aluminium foil and stored in the refrigerator (4^{o}C) with a shelf life of 6 months. On the day of experiment, 10 μl of PVP solution was used to make a few microdroplets (2 to 3 μl for each microdroplet) on the manipulation dish (Table 3.10).

Table 3.10: Composition of PVP solution with a shelf life of 6 months (stored at 4^{o}C)

Chemical component	Catalogue number	Concentration	Quantity/ml
PVP	PVP360	100 mg/ml	0.1 g
TL-Hepes working solution	-	-	1 ml

3.4.2.3(f) Preparation of fusion medium

Fusion medium was used to facilitate the fusion between the donor cell and enucleated oocyte cytoplasm.

(i) Preparation of fusion stock solution

Typically, fusion stock solution (20 ml) was prepared each time. All the chemicals, except for BSA-FV were weighed as shown in Table 3.11, using a digital balance and dispensed in a sterile conical tube (50 ml). Milli-Q water was then added by using a sterile disposable plastic pipette (10 ml) to make the final volume of 20 ml solution. The chemicals were dissolved slowly by stirring gently using a magnetic stirrer on a stirrer machine. The resulting fusion stock solution was filter-sterilised by using syringe filter (0.22 μm pore size) and stored in the refrigerator (4^{o}C) with a shelf life of 3 months.

Table 3.11: Composition of fusion stock solution with a shelf life of 6 months (stored at 4^{o}C)

Chemical component	Catalogue number	Concentration	Quantity/20 ml
Sorbitol	S3889	10.0 mM	0.911 g
$Mg(CH_3COO)_2$	M0631	0.02 mM	0.0021 g
Hepes (free acid)	H3375	0.02 mM	0.0024 g
Milli-Q water	-	-	20 ml

(ii) Preparation of fusion working solution

Typically, 5 ml of fusion working solution was prepared each time. Using a sterile disposable micropipette tips (1 ml), fusion stock solution were measured and dispensed into a sterile conical tube (15 ml). Using a digital balance, BSA-FV was weighed and dissolved in the solution. The resulting fusion working solution was filter-sterilised again by using syringe filter (0.22 μm pore size) and aliquot (2.5 ml each) into sterile

conical tube (15 ml) prior to use. The working solution was kept in the refrigerator (4^{o}C) with a shelf life of 1 to 2 weeks (Table 3.12).

Table 3.12: Composition of fusion working solution with a shelf life of 1 to 2 weeks (stored at 4^{o}C)

Chemical component	Catalogue number	Quantity/5 ml
Fusion stock solution	-	5 ml
BSA-FV	A7030	0.01 g

3.4.2.4 Preparation of activation medium

In the present study, double activation was used to activate the reconstructed oocytes after nuclear transfer. The activation chemicals used were calcium ionophore (CaI) and 6-dimethylaminopurine (6-DMAP).

3.4.2.4(a) Preparation of calcium ionophore solution

Calcium ionophore solution was used to activate the oocytes after nuclear transfer in interspecies SCNT. Calcium ionophore is important to increase the concentration of free calcium in the cytosol, thereby mimicking the physiological cell-signaling mechanism and form of calcium oscillations. This calcium oscillations pattern during oocyte activation may influence not only fertilisation but also embryo development and, therefore, the implantation (Rout *et al*., 1997; Ozil *et al*., 2006).

(i) Preparation of calcium ionophore stock solution

As shown in Table 3.13, calcium ionophore stock solution was prepared by dissolving calcium ionophore powder (0.001 g) in DMSO (3.82 ml). The prepared solution was then aliquot (10 µl each) in microcentrifuge tubes, labelled on the cover, sealed with parafilm, wrapped in aluminium foil and stored in the freezer (-20^{o}C) with a shelf life of 6 months.

Table 3.13: Composition of calcium ionophore stock solution with a shelf life of 6 months (stored at -20^{o}C)

Chemical component	Catalogue number	Quantity/ml
DMSO	D5879	3.82 ml
Calcium ionophore	C7522	0.001 g

(ii) Preparation of calcium ionophore working solution

On the day of experiment, 1 tube of calcium ionophore stock solution (10 µl) was withdrawn from the freezer and diluted with KSOM A working solution (990 µl) to make a final volume of 1 ml. The final concentration of the working solution was 5 µM (Table 3.14).

Table 3.14: Composition of calcium ionophore working solution with a shelf life of 6 months (stored at -20^{o}C)

Composition	Concentration	Quantity/ml
Calcium ionophore stock solution	5 µM	10 µl
KSOM A working solution	-	990 µl

3.4.2.4(b) Preparation of 6-dimethylaminopurine (6-DMAP)

6-DMAP was used together with calcium ionophore to activate the oocytes following nuclear transfer in interspecies SCNT.

(i) Preparation of 6-DMAP stock solution

6-DMAP stock solution was prepared by dissolving 6-DMAP powder (0.1 g) in Milli-Q water (3.08 ml), as shown in Table 3.15. The prepared solution was then aliquot (10 µl each) in microcentrifuge tubes, labelled on the cover, sealed with parafilm, wrapped in aluminium foil and stored in the freezer (-20^{o}C) with a shelf-life of 6 months.

Table 3.15: Composition of 6-DMAP stock solution with a shelf life of 6 months (stored at -20^{o}C)

Chemical component	Catalogue number	Quantity/3.08 ml
Milli-Q water	-	3.08 ml
6-DMAP	D2629	0.1 g

(ii) Preparation of 6-DMAP working solution

On the day of experiment, 1 tube of 6-DMAP stock solution (10 µl) was withdrawn from the freezer and diluted with KSOM A working solution (990 µl) to make a final volume of 1 ml. The final concentration of the working solution was 2 mM (Table 3.16).

Table 3.16: Composition of 6-DMAP working solution with a shelf life of 6 months (stored at -20^{o}C)

Composition	Concentration	Quantity/ml
6-DMAP stock solution	2 mM	10 µl
KSOM A working solution	-	990 µl

3.4.2.5 Preparation of KSOM medium

Simplex Optimisation Medium (SOM) was developed by Lawitts and Biggers (1991) and modified to formulate KSOM (K Simplex Optimisation Medium) (Lawitts and Biggers, 1993). KSOM medium was used as the base medium for activation and *in vitro* culture (IVC). In the present study, 2 types of KSOM medium were prepared and used for embryos culture, namely, (a) KSOM A – classical/standard KSOM, (b) KSOM B – KSOM supplemented with glucose (0.04%, 2.2 mM).

(i) Preparation of KSOM stock solution

Typically, KSOM stock solution (100 ml) was prepared each time. All the chemicals, except for sodium lactate (60% syrup), were weighed as shown in Table 3.17 using a digital balance and dispensed into a measuring cylinder (100 ml). Milli-Q water was then measured and added by using a sterile disposable plastic pipette (10 ml) to make the final volume of 100 ml solution. The chemicals were dissolved slowly by stirring gently using a magnetic stirrer on a stirrer machine and at the same time, sodium lactate (60% syrup) was slowly added to the solution with the use of sterile disposable micropipette tips (1 ml). The resulting KSOM stock solution was filter-sterilised by using syringe filter (0.22 μm pore size) and stored in the refrigerator (4^{o}C) with a shelf life of 1 month.

Table 3.17: Composition of KSOM stock solution with a shelf life of 1 month (stored at 4^{o}C)

Chemical component	Catalogue number	Concentration	Quantity/100 ml
NaCl	S5886	95 mM	0.5553 g
KCl	P5405	2.5 mM	0.0186 g
KH_2PO_4	P5655	0.35 mM	0.0048 g
$MgSO_4$	M7506	0.2 mM	0.0024 g
Sodium pyruvate	P4562	0.2 mM	0.0022 g

(continued)

(continued)

D-glucose	G6152	0.2 mM	0.0036 g
$NaHCO_3$	S5761	25 mM	0.2101 g
$CaCl_2$	C5670	1.71 mM	0.0190 g
L-glutamine	G3126	1 mM	0.0146 g
EDTA	E9884	0.01 mM	0.0004 g
Na Lactate (60% syrup)	L7900	10.0 mM	0.1860 ml
Milli-Q water	-	-	99.814 ml

(ii) Preparation of KSOM working solution

Two types of KSOM working solutions were prepared for the present study to culture the reconstructed oocytes after cloning, which namely, KSOM A (early stage of embryos) and KSOM B (later stage of embryos). For KSOM A, KSOM A working solution (10 ml) was prepared each time. Using a sterile disposable micropipette tips (1 ml), KSOM stock solution (9.85 ml) were measured and dispensed into a sterile conical tube (15 ml). Using a sterile disposable micropipette tips, BME (100 µl) and MEM (50 µl) were measured and dispensed into the solution. Using a digital balance, BSA (0.04 g) was weighed and dissolved in the mixture. The resulting KSOM A working solution was filter-sterilised again by using syringe filter (0.22 µm pore size) prior to use (Table 3.18).

Table 3.18: Composition of KSOM A medium with a shelf life of 1 to 2 weeks (stored at 4^oC)

Chemical component	Catalogue number	Quantity/10 ml
KSOM stock solution	-	9.85 ml
BME amino acids solution	B6766	100 µl
MEM non-essential amino acids solution	M7145	50 µl
BSA	A6003	0.04 g

For KSOM B, KSOM B working solution (5 ml) was prepared each time. Using a sterile disposable micropipette tips (1 ml), KSOM stock solution (4.925 ml) were measured and dispensed into a sterile conical tube (15 ml). Using a sterile disposable micropipette tips, BME (50 µl) and MEM (25 µl) were measured and dispensed into the solution. Using a digital balance, BSA (0.02 g) and D-glucose (0.04%, 0.002 g, 2.2 mM) were weighed and dissolved in the mixture. The resulting KSOM B working solution was filter-sterilised again by using syringe filter (0.22 µm pore size) prior to use (Table 3.19).

Table 3.19: Composition of KSOM B medium with a shelf life of 1 to 2 weeks (stored at $4^{o}C$)

Chemical component	Catalogue number	Quantity/5 ml
KSOM stock solution	-	4.925 ml
BME amino acids solution	B6766	50 µl
MEM non-essential amino acids solution	M7145	25 µl
BSA	A6003	0.02 g
D-glucose (0.04%, 2.2 mM)	G6152	0.002 g

(P.J. Kwong, personal communication)

3.4.2.6 Preparation of somatic cell (donor karyoplast) culture medium

In the present study, the media were used to establish somatic cell line, and somatic cell-bank which was stored in cryotube containing freezing medium under liquid nitrogen (-196^{o}C) and would be thawed for further used. The media involved were (a) Tissue culture medium, (b) PBS (-) solution, (c) Trypsin-EDTA solution, and (d) Freezing medium.

3.4.2.6(a) Preparation of tissue culture medium

In the present study, tissue culture medium was used as a nutrient medium which provided the necessary nutrients to the cell to grow in monolayers *in vitro*. This medium consisted of α-Minimum Essential Medium (α-MEM), FBS and Penicillin-Streptomycin (P-S) and is also known as the synthetic cell culture medium.

(i) Preparation of tissue culture stock solution

As shown in Table 3.20, tissue culture stock solution (1 litre) was prepared each time. One bottle of α-MEM contained 10.1 g of powder and which was ready to prepare 1 litre of the stock solution. Using a sterile spatula, α-MEM powder (1 bottle) was transferred and dissolved in Milli-Q water (1 litre) in a sterile Schott bottle. Using a digital balance, $NaHCO_3$ (2.2 g) was weighed and dissolved into the mixture. The chemicals were dissolved slowly by stirring gently using a magnetic stirrer on a stirrer machine. The resulting tissue culture stock solution was filter-sterilised by using syringe filter (0.22 μm pore size) and stored in the refrigerator (4^{o}C) with a shelf life of 3 to 4 months.

Table 3.20: Composition of tissue culture stock solution with a shelf life of 3 to 4 months (stored at 4^{o}C)

Chemical component	Catalogue number	Concentration	Quantity/litre
α-MEM powder	M0644	10.1 g/litre	10.1 g
$NaHCO_3$	S5761	2.0 mM	2.2 g
Milli-Q water	-	-	1 litre

(ii) Preparation of tissue culture working solution

Typically, tissue culture working solution (100 ml) was prepared each time. Using a sterile disposable plastic pipettes (10 ml), tissue culture stock solution (90 ml) and FBS (10 ml) were measured and dispensed into a sterile Scott bottle (100 ml). Using a sterile disposable micropipette tips, P-S stock solution (100 µl) was measured and dispensed into the solution. The resulting tissue culture working solution was filter-sterilised again by using syringe filter (0.22 µm pore size) prior to use. The working solution was kept in the refrigerator (4^{o}C) with a shelf life of 1 to 2 months (Table 3.21).

Table 3.21: Composition of tissue culture stock solution with a shelf life of 1 to 2 weeks (stored at 4^{o}C)

Chemical component	Catalogue number	Quantity/100 ml
Tissue culture stock solution	-	90 ml
FBS	Gibco 10270	10 ml
P-S stock solution	-	100 µl

3.4.2.6(b) Preparation of PBS (-) solution

PBS (-) solution was used to wash the somatic cells prior to trypsinisation to reduce the concentration of divalent cations from the tissue culture medium and proteins from the FBS that inhibit trypsin action. Typically, PBS (-) solution (100 ml) was prepared each time. Using a digital balance, NaCl (1.0 g), KCl (0.025 g), KH_2PO_4 (0.144 g) and Na_2HPO_4 (0.025 g) were weighed and dissolved in Milli-Q water (100 ml) in a sterile Scott bottle. The chemicals were dissolved slowly by stirring gently using a magnetic stirrer on a stirrer machine. The resulting PBS (-) solution was filter-sterilised by using syringe filter (0.22 μm pore size) and stored at room temperature with a shelf life of 3 months (Table 3.22).

Table 3.22: Composition of PBS (-) solution with a shelf life of 3 months (stored at room temperature, 25^{o}C)

Chemical component	Catalogue number	Concentration	Quantity/100 ml
NaCl	S5886	171.1 mM	1.0 g
KCl	P5405	3.35 mM	0.025 g
KH_2PO_4	P5655	10.58 mM	0.144 g
Na_2HPO_4	S5136	1.76 mM	0.025 g
Milli-Q water	-	-	100 ml

3.4.2.6(c) Preparation of trypsin-EDTA solution

Trypsin-EDTA solution was used for the dissociation of adherent cell and cell aggregates into single cell suspensions that could be used as donor cell in nuclear transfer. Typically, trypsin-EDTA solution (100 ml) was prepared each time, as shown in Table 3.23. Using a digital balance, trypsin (0.25 g) and EDTA (0.04 g) were weighed and dissolved in PBS (-) solution (100 ml) in a sterile Scott bottle. The chemicals were dissolved slowly by stirring gently using a magnetic stirrer on a stirrer machine. The resulting trypsin-EDTA solution was filter-sterilised by using syringe filter (0.22 μm pore size) and stored in the refrigerator (4^{o}C) with a shelf life of 3 months.

Table 3.23: Composition of trypsin-EDTA solution with a shelf life of 3 months (stored at 4^{o}C)

Chemical	Catalogue number	Concentration	Quantity/100 ml
Trypsin	T4799	2.5 mg/ml	0.25 g
EDTA	E4884	0.4 mg/ml	0.04 g
PBS (-) solution	-	-	100 ml

3.4.2.6(d) Preparation of freezing medium

Freezing medium was used to store the somatic cell in a very low temperature, for example, liquid nitrogen (-196^{o}C). This medium consisted of DMSO (10%), which act as a cryoprotectant to prevent the formation of ice crystal in the cell when freezing. Typically, freezing medium (100 ml) was prepared each time. Using a sterile disposable plastic pipette (10 ml), tissue culture medium supplemented with FBS (10%) (90 ml) and DMSO (10 ml) were measured and dispensed into a sterile Scott bottle (100 ml). The resulting freezing medium was mixed well and filter-sterilised by using syringe filter (0.22 µm pore size) prior to use. The medium was kept in the refrigerator (4^{o}C) with a shelf life of 1 to 2 months (Table 3.24).

Table 3.24: Composition of freezing medium with a shelf life of 1 to 2 months (stored at 4^{o}C)

Chemical	Catalogue number	Quantity/100 ml
DMSO	D5879	10 ml
Tissue culture medium supplemented with 10% FBS	-	90 ml

3.4.2.7 Preparation of other stock solution

3.4.2.7(a) Preparation of Penicillin-Streptomycin (P-S) stock solution

Penicillin-Streptomycin (P-S) solution was used to supplement in cell culture medium to control bacterial contamination. Typically, P-S solution (10 ml) was prepared each time. Using a digital balance, penicillin-G (0.3 g) and streptomycin (0.5 g) were weighed and dissolved in PBS (-) solution (10 ml) in a sterile conical tube (15 ml). The chemical were dissolved slowly by gently shaking the conical tube. The resulting P-S solution was filter-sterilised by using syringe filter (0.22 μm pore size), aliquot (100 μl each) in microcentrifuge tubes, labelled on the cover, sealed with parafilm, wrapped in aluminium foil and stored in the freezer (-20^{o}C) with a shelf life of 6 months (Table 3.25).

Table 3.25: Composition of Penicillin-Streptomycin (P-S) stock solution with a shelf life of 6 months (stored at -20^{o}C)

Chemical component	Catalogue number	Quantity/10 ml
Penicillin-G	P7794	0.3 g
Streptomycin	S9137	0.5 g
PBS (-)	-	10 ml

3.4.2.8 Preparation of staining solution

In the present study, fixative solution and bisBenzimide or Hoechst 33342 dye were prepared and used to stain the embryos for evaluation of their developmental stages. Fixative solution, is a useful step because it could be stored the stained embryos for several weeks before view under fluorescent microscope for the cell number. Typically, fixative solution (10 ml) was prepared each time. Using a sterile disposable micropipette tips (1 ml), PBS (-) (10 ml), Formaldehyde (40%, 200 µl) and Glutaraldehyde (25%, 25 µl) were measured and dispensed into a sterile conical tube (15 ml). Using a digital balance, PVP (0.01 g) was weighed and dissolved in the mixture. The resulting fixative solution was wrapped with aluminium foil and stored in the refrigerator (4^{o}C) with a shelf life of 3 months.

Hoechst 33342 was used to stain the nucleus or DNA in the embryos. Typically, Hoechst 33342 dye (2 ml) was prepared each time. Using a digital balance, Hoechst 33342 (0.0025 g) was weighed and dissolved in PBS (-) solution (10 ml) in a sterile conical tube (15 ml). The chemicals were dissolved slowly by gently shaking the conical tube. The resulting Hoechst 33342 stock solution was aliquot (100 µl each) in microcentrifuge tube (1.5 ml) and labelled as “Stock A”. Subsequently, using a sterile disposable micropipette tips (1 ml), PBS (-) solution (900 µl) was measured and added into the “Stock A” of Hoechst 33342 and labelled as “Stock B”. Finally, the working solution of Hoechst 33342 was prepared by mixing the “Stock B” (1 ml) and glycerol (1 ml) together by using the sterile disposable micropipette tips into a conical tube (15 ml). The working solution of Hoechst 33342 was aliquot in microcentrifuge tube (1.0 ml each) and stored in the refrigerator (4^{o}C) with a shelf life of 3 months.

3.4.3 Preparation of Mouthpiece-controlled Pipette and Microneedles

A mouthpiece-controlled pipette was used for handling the oocytes and embryos during manipulation work. It consisting of an aspirator mouthpiece, tubing and glass Pasteur pipette pulled on a flame. Microneedles used for the present study included holding pipette, cutting needle, aspiration pipette and injection pipette. All the pipettes and needles used in different experiments were prepared 'in-house' in the laboratory.

3.4.3.1 Capillary cleaning and sterilisation

The glass Pasteur pipettes for making mouthpiece-controlled pipettes were soaked overnight in RO water, rinsed with Milli-Q 5 times, sterilised by autoclaving and last dried thoroughly in the oven (56^{o}C) overnight prior to use. While, the borosilicate capillaries for making microneedles were soaked in hydrochloric acid solution (10%) for 24 hours in a glass cylinder, rinsed with Milli-Q water 20 times to remove all traces of the acid, sterilised by autoclaving and thereafter, dried thoroughly in the oven (56^{o}C) overnight before used.

3.4.3.2 Preparation of mouthpiece-controlled pipette

The glass Pasteur pipette could be pulled to create a narrow opening by rotating the middle portion in a fine spirit burner flame until the glass became soft. After that, the glass was immediately withdrawn from the heat and both ends were quickly pulled smoothly in opposite directions so that the middle portion had an inner diameter (ID) of approximately 180 to 350 μm along the pulled pipette. The pulled portion of the glass pipette was scribed with a diamond stone and snapped at the scribed portion according to the inner diameter that needed for the experiments, for example, 160 to 180 μm of inner diameter were used for oocytes or embryos and 330 to 350 μm of inner diameter were used for COC. Subsequently, the tip of the glass pipette was fire-polished by

quickly touching the flame to achieve a smooth edge of the pipette tip. This step is important to prevent the injury of the oocytes and embryos when transferring from droplet to droplet.

3.4.3.3 Preparation of microneedles

In the present study, there were 5 different types of microneedles needed to prepare 'in-house' according to the treatments designed. All types of microneedles were made from thin-walled borosilicate capillaries (Drummond, Broomall, USA) with an inner and outer diameter of 0.69 and 0.97 mm, respectively, and a length of 10 cm. Three instruments were necessary to prepare the needles, which namely, micropuller, microforge and microgrinder (Figure 3.1). A horizontal micropuller (P-97, Sutter Instrument, USA) is preferred to the vertical as the former produced needles of uniform shapes as shown in Figure 3.1. The quantity of heat, pull speed and strength had been established and stored in the program (heat = 665 units, pull = 150 units, time = 150 units, pressure = 500 units). The ideal shape of the needle should be one that has a long and uniform tapering end with a length of approximately 10 mm. After pulling, microfoge (Technical Products Internationals, USA) and microgrinder (EG-4. Narashige Co. Ltd., Japan) were used to finalise the preparation of the needles. A microforge is an instrument to make holding, cutting and injection needles of a specific size which contains an electrical air blower and electrical network for control of the filament and the illuminator. Generally, the function of the air blower is to converge on the wire heating filament to provide for "spot" cooling of the heated element when preparing any microneedles. While, an electrical network is function to control the heat of the filament (the heat was ranging from levels 0 to 12). The "heat" can be determined by observing the colour of the glowing filament. The "lighter" and "whiter" the filament, the hotter it is. In the present study, the maximum heat needed for preparing the microneedles was

level 4, because above the level 4, the platinum-iridium filament would becomes a "dazzling" white and will quickly melt.

Before needle processing could begin, it was necessary to place a small bead of glass on top of the heater element. It was to ensure the filament itself never came into direct contact with the needle. To make the glass bead, first, the filament glowed dull red (heat level = 4) with the heater control adjusted, and then a needle lowered on to the hot filament. The needle was lowering continuously as the glass melted until a bead of approximately 20 to 30 µm diameter has formed; and the heat switched off immediately. The needle closed to the bead was broke with a pair of fine forceps, and then broken pipette withdrawn as well as discarded. Finally, the bead was heated gently until the jagged portion absorbed. The microforge was now ready to process holding and injection pipettes.

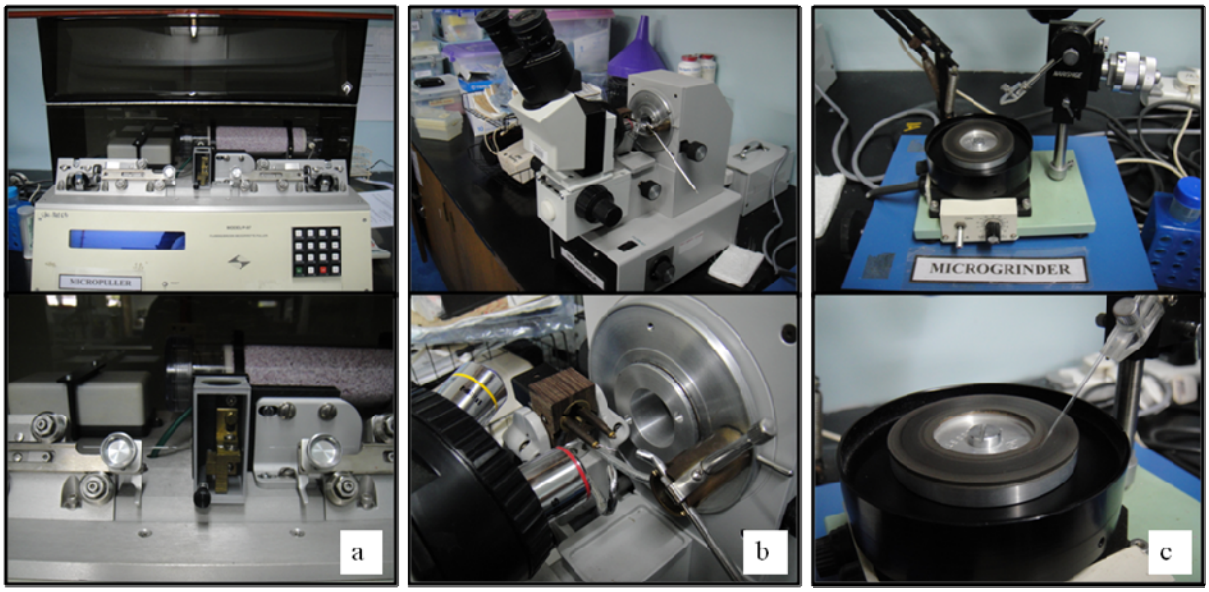

Figure 3.1: Preparation of microneedles. (a) Micropuller; (b) Microforge; and (c) Microgrinder.

3.4.3.3(a) Preparation of holding pipette

Holding pipette was used to hold or stabilise the oocye during enucleation and injection of donor cell. There are 2 different diameters in the holding pipette, which were known as outer diameter and inner diameter. Basically, holding pipette was prepared by scoring the pulled capillary with an ampoule cutter, breaking it and fire polishing (heat level = 3) the tips with a microforge. The inner and outer diameters of the holding needle were approximately 25 μm and 120 to 140 μm, respectively. The holding pipette was then bent at 30^{o} (heat level = 4) to allow a horizontal displacement on the microscope stage.

3.4.3.3(b) Preparation of cutting needle

Cutting needle was used to make a cutting point on the oocyte's zona pellucida and subsequently squeeze out the polar body as well as 10% of cytoplasm which containing DNA chromosome. Typically, the pulled capillary was broke by using a diamond stone and placed vertically above the glass bead. The needle was brought slowly to touch the heated glass bead (heat level = 3), at the same time, repeatedly pull the needle up and down until it formed a sharp end needle. The cutting needle was then bent at 30^{o} (heat level = 4) to allow a horizontal displacement on the microscope stage.

3.4.3.3(c) Preparation of aspiration pipette

Aspiration pipette was another type of microneedle that used to remove the polar body and DNA chromosome which contained in the cytoplasm of an oocyte and subsequently using the same aspiration pipette to inject a single donor cell into the oocyte's perivitellin space. Aspiration pipette was prepared by cutting the tip of a pulled capillary on a heated glass bead (heat level = 1) of the microforge at an inner and outer diameters were approximately 20 to 22 μm and 22 to 24 μm, respectively. The needle tip was then ground to produce a bevelled edge with a microgrinder (Figure 3.1) at 45^{o} desired angle

for approximately 3 minutes. After that, the aspiration pipette was fixed back to the microforge vertically above the glass bead. The filament was switched on (with a small amount of heat) and the aspiration pipette was brought near to the glass bead (heat level = 1). The tip of the aspiration pipette was touched on the glass bead and slowly pulled up to form a spike. The aspiration pipette with spike was bent at 18^{o} (heat level = 1.5) for easier manipulation when doing cloning on the microscope stage. Lastly, the dust accumulated in the needle was washed away with alcohol (70%) for 5 seconds and rinsed thoroughly with Milli-Q water for another 5 seconds.

3.4.3.3(d) Preparation of injection pipette

Injection pipette was used to inject a single donor cell into the oocyte's perivitellin space. The preparation of this injection pipette was similar to the aspiration pipette that had mentioned before but without a spike at the tip of the pipette. Besides that, the inner and outer diameters were smaller with approximately 16 to 18 μm and 18 to 20 μm, respectively. Briefly, after made a cut (heat level = 1) on the pulled capillary, the needle tip was ground to produce a bevelled edge with a microgrinder at 45^{o} desired angle for approximately 3 minutes. The aspiration pipette was then bent at 18^{o} (heat level = 1) for easier manipulation when doing cloning on the microscope stage. The dust accumulated in the needle was washed away with alcohol (70%) for 5 seconds and rinsed thoroughly with Milli-Q water for another 5 seconds.

3.4.3.3(e) Preparation of intracytoplasmic injection (ICI) pipette

ICI pipette was another type of injection pipette that used to inject a donor cell directly into the oocye's cytoplasm. Basically, an ICI pipette was prepared by cutting the tip of a pulled capillary on a heated glass bead (heat level = 1) of the microforge at an inner and outer diameters were approximately 8 to 9 μm and 9 to 10 μm, respectively. The ICI

pipette was then bent at 18^{o} (heat level = 1) for easier manipulation when doing cloning on the microscope stage.

3.4.4 Preparation of Caprine Donor Cell (Donor Karyoplast)

In the present study, caprine foetal fibroblast cell was used as donor cell. Typically, the caprine foetuses were derived at days 28 and 35, in which foetuses were produced by natural mating (Figures 3.2 and 3.3). On the day of caesareans, foetuses were surgically removed and placed in a culture dish (90 mm) containing equilibrated phosphate-buffered saline (-) (PBS, Ca^{2+} /Mg^{2+}–free). In the PBS (-) medium, the head and internal organs of the foetuses were removed by using a pair of surgical forceps. After removal the head and internal organs, the remaining tissues were mechanically dissociated. The explants were cultured in a culture dish (60 mm) containing α-modified Eagle medium (α-MEM) supplemented with foetal bovine serum (20%, FBS) and penicillin-streptomycin (P-S) under a humidified atmosphere of CO_2 (5%) in air at 37^{o}C (Figure 3.4). While the explants cultures contained a mixed population of cells, foetal fibroblasts were considered predominant. When the cells from the explants reached confluency (70%) (approximately 8 to 9 days), they were harvested using trypsin-EDTA (0.25%) and then subcultured to passage 1 or 2. The foetal fibroblast cells at passage 1 or 2 were harvested and cryopreserved using dimethyl sulfoxide (10%, DMSO) mixed in the tissue culture medium and stored in liquid nitrogen. The frozen cells were thawed and cultured up to confluence (80%) to use as donor cells. However, subsequent sub-cultured donor cells were made to use as donor cells in nuclear transfer (passages 2 to 5).

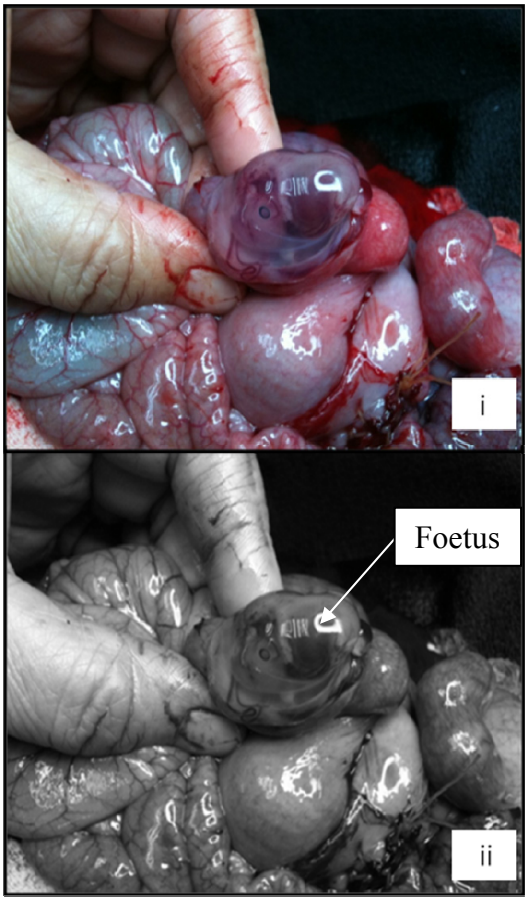

Figure 3.2: Foetus at day 35. (i) Original photograph. (ii) Labelled photograph.

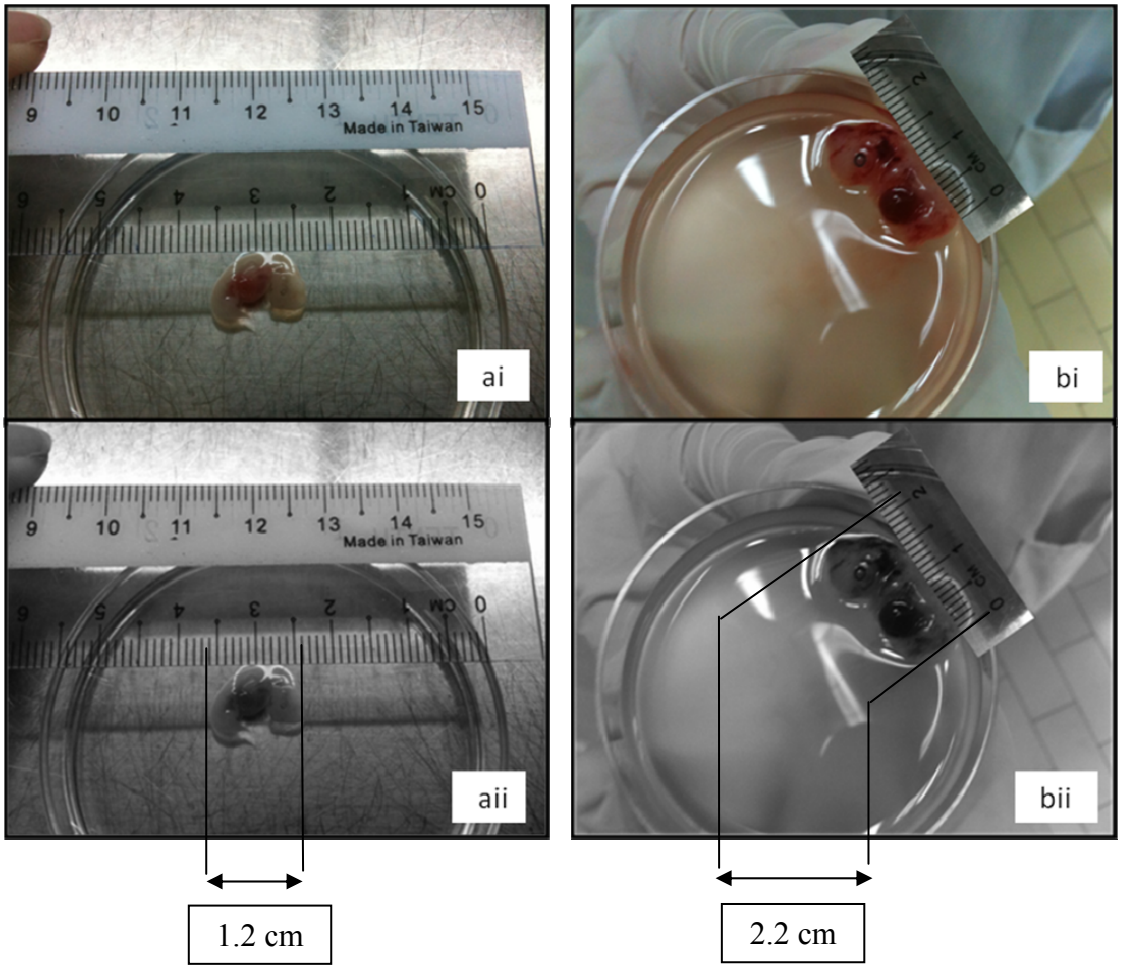

Figure 3.3: Collected foetuses. (i) Original photograph. (ii) Labelled photograph. (a) Foetus collected on day 28 with the length of 1.2 cm; (b) Foetus collected on day 35 with the length of 2.2 cm.

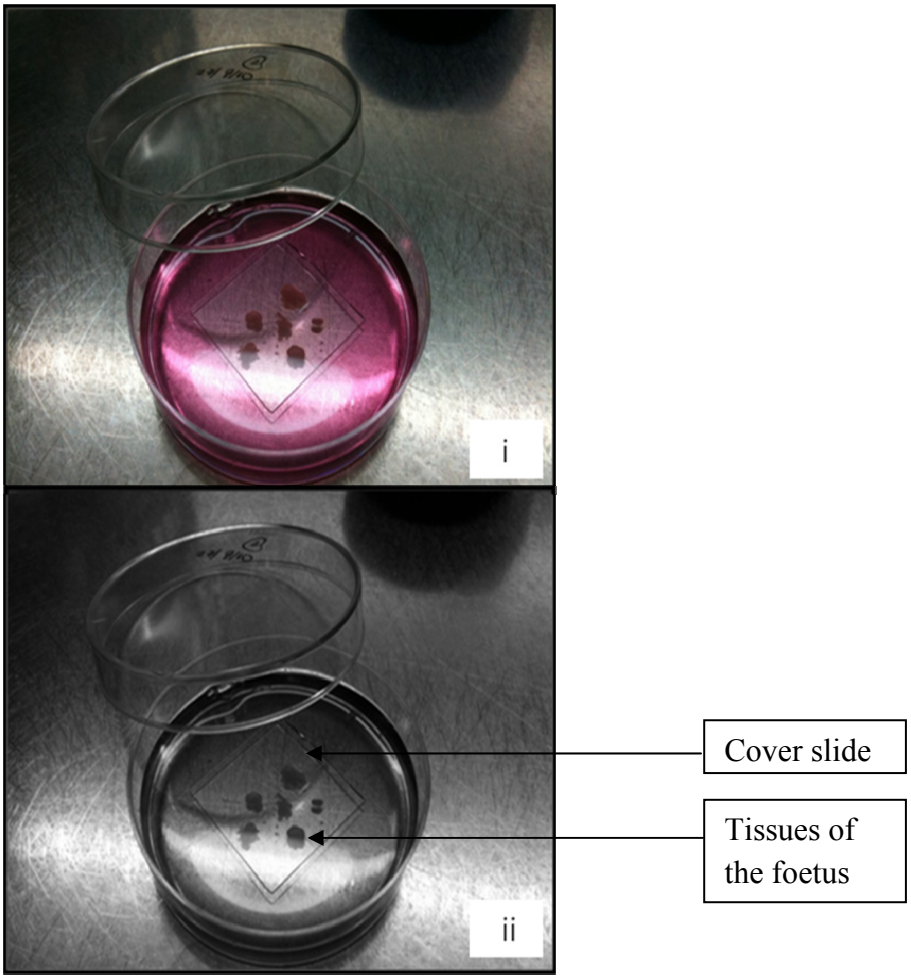

Figure 3.4: Arrangement of foetus tissues in culture dish. (i) Original photograph. (ii) Labelled photograph.

3.4.5 Preparation of Bovine Recipient Cytoplast

In the present study, the bovine recipient cytoplasts were retrieved from local abattoir-derived ovaries.

3.4.5.1 Oocyte retrieval from abattoir-derived ovaries

The bovine ovaries were transported back to the laboratory in ovary collecting medium which consisted of NaCl (0.9%) supplemented with penicillin-G (60 μg/ml) and streptomycin (50 μg/ml) (Table 3.1) at 30 to 35^{o}C (Figure 3.5). Soon after reached the laboratory, ovaries were washed and rinsed few times with warm ovary collecting medium to wash off the blood. Two sterile beakers (100 ml) and a polystyrene culture dish (90 mm) were filled with warm TL-Hepes medium (35 to 37^{o}C) and placed on a stage warmer that was set at 38.5^{o}C. Each ovary was held with a sterile curved haemostat and checkerboard slicing was made to the entire surface of the ovary inside the culture dish by using the razor blade (Figure 3.6). The sliced ovary was rinsed in the beaker containing TL-Hepes medium. All the steps in the process were repeated until all the ovaries were sliced. The polystyrene culture dish containing COC and debris were scanned under a stereomicroscope for COC. After finishing the search for the COC in culture dish (90 mm), the TL-Hepes medium containing debris and COC was poured from the beaker into a sterile polystyrene culture dish (60 mm) to continue searching. All collected COC were placed in a culture dish (35 mm) containing TL-Hepes medium (2 ml) and then washed 2 times in TL-Hepes medium followed by 3 times in *in vitro* maturation (IVM) medium before cultured in IVM medium droplets overlaid with mineral oil.

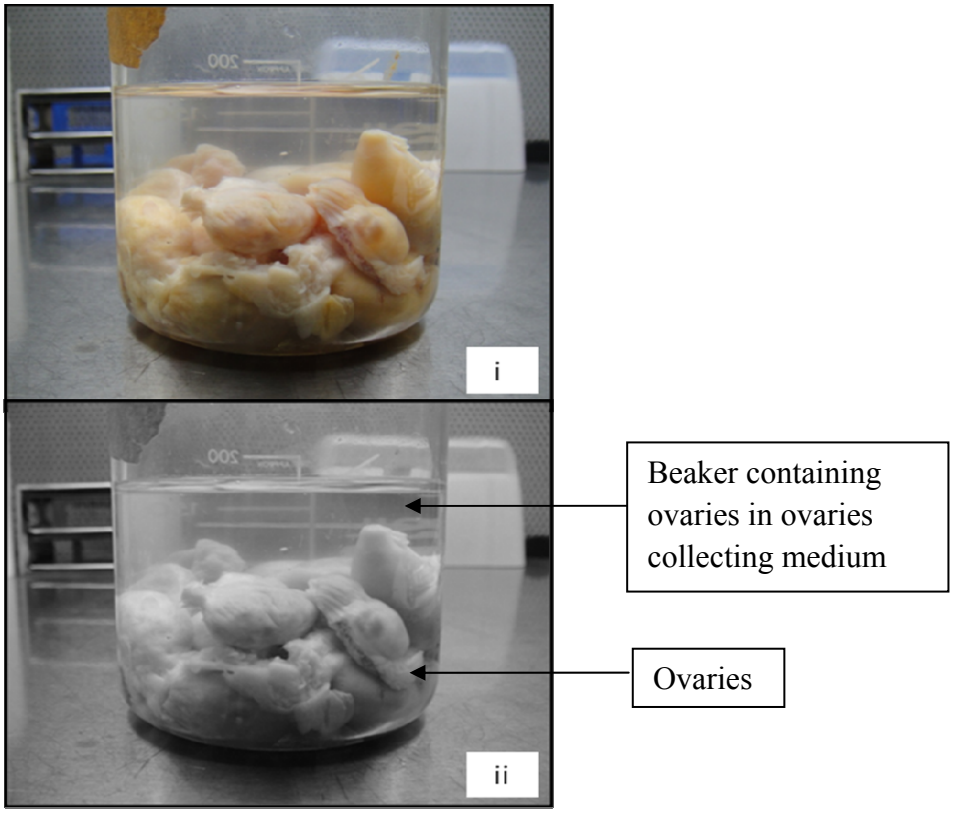

Figure 3.5: Ovaries collected from the local abattoir. (i) Original photograph. (ii) Labelled photograph.

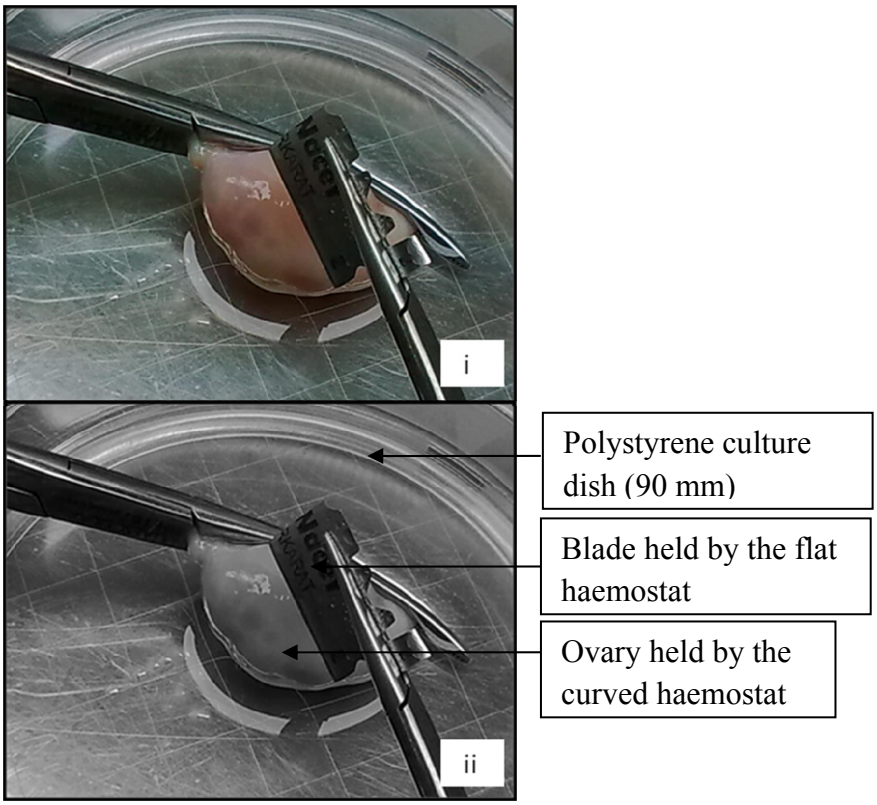

Figure 3.6: Slicing of ovary. (i) Original photograph. (ii) Labelled photograph.

3.4.5.2 *In vitro* maturation procedure

The IVM medium was prepared in microdroplets (80 µl for each droplet; 6 microdroplets for each polystyrene culture dish) and overlaid with equilibrated light mineral oil in a small polystyrene culture dish (35 mm). After that, placed in the CO_2 (5%) incubator at 38.5^{o}C in humidified atmosphere and equilibrated for at least 3 hours or overnight (12 hours) prior to use. After washing the collected COC, the selected oocytes were placed in IVM microdroplets (15 to 20 oocytes per each microdroplet). Typically, the oocytes surrounded by at least 3 layers of granulose cells were selected for IVM. The oocytes were cultured for 22 hours at 38.5^{o}C in presence of CO_2 (5%) in air in a humidified atmosphere of a CO_2 incubator.

3.4.6 Protocol of Interspecies Somatic Cell Nuclear Transfer (interspecies SCNT)

The interspecies SCNT procedure including preparation of micromanipulation dish, microneedles alignment, oocytes denuding, enucleation, nuclear transfer, electrofusion and activation has been described in the following subsections.

3.4.6.1 Preparation of micromanipulation dish

The micromanipulation dish was prepared on the cover of a polystyrene culture dish (35 mm). One microdroplet (10 µl) of TL-Hepes medium was placed on the centre, closed to the top of the dish for needles alignment. Three microdroplets (10 µl) of TL-Hepes medium with cytochalacin B was placed on the right side, closed to the centre of the dish. These microdroplets were used for the oocytes when doing enucleation. Another 3 microdroplets (10 µl) of TL-Hepes medium was placed on the left side, closed to the centre of the dish. These microdroplets were used for oocytes when doing the injection of donor cell. Then, 4 to 5 microdroplets (2 to 3 µl) of PVP or TL-Hepes medium (according to the treatments carried out) for donor cells were prepared on the left side of

TL-Hepes medium microdroplets. Lastly, the whole dish was overlaid with mineral oil as soon as possible and the oil should just over the microdroplets completely to avoid evaporation. Micromanipulation dish was equilibrated on the stage warmer (38.5^{o}C) (Figure 3.7).

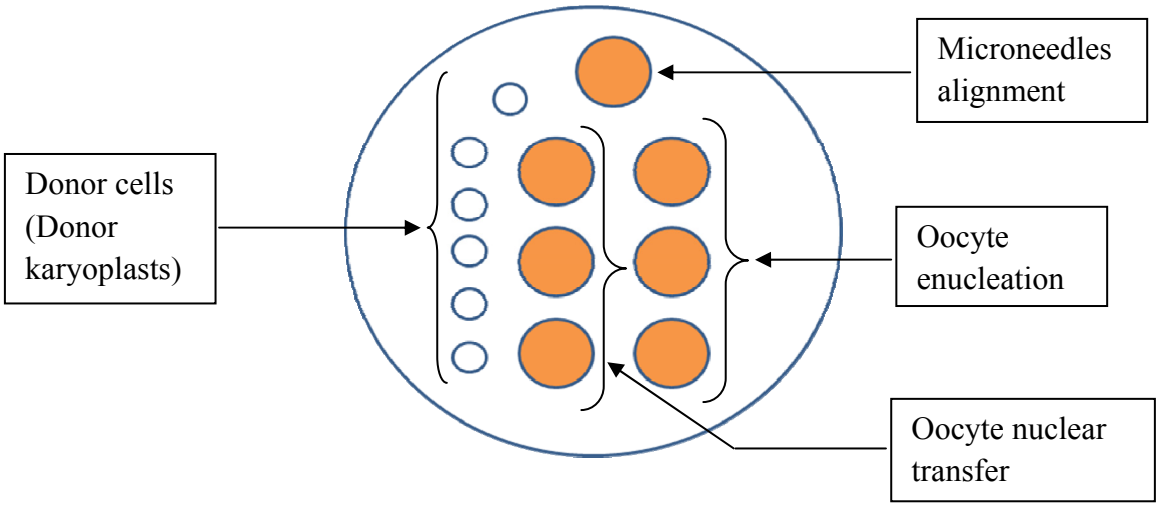

Figure 3.7: Arrangement of microdroplets on the micromanipulation dish.

3.4.6.2 Alignment of microneedles

In the present study, an alignment of microneedles on the micromanipulation dish was important and needed to ensure a smooth and easy performance of the interspecies SCNT. Firstly, all the knobs (X-, Y- and Z-control) and the syringes (3 ml) were adjusted to the centre of the scale. The micromanipulation dish was placed on the heated stage warmer with 38.5^{o}C of the micromanipulator. From the microscope (under 4x objective), focus the microdroplet with TL-Hepes medium that prepared for the needle alignment. The holding pipette was inserted to the needle holder (left micromanipulator), tightened well and placed above the TL-Hepes medium microdroplet. The tip of the holding pipette was touched in the oil and kept for few minutes so that the end of the tip

was filled with oil by capillary action. Then the edge of the TL-Hepes medium microdroplet was sharply focused, the needle was brought inside the droplet near the edge and aligned properly. After that, the cutting needle or injection pipette (according to the experiments needed) was inserted to the needle holder (right micromanipulator) and tightened well. Like the holding pipette, the tip of the injection pipette was touched in the oil and kept for few minutes so that the end of the tip was filled with oil by capillary action (except for the cutting needle). The cutting needle or injection pipette was focused with the holding pipette and was aligned so that the working tips were parallel to the microscope stage (under 4x objective). Finally, both the holding pipette and cutting needle or injection pipette were checked under high magnification to ensure the accurate alignment (sharply in focus) and parallel.

3.4.6.3 Preparation of oocytes for enucleation

After 22 hours of maturation, cumulus cell were removed from oocytes by repeated pipetting in TL-Hepes medium containing hyaluronidase (0.1%) within 5 minutes. These denuded oocytes were washed through 5 times in TL-Hepes medium to wash off the hyaluronidase and accessed for maturation under stereomicroscope by rotating the oocytes using mouthpiece-controlled pipette. Oocytes with a clear first polar body (PB-1) were considered as metaphase II (MII) stage and meiotic competent. These matured oocytes were then treated with TL-Hepes medium supplemented with cytochalasin B (5 μg/ml) for 10 minutes prior to enucleation.

3.4.6.4 Preparation of donor cells (donor karyoplasts)

Only the cultured donor cells reached in 70 to 80% of confluency were used as donor karyoplast in this study. Typically, PBS (-) and trypsin-EDTA were used to harvest the cultured donor cells from the culture dish. Washed twice in PBS (-) and incubate the donor cells for 3 to 5 minutes with trypsin-EDTA. Then, the donor cells with TL-Hepes medium were centrifuged to get a pellet form of donor cells. The unwanted supernatant medium was removed, and using a sterile disposable micropipette tips (100 µl), TL-Hepes medium (100 µl) was added into the pellet form of donor cells. Slowly and gently, the donor cells were mixed with the medium and prepared to use for injection.

3.4.6.5 Enucleation of the matured oocyte

In the present study, optimisation on enucleation techniques was carried out to improve the efficiency of interspecies cloned-caprine embryos: (i) Squeezing method and (ii) Aspiration method.

(i) Squeezing method:

Typically, all selected matured oocytes were transferred to the microdroplet containing TL-Hepes medium supplemented with cytochalasin B (5 µg/ml) of (enucleation medium) for 10 minutes before enucleation. All cytochalasin B treated matured oocytes were then transferred to the microdroplet (enucleation medium) on the micromanipulation dish for enucleation. Firstly, the first polar body of the matured oocyte was placed at 12 o'clock and achieved by rotating the oocyte at the tip of the holding needle with the cutting needle. While the oocyte was held firmly by the holding needle, a cut was made on the zona pellucida above the first polar body and 10% of the cytoplasm beneath the first polar body was gently squeezed out (Figure 3.8). All the steps in the process were repeated until all the oocytes were enucleated. The enucleated

oocytes were then washed 3 times in TL-Hepes medium, 3 times in waiting medium (TCM-199 medium supplemented with 10% FBS) and lastly kept in waiting medium at 38.5^{o}C in presence of CO_2 (5%) in air in a humidified atmosphere of a CO_2 incubator for 30 to 45 minutes prior to nuclear transfer.

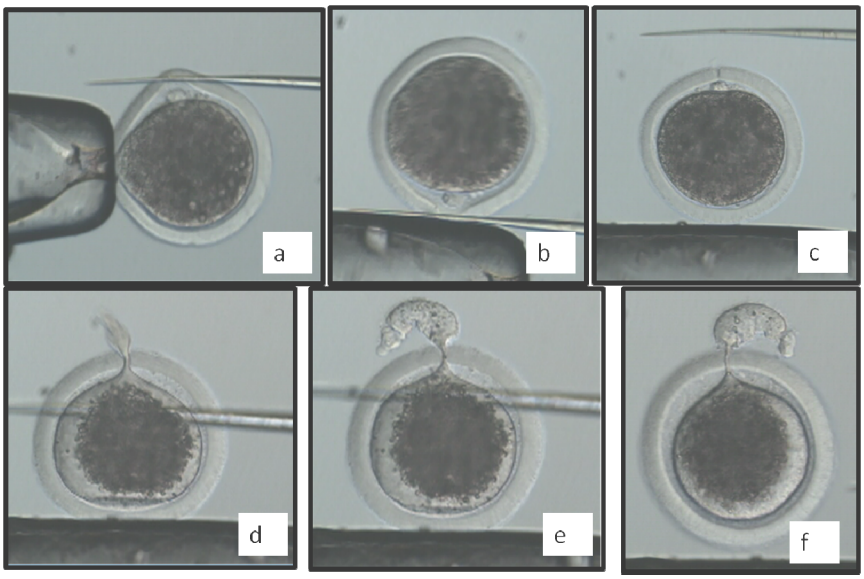

Figure 3.8: Oocyte enucleation by squeezing method. (a) A MII oocyte was held firmly by the holding pipette with PB-1 placed at 12 o'clock and a cut was made by the cutting needle; (b) The oocyte was released from the holding pipette and brought down to opposite side, few attritions were made between the cutting needle and holding pipette to form a cutting point; (c) The cutting point was placed at 12 o'clock (above the PB-1); (d) and (e) The PB-1 and cytoplasm (10%) beneath the PB-1 were gently squeezed out; and (f) Enucleated oocyte.

(ii) Aspiration method:

For aspiration method, both enucleation and nuclear transfer (sub-zonal injection) were done together. Typically, the donor cells were prepared before enucleation had been carried out. After harvested from the culture dish, the donor cells were placed in the microdroplet containing TL-Hepes medium. Subsequently, all selected matured oocytes were transferred to the microdroplet containing TL-Hepes medium supplemented with cytochalasin B (5 μg/ml) (enucleation medium) for 10 minutes before enucleation. All cytochalasin B treated matured oocytes were then transferred to the microdroplet (enucleation medium) on the micromanipulation dish for enucleation. The first polar body of the oocyte was placed at 4 o'clock and held by the holding pipette. The aspiration pipette, bevelled with spike (ID: 20 to 22 μm and OD: 22 to 24 μm), was slowly advanced through the zona pellucida and gently aspirated the polar body with 10% cytoplasm beneath the first polar body (Figure 3.9). After that, a single donor cell was placed at the perivitelline space by passing through the same cutting point (Figure 3.10). The injected oocytes were washed 3 times in TL-Hepes medium, 3 times in waiting medium and incubate in the final microdroplet of waiting medium for 30 to 45 minutes prior to fusion.

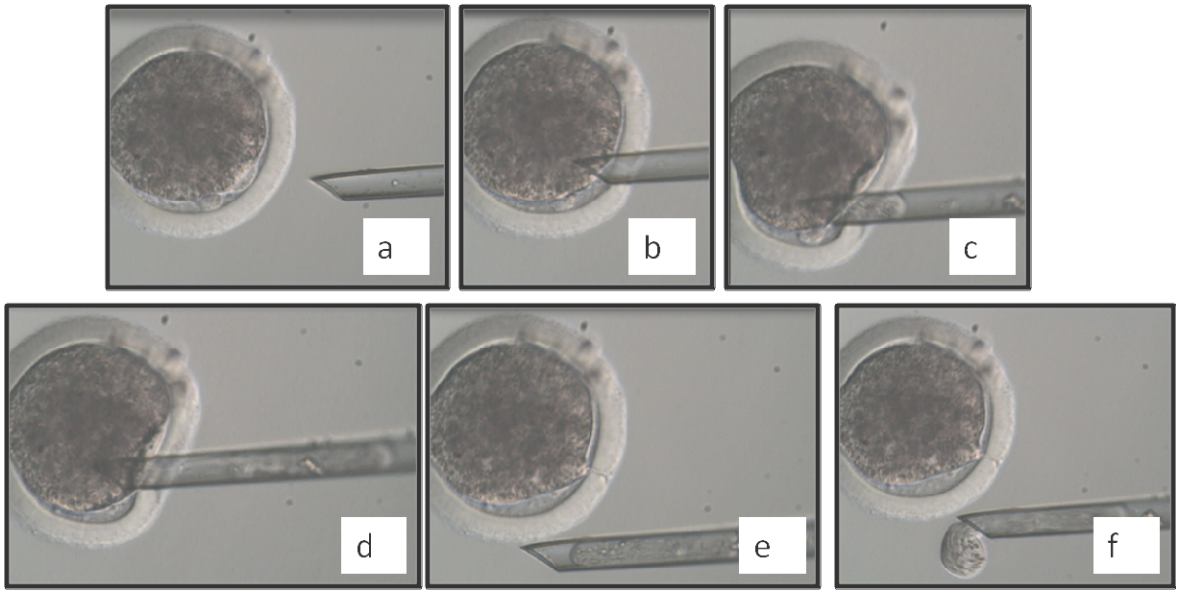

Figure 3.9: Oocyte enucleation by aspiration technique. (a) A MII oocyte was held firmly by the holding pipette with PB-1 placed at 5 o'clock; (b), (c) and (d) The aspiration pipette, beveled with spike (ID: 20 to 22 μm and OD: 22 to 24 μm), was slowly advanced through the zona pellucida and gently aspirates the polar body with 10% cytoplasm beneath the first polar body; (e) The aspiration needle was gently removed from the oocyte; (f) The aspirated PB-1 and cytoplasm was released from the aspiration needle.

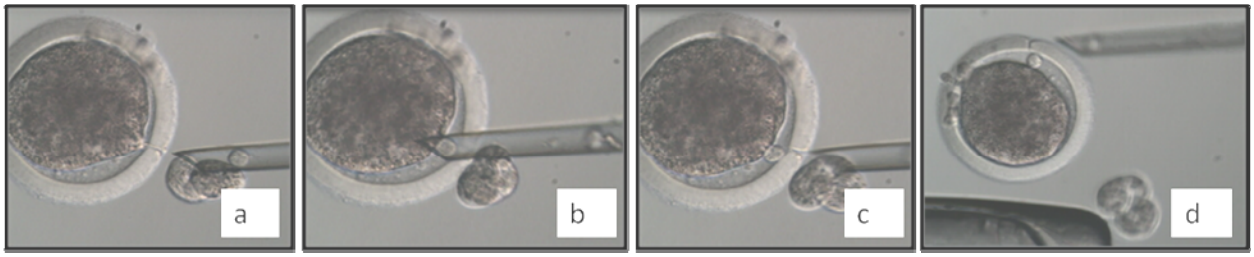

Figure 3.10: Nuclear transfer by sub-zonal injection technique. (a) Once the PB-1 and cytoplasm was released, followed, a single donor cell in the aspiration needle was brought to the tip of the needle; (b) The injection needle is pushed through the zona pellucida via the cutting point made during aspiration; (c) and (d) A single donor cell was placed at the perivitelline space of an enucleated oocyte.

3.4.6.6 Nuclear transfer of the enucleated oocyte

Besides that, an optimisation on nuclear transfer techniques was also carried out to improve the efficiency of interspecies cloned-caprine embryos: (i) Sub-zonal injection and (ii) Intracytoplasmic injection.

(i) Sub-zonal injection method:

For sub-zonal injection, the donor cells were placed in TL-Hepes medium microdroplets (2 to 3 µl). Briefly, after 30 to 45 minutes of enucleation (squeezing method), the incubated enucleated oocytyes were transferred and placed in TL-Hepes medium microdroplets (10 µl) of the micromanipulation dish. An injection needle with the donor cell was brought to the microdroplet containing oocyte. The cutting point that was made during enucleation was placed at 1 or 2 o'clock and achieved by rotating the oocyte at the tip of the holding pipette with the injection needle. While the oocyte was held firmly by the holding needle, the injection needle was brought near to the oocyte, gently passed through the cutting point and placed the donor cell at the perivitelline space (Figure 3.11). The injected oocytes were washed 3 times in TL-Hepes medium, 3 times in waiting medium and incubate in the final microdroplet of waiting medium for 30 to 45 minutes prior to fusion.

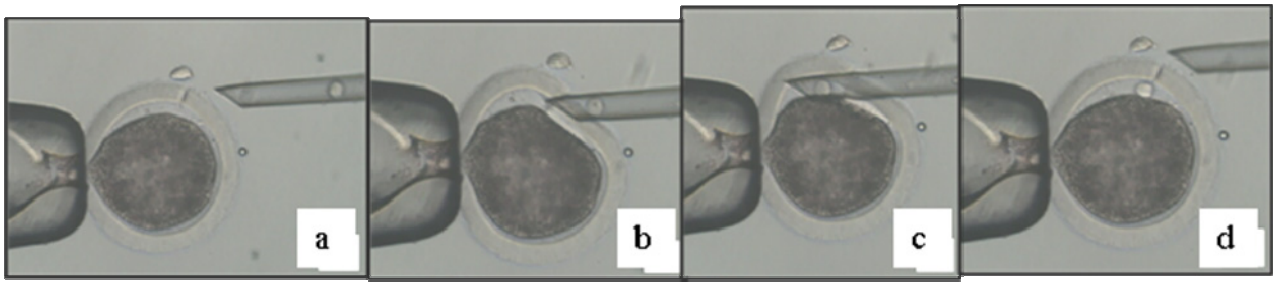

Figure 3.11: Nuclear transfer by sub-zonal injection. (a) An enucleated oocyte was held firmly by the holding pipette with cutting point placed at 1 to 2 o'clock; (b) The injection needle with single donor cell was brought near to the enucleated oocyte; (c) The injection needle is pushed through the zona pellucida via cutting point; (d) The donor cell was placed at the perivitelline space of the enucleated oocyte.

(ii) Intracytoplasmic injection method:

For intracytoplasmic injection method, the recipient oocytes were placed in the TL-Hepes medium microdroplets (10 µl) and the donor cells were placed in the PVP (10%) medium (2 to 3 µl). The plasma membrane of the donor cell was broke by pipette in and out few times in PVP (10%) medium using a blunt mouth injection needle (ID: 8 to 9 µm; OD: 9 to 10 µm). The donor cell will become elongated shape (Figure 3.12). The oocyte was held by the holding pipette and placing the cutting point at 3 o'clock. The donor cell in the injection needle was gently pushed towards the tips. The injection needle was slowly advanced through the zona pellucida and get into the end point of the cytoplasm (9 o'clock position). A small amount of cytoplasm was gently aspirated into the injection needle until a sudden flux of cytoplasm goes into the needle observed. This is to confirm the breakage of the plasma membrane, thereby facilitating donor cell injection. A single donor cell was then gently deposited into the cytoplasm. After the injection, the needle was gently removed and the oocyte was released from the holding

pipette (Figure 3.13). The injected oocytes were washed 3 times in TL-Hepes medium, 3 times in waiting medium and incubate in the final microdroplet of waiting medium for 30 to 45 minutes prior to activation.

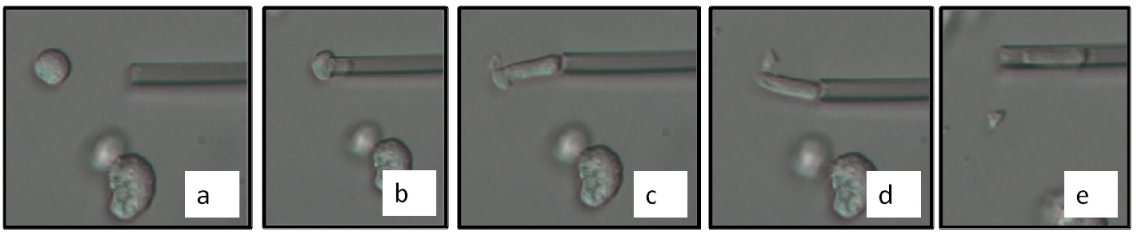

Figure 3.12: Removing of cell plasma membrane. (a) A blunt mouth injection needle with ID: 8 to 9 µm and OD: 9 to 10 µm was brought near to the donor cell; (b) The donor cell was aspirated into the injection needle slowly; (c) The donor cell was pipette in and out few times in 10% PVP medium; (d) and (e) The cell plasma membrane of donor cell was broke and become elongated shape.

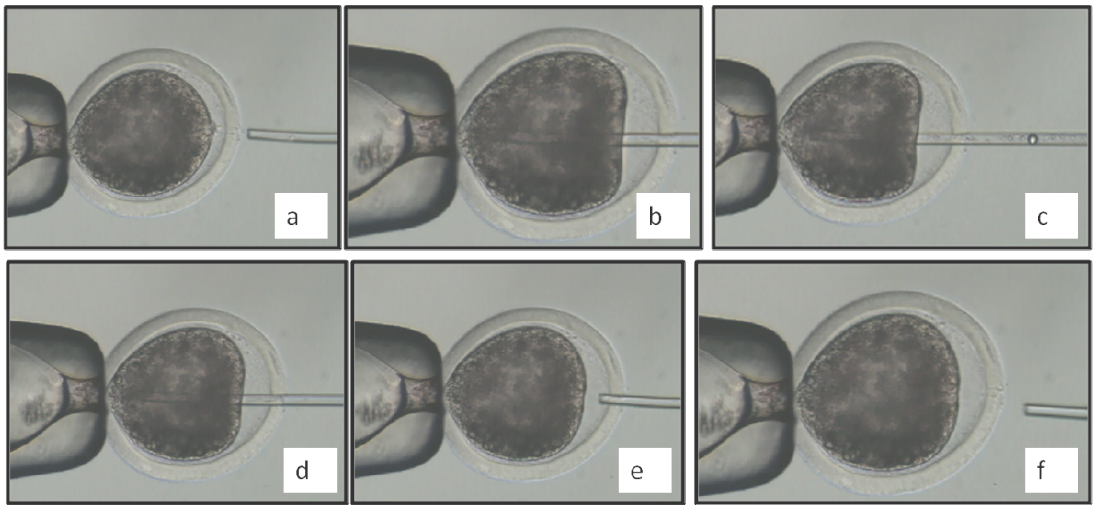

Figure 3.13: Nuclear transfer by intracytoplasmic injection technique. (a) The oocyte was held firmly by the holding pipette and the cutting point was placed at 3 o'clock; (b) The donor cell in the injection needle was gently pushed towards the tips. The injection needle was slowly advanced through the zona pellucida and get into the end point of the cytoplasm (9 o'clock position); (c) A small amount of cytoplasm was gently aspirated into the injection needle until a sudden flux of cytoplasm goes into the needle observed; (d) The aspirated cytoplasm and a single donor cell was then gently deposited back into the cytoplasm; (e) and (f) After the injection, the needle was gently removed.

3.4.6.7 Electrofusion

Only the sub-zonal injection oocytes were undergone electrofusion to fuse the donor karyoplast into the cytoplasm of the recipient oocytes. Typically, 2 microdroplets (50 µl) of fusion medium as described in Table 3.12 were prepared in a polystyrene culture dish (35 mm), and overlaid with mineral oil prior to fusion. Before fusion, all the oocytes were washed 3 times in the fusion medium (5 minutes). In the present study, the fusion machine used was namely SUTF-1, which was manufactured by Suranaree University of Technology, Thailand. The parameter of the fusion was 20 voltages, 2 direct current (DC) pulses and 15 µseconds. After fusion, all the fused oocytes were washed 5 times in TL-Hepes medium, 3 times in waiting medium followed by transferred into the final microdroplet of waiting medium and incubate for at least 30 to 45 minutes prior to activation.

3.4.6.8 Activation of the injected or fused oocytes

All the injected or fused oocytes were subjected to activation with calcium ionophore and 6-DMAP. Typically, the activation dish was prepared at least 3 hours before activating the oocytes and equilibrated in the CO_2 (5%) incubator at 38.5^{o}C in humidified atmosphere. Microdroplets of activation medium (80 µl) were prepared on a polystyrene culture dish (35 mm) and all the injected and fused oocytes (10 to 15 oocytes) were placed in each microdroplets overlaid with mineral oil to permit temperature and gas equilibration. All the injected or fused oocytes were activated in 5 µm of calcium ionophore inside the CO_2 (5%) incubator for 5 minutes. After that, the oocytes were wash 3 times in 6-DMAP microdroplets to wash off the calcium ionophore medium prior to culture in 6-DMAP for another 4 to 5 hours in CO_2 (5%) incubator.

3.4.7 *In Vitro* Culture (IVC) of Cloned Embryos

The IVC dish was prepared at least 3 hours before culture the reconstructed oocytes and equilibrated in the CO_2 (5%) incubator. Typically, microdroplets of embryo culture (80 µl) were prepared on a polystyrene culture dish (35 mm) and reconstructed oocytes (10 to 15 oocytes) were placed in each microdroplets overlaid with mineral oil. All the activated oocytes were washed 3 times in IVC medium before being transferred in the final IVC microdroplets under a humidified atmosphere of CO_2 (5%) in air at 38.5^{o}C. This is to wash off the activation medium that might affect the development of embryos. All reconstructed cloned-caprine embryos were culture in KSOM A for the first 3 days. In the present study, 3 different changing medium treatments (Experiment 3) were carried out on the combination of squeezing with sub-zonal injection method: a) Group 1 (KSOM A throughout culture), the embryos were observed and recorded on days 3, 5, 7 and 8 without changing the medium; Group 2 (KSOM A on days 1-3, change KSOM A on days 3 and 5), the embryos were observed and recorded on days 3, 5, 7 and 8; and Group 3 (KSOM A on days 1-3, change KSOM B on days 3 and 5), the embryos were observed and recorded on days 3, 5, 7 and 8. Correspondingly, the development of embryos *in vitro* was observed under inverted microscope, and the embryo numbers were recorded.

3.4.8 Assessment of Cloned Embryos using Hoechst Staining

The embryos that were cleaved *in vitro* were observed under an inverted microscope and recorded. The stages of embryonic development of the cloned embryos were assessed by using Hoechst 33342 staining to determine the number of nuclei in the blastomeres. For Hoechst 33342 staining protocol, briefly, the embryos were washed 5 times in PBS (-) (100 µl for each droplet without overlaid mineral oil) followed by 3 times in fixative solution (100 µl for each droplet without overlaid mineral oil) on a

heated stage of a stereomicroscope. Finally, the embryos were transferred to the last droplet of fixative solution for 5 minutes at room temperature (25^{o}C). While waiting, 4 drops of Vaseline-wax were placed on the centre of the slide. After 5 minutes, all the embryos were transferred on the glass slide and mounted with coverslip. The coverslip was sealed with adhesive (cutex), labelled on the side of the glass slide and kept in the refrigerator (4^{o}C) before being examined using an epifluorescent microscope.

3.4.9 Embryo Transfer

Oviduct embryo transfer (ET) was carried out in the recipient does with mid-ventral laporatomy surgery. A total of 5 laparotomy sessions were performed on 12 does. Four to 8 cell stage embryos were transferred at day 2 of IVC culture into the oviduct ipsilateral to the corpus luteum. Pregnancy was diagnosed at 30 days after ET using real-time ultrasound scanning.

3.4.9.1 Treatment of recipient

All the recipient does underwent oestrus synchronisation to manipulate the oestrous cycle for embryo transfer. Briefly, a controlled internal drug release device (EAZI BREED^{TM}, CIDR$^{®}$) was inserted into the vagina of the doe with the help of a clean CIDR applicator and a veterinary obstetrical lubricant (K-Y Jelly). The device contained progesterone (0.3 g) and remained in the vagina for 16 days before being removed. At approximately 8 hours prior to CIDR removal, PMSG (Folligon, 300 IU) was administered intramuscularly to stimulate a cohort of follicular growth for ovulation. At approximately 15 hours after CIDR removal, the recipient does were observed for the onset of oestrus behaviour such as switching of the tail, increase vaginal secretion and willingness to be mounted on by a teaser buck. The oestrus behaviour of the recipient

does was checked continuously 3 days after CIDR removal with 3 sessions (morning, 0830 hours; afternoon, 1330 hours and evening, 1830 hours) per day.

3.4.9.2 Disinfection of surgical instruments

On the day of surgery, all the surgical instruments and accessories used for embryo transfer such as paediatric grasper, fibre optic cable, light probe and scalpel were disinfected in Hibiscrub (10%) for 10 minutes and subsequently rinsed in RO water before being placed on a clean surgical trolley that was already covered with a sterile drape. While, the surgical set, trocar, embryo transfer tube and beakers were disinfected under UV light for 30 minutes.

3.4.9.3 Anaethesia and sedation of recipient does

The recipient does were off-fed for 18 to 24 hours prior to embryo transfer. On the day of performing the embryo transfer, anaethesia was induced with intramuscular administration of mixed Xylazine hydrochloride (1 ml) (Illium Xylazine-20) and Ketamine hydrochloride (50 ml) (Ketamil) (from the mixture, aspirated 1 ml for the first injection). After that, the doe was maintained under general anaethesia with Illium-Ketamine (0.5 ml) administered intramuscularly as maintenance doeses every 20 to 30 minutes or as required.

3.4.9.4 Disinfection of skin area of doe

When the recipient doe has been immobolised, it was fastened on a cradle (surgical table) at dorsal recumbence tilted at approximately 45^{o} angle. The abdominal and inguinal skins were shaven, scrubbed and cleaned with Hibiscrub (10%). Surgical iodine solution (weak iodine) was applied on the surgical surface before starting the

embryo transfer procedure. The recipient doe was then covered with sterile drape with an opening that revealed the shaved bare skin and was ready for embryo transfer.

3.4.9.5 Embryo transfer procedure

First of all, the ovaries were checked for the presence of at least 1 corpus luteum (CL) via laporoscopic procedure. Briefly, a small incision was made on the bare skin and the verrus needle attached to plastic tubing that was connected to a CO_2 tank via CO_2 gas insufflators units was inserted into the incision to create a pneumoperitoneum. Once the pneumoperitoneum was made, small incisions (3 to 5 mm) were made, one near the umbilicus to facilitate insertion of trocar for passing the laparoscope and one on the right side of lower-ventral abdomen to insert the trocar for passing the grasping forceps. The paediatric Storz laparoscope that was connected to the CCD camera was inserted into the abdominal cavity through the trocar sheath. Then a paediatric grasper was passed through the small trocar sheath. The ovary was visualised with the help of laparoscope and exposed by pulling the oviduct in different directions with the grasper. The number of visible corpus luteum on both ovaries was counted and recorded. After that, only the recipient does with the presence of corpus luteum (n = 1 to 3 corpus luteum) were undergoing embryo transfer. Generally, the verrus needle and trocars were removed from the body, a mid-ventral incision (approximately 5 to 6 mm) was made at the lower abdomen near the udder. The ovaries with corpus luteum were located and exteriorised. Embryo transfer was carried out with a customised embryo transfer catheter, a flexible polythene tubing, threaded through a 25-G hypodermic needle (Figure 3.14). Briefly, a 1 ml insulin syringe that was filled with EmCare medium was connected to the embryo transfer catheter. The embryo transfer catheter was then flushed with EmCare medium until 0.001 ml of the medium was left in the syringe. A small column of air was then aspirated into the catheter. Embryos (n=2 to 4, 4- to 8 cell

stage embryos) were then aspirated from the culture dish (35 mm) resulting in a 5 mm column containing the embryos and the medium. A further 5 mm air column was made. Embryos were transferred to the oviduct ipsilateral to the corpus luteum. The catheter was inserted through the ostium advancing the catheter as far as possible into the infundibulum of the oviduct and interspecies cloned-caprine embryos were gently released by completely depressing the plunger of the syringe (Figure 3.15). After transferred, the ovary was then returned back to the inner part of the body and the incisions on the abdomen were sutured; and finally the donor goat was carefully removed from the cradle. The sutured incision area was sprayed with antiseptic and insecticide containing cyphenothrin. The donor goat was administered with oxytetracycline (20 mg/kg body weight) via intramuscular injection once in 4 days within the duration of 2 weeks to prevent possible post-surgical infection.

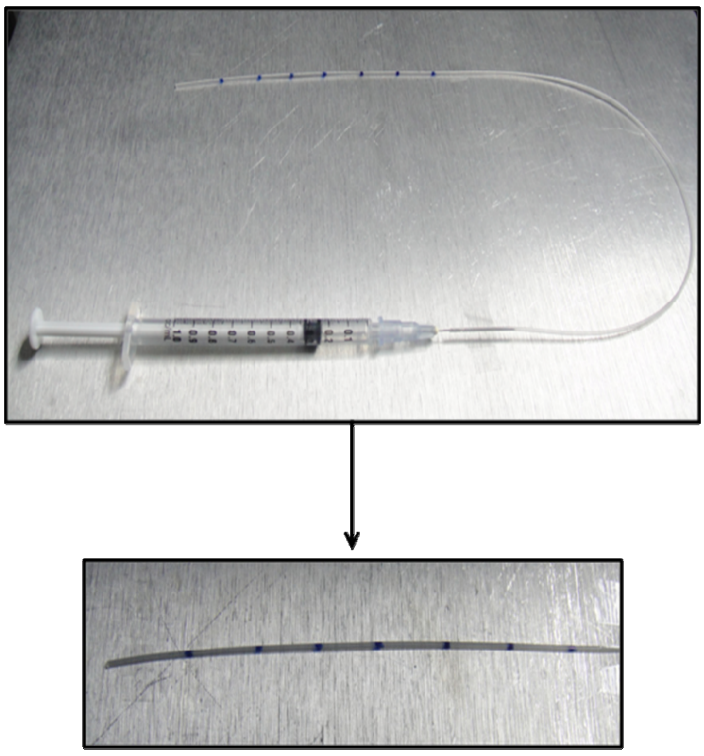

Figure 3.14: Embryo transfer tube.

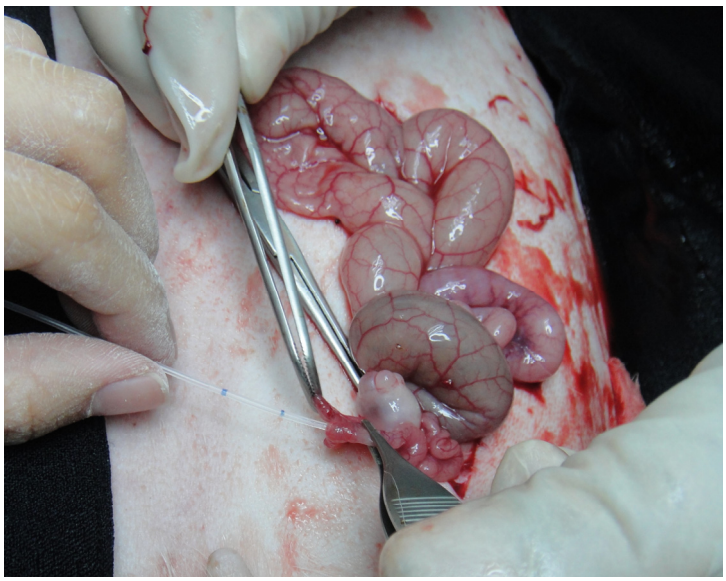

Figure 3.15: Transferring of embryos through oviduct.

3.4.10 Pregnancy Diagnosis

Pregnancy of the recipient does was diagnosed at 30 days after embryo transfer. A real-time ultrasound scanner (Aloka SSD500V, Tokyo, Japan) equipped with a 5.0 mHz linear array transducer for the transrectal approach was used. The coupling agent (contact fluid) for ultrasound transmission used was a carboxymethylcellulose gel.

3.5 EXPERIMENTAL DESIGN

The author attempted to produce *in vitro* caprine embryos through interspecies somatic cell nuclear transfer. The present study was divided into 4 experiments. The design of each experiment has been described in the following sections.

3.5.1 Effects of Enucleation Methods on *In Vitro* Cloned-Caprine Embryo Developmental Competence Following Interspecies SCNT (Experiment 1)

The objective of this experiment was to examine the effect of 2 enucleation methods on cloned-caprine embryo developmental competence after interspecies SCNT. A total of 69 bovine ovaries were collected from local abattoir. After recovery, the cumulus oocyte complexes were matured in IVM medium for 22 hours in the CO_2 incubator in presence of CO_2 (5%) at 38.5^{o}C with humidified atmosphere. The cumulus oocyte complexes were denuded by pipetting in and out using a micropipette (100 µl) in hyaluronidase (0.1%) for 5 minutes and then transferred to the TL-Hepes microdroplet to select the MII oocyte under stereomicroscope. The selected denuded MII oocytes were washed 3 times in TL-Hepes, 3 times in waiting medium and finally cultured in the CO_2 (5%) inbucator until enucleation could be performed. Prior to enucleation, the MII oocytes were treated with cytochalasin B (5 µg/ml) for 10 minutes. Two different enucleation methods were carried out, a) Group 1 (squeezing method): A cut was made on the zona pellucida above the first polar body and cytoplasm (10%) beneath the first polar body {containing metaphase II (MII) chromosomes} was gently squeezed out, b) Group 2 (aspiration method): A aspiration pipette, bevelled with spike (ID: 20 to 22 µm and OD: 22 to 24 µm), was slowly advanced through the zona pellucida and gently aspirated the polar body with cytoplasm (10%) beneath the first polar body. After enucleation, a donor cell was then injected into the perivitelline space of an enucleated oocyte. All injected oocytes were subjected to electrofusion with 20 voltages, 2 direct

current pulses and 15 μseconds. After fusion, all the fused oocytes were washed 5 times in TL-Hepes medium, 3 times in waiting medium followed by transferred into the final microdroplet of waiting medium and incubate for at least 30 to 45 minutes prior to oocyte activation. In this study, double activation procedure was carried out with culturing the fused oocytes in calcium ionophore for 5 minutes and then cultured in 6-DMAP for 4 to 5 hours. All the activated oocytes were finally cultured in KSOM A medium in CO_2 (5%) incubator for the first 3 days and culture medium was changed on days 3 and 5 with KSOM B. The embryos were checked when changing the culture medium and the developmental stages were recorded. The successfully enucleated, injected, reconstructed oocytes and developmental competence of embryos were recorded in percentages. Data were analysed by analysis of variance (ANOVA), followed by Duncan's multiple range tests (DMRT) to determine differences between the treatments.

3.5.2 Effects of Nuclear Transfer Methods on *In Vitro* Cloned-Caprine Embryo Developmental Competence Following Interspecies SCNT (Experiment 2)

This study was designed to compare the 2 nuclear transfer methods on cloned-caprine embryo developmental competence after interspecies SCNT. A total of 73 bovine ovaries were collected from local abattoir. After recovery, the cumulus oocyte complexes were matured in IVM medium for 22 hours in the CO_2 incubator in presence of CO_2 (5%) at 38.5^{o}C with humidified atmosphere. The cumulus oocyte complexes were denuded by pipetting in and out using a micropipette (100 μl) in hyaluronidase (0.1%) for 5 minutes and then transferred to the TL-Hepes microdroplet to select the MII oocyte under stereomicroscope. The selected denuded MII oocytes were washed 3 times in TL-Hepes, 3 times in waiting medium and finally cultured in the CO_2 inbucator until enucleation could be performed. Prior to enucleation, the MII oocytes were treated

with cytochalasin B (5 μg/ml) for 10 minutes. For enulceation, a cut was made on the zona pellucida above the first polar body and cytoplasm (10%) beneath the first polar body {containing metaphase II (MII) chromosomes} was gently squeezed out. After enucleation, a single donor cell was then injected into an enucleated oocyte either by sub-zonal injection (Group 1) or intracytoplasmic injection (Group 2). For Group 1, a donor cell was placed at the perivitelline space of an enucleated oocyte and then subjected to the electrofusion with 20 voltages, 2 direct current pulses and 15 μseconds. For Group 2, a single donor cell was gently deposited into the oocyte cytoplasm. After that, all the fused or injected oocytes were washed 5 times in TL-Hepes medium, 3 times in waiting medium followed by transferred into the final microdroplet of waiting medium and incubate for at least 30 to 45 minutes prior to activation. In this study, double activation procedure was carried out with cultured the fused oocytes in calcium ionophore for 5 minutes and then cultured in 6-DMAP for 4 to 5 hours. All the activated oocytes were finally cultured in KSOM A medium for the first 3 days and culture medium was changed on days 3 and 5 with KSOM B. The embryos were checked when changing the culture medium and the developmental stages were recorded. The successfully enucleated, injected, reconstructed oocytes and developmental competence of embryos were recorded in percentages. Data were analysed by analysis of variance (ANOVA), followed by Duncan's multiple range tests (DMRT) to determine differences between the treatments.

3.5.3 Effects of *In Vitro* Culture Medium on Cloned-Caprine Embryo Developmental Competence Following Interspecies SCNT (Experiment 3)

In this experiment, the effects of 3 *in vitro* culture media on cloned-caprine embryo developmental competence after interspecies SCNT were investigated. A total of 107 bovine ovaries were collected from local abattoir. After recovery, the cumulus oocyte complexes were matured in IVM medium for 22 hours in the CO_2 incubator in presence of CO_2 (5%) at 38.5^{o}C with humidified atmosphere. The cumulus oocyte complexes were denuded by pipetting in and out using a micropipette (100 µl) in hyaluronidase (0.1%) for 5 minutes and then transferred to the TL-Hepes microdroplet to select the MII oocyte under stereomicroscope. The selected denuded MII oocytes were washed 3 times in TL-Hepes, 3 times in waiting medium and finally cultured in the CO_2 inbucator until enucleation could be performed. Prior to enucleation, the MII oocytes were treated with cytochalasin B (5 µg/ml) for 10 minutes. For enulceation, a cut was made on the zona pellucida above the first polar body and cytoplasm (10%) beneath the first polar body {containing metaphase II (MII) chromosomes} was gently squeezed out. After enucleation, a donor cell was then injected into the perivitelline space of an enucleated oocyte. All injected oocytes were underwent electrofusion with 20 voltages, 2 direct current pulses and 15 µseconds. After fusion, all the fused oocytes were washed 5 times in TL-Hepes medium, 3 times in waiting medium followed by transferred into the final microdroplet of waiting medium and incubate for at least 30 to 45 minutes prior to activation. In this study, double activation procedure were carried out with cultured the fused oocytes in calcium ionophore for 5 minutes and then cultured in 6-DMAP for 4 to 5 hours. All reconstructed cloned-caprine embryos were culture in KSOM A for the first 3 days. In the present study, 3 different changing medium treatments were carried out: Group 1 (KSOM A throughout culture); Group 2 (KSOM A on days 1-3; change KSOM A on days 3 and 5); and Group 3 (KSOM A on days 1-3; change KSOM B on

days 3 and 5). The embryos were observed and recorded for the respective groups on days 3, 5, 7 and 8. The developmental competence of embryos for each culture medium treatments were recorded in percentages. Data were analysed by analysis of variance (ANOVA), followed by Duncan's multiple range tests (DMRT) to determine differences between the treatments.

3.5.4 An Attempt on Oviduct Embryo Transfer of Interspecies SCNT Embryos and Subsequent Pregnancy Diagnosis Using Ultrasound Scanning (Experiment 4)

This experiment was attempted to produce pregnancy via oviduct embryo transfer on cloned-caprine embryos obtained from interspecies SCNT technique. A total of 12 recipient does were subjected to oestrus synchronisation and superovulation. The oestrus was synchronised with the insertion of a CIDR device (progesterone, 0.3 g) for 16 days and at approximately 8 hours prior to CIDR removal, PMSG (Folligon, 300 IU) was administered intramuscularly to stimulate a cohort of follicular growth for ovulation. After 15 hours of CIDR removal, the recipient does were observed for the onset of oestrus behaviour such as switching of the tail, increase vaginal secretion and willingness to be mounted on by a teaser buck. The oestrus behaviour of the recipient does was checked continuously 3 days with 3 sessions (morning, 0830 hours; afternoon, 1330 hours and evening, 1830 hours) per day. On day 5 after CIDR removal, the recipient does were brought to the surgery room for embryo transfer procedure. Only the recipient does with the presence of corpus luteum (n = 1 to 3) were undergoing embryo transfer. On the other hand, the cloned-caprine embryos with 4- to 8 cell stage were transferred on day 2 of IVC culture into the recipient does by oviduct ipsilateral transfer. Pregnancy of the recipients was diagnosed at 30 days by real-time ultrasound scanning (SSD500V; Aloka, Japan). The successful ovulation (presence of corpus

luteum) and pregnancy rate were recorded in percentages. Data were analysed statistically to determine the significance of this experiment.

The experiments involved in this study were summarised and presented in the flow chart as illustrated in Figure 3.16.

3.6 STATISTICAL ANALYSIS

The effect of enucleation methods (squeezing and aspiration) on cloned-caprine embryo developmental competence (cleavage rate) following interspecies SCNT (Experiment 1); effect of nuclear transfer methods (sub-zonal injection and intracytoplamic injection) on cloned-caprine embryo developmental competence (cleavage rate) following interspecies SCNT (Experiment 2); and effect of *in vitro* culture medium (KSOM A and B) on cloned-caprine embryo developmental competence (cleavage rate) following interspecies SCNT (Experiment 3) were all analysed by using one-way analysis of variance (ANOVA). Using SPSS statistical programme, means were obtained and analysed using ANOVA and differences among the means were determined using DMRT. As for an attempt on oviduct embryo transfer and subsequent pregnancy diagnosis using ultrasound scanning (Experiment 4), the data were analysis statistically to evaluate the significance of this experiment.

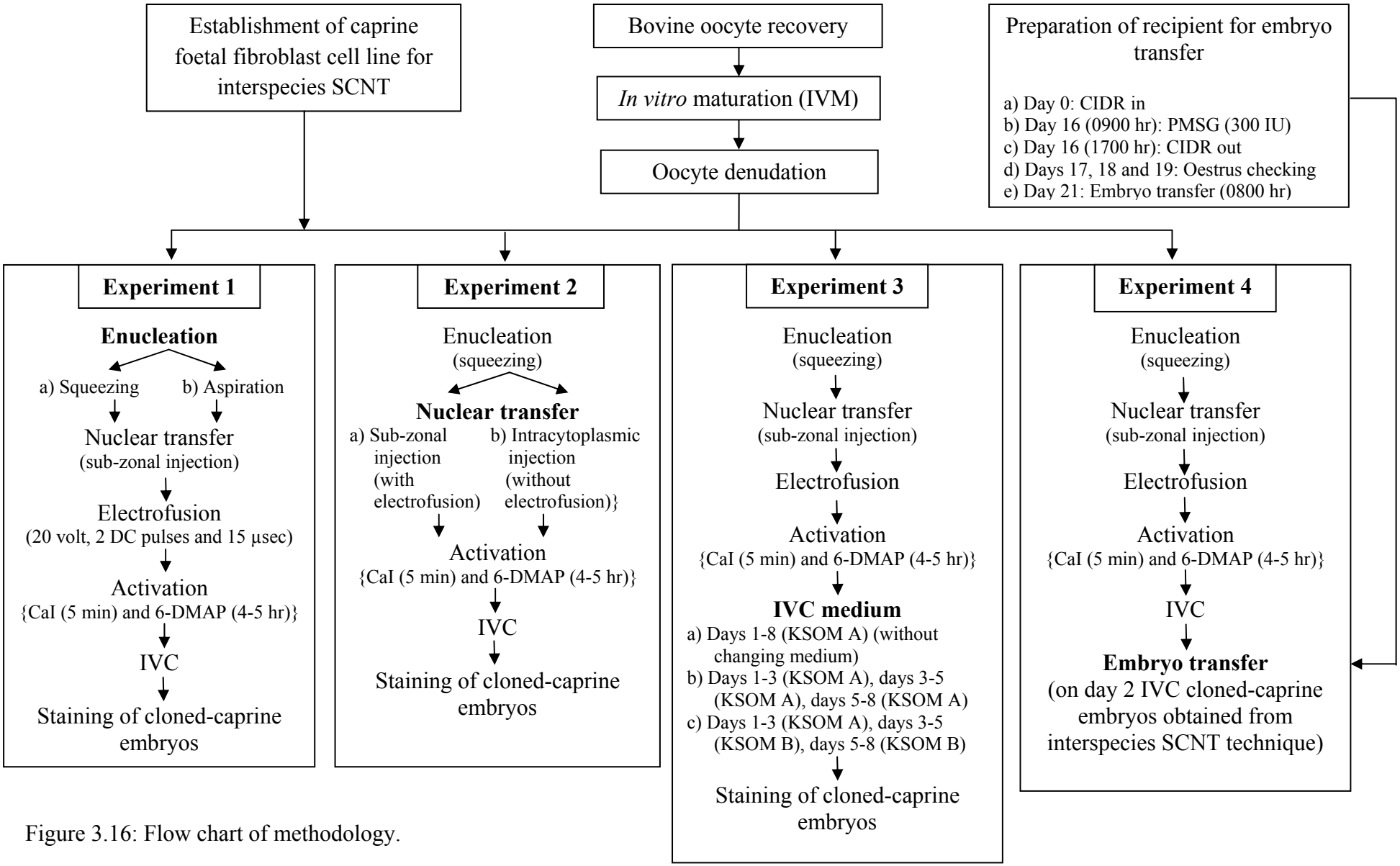

Figure 3.16: Flow chart of methodology.

Chapter 4

4.0 RESULTS

4.1 EFFECTS OF ENUCLEATION METHODS ON *IN VITRO* CLONED-CAPRINE EMBRYO DEVELOPMENTAL COMPETENCE FOLLOWING INTERSPECIES SCNT (EXPERIMENT 1)

This experiment was designed to compare the efficacy of 2 enucleation methods and the effects of enucleation methods on *in vitro* cloned-caprine embryo development competence following interspecies somatic cell nuclear transfer (interspecies SCNT). The rates of oocytes successfully enucleated, injected and duration needed were calculated and depicted in Table 4.1. The results of *in vitro* cloned-caprine embryo developmental competence obtained from both enucleation methods were analysed and presented in Table 4.2.

4.1.1 Effects of Enucleation Methods on the Manipulation Efficiency to the Production of Interspecies SCNT Cloned-caprine Embryos

A total of 765 cumulus oocyte complexes (COC) were recovered from 69 bovine ovaries (abattoir source) and matured *in vitro* for 20 to 22 hours (Table 4.1). Out of 765 COC, there were 414 (54.62±1.26%) matured oocytes indicated by the partial extrusion of first polar body. Squeezing and aspiration enucleation methods were carried out to evaluate their efficiencies on interspecies SCNT cloned-caprine embryos performance. The total percent successfully enucleated and injected oocytes were 89.84±1.79 and 96.63±1.62%, respectively.

There was no significant difference in the percent successfully enucleated oocytes ($P>0.05$) for both enucleation methods (squeezing vs. aspiration: 88.01±3.00%

vs. 91.68±1.92%, respectively). However, the percent successfully injected with donor karyoplasts after enucleation using aspiration method showed significantly higher ($P<0.05$) than the squeezing method (100.00±0.00 and 93.26±2.65%, respectively). In terms of manipulation efficiency, the duration needed to complete the enucleation together with injection (calculation was based on 30 oocytes used), the speed for aspiration with sub-zonal injection was significantly faster ($P<0.05$) than squeezing with sub-zonal injection (41.18±2.77 and 83.82±3.16 minutes, respectively). To standardise the experiment, sub-zonal injection method was chosen and used to transfer the donor karyoplast into an enucleated oocyte.

4.1.2 Percent *In Vitro* Embryo Development from Two Different Enucleation Methods in Interspecies SCNT

From Table 4.2, there were 314 (86.18±1.60%) successfully reconstructed couplets obtained after activation and subsequently cultured *in vitro* up to blastocyst stage. The total percent *in vitro* embryo development from 2- to 4-, 8 cell, morula and blastocyst stages for both enucleation methods were 60.86±2.32, 51.83±2.36, 38.46±2.58, 22.76±2.33 and 11.51±1.67%, respectively.

In *in vitro* embryo development, the percent interspecies SCNT cloned-caprine embryos from 2 cell stage up to blastocyst stage using squeezing and aspiration methods did not differ significantly ($P>0.05$). In all cases, the values of *in vitro* development of cloned-caprine embryos obtained from interspecies SCNT were apparently reduced from 2 cell stage up to blastocyst stage. For the squeezing method, there were significant differences in embryo development among the stages from 2 cell up to blastocyst ($P<0.05$). In the aspiration method, the *in vitro* cloned-caprine embryo development showed significant differences ($P<0.05$) among the embryo stages, except

for morula and blastocyst stages (P>0.05). Figure 4.1 shows the cleavage rates after interspecies SCNT for both enucleation methods. It was clearly shown that there were not significant differences (P>0.05) between these 2 enucleation methods.

Table 4.1: Successfully enucleated and injected rates (%, mean±SEM), and duration (min, mean±SEM) needed to complete the enucleation together with injection in interspecies SCNT cloned-caprine embryos

Type of enucleation methods	Total no. of ovaries	Total no. of oocytes	Percent of matured oocytes (MII)	Percent oocytes successfully enucleated (n)	* Percent oocytes successfully injected with donor karyoplast (n)	** Duration needed to complete enucleation with injection
Squeezing	36	360	56.78±2.14[a] (201/360)	88.01±3.00[a] (179/201)	93.26±2.65[a] (166/179)	83.82±3.16[b]
Aspiration	33	405	52.47±0.73[a] (213/405)	91.68±1.92[a] (197/213)	100.00±0.00[b] (197/197)	41.18±2.77[a]
Total	69	765	54.62±1.26 (414/765)	89.84±1.79 (376/414)	96.63±1.62 (363/376)	62.50±6.73

* The injection methods used in this experiment was standardised to sub-zonal injection method.
** Calculation was based on 30 oocytes used.
[ab] Means with different superscripts in a column were significantly different (P<0.05).

Table 4.2: Percentages (%, mean±SEM) of reconstructed couplets and *in vitro* embryo development for different enucleation methods in interspecies SCNT cloned-caprine embryos

Type of enucleation methods	Total no. of oocytes	Total no. of matured oocytes (MII)	Percent reconstructed couplets (n)	Percent cleaved interspecies SCNT cloned-caprine embryos at different cell stages (n)				
				2 cell	4 cell	8 cell	Morula	Blastocyst
Squeezing	360	201	87.52±1.57[a] (146/166)	60.18±2.43[az] (88/146)	53.80±2.84[az] (79/146)	37.71±3.30[ay] (56/146)	24.45±2.71[ax] (36/146)	12.08±2.95[aw] (18/146)
Aspiration	405	213	84.84±2.85[a] (168/197)	61.55±4.20[az] (96/168)	49.86±3.87[ay] (80/168)	39.22±4.26[ay] (63/168)	21.07±3.94[ax] (31/168)	10.93±1.87[ax] (13/168)
Total	765	414	86.18±1.60 (314/363)	60.86±2.32 (184/314)	51.83±2.36 (159/314)	38.46±2.58 (119/314)	22.76±2.33 (67/314)	11.51±1.67 (31/314)

[a] Means with different superscripts in a column were not significantly different (P>0.05).
[wxyz] Means with different superscripts in a row were significantly different (P<0.05).

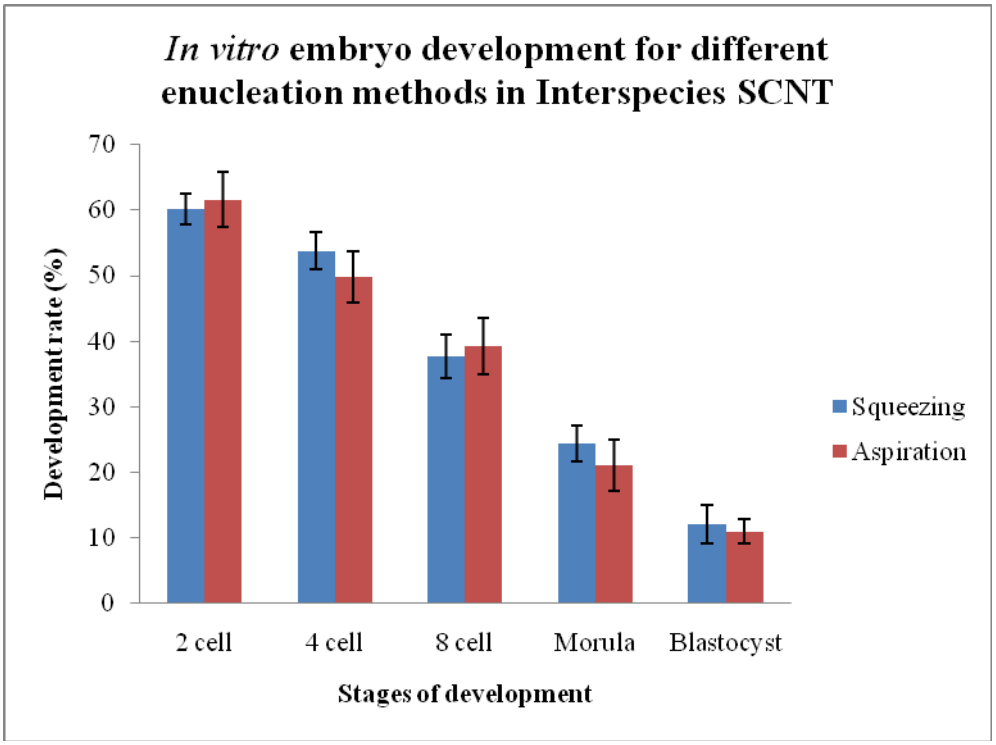

Figure 4.1: Comparison between two enucleation methods on the cleavage rates after interspecies SCNT.

4.2 EFFECTS OF NUCLEAR TRANSFER METHODS ON *IN VITRO* CLONED-CAPRINE EMBRYO DEVELOPMENTAL COMPETENCE FOLLOWING INTERSPECIES SCNT (EXPERIMENT 2)

This experiment was carried out to compare the effects of 2 nuclear transfer methods on the efficiency of successfully injected and reconstructed couplets as well as subsequent *in vitro* cloned-caprine embryo developmental competence following interspecies somatic cell nuclear transfer (interspecies SCNT). The percent oocytes successfully enucleated, injected and reconstructed were analysed and summarised in Table 4.3. The data on *in vitro* cloned-caprine embryo developmental competence obtained from both nuclear transfer methods were calculated and tabulated in Table 4.4.

4.2.1 Percent Oocytes Successfully Enucleated, Injected, Reconstructed Couplets and Cleaved Embryo Obtained from Two Nuclear Transfer Methods in Interspecies SCNT

A total of 725 cumulus oocyte complexes (COC) were recovered from 73 bovine ovaries (abattoir source) and subsequently matured *in vitro* for 20 to 22 hours (Table 4.3). There were 400 (54.76±1.70%) matured oocytes indicated by the partial extrusion of first polar body. Two different types of nuclear transfer methods {sub-zonal injection (SUZI) and intracytoplasmic injection (ICI)} were carried out to evaluate the efficiency of interspecies SCNT on production of cloned-caprine embryos.

The total percent successfully enucleated, injected and reconstructed couplets were 90.31±1.67, 91.80±1.71 and 89.67±1.43%, respectively. To standardise the experiment, squeezing method was chosen and used to remove the DNA materials from the oocytes for both nuclear transfer methods. There were no significant differences ($P>0.05$) in the injection and reconstruction rates for both nuclear transfer methods.

4.2.2 Percent *In Vitro* Embryo Development from Two Different Nuclear Transfer Methods in Interspecies SCNT

In Table 4.4, the total percent *in vitro* embryo development from 2- to 4-, 8 cell, morula and blastocyst stages for both nuclear transfer methods were 56.73±1.63, 45.12±2.69, 34.09±2.43, 19.65±1.78 and 7.75±1.78%, respectively. The percent cloned-caprine embryos obtained from interspecies SCNT at 2- and 8 cell using SUZI and ICI methods did not differ significantly ($P>0.05$). However, the percentages of cloned-caprine embryos at 4 cell, morula and blastocyst derived from SUZI method were significantly higher ($P<0.05$) compared to the ICI method (53.80±2.84% vs. 38.60±2.25%, 24.45±2.71% vs. 16.05±1.43% and 12.08±2.95% vs. 4.51±1.45%, respectively).

In all cases, there was a decline trend of *in vitro* cloned-caprine embryo development obtained from interspecies SCNT from 2 cell up to blastocyst stages. For the SUZI method, the *in vitro* cloned-caprine embryo development showed significant differences ($P<0.05$) among the embryo stages, except for 2- and 4 cell stages. For the ICI method, there were significant differences in embryo development among the stages from 2 cell up to blastocyst ($P<0.05$). Figure 4.2 shows the cleavage rates after interspecies SCNT for both nuclear transfer methods. It was clearly shown that the sub-zonal injection (SUZI) method when used in interspecies SCNT gave higher cleavage rates in all cases compared with intracytoplasmic injection (ICI) method.

Table 4.3: Successfully enucleated, injected, reconstructed couplets and embryos cleaved rates (%, mean±SEM) for different injection methods in interspecies SCNT cloned-caprine embryos

Type of injection methods	Total no. of ovaries	Total no. of oocytes	Percent of matured oocytes (MII)	* Percent oocytes successfully enucleated (n)	Percent oocytes successfully injected with donor karyoplast (n)	Percent reconstructed couplets (n)
Sub-zonal injection	36	360	56.78±2.14[a] (201/360)	88.00±3.00[a] (179/201)	93.26±2.65[a] (166/179)	87.52±1.57[a] (146/166)
Intracytoplasmic injection	37	365	53.24±2.49[a] (199/365)	92.04±1.80[a] (186/199)	90.70±2.30[a] (166/186)	91.28±2.11[a] (150/166)
Total	73	725	54.76±1.70 (400/725)	90.31±1.67 (365/400)	91.80±1.71 (332/365)	89.67±1.43 (296/332)

* The enucleation methods used in this experiment was standardised to squeezing method.
[a] Means with different superscripts in a column were not significantly different (P>0.05).

Table 4.4: Percentages (%, mean±SEM) of reconstructed couplets and *in vitro* embryo development for different injection methods in interspecies SCNT cloned-caprine embryos

Type of injection methods	Total no. of oocytes	Total no. of matured oocytes (MII)	Percent of reconstructed couplets (n)	Percent of cleaved interspecies SCNT cloned-caprine embryos at different cell stage (n)				
				2 cell	4 cell	8 cell	Morula	Blastocyst
Sub-zonal injection	360	201	87.52±1.57[a] (146/166)	60.18±2.43[az] (88/146)	53.80±2.84[bz] (79/146)	37.71±3.30[ay] (56/146)	24.45±2.71[bx] (36/146)	12.08±2.95[bw] (18/146)
Intracytoplasmic injection	365	199	91.28±2.11[a] (150/166)	54.14±1.79[az] (81/150)	38.60±2.25[ay] (57/150)	31.38±3.32[ax] (47/150)	16.05±1.43[aw] (23/150)	4.51±1.45[av] (6/150)
Total	725	400	89.67±1.43 (296/332)	56.73±1.63 (169/296)	45.12±2.69 (136/296)	34.09±2.43 (103/296)	19.65±1.78 (59/296)	7.75±1.78 (24/296)

[ab] Means with different superscripts in a column were significantly different (P<0.05).
[vwxyz] Means with different superscripts in a row were significantly different (P<0.05).

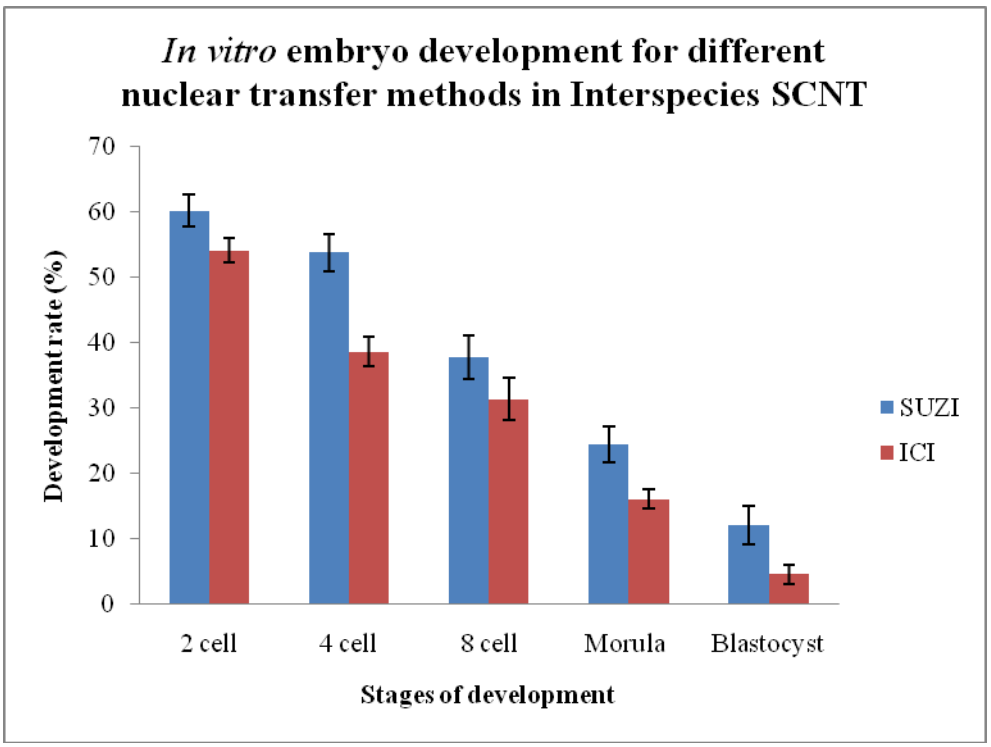

Figure 4.2: Comparison between two nuclear transfer methods on the cleavage rates after interspecies SCNT.

4.3 EFFECTS OF *IN VITRO* CULTURE MEDIUM ON CLONED-CAPRINE EMBRYO DEVELOPMENTAL COMPETENCE FOLLOWING INTERSPECIES SCNT (EXPERIMENT 3)

This experiment was to evaluate the effects of culture medium on the *in vitro* cloned-caprine embryo developmental competence following interspecies somatic cell nuclear transfer (interspecies SCNT). The percent oocytes successfully enucleated, injected and reconstructed rates are presented in Table 4.5. The effect of culture medium on *in vitro* cloned-caprine embryos developmental competence obtained from interspecies SCNT is shown in Table 4.6.

4.3.1 Percent Oocytes Successfully Enucleated, Injected and Reconstructed Couplets for Different *In Vitro* Culture Systems in Interspecies SCNT

A total of 1,050 cumulus oocyte complexes (COC) were recovered from 107 bovine ovaries (abattoir source) and subsequently matured *in vitro* for 20 to 22 hours. There were 594 (56.84±1.04%) matured oocytes indicated by the partial extrusion of first polar body. The total percent oocytes successfully enucleated, injected and reconstructed couplets were 90.95±1.31, 91.97±1.19 and 86.96±0.95%, respectively. There were no significant differences ($P>0.05$) in the enucleation, injection and reconstruction rates ($P>0.05$) for all stages of manipulation methods in interspecies SCNT.

4.3.2 Percent *In Vitro* Embryo Development for Different *In Vitro* Culture Systems in Interspecies SCNT

For the *in vitro* development, there were 434 (86.96±0.95%) successfully reconstructed couplets which were further divided into 3 groups and subsequently cultured up to

blastocyst stage. The total percent *in vitro* embryo development from 2- to 4-, 8 cell, morula and blastocyst were 53.01±1.88, 43.47±2.37, 31.17±1.74, 14.36±2.00 and 4.03±1.66%, respectively (Table 4.6).

From the results obtained, Group 3 showed significantly higher ($P<0.05$) in the *in vitro* development competence from 2 cell up to blastocyst stages compared to Groups 1 and 2. There was no significant difference ($P>0.05$) in the *in vitro* cloned-caprine embryo development from 2 cell up to blastocyst stages between Groups 1 and 2.

In all cases, the cleavage rates of *in vitro* cloned-caprine embryos obtained from interspecies SCNT were successively reduced from 2 cell stage up to blastocyst stage. For the Groups 1 and 2, there were significant differences ($P<0.05$) in embryo development among the stages from 2 cell up to morula. In Group 3, the *in vitro* development of interspecies SCNT cloned-caprine embryos showed significant differences ($P<0.05$) among the embryo stages, except for 2- and 4 cell stages. Figure 4.3 shows the cleavage rates after interspecies SCNT for different *in vitro* culture systems. It was clearly shown that the Group 3 was significant higher ($P<0.05$) cleavage rates in all cases compared with Groups 1 and 2. Morphology of COC and mature oocytes and donor karyoplast are shown in Figures 4.4 and 4.5, respectively. The nuclear transfer method and subsequent embryo development are depicted in Figures 4.6 and 4.7, respectively. In addition, detailed embryo development for each stage, blastocyst stage and nucleus staining of the embryos are presented in Figures 4.8, 4.9 and 4.10, respectively.

Table 4.5: Successfully enucleated, injected and reconstructed couplets rates (%, mean±SEM) for different *in vitro* culture systems in interspecies SCNT cloned-caprine embryos

Groups	Total no. of ovaries	Total no. of oocytes	Percent matured oocytes (n)	Percent oocytes successfully enucleated (n)	Percent oocytes successfully injected with donor karyoplast (n)	Percent reconstructed couplets (n)
1	33	395	57.86±1.09[a] (229/395)	91.47±1.14[a] (210/229)	90.97±1.17[a] (191/210)	87.57±1.51[a] (168/191)
2	38	295	55.87±2.18[a] (164/295)	93.38±2.05[a] (153/164)	91.69±2.35[a] (142/153)	85.80±2.01[a] (120/142)
3	36	360	56.78±2.14[a] (201/360)	88.01±3.00[a] (179/201)	93.26±2.65[a] (166/179)	87.52±1.57[a] (146/166)
Total	107	1,050	56.84±1.04 (594/1,050)	90.95±1.31 (542/594)	91.97±1.19 (499/542)	86.96±0.95 (434/499)

* Culture system groups: Group 1 (KSOM A; no medium changing),
Group 2 {KSOM A (day 0-3), KSOM A (day 3-9); change medium on day 3 and 5}, and
Group 3 {KSOM A (day 0-3), KSOM B (day 3-9); change medium on day 3 and 5}

** All embryos cultured were observed only on day 3, day 5, day 7, day 8 and day 9.

*** KSOM A : KSOM

**** KSOM B : KSOM additional supplemented with glucose

[ab] Means with different superscripts in a column were significantly different ($P<0.05$).

Table 4.6: Percentages (%, mean±SEM) of reconstructed couplets and *in vitro* embryo development for different *in vitro* culture systems in interspecies SCNT cloned-caprine embryos

Groups	Total no. of oocytes	Total no. of matured oocytes (MII)	Percent reconstructed couplets (n)	Percent cleaved interspecies SCNT cloned-caprine embryos at different cell stage (n)				
				2 cell	4 cell	8 cell	Morula	Blastocyst
1	395	229	87.57±1.51^{a} (168/191)	49.01±2.02^{az} (81/168)	36.92±3.02^{ay} (61/168)	26.46±1.74^{ax} (44/168)	8.42±0.47^{aw} (14/168)	0.00±0.00^{av} (0/168)
2	295	164	85.80±2.01^{a} (120/142)	49.85±3.27^{az} (60/120)	39.68±2.72^{ay} (49/120)	29.34±1.87^{ax} (36/120)	10.22±1.49^{aw} (13/120)	0.00±0.00^{av} (0/120)
3	360	201	87.52±1.57^{a} (146/166)	60.18±2.43^{bz} (88/146)	53.80±2.84^{bz} (79/146)	37.71±3.30^{by} (56/146)	24.45±2.71^{bx} (36/146)	12.07±2.95^{bw} (18/146)
Total	1,050	594	86.96±0.95 (434/499)	53.01±1.88 (229/434)	43.47±2.37 (189/434)	31.17±1.74 (136/434)	14.36±2.00 (63/434)	4.03±1.66 (18/434)

* Culture system groups: Group 1 (KSOM A; no medium changing),
Group 2 {KSOM A (day 0-3), KSOM A (day 3-9); change medium on day 3 and 5}, and
Group 3 {KSOM A (day 0-3), KSOM B (day 3-9); change medium on day 3 and 5}

** All embryos cultured were observed only on day 3, day 5, day 7, day 8 and day 9.

*** KSOM A : KSOM

**** KSOM B : KSOM additional supplemented with glucose

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

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

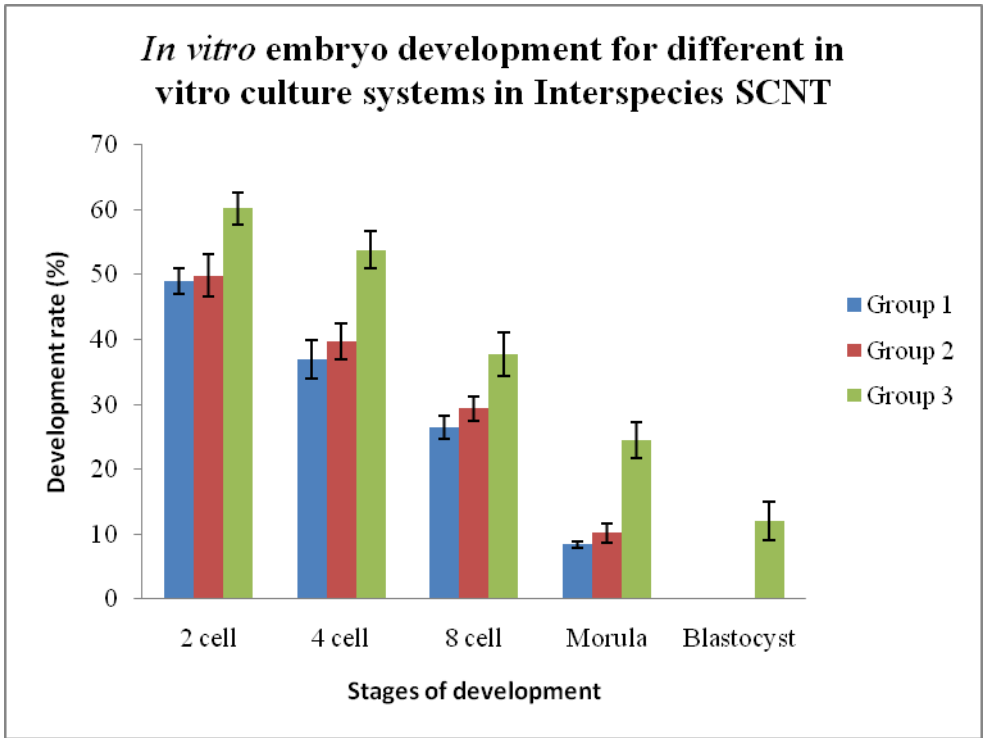

Figure 4.3: Comparison between different *in vitro* culture systems on the cleavage rates after interspecies SCNT.

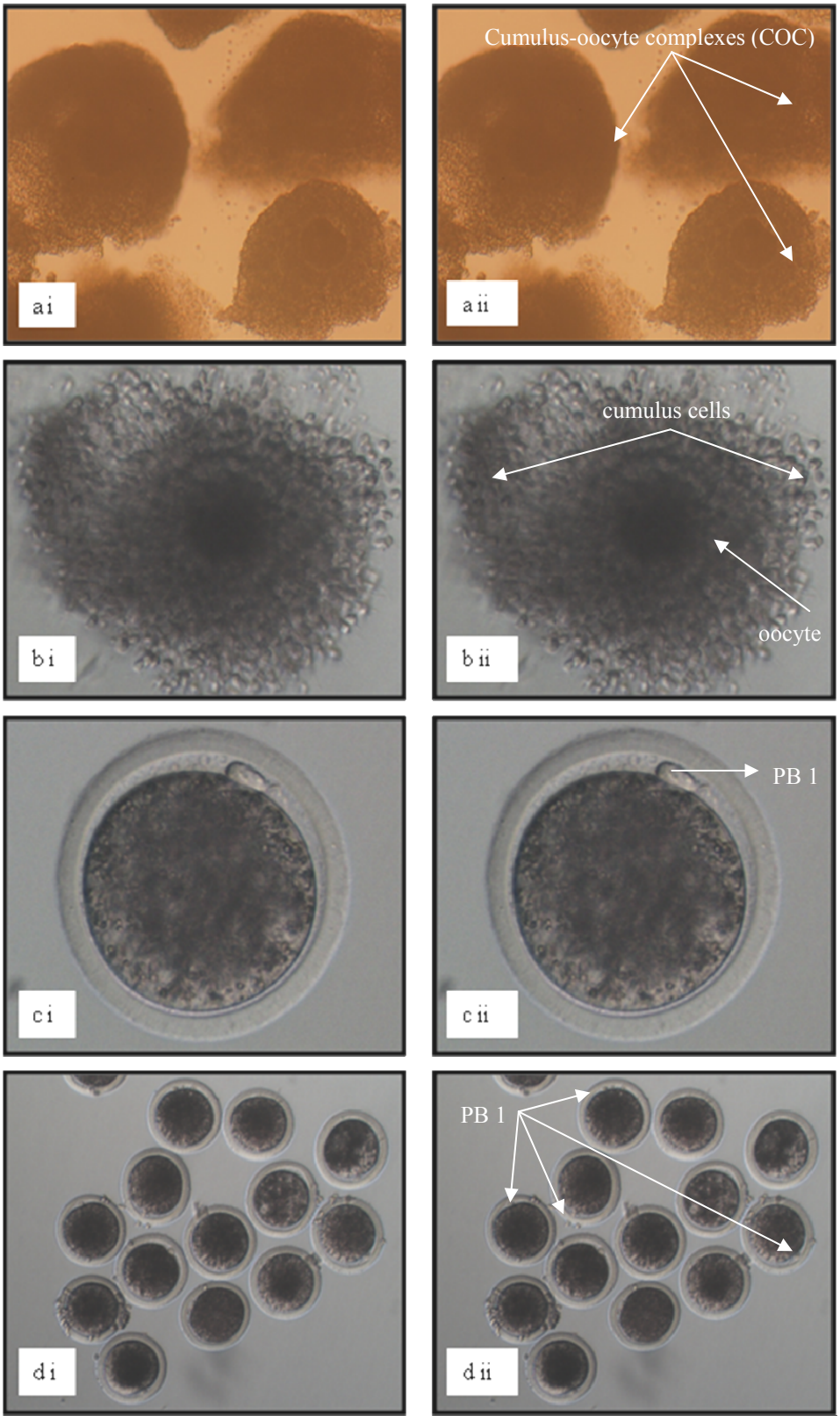

Figure 4.4: Morphology of COC and mature oocytes (i- original photomicrograph, ii- labeled photomicrograph). (a) COC before *in vitro* maturation, (b) COC after *in vitro* maturation with sunburst formation, (c, d) matured oocyte with the extrusion of polar body.

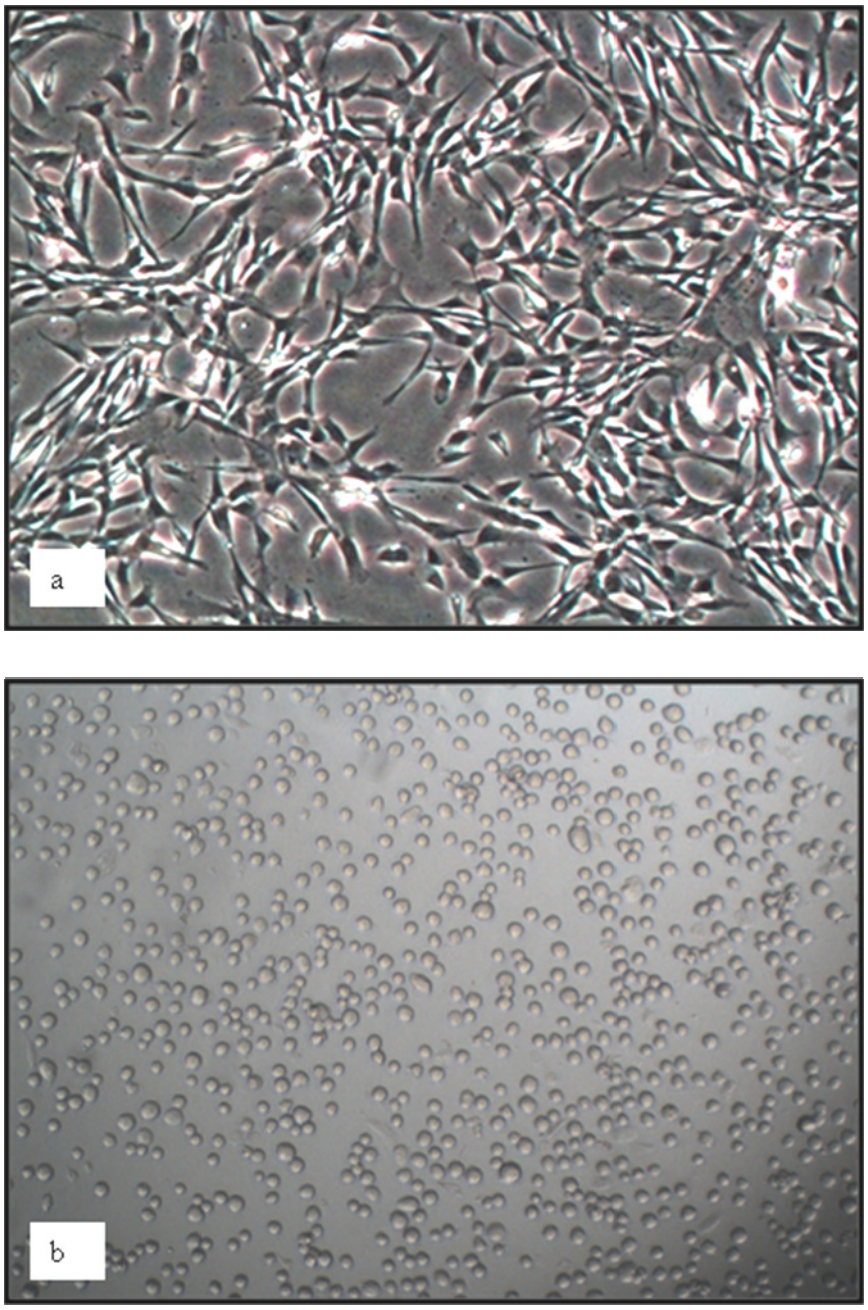

Figure 4.5: Morphology of donor karyoplast. (a) Foetal fibroblast cell line at day 2 (60% of confluency) and (b) Single foetal fibroblast cell.

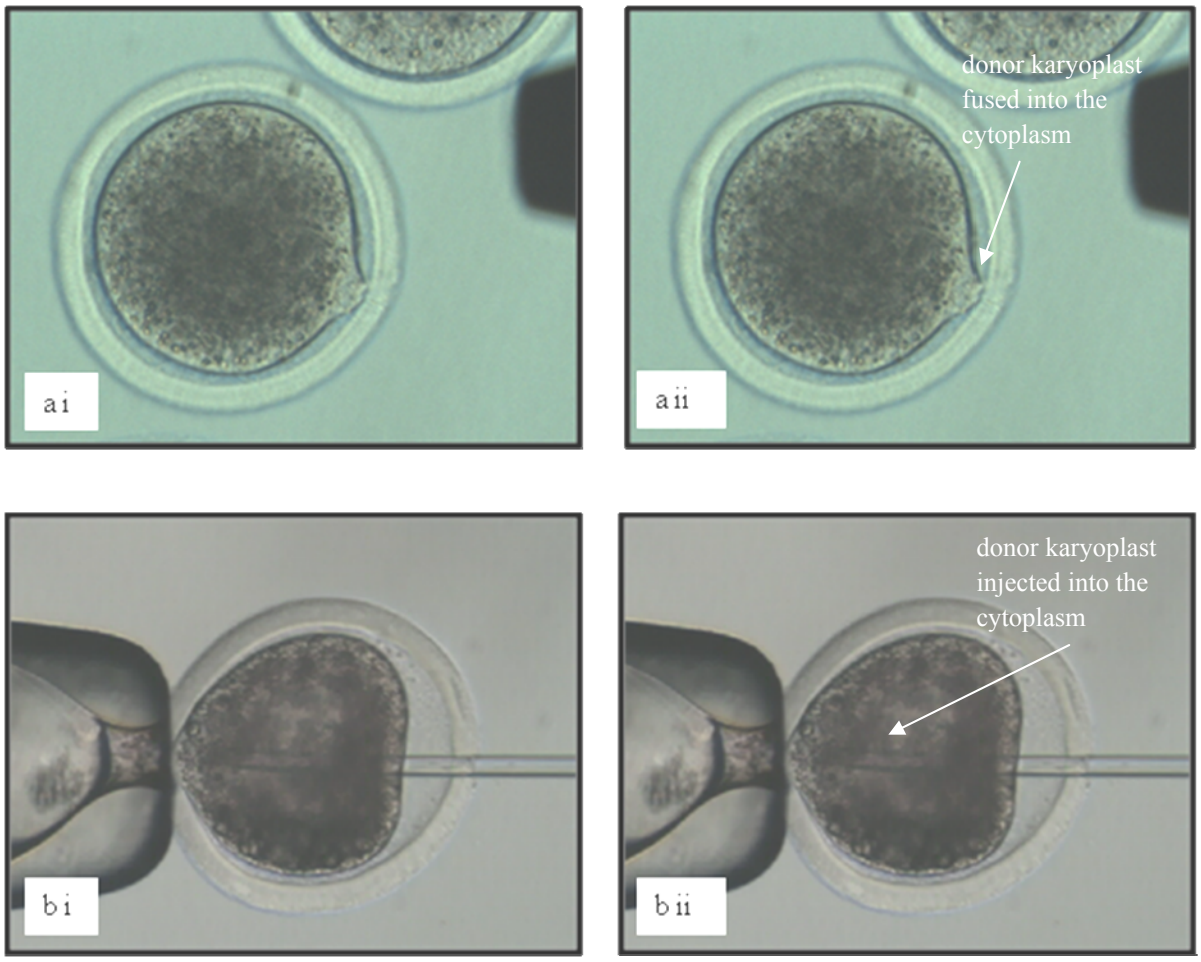

Figure 4.6: Nuclear transfer methods (i- original photomicrograph, ii- labeled photomicrograph). (a) The donor karyoplast is fused into the oocyte cytoplasm using the electrical pulse after sub-zonal injection, and (b) The donor karyoplast is injected into the oocyte cytoplasm by intracytoplasmic injection method.

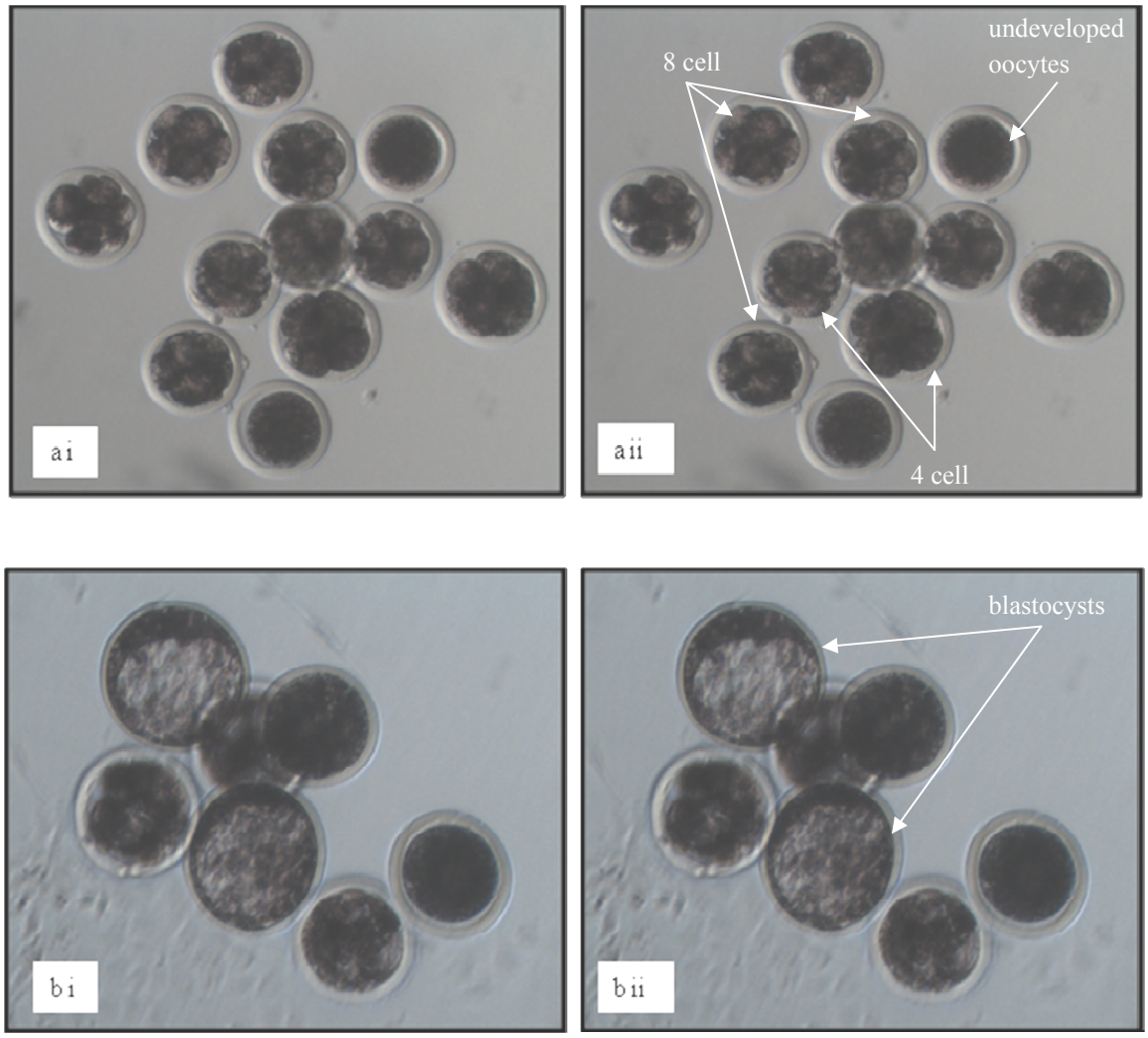

Figure 4.7: *In vitro* embryo development (i- original photomicrograph, ii- labeled photomicrograph). (a) interspecies SCNT cloned-caprine embryos at day 3 (4- to 8 cell stages), and (b) interspecies SCNT cloned-caprine embryos at day 7 (blastocyst stage).

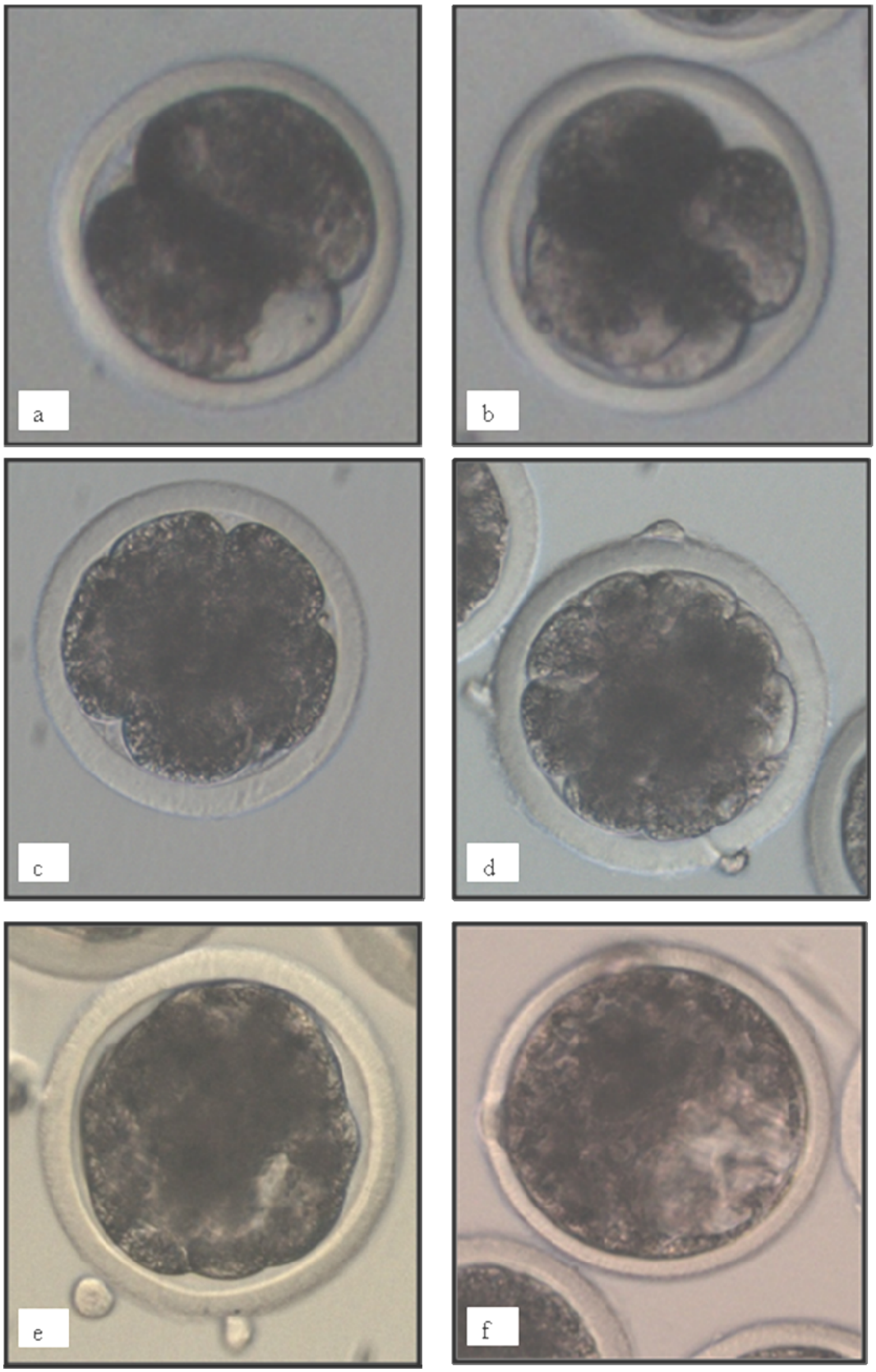

Figure 4.8: Developmental stages *in vitro* of cloned-caprine embryos after interspecies SCNT. (a) 2 cell embryo, (b) 4 cell embryo, (c) 8 cell embryo, (d) 16 cell or early morula, (e) Compact morula, and (f) Early blastocyst.

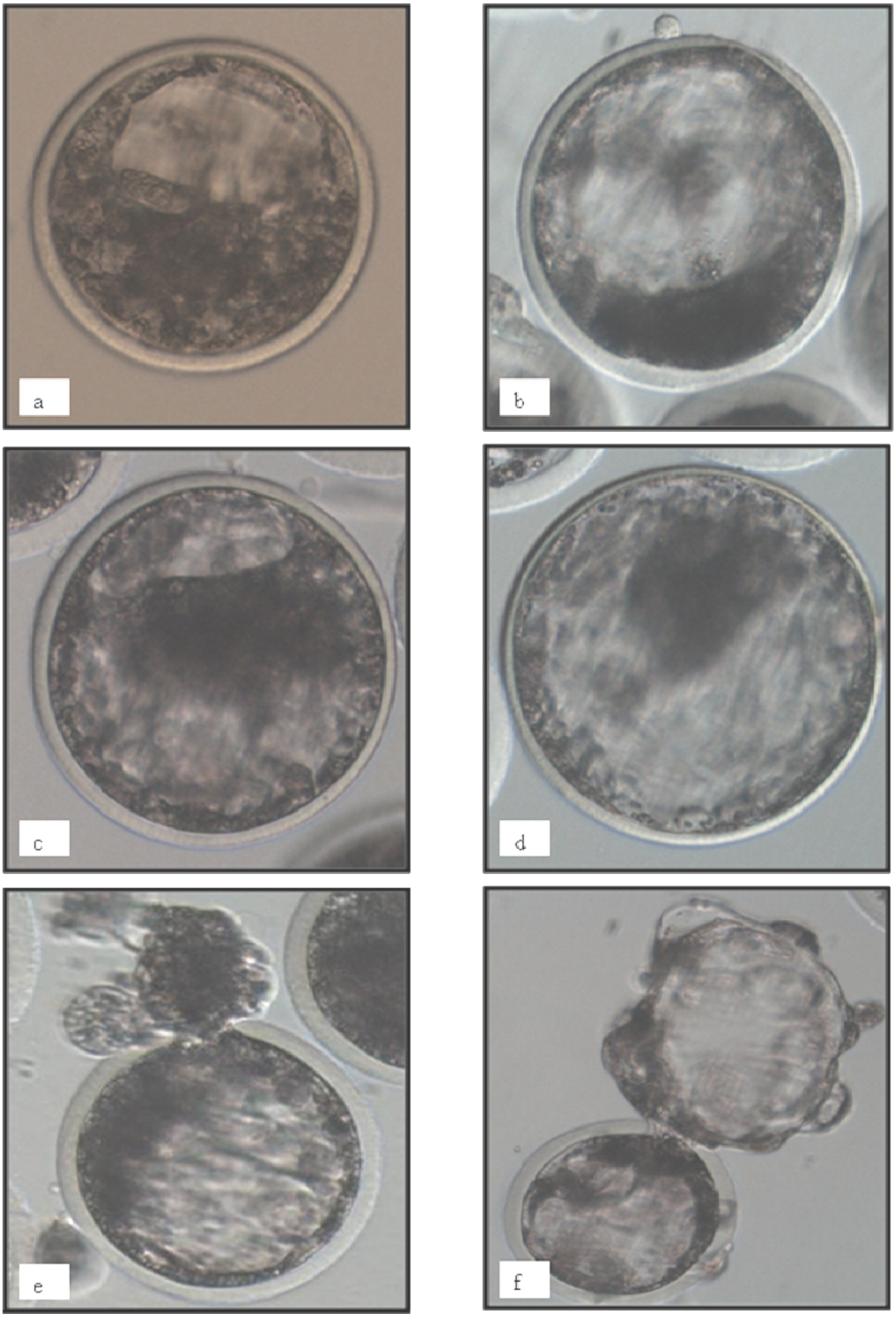

Figure 4.9: *In vitro* development of blastocyst at different stages. (a) Mid blastocyst, (b and c) Late blastocyst, (d) Compacting blastocyst, (e and f) Hatching blastocyst.

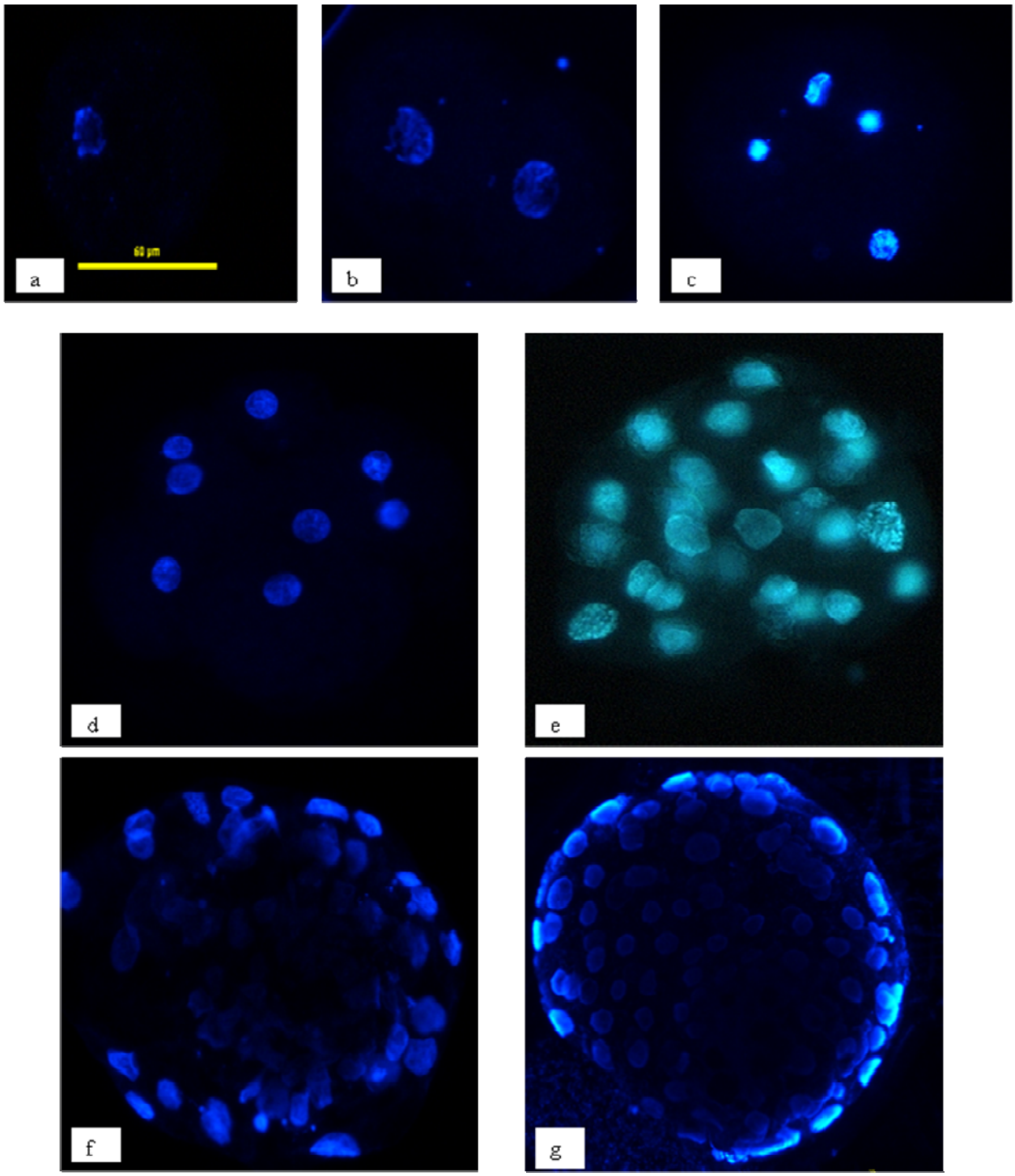

Figure 4.10: Hoechst staining. (a) DNA materials after enucleation, (b) 2 cell, (c) 4 cell, (d) 8 cell, (e) Morula, (f) Early blastocyst, and (g) Mid blastocyst.

4.4 AN ATTEMPT ON OVIDUCT EMBRYO TRANSFER OF INTERSPECIES SCNT EMBRYOS AND SUBSEQUENT PREGNANCY DIAGNOSIS USING ULTRASOUND SCANNING (EXPERIMENT 4)

This experiment was an attempt to produce pregnancy after oviduct embryo transfer of interspecies SCNT embryos and subsequent pregnancy diagnosis using ultrasound scanning. The percent oocytes successfully enucleated, injected, reconstructed and cleaved embryos rates are presented in Table 4.7. The number of embryo transfer replicates was showed in Table 4.8.

4.4.1 Percent Oocytes Successfully Enucleated, Injected, Reconstructed Couplets and Cleaved Embryos Rates for Embryo Transfer

A total of 265 cumulus oocyte complexes (COC) were recovered from 20 bovine ovaries (abattoir source) and subsequently matured *in vitro* for 20 to 22 hours. There were 153 (57.74%) matured oocytes indicated by the partial extrusion of first polar body. The total percent oocytes successfully enucleated, injected, reconstructed couplets and cleaved embryos rates were 92.16, 89.36, 88.10 and 56.76 %, respectively.

4.4.2 Embryo Transfer of Interspecies SCNT Cloned-caprine Embryos

A total of 63 cloned-caprine embryos were obtained from interspecies SCNT experiment. However, only 55 embryos with quality at grades 1 and 2 (4- and 8 cell stages) were chosen and transferred into 9 recipients with at least 1 CL observed in the ovaries (Figure 4.11). After ultrasound scanning on day 30, there is not pregnancy observed on the recipient goats after embryo transfer experiment (Figure 4.12). As a reference, Figure 4.13 shows the positive image of pregnancy in a doe as visualised via transrectal ultrasonography on day 30 (Mohd Nizam, 2011).

Table 4.7: Successfully enucleated, injected, reconstructed couplets and cleaved embryos rates (%) for an attempt of embryo transfer

No. of replicates	Total no. of ovaries	Total no. of oocytes	Percent matured oocytes (n)	Percent oocytes successfully enucleated (n)	Percent oocytes successfully injected with donor karyoplast (n)	Percent reconstructed couplets (n)	*Percent cleaved embryos on day 2 (n)
1	4	60	55.00 (33/60)	90.91 (30/33)	86.67 (26/30)	84.62 (22/26)	54.55 (12/22)
2	5	65	53.85 (35/65)	94.29 (33/35)	87.88 (29/33)	86.21 (25/29)	56.00 (14/25)
3	5	60	58.33 (35/60)	91.43 (32/35)	87.50 (28/32)	85.71 (24/28)	54.17 (13/24)
4	6	80	62.50 50/80	92.00 (46/50)	93.48 (43/46)	93.02 (40/43)	60.00 (24/40)
Total	20	265	57.74 (153/265)	92.16 (141/153)	89.36 (126/141)	88.10 (111/126)	56.76 (63/111)

* Cleaved embryos on day 2, included 4- to 8 cell.

Table 4.8: Embryo transfer of Interspecies SCNT cloned-caprine embryos

No. of replicates	Recipient underwent synchronisation*	No. of corpus luteum		No. of embryos transferred		Grading of embryos				Pregnancy rate
						4 cell		8 cell		
		Left	Right	Left	Right	A	B	A	B	
1	B0201	3	0	2	2	1	-	1	2	0
	0103	0	1	4	4	3	1	2	2	0
2	0134	1	0	2	2	1	-	2	1	0
	0154	2	0	2	2	2	1	1	-	0
3	0140	0	2	-	4	1	1	2	-	0
	B0183	1	1	3	3	1	-	3	2	0
	B0175	2	1	3	2	1	-	3	1	0
4	0032	1	2	5	5	2	-	5	3	0
	5240	1	2	5	5	-	-	7	3	0
	Total	11	9	26	29	12	3	26	14	0

* Nine out of 12 synchronised recipient goats showed the presence of CL.

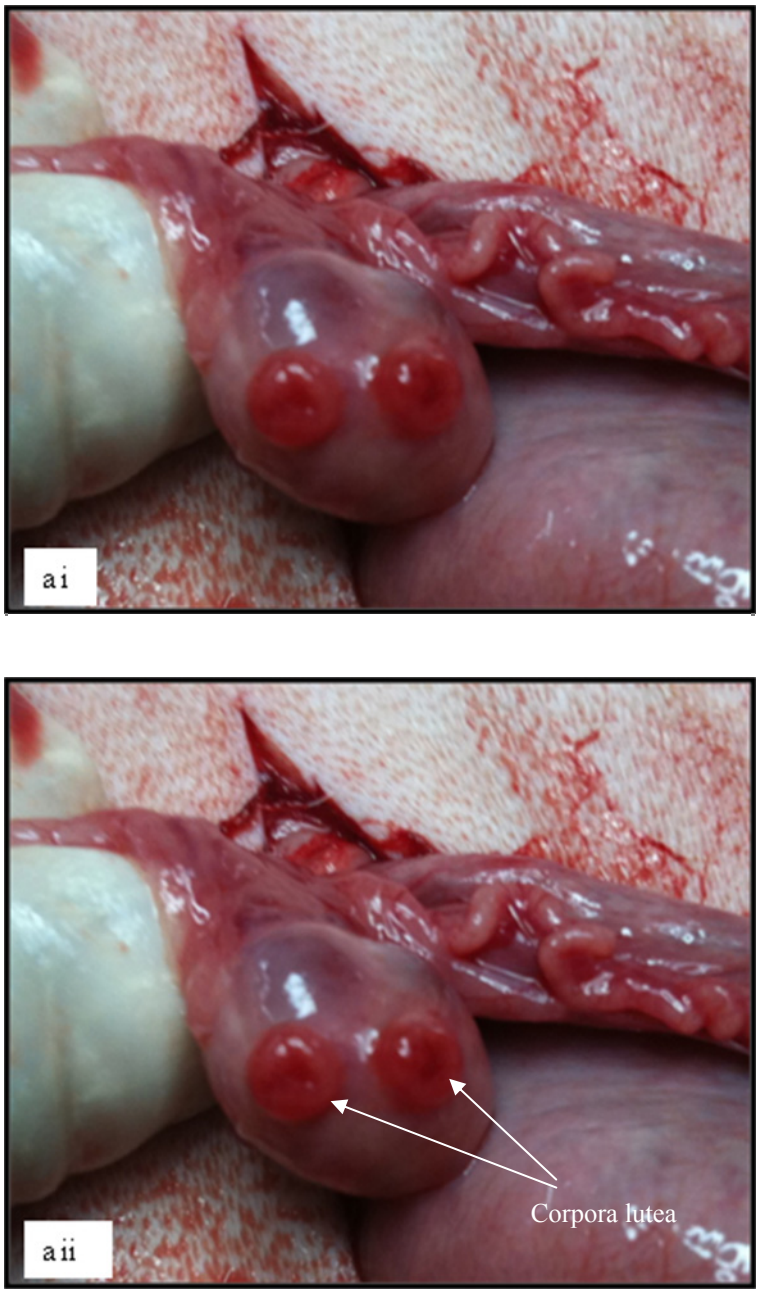

Figure 4.11: Corpora lutea (i- original photomicrograph, ii- labelled photomicrograph). (a) ovary with the presence of CL.

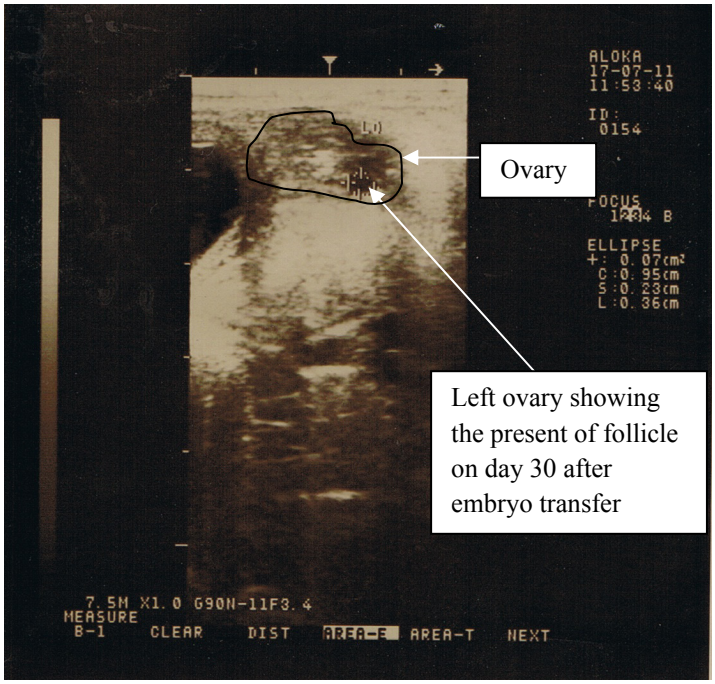

Figure 4.12: Original image of an open doe via transrectal ultrasonography. No pregnancy related structure (such as umbilical cord and foetal heart) was detected (left ovary) on day 30 after embryo transfer. Ovary with follicle could be detected easily.

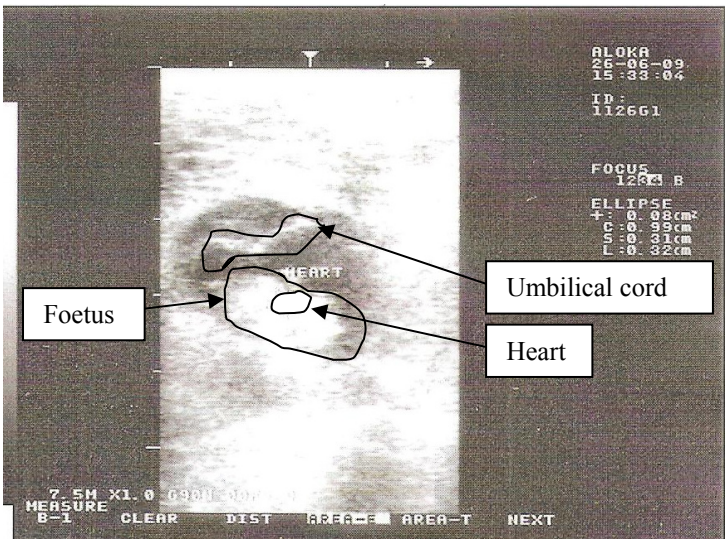

Figure 4.13: Example image of pregnancy doe as visualised via transrectal ultrasonography (day 30 of pregnancy). Pregnancy related structure (such as umbilical cord and foetal heart) was detected (image adapted from Mohd Nizam B. Abd. Rashid, 2011).

Chapter 5

5.0 DISCUSSION

5.1 INTRODUCTION

Somatic cell nuclear transfer (SCNT) is a new reproductive technique that has great potentials to increase the number of a species at a rapid rate as an effort to prevent extinction of endangered species, to enhance the propagation and sustaining the current levels of genetics biodiversity, to multiply livestock animals for human animal protein consumption as well as to produce proteins, cells, tissues and organs for biomedical therapeutic purposes. Live offspring have been successfully produced by intraspecies SCNT in numerous species of animals including sheep (Wilmut *et al*., 1997), mouse (Wakayama *et al*., 1998), cattle (Cibelli *et al*., 1998), goat (Baguisi *et al*., 1999), pig (Polejaeva *et al*., 2000), horse (Galli *et al*., 2003), dog (Lee *et al*., 2005) and cat (Yin *et al*., 2007); however, the successful birth rate of SCNT is limited ranging from 5 to 15%. As for the new approach using interspecies SCNT, the live offspring delivered is particularly only in the closely related species with the success rate ranges from 1 to 8%, represented by argali-sheep (White *et al*., 1999), gaur-cattle (Lanza *et al*., 2000), European mouflon-sheep (Loi *et al*., 2001), African wild cat-domestic cat (Gomez *et al*., 2004) and river buffalo-swamp buffalo (Yang *et al*., 2010). Other successful interspecies SCNT experiments only being reported up to *in vitro* embryo development, which included buffalo-cattle {Kitiyanant *et al*., 2001 (blastocyst: 33%)}, giant panda-rabbit {Chen *et al*., 2002 (blastocyst: 19%)}, banteng-cattle {Sansinena *et al*., 2005 (blastocyst: 15 to 28%)}, yak-cattle {Li *et al*., 2006 (blastocyst: 28%)}, pig-cattle {Uhm *et al*., 2007 (blastocyst: 4%)}, sheep-cattle {Dominko *et al*., 1999 (blastocyst: 18%)}, goat-sheep {Ma *et al*., 2008 (blastocyst: 7%)}, dog-pig {Sugimura *et al*., 2009

(blastocyst: 6 to 52%)}, marbled cat-domestic cat {Thongphakdee *et al*., 2010 (blastocyst: 5%)} and goat-cattle {Song *et al*., 2008 (blastocyst: 8%); Abdullah *et al*., 2011 (morula: 7%); Soh *et al*., 2011 (blastocyst: 12%)}.

SCNT involved a series number of steps that might affect the efficiency and outcome of a successful cloned animal. To date, these SCNT steps have already been improved by monitoring the types of donor cells used (Wakayama and Yanagimachi, 2001; Lagutina *et al*., 2005); selecting procedures for cell passage (Kubota *et al*., 2000; Li *et al*., 2003; Zhao *et al*., 2007; Zhang *et al*., 2008); treatment of the SCNT embryos with histone deacetylase inhibitors such as trichostatin A (TSA) (Kishigami *et al*., 2006) and scriptaid (Van Thuan *et al*., 2009); *in vitro* culture medium (Tang *et al*., 2011). However, most of the optimisations for these steps were particularly focusing on the intraspecies SCNT such as cattle, mouse and pig compared to goat, especially in interspecies SCNT experiments. The goals of this dissertation were obtained through experimentations to evaluate the effects of enucleation methods, nuclear transfer methods and *in vitro* culture systems on interspecies cloned goat-cattle *in vitro* embryo developmental competence as well as an attempt to produce cloned goat-cattle pregnancy after embryos transfer at early cell stages through oviduct transfer. Besides that, in the present research, intraspecies SCNT experiment on caprine, bovine and porcine were carried out as preliminary studies as preparation for actual experiments in order to specifically gain the nuclear transfer skill as well as to establish the cloning protocols in our laboratory. Consequently, the summarised data are presented in Appendix Table 3.1.

5.2 EFFECTS OF ENUCLEATION METHODS ON *IN VITRO* CLONED-CAPRINE EMBRYO DEVELOPMENTAL COMPETENCE FOLLOWING INTERSPECIES SCNT (EXPERIMENT 1)

In this experiment, squeezing and aspiration methods for oocyte enucleation were carried out to determine the efficiency of *in vitro* embryo developmental competence following interspecies SCNT. For the percentage of successful enucleation, no significant difference was observed between squeezing and aspiration methods with the values of 88 and 92%, respectively. The percentage for successful enucleation using squeezing method was found to be similar with the previous studies reported by Liu *et al*. (2002) (84% in bovine) and Abdullah *et al*. (2011) (96% in caprine and 89% in bovine). However, the percent successful enucleation using aspiration method in the present study was shown to be improved compared to that of Reggio *et al*. (2001) (72% in caprine), Lee and Campbell (2006) (78% in ovine) and Hosseini *et al*. (2008) (79% in ovine).

In the squeezing method, a small cut was created by an elongated sharp end enucleation needle in the zona pellucida above the first extrusion of polar body. Pressure was needed to press out the DNA materials through the cutting point that made by the enucleation needle. Therefore, some oocytes might degenerate due to damage in ooplasmic membrane generated by the pressure when pressing out the first polar body and the surrounding cytoplasm (10%). While, in the aspiration method, the zona pellucida was punctured by using a spike with beveled pipette (outer diameter: 22 μm) and the DNA materials of an oocyte were removed by aspirating out the first polar body together with minimal amount of surrounding cytoplasm (10%). This technique resulted in minimal injury to the oocyte, however aspiration out of more than 15% of the

surrounding cytoplasm was unavoidable and consequently causing the death of the oocyte.

Our results demonstrated that in terms of duration needed for enucleation up to donor karyoplast injection, the speed for aspiration with sub-zonal injection method was faster than squeezing with sub-zonal injection method with the values of 41 and 84 minutes (calculation was based on 30 oocytes used), respectively. This was in agreement with the findings reported by Lee *et al*. (2008) on the porcine SCNT. In their studied, the duration needed to complete the enucleation with injection method for squeezing and aspiration was 307 and 113 minutes (calculation was based on 100 oocytes used), respectively. For the squeezing with sub-zonal injection method, 2 different microtools were needed to complete the enucleation and injection methods namely, elongated sharp end enucleation needle and injection pipette (inner diameter: 16 to 18 μm and outer diameter: 18 to 20 μm). Therefore, both steps have to be carried out separately throughout the cloning process. After squeezing, the enucleated oocytes were transferred into the waiting medium and incubated for 1 to 2 hours before injection. During the injection step, the slit made during enucleation had to be identified to insert donor karyoplast into the perivitelline space. This procedure was time-consuming and subsequently resulted in extended manipulation time (Lee *et al*., 2008). As for the aspiration with sub-zonal injection method, both steps were carried out simultaneously by using the same aspiration pipette (inner diameter: 20 to 22 μm and outer diameter: 22 to 24 μm). The donor karyoplast was deposited in the aspiration pipette prior to enucleation. Therefore, after enucleation, the donor karyoplast was directly injected and placed in the perivitelline space through the same slit made during enucleation of an oocyte. In relationship to this, the squeezing method needed at least double as much time as the aspiration method in making the same number of SCNT embryos (Lee *et al*.,

2008). At the same time, the percentage of successful injection for aspiration method was significantly higher (100%) than in the squeezing method (93%).

In terms of cell-oocyte fusion and *in vitro* embryo development competence, there were no significant differences between these 2 enucleation methods in this study. However, Lee *et al*. (2008) reported that the blastocyst formation obtained from aspiration method was significantly higher than squeezing method (9 and 5%, respectively). In this experiment, we successfully obtained the interspecies cloned goat-cattle embryo up to blastocyst stage with the percentage of 12% (squeezing method) and 11% (aspiration method), respectively, using foetal fibroblast cell as donor karyoplast. This result was higher compared to the findings reported by Abdullah *et al*. (2011) on interspecies cloned goat-cattle embryo using squeezing method and the donor karyoplast was ear fibroblast cell. In their study, they only managed to produce the cloned embryos up to morula stage (7%). Yang *et al*. (2010) obtained a 18% of blastocyst rate on the interspecies cloned river buffalo-swamp buffalo using aspiration method and ear fibroblast cells as the donor karyoplast.

Squeezing and aspiration methods were routinely used to remove the DNA materials of a matured oocyte in cloning procedure. However, there is still a lack of information on whether which methods can be applied more efficiently in the interspecies SCNT on goat-cattle. Present study showed that there was no significant difference on the outcome of *in vitro* embryo developmental competence between these 2 enucleation methods, resulting in production of cloned blastocysts. Other enucleation methods such as Spindle-View System, Xyclone machine and enucleation on stained oocytes under UV light microscope were also proposed, but these methods were used in limited laboratories due to high cost of these machines. Furthermore, applying ultraviolet (UV) light during enucleation may cause damage to the maternal cytoplast

(Li *et al*., 2004). Chen *et al*. (2006) conducted studies on buffalo SCNT by comparing 3 enucleation protocols, namely, blind-sucking, point-hitting and Spindle-View System. The enucleation rate of oocytes by the Spindle-View System (95%) was significantly higher than that achieved by blind-sucking (65%) and the point-hitting (82%). However, there was no significant difference in fusion rate, cleavage rate and blastocyst yield of the reconstructed embryos among the 3 enucleation methods. In their findings, they suggested that the point-hitting could be employed for enucleation of oocytes in consideration of the costs of the Spindle-View System. Currently, in our research laboratory, using Xyclone machine as an enucleation method has been carried out to compare with the squeezing technique. In this study, the Xyclone machine gave a better percentage of successful enucleation than squeezing method, 96.61 and 95.23%, respectively (Goh, 2011).

5.3 EFFECTS OF NUCLEAR TRANSFER METHODS ON *IN VITRO* CLONED-CAPRINE EMBRYO DEVELOPMENTAL COMPETENCE FOLLOWING INTERSPECIES SCNT (EXPERIMENT 2)

Nuclear transfer of a donor karyoplast to an enucleated oocyte and subsequent activation of the resulting couplet are important steps required to successfully generate embryos as well as live offspring by somatic cell nuclear transfer. In the past decades, various methods of nuclear transfer have been reported in order to improve the efficiency of SCNT, such as sub-zonal with electro-fusion (SUZI: Wilmut *et al*., 1997), intracytoplasmic injection (ICI: Wakayama *et al*., 1998) and whole cell intracytoplasmic injection (WCICI: Lee *et al*., 2003). In the present experiment, the effects of nuclear transfer methods, namely, SUZI electro-fusion and ICI on *in vitro* cloned embryo developmental competence following interspecies SCNT were determined. The former

method seems to be more commonly used by many intraspecies SCNT researchers. However, effectiveness of both methods still remains to be evaluated, especially in the interspecies SCNT.

In our study, percentage of successful reconstructed oocytes was not significantly different between SUZI and ICI methods. In terms of *in vitro* embryo development following interspecies SCNT, the overall success rates for 2 cell up to blastocyst were higher in the SUZI with electro-fusion compared to ICI; while significant differences were detected only at 4 cell (54% vs. 39%), morula (24% vs. 16%) and blastocyst (12% vs. 5%) stages. Therefore, based on the present results, SUZI with electro-fusion was considered to be a better method than ICI in nuclear transfer on the production of cloned goat-cattle interspecies embryos using foetal fibroblast cell as donor karyoplast. The present comparative methods are in agreement with Nagashima *et al*. (2003) on the production of pig intraspecies SCNT using foetal fibroblast cell. They reported that the rate of normal cleavage and blastocyst formation were significantly higher in the SUZI with electro-fusion than ICI method (46% vs. 32% and 19% vs. 5%, respectively). Another similar study on the comparison of different nuclear transfer methods was conducted by Kurome *et al*. (2003) on pig intraspecies SCNT. They reported that using foetal fibroblast cell, former method gave significantly higher production blastocyst rate (19% vs. 5%, respectively). In contrast, Zou *et al*. (2001) demonstrated that the survival rate of cloned embryos derived from direct injection method was higher than the fusion method (63 and 46%, respectively) on caprine intraspecies nuclear transfer.

The first interspecies SCNT using bovine cytoplast and caprine karyoplast was reported by Song *et al*. (2008). They suggested that the cytoplasts of bovine oocyte could support blastocyst development of cloned embryos with porcine (3%) and caprine

(8%) donor cells, but they were not suitable for monkey donor cells (0%). Similarly in their study, foetal fibroblast cells were used as the donor karyoplast, and the nuclear transfer method was SUZI with electro-fusion. The blastocyst formation in the present study was slightly higher compared to the results obtained by Song *et al*. (2008) (12 and 8%, respectively). However, the *in vitro* culture medium used in both studies was different, namely KSOM (present study) and CR1aa (Song *et al*., 2008). Therefore, further studies on different *in vitro* culture media should be carried out in the future in order to evaluate the relative efficiency of the nuclear transfer methods.

In recent years, different combinations of animal species for interspecies SCNT have been reported with specific nuclear transfer methods. For the SUZI with electro-fusion, the combinations are: monkey-cattle (Dominko *et al*., 1999), sheep-cattle, (Dominko *et al*., 1999), pig-cattle (Dominko *et al*., 1999), red panda-rabbit (Chen *et al*., 2002; Li *et al*., 2002b; Tao *et al*., 2009a), goat-cattle (Song *et al*., 2008; Soh *et al*., 2011), ibex-rabbit (Jiang *et al*., 2005), camel-rabbit (Zhao *et al*., 2006), camel-sheep (Zhou and Guo, 2006) and gaur-cattle (Mastromonaco *et al*., 2007). By using this method, they managed to produce the interspecies cloned embryos up to blastocyst (7-33%) stage. Yin *et al*. (2006) and Sainsinena *et al*. (2005) reported the pregnancy on leopard cat-domestic cat (1%) and banteng-cattle (17%), respectively, using SUZI with electro-fusion. Meanwhile, Lanza *et al*. (2000), Loi *et al*. (2001), Gomez *et al*. (2003) and Yang *et al*. (2010) reported the interspecies offspring using this method. As for the ICI method, the information of this technique is much limited compared to electro-fusion. The examples of interspecies studies by using this method include: buffalo-cattle (Lu *et al*., 2005), cattle-buffalo (Lu *et al*., 2005), sheep-cattle (Hua *et al*., 2008), canine-pig (Sugimura *et al*., 2009) and goat-cattle (Soh *et al*., 2011). In their results, the blastocyst formation ranged between 5 to 25%, however, none of them carried out the

embryo transfer. Based on the percentage of blastocyst formation in the present study and previous report, it is suggested that SUZI with electro-fusion gives better interspecies performance compared to ICI method.

Results obtained from comparing the nuclear transfer techniques, it was shown that the low efficiency of cloned-caprine embryo development in the ICI method might be due to the damage of the isolated nucleus before injection (Lee *et al*., 2003; Chen *et al*., 2007). In the ICI method, plasma membrane of a donor cell is necessary to be ruptured by pipetting in and out for a few times using a narrow microinjection needle in PVP (10%). Therefore, it is believed that the nucleus of the donor cells might be damaged during this micromanipulation procedure. As a result, donor cell nucleus failed to reprogramme by the oocyte cytoplasm after injection.

As for the SUZI with electro-fusion method, the donor karyoplast was placed in the perivitelline space of an enucleated oocyte and subsequently subjected with electrical fusion in fusion medium {10 mM sorbitol containing 0.02 mM $Mg(CH_3COO)_2$, 0.02 mM Hepes (free acid) and 2 mg/ml BSA-FV} under electrical pulses of 20 voltages, 2 direct current (DC) pulses and 15 μseconds. The percentages of successful injection and reconstruction by using this method were 93 and 88%, respectively. This may suggest that the donor karyoplast could easily be fused into the oocyte cytoplast, and at the same time the electrical fuse applied on the oocyte might also trigger the preparation of calcium oscillation within the cytoplasm. The fused oocyte was then subjected to the activation medium for further biological event to occur between the donor cell nucleus and the cytoplasm in an oocyte, such as inhibiting the level of maturation promoting factor (MPF) and calcium oscillation.

It was reported that different donor cell types compose different membrane surface properties, and this may contribute to the variation in the efficiency of the

reconstructed oocytes rate for nuclear transfer (Daniel *et al*., 2008). The present study showed a higher reconstructed oocytes rate (88%) compared to previous interspecies studies using bovine oocyte as the recipient cytoplast, such as Dominko *et al*. (1999), Sansinena *et al*. (2005) and Thongphakdee *et al*. (2008) with the values of 58, 73, and 53%, respectively. In the present study, caprine foetal fibroblast cell was chosen as the donor karyoplast to produce cloned-caprine embryo using bovine cytoplast. While Dominko *et al*. (1999), Sansinena *et al*. (2005) and Thongphakdee *et al*. (2008) used ear skin fibroblast (sheep, pig, monkey and rat), adult skin fibroblast (banteng) and oviduct cells (cat) as the donor karyoplast, respectively. In contrast, Srirattana *et al*. (2010) reported a similar successful reconstructed oocytes rate using different types of donor cells in bovine intraspecies SCNT: foetal fibroblast cell (86%), ear fibroblast cell (92%), granulosa cell (90%) and cumulus cell (81%). In their studies, they demonstrated that the cumulus cells gave a lower fusion rate among all the donor cell types reconstructed with bovine oocytes. The reason for the low fusion rate of cumulus cell is not clear, but it may be due to the smaller size of compared to other fibroblasts. Therefore, the cumulus cell cannot withstand the high electrical pulse during fusion.

5.4 EFFECTS OF *IN VITRO* CULTURE MEDIUM ON CLONED-CAPRINE EMBRYO DEVELOPMENTAL COMPETENCE FOLLOWING INTERSPECIES SCNT (EXPERIMENT 3)

The culture system plays a very important factor affecting the development efficiency of *in vitro* embryos in interspecies SCNT. However, it is unclear whether the culture system is dependent on the donor karyoplast or the recipient cytoplast or interaction of both for interspecies nuclear transfer embryos (Zhao *et al*., 2006). The present experiment was specifically conducted to evaluate *in vitro* culture system using various

modifications of standard KSOM medium on embryos developmental competence following interspecies SCNT. Briefly, all reconstructed interspecies SCNT cloned-caprine oocytes were cultured in KSOM A (also known as standard KSOM) for the first 3 days of cleavage. Three different changing medium treatments were carried out: Group 1 (KSOM A throughout culture), the embryos were observed and recorded on days 3, 5, 7 and 8 without changing the medium; Group 2 (KSOM A days 1-3, change KSOM A on days 3 and 5), the embryos were observed and recorded on days 3, 5, 7 and 8; and Group 3 (KSOM A days 1-3, change KSOM B on days 3 and 5), the embryos were observed and recorded on days 3, 5, 7 and 8. KSOM A defined as the "K Simplex Optimisation Medium" (standard KSOM) which was modified by Lawitts and Biggers (1993). While KSOM B is the KSOM A supplemented with additional glucose (0.04%, 0.4 mg/ml, 2.2 mM), and was modified by our senior researcher, Kwong Phek Jin.

The aim of the present study was to evaluate the performance of *in vitro* culture system to support the maximum *in vitro* development of interspecies SCNT cloned goat-cattle embryos. Our results indicate that Group 3 was significantly higher in *in vitro* embryo developmental competence from 2 cells up to blastocyst stage compared to Groups 1 and 2, suggesting that additional supplementation of glucose in the KSOM medium is important to provide the support of *in vitro* development of interspecies SCNT caprine embryos. In terms of blastocyst production, only Group 3 culture system was able to successfully cleave the interspecies SCNT cloned embryos with 12% blastocyst rate. In spite of that, in Groups 1 and 2, similar embryos were able to develop up to morula stage (8 and 10%, respectively), even though lower than that of Group 3 (24%). It is clearly suggesting the importance of additional glucose supplementation in KSOM medium at later stages of preimplantation embryo development since there was still no blastocyst formation obtained in Groups 1 and 2. However, more detailed and

refined studies are needed in the future in order to obtain maximum blastocyst rate of interspecies SCNT cloned goat-cattle embryos, for examples, the optimum concentration of glucose at specific developmental stages of embryos as well as the exact timing for specific glucose concentration for particular embryo stages.

It is well established that embryos from different mammalian species require species-specific embryo culture conditions (Campbell *et al*., 2007). The commonly used culture media for caprine species were CR1aa and mSOF, however, the results of the *in vitro* embryo production are still variable. In our present study, we used KSOM medium to culture all our interspecies SCNT cloned embryos. It is interesting to observe that the cleavage of embryos in our laboratory was better in KSOM medium compared to previously used mSOF medium (Rahman, 2008). It is worth mentioning that in our laboratory caprine blastocyst was obtained for the first time after using KSOM medium for embryo culture *in vitro*. In one previously brief report on goat-cattle interspecies cloning demonstrated by Song *et al*. (2008), they used CR1aa medium supplement with BSA (0.3%) for the first 3 days. After 3 days, cleaved embryos were transferred to CR1aa medium supplemented with FBS (10%) and cultured for an additional 4 days. In their study, the rate of blastocyst formation was 8%. Fischer-Brown *et al*. (2002) reported that the KSOM produced more blastocyst embryos than mSOF medium in bovine intraspecies SCNT. Similar finding was reported by Bhuiyan *et al*. (2004), they observed that using KSOM medium provide a satisfactory embryo development rate in bovine intraspecies SCNT. In contrast, Niemann and Wrenzycki (2000), Wrenzycki *et al*. (2001b) and Sagirkaya *et al*. (2006) suggested that the *in vitro* derived bovine embryos generated in the mSOF system were reported to be more similar to their *in vivo* counterparts with regard to gene expression pattern reflecting viability of the embryos. Lorthongpanich *et al*. (2008) demonstrated that the mSOF medium provided better

support when embryos were co-cultured with bovine oviductal epithelial cells (BOVD) (blastocyst: 40%). It is reported that the oviduct epithelium exerts a specific action even in culture, combining the stimulation of cleavage with the preservation of viability. Therefore, the oviduct cell culture system could be used with confidence in a variety of experiments, including those designed to study the mechanisms underlying early embryogenesis and those involving genetic manipulation and nuclear transplantation (Gandolfi and Moor, 1987).

Besides that, previous reports on the interspecies SCNT using rabbit oocyte as the recipient cytoplast have shown that the M199 with FCS (10%) supports *in vitro* development of panda-rabbit, cat-rabbit, chicken-rabbit and camel-rabbit embryos to the blastocyst stage. Another type of culture medium, namely G1/G2 medium which is one of the commercially available culture systems for human embryos, is widely used for the *in vitro* culture of mammalian embryos (Tang *et al*., 2011). Previous studies have shown that G1/G2 medium could support the development of bovine (Lane *et al*., 2003), caprine (Bormann *et al*., 2003; Koeman *et al*., 2003) and porcine (Swain *et al*., 2001) *in vitro* fertilisation (IVF) embryos. However, the hatching rates and cryotolerance of sheep blastocysts cultured in the G1/G2 sequential medium were reported to be lower than that of mSOF medium (Garcia-Garcia *et al*., 2007). Tang *et al*. (2011) investigated the effects of culture conditions on the developmental competence of cloned caprine embryos. Their results indicated that mSOF was superior to the sequential G1/G2 medium in terms of hatching rate, while the supplementation of mSOF-BSA with FBS (10%) also increased the blastocyst hatching rate. Therefore, they suggested that the G1/G2 medium has the same accelerative effect on the development of caprine embryos, but further studies are required.

The *in vitro* culture medium has been reported to be critical in determining the ability of an embryo to survive (Martino *et al*., 1993; Lonergan *et al*., 2001). The use of different culture systems for embryo production provides the necessary specific conditions for embryo development. Likewise, the morphological and biochemical embryo characteristics are conditioned by the culture system employed (Massip *et al*., 1995; Ohboshi *et al*., 1997; Kaidi *et al*., 1998; Pugh *et al*., 1998; Yamashita *et al*., 1999). Therefore, due to lack of information, it is important to take into consideration the nutrient requirements with regards interspecies SCNT-derived cloned-caprine embryos in order to improve the current *in vitro* production system including our laboratory. Mammalian embryos produced *in vitro* differ from their *in vivo* counterparts due in part to the sub-optimal nature of *in vitro* culture systems. *In vitro* produced preimplantation embryos are sensitive to environmental conditions that can affect embryo morphology, gene expression, embryonic growth and developmental potential both pre- and postnatally (Summers and Biggers, 2003; Fleming *et al*., 2004). It is indicated that mammalian embryos display environmental sensitivity to *in vitro* culture system which manifests in phenotypic condition known as large offspring syndrome (LOS) (Young *et al*., 1998; Wrenzycki *et al*., 2004). The LOS can be identified as obvious abnormalities, such as increased incidence of oversize foetuses, increased foetal myogenesis, dystocia, dysfunctional perinatal pulmonary activity, abnormalities in placental development and reduced pregnancy rates (Summers and Biggers, 2003; Fleming *et al*., 2004). These abnormalities are induced during critical periods of preimplantation development as a result of inadequate *in vitro* production system Campbell *et al*. (2007).

In the present study, supplementation of glucose level (0.04%, 2.2 mM) at the late stage of embryo culture has significantly improved the blastocyst formation. In many species, glucose has been widely used as a major energy substrate in embryo

culture media. The role of glucose in preimplantation development has been demonstrated in various species, but the rationale for glucose supplementation is still ambivalent. In bovine embryo development, glucose is known as an important energy substrate for blastocyst formation (Rieger *et al*., 1992). According to Rieger *et al*. (1992), the first marked increase in glucose metabolism in the cattle embryos in their study occurred between the 8- and 16 cell stages, whereas the first marked increase occurs between the 4- and 8 cell stages in pig (Flood and Wiebold, 1988) and human (Wales *et al*, 1987) embryos, and between the 2- and 8 cell stages in sheep embryos (Thompson *et al*., 1991). To our knowledge, there is no report regarding the marked requirement of glucose for caprine embryos stage. Conversely, based on various studies, it is suggested that for the pig, human, sheep and cattle embryos, the first marked increase in glucose metabolism approximately coincides with the time of activation of the embryonic genome (Telford *et al*., 1990). It is interesting to note that the marked requirement of glucose at specific developmental stage of embryos for the caprine species as indicated by the findings of this study is an agreement with those of other domestic animals which may also coinciding with the activation of embryonic genome. This confirmed the role of glucose as important nutrient during preimplantation embryo development in domestic animals including caprine species. The extra glucose is needed during the caprine embryo *in vitro* culture to produce enough ATP to support the embryos in the zygotic transition stage (8- and 16 cell stages) in order for further development up to blastocyst stage. In contrast, it has also been reported that exposure to high concentrations of glucose during early embryonic stages caused developmental retardation in many species including hamster (Schini and Bavister, 1988; Barnett and Bavister, 1996; Barnett *et al*., 1997), mouse (Chatot *et al*., 1989; Lawitts and Biggers, 1991; Scott and Whittingham, 1996), rat (Kishi *et al*., 1991; Miyoshi *et al*., 1994), cattle

(Kim *et al*., 1993), sheep (Thompson *et al*., 1992), and human (Conaghan *et al*., 1993). Consequently, in the present study, the reconstructed oocytes were cultured in KSOM A with low concentration of glucose in order to maintain the normal development of embryos at early stage.

Typical chemically defined culture medium have been developed such as KSOM, CR1aa, mSOF, TCM199, NCSU 23 and G1/G2 (commercial medium), and were used to culture mammalian embryos *in vitro*. However, these *in vitro* culture media generally require supplementation with additional nutrients which can aid *in vitro* development, but in long term, these chemicals can compromise embryo quality. Serum and BSA are complex undefined mixtures containing hormones, growth factors, vitamins and numerous other factors. For examples, serum such as foetal bovine serum (FBS) and foetal calf serum (FCS) are known to be detrimental to embryonic and foetal development. While, the effects of serum compared to BSA supplementation during *in vitro* mouse development results in lower preimplantation development and neonatal offspring (Fernandez-Gonzalez *et al*., 2004). However, the role of serum on embryonic development is still controversial. Previous reports have shown that FBS has the beneficial effect to blastocyst development and hatching rate of embryos (Wang *et al*., 1997; Kim *et al*., 2004; McElroy *et al*., 2008). However, the exposure of early embryos to FBS has been shown to be detrimental to the quality of blastocysts produced (Thompson *et al*., 1995; Rizos *et al*., 2002, 2003). Besides that, results of studies in cattle have suggested the enhancement of preimplantation development of cloned bovine embryos in FBS supplemented medium, even though it was not improving the calving outcome (Choi *et al*., 2002a). FBS has widely been used for the culture of nuclear transfer caprine embryos (Baguisi *et al*., 1999; Behboodi *et al*., 2004, 2005).

Even though controversial, most of the researches including our study prefer to use the BSA as a chemical supplement to the culture medium.

In a nutshell, there is still a large gap of information among the media used for the culture of embryos obtained from various manipulation techniques. For an example, in one extreme case, Lorthongpanich *et al*. (2008) pointed out that different culture media did not enhance embryo growth, which was largely affected by nuclear manipulation. However, as clearly shown by our results of blastocyst production in caprine interspecies SCNT after additional supplementation of glucose in KSOM medium, we believe that maximum embryo developmental competence *in vitro* can be achieved if all the myriads of intrinsic and extrinsic factors are taken into consideration in formulating the culture media.

5.5 AN ATTEMPT ON OVIDUCT EMBRYO TRANSFER OF INTERSPECIES SCNT EMBRYOS AND SUBSEQUENT PREGNANCY DIAGNOSIS USING ULTRASOUND SCANNING (EXPERIMENT 4)

The final goal of any research involving culture of embryos *in vitro* is to produce live born offspring after embryo transfer. Embryo transfer is considered to be a terminal integral component of reproductive techniques including both intraspecies and interspecies SCNT. In the present study, active and enthusiastic attempts have been made in an effort to produce viable cloned offspring after the transfer of interspecies SCNT cloned-caprine embryos. Unfortunately, none of the recipients were detected pregnant after diagnosis using ultrasound scanning after 30 days of expected gestation. The reasons for the failures in pregnancy in this experiment are unexplained at this time and warrant intensive further investigations involving numerous intrinsic and extrinsic factors in future studies.

Interspecies SCNT embryo transfer always comes with a crucial issue, which is the availability of suitable foster mothers for interspecies SCNT embryos. In this experiment, caprine DNA donor cells was used as karyoplast and bovine oocyte was used as cytoplast while caprine doe was used as the foster mother for the embryo transfer. In interspecies embryo transfer, the genetic background of the foster mother and the embryos may be a roadblock even more serious than the genomic/mitochondrial DNA compatibility or the zygotic genome activation (ZGA) activation in order to ensure normal developmental competence of embryos during *in vitro* culture, survival of foetus during gestation and the birth of healthy offspring. Therefore, the removal or intervention of species-specific boundaries for embryo transfer is a fundamental requirement for the successful multiplication of reconstructed embryos, for example via reproductive techniques including interspecies cloning. Up to date, however, there is a very little has been done with inter-specific embryo transfer in large animal (Loi *et al*., 2011).

In our study, prior to the surgical operation, the ovaries of the recipients were checked laparoscopically for the presence of corpus luteum. Only those recipients with at least 1 well developed corpus luteum were selected for embryo transfer. Each recipient received embryos 2 or 3 days after the beginning of oestrus. The embryos were surgically transferred into the oviducts of a synchronised recipient at the 4- to 8 cell stages. A total of 9 recipients received reconstructed embryos by surgical operation. Studies suggested that transferred the embryos at early cell stages might allow the reconstructed embryos underwent a relatively short *in vitro* culture period. It may be that avoidance of longer term culture may have alleviated some of the detrimental effects of *in vitro* culture (Keefer *et al*., 2001; Reggio *et al*., 2001). In intraspecies SCNT on caprine, most viable cloned caprine offspring were obtained from the transfer

of early stage cloned embryos into the foster mothers (Baguisi *et al*., 1999; Reggio *et al*., 2001; Zou *et al*., 2002; Behboodi *et al*., 2004; Tang *et al*., 2011). Generally, caprine intraspecies embryos are known to have a comparatively lower *in vitro* developmental potential towards the late preimplantation stage compared to those of other domestic species. Most of the reports of success in producing cloned kids involved the transfer of embryos at early cloned embryos stages from 2- to 8 cell stages (Abdullah *et al*., 2011). Tang *et al*. (2011) showed that the pregnancy rate by transfer of early stage embryos was higher than that obtained in the *in vitro* cultured blastocysts group on intraspecies caprine SCNT. Behboodi *et al*. (2004) reported that the main reason for the difference in embryo transfer performance with regard to embryo stages may be due to the embryo damage during *in vitro* culture resulting in poor embryo quality before the embryo transfer. However, Ohkoshi *et al*. (2003) produced the first nuclear transfer-derived cloned caprine following transfer of blasotcysts, although it died 16 days after birth. Tang *et al*. (2011) demonstrated viable offspring after the transfer of *in vitro* produced blastocysts. They monitored the health status of the cloned kid for 6 months and observed that there were no abnormalities of the kid. Until now, most researchers prefer to transfer caprine embryos prior to blastocyst stage through oviduct embryo transfer due to the difficulty of culturing viable embryos *in vitro* to the blastocyst stage. Therefore, in the present study, we decided to transfer the cloned caprine embryos at early cell stages through oviduct transfer.

From our results, together with the findings of cloned embryos in other interspecies, we speculate that the bovine cytoplast can be reprogrammed by the donor karyoplasts (Dominko *et al*., 1999; Sansinena *et al*., 2005; Song *et al*., 2008; Abdullah *et al*., 2011). However, up to now, embryo implantation was not detected after these goat-cattle interspecies cloned embryos were transferred to synchronised recipient

caprine, even though bovine cytoplast was shown to support the dedifferentiation of caprine somatic nuclei (Abdullah *et al*., 2011). During embryo implantation, the signals from embryos to a recipient uterus for implantation are controlled by the donor nucleus, and these signals may be species-specific (Chen *et al*., 2002). To be successfully implanted, the signals from both embryo trophectoderm and recipient uterus must compatible to trigger the process of implantation. Compatibility between nucleus and cytoplasm may be limited to closely related species (Wells *et al*., 1998), while more diverse relationships may result in early embryonic loss (Dominko *et al*., 1999; White *et al*., 1999). The birth of cloned gaur by interspecies SCNT and successful implantation of a yak interspecies SCNT embryo using bovine oocytes as recipient cytoplasm proves that a close phylogenetic distance between the donor nucleus and recipient cytoplasm results in a higher likelihood of success in delivering live offspring. Nevertheless, more evidence for the improvement of embryo transfer performance in interspecies implantation is timely in order to establish interspecies cloning in goat using goat karyoplast and cattle cytoplast a reality in the near future.

Factors such as the genetic differences between the embryo and the recipient could have affected pregnancy establishment in the present study. Andrabi and Maxwell, (2007) suggested that the interspecies embryo transfer between endangered species and domestic surrogates is limited by a low pregnancy success, and the foeto-maternal recognition mechanism is still remained unclear. Therefore, future research should focus on defining factors affecting embryo development and pregnancy establishment after transferring the interspecies SCNT embryos to phylogenetically related animals. This is to increase the efficiency of using interspecies SCNT for the conservation of wildlife in the future (Thongphakdee *et al*., 2010). Contrarily, this lack of prenatal loss may be a

species related phenomenon reflected in the cytoplast's ability to reprogramme the donor nucleus.

5.6 GENERAL DISCUSSION

The present research is a comprehensive study on the manipulation methods on the production of interspecies goat-cattle *in vitro* embryo development as well as an attempt to produce pregnancy after embryo transfer. Interspecies SCNT has been an invaluable tool for studying donor karyoplast (nucleus)-recipient cytoplast (oocyte) interaction, and it may be a method for rescuing endangered species whose oocytes are difficult to obtain (Tao *et al*., 2009a). Many factors influencing the optimum embryo and foetus developmental competence of the present study have been discussed in the previous section such as the enucleation methods, nuclear transfer methods, *in vitro* culture systems and sustaining pregnancy. In spite of the above-mentioned constraints, it is believed that this is the first report in Malaysia in regard to successful in obtaining *in vitro* development of interspecies goat-cattle embryos, involving optimisation of the micromanipulator techniques as previously described. The following section will provide brief discussion on other specific factors affecting interspecies SCNT performance not covered in this study.

5.6.1 Type of Oocytes Used as the Recipient Cytoplast

In general, the use of alternative nuclear transfer techniques such as interspecies nuclear transfer has interesting potential for species constrained by a limited availability of oocytes source (Yin *et al*., 2006). Interspecies SCNT has successfully shown the evidence that one species oocytes (cytoplast) could reprogramme the highly differentiated somatic cells (karyoplast) of the other species (Tao *et al*., 2009a). In the

present study, bovine oocytes were used as the recipient cytoplasts in caprine interspecies SCNT. The bovine oocytes have been successfully used as recipient cytoplasts for producing interspecies cloned blastocysts of gaur (Lanza *et al*., 2000); buffalo (Kitiyanant *et al*., 2001; Saikhun *et al*., 2002); yak (Li *et al*., 2007); banteng (Sansinena *et al*., 2005); black bear (Ty *et al*., 2003); monkey (Dominko *et al*., 1999) and goat (Song *et al*., 2008; Soh *et al*., 2011) with reasonably high blastocyst rates (8-35 %) regardless of species. Besides using bovine as the recipient cytoplast, some other combinations of the species in interspecies SCNT have also being carried out such as panda-rabbit (Chen *et al*., 2002), cat-rabbit (Wen *et al*., 2003), goat-sheep (Ma *et al*., 2008) and dog-pig (Sugimura *et al*., 2009) (7-25%). To date, rabbit was also widely used as the recipient oocytes due to their small animal in size, short reproductive life span, and easy manipulation and inducement of ovulation. Besides that, Zhao *et al*. (2006) reported that the rabbit oocyte was an ideal model for many types of studies due to its large size, elasticity and easy handling as well as the oocyte cytoplasm is capable of dedifferentiating somatic cell nuclei from other species.

One of the main reasons of choosing the bovine oocytes as the recipient cytoplasts in the current study is due to the number of chromosome present in bovine was similar to the caprine donor karyoplast, which is 60. It is believed that the chromosome number between the species used in interspecies SCNT might affect the reprogramming of donor nucleus to the cytoplast. In contrast, Dominko *et al*. (1999) reported that the disparity in the number of chromosomes between species does not seem to be limiting for the embryo developmental success (60 in cattle, 54 in sheep, 38 in pig, 42 in monkey, 42 in rat). They demonstrated that blastocysts are produced *in vitro* from interspecies SCNT from species with different chromosome numbers. Besides the equal chromosome numbers of caprine and bovine, the reason for bovine

cytoplasts (oocytes) was chosen in the present study due to difficulty obtaining sufficient number of oocytes from other species such as ovine, porcine or equine. Therefore, bovine oocyte is suggested to be a preferred alternative for the production of caprine embryo through interspecies SCNT.

In addition, using bovine ooplasm as the recipient cytoplast can support differentiation of the introduced nuclei (donor karyoplast) from different species such as the caprine; however, the genetic divergence between donor karyoplast and recipient cytoplast may indeed represent a limiting factor influencing interspecies SCNT outcome (Dominko *et al*., 1999; Thongphakdee *et al*., 2008). Li *et al*. (2006) suggested that a closer genetic background between the donor karyoplast and recipient cytoplast could enhance blastocyst development *in vitro* better than the diverse genetic background. In the present study, the encouraging of blasotcyst formation rate in the goat-cattle interspecies SCNT (12%) has opened a window of opportunity for interspecies cloning using 2 different species in our laboratory as well as in Malaysia. In spite of this, the quality of blastocyst obtained in this present study is uncertain as indicated in our embryo transfer experiment that none of the goat recipient was detected pregnant. The issue is whether the recipient factor or the embryo factor or both that the desired results were not obtained. Therefore, detailed studies are needed in the future to elucidate this phenomenon. There were still some issues that might be involved in the low production of interspecies blastocyst such as the genetic distance between bovine and caprine, which may result in incompatible genomic regulation and metabolic mechanism. As compatibility between the recipient ooplasm and the donor nucleus must be reflected in some part in the overall effectiveness of nuclear reprogramming, nucleo-cytoplasmic interactions may be responsible for some of the detrimental effects observed following nuclear transfer (Dominko *et al*., 1999). However, our results have shown that

improvement in the culture system such as appropriate culture medium by additional supplementation of glucose has improved significantly the blastocyst production in caprine interspecies SCNT.

5.6.2 Types of Donor Cell Used as the Donor Karyoplast

In the present study, caprine foetal fibroblast cell was used as the donor karyoplast in the production of interspecies cloned caprine embryos. To our knowledge, this is the second report on the interspecies SCNT between goat-cattle by using foetal fibroblast cells in the world next after Song *et al*. (2008). In our study, the percentage of blastocyst (12%) obtained was slightly improved to the results reported by Song *et al*. (2008) (8%). Foetal fibroblast cells were believed to have less genetic damage and more proliferative ability (as measured by cell doublings) than adult somatic cells. Therefore, they have been the cell type of choice as nuclear donors (Hill *et al.,* 2000a). However, this presents a limitation in that the individual the donor cells are derived from (a foetus) has not had the chance to demonstrate its genetic merit (an adult) prior to somatic cell nuclear transfer.

Generally, most of the reports on both intraspecies and interspecies SCNT were done by using either cumulus or ear skin fibroblast cells as they are easy to obtain and result in no injuries to animals (Yang *et al.,* 2010). Selokar *et al*. (2011) reported that in their study the reason for low blastocyst rate could be the use of adult fibroblasts as donor cells, which have been reported to give far lower cloning efficiency compared to those obtained with foetal or new born fibroblasts in buffalo (Shah *et al*., 2009), cattle (Saikhun *et al*., 2002) and pig (Lee *et al*., 2007).

When the efficiency of various cell types from adult, newborn and foetal male and female donor fibroblast cells was compared in intraspecies SCNT experiment, the

percentage of blastocysts produced from each cell type was found not to be significantly different (Kato *et al.,* 2000). Similar results were obtained using various cell types derived from mice of different strains, sexes and ages (Wakayama and Yanagimachi, 2001). Besides that, no significant differences were found in bovine embryo developmental rates to the blastocyst stage with adult, newborn or foetal cell nuclei; however, abortion in later stages of pregnancy was higher for cloned foetuses derived from adult cells (Kato *et al.,* 2000). Similarly, no differences among embryos derived from foetal and adult bovine fibroblasts with regard to fusion, cleavage and blastocyst formation were detected (Niemann *et al.,* 2002). However, more foetal losses after transfer into recipients were noted with embryos reconstructed with adult bovine donor cells (Hill *et al.,* 2000b; Niemann *et al.,* 2002). In terms of the production of transgenic animal, foetal fibroblast cells have been used most often (Schnieke *et al*., 1997; Cibelli *et al*., 1998; Hyun *et al*., 2003). This is because they have been considered the most suitable cell type for transgenic animal production. Therefore, it is suggested that foetal fibroblast cells could be the ideal donor karyoplasts in both SCNT and transgenic approach in the future.

5.6.3 Epigenetic Reprogramming in Somatic Cell Nuclear Transfer

Epigenetic remodeling must occur for normal embryo development to proceed (Dean *et al*., 2003). However, the most extreme version of this remodeling must take place when a differentiated somatic cell (donor karyoplast) is returned to an oocyte (recipient cytoplast) and challenged to undergo de-differentiation to restore totipotency of the cloned embryo (Li, 2002; Wade and Kikyo, 2002). Although a wide variety of nuclear donor types have been found to be successful in a number of different mammalian species, they all share the common problems of low efficiency and high levels of early

and later embryonic mortality, suggesting that the errors that arise are fundamental and systematic in nature. In order to achieve the reprogramming of the somatic nucleus (donor karyoplast), a number of critical processes must occur which include transcriptional silencing of the donor nucleus (karyoplast), erasure of differentiated cellular memory (cytoplast), appropriate activation of the reconstructed interspecies "one-cell embryo", and appropriate embryonic gene expression at all later stages. Each of these steps involves a series of complex epigenetic alterations (Dean *et al*., 2003). Wade and Kikyo (2002) and Reik *et al*. (2001) reported that the changes in chromatin, involving the covalent modification of histones and in DNA methylation would seem to be prime factors determine the successful outcome for nuclear reprogramming.

Dean *et al*. (2003) demonstrated that the DNA methylation and chromatin errors may contribute significantly to the low rates of efficiency of somatic nuclear transfer. In their study, they used antibodies to 5-methyl cytosine to evaluate the genome-wide pattern of DNA methylation in cloned intraspecies bovine embryos. They found out that partial reprogramming was provided by the change in the organisation of methylation in "one-cell" reconstructed embryos, possibly as a result of active demethylation. However, it is interesting to note that the absence of passive demethylation and the inappropriately early *de novo* methylation at the 4- to 8 cell stages provided a strong indication that inadequate nuclear reprogramming had occurred (Dean *et al*., 2001a). While, in the blastocyst stage, the patterns normally observed between the differentiated lineages of ICM and trophectoderm were much less obvious as overall increases in DNA methylation had taken place, resulting in particular in aberrant hypermethylation in trophectoderm. Therefore, they concluded that aberrant epigenetic reprogramming occurs in the majority of both intraspecies and interspecies cloned preimplantation embryos. Boiani *et al*. (2002), Byrne *et al*. (2002) and Inoue *et al*. (2002) proposed that

the large scale reprogramming failure underlies, at least in part, the developmental failures of cloned embryos by interfering with appropriate gene expression.

It is believed that major barriers to the development of interspecies embryos are first manifested at the time when the genome of the somatic cell (donor karyoplast) becomes independent from the maternal transcripts (recipient cytoplast) and initiates transcription on its won (reconstructed embryo) (Beyhan *et al*., 2007), the failure of occurrence of which may lead to imposition of developmental arrest (Latham, 2005). Therefore, it is critical in the caprine embryos in which the maternal-embryonic transition occurs at 8-16 cell stage. Dominko *et al*. (1999) reported that the use of alternative nuclear transfer techniques such as interspecies nuclear transfer has interesting potential for species constrained by a limited availability of oocytes as recipient cytoplast. However, an *in vitro* embryo developmental block as has been previously reported as well as other factors influencing interpsecies cloning procedure should be taken into consideration to ensure normal developmental competence of cloned embryos, particularly when the species of the donor karyoplasts and recipient cytoplasts are far apart in taxonomic classification. It is suggested in the present study that further experiment may carry out to compare the efficiency between both oviduct and uterine embryo transfer.

5.6.4 Summary

In summary, blastocyst caprine embryos could be produced *in vitro* for the first time in Malaysia through interspecies SCNT under local setting. Bovine cytoplast could be reprogrammed by the caprine nuclei foetal fibroblast karyoplast. Both enucleation methods, *i.e.* squeezing and aspiration were able to produce *in vitro* cloned-caprine embryos up to blastocyst stage. Nuclear transfer by using sub-zonal injection with

electro-fusion gave a significantly higher blastocyst results than intracytopalsmic injection. Relatively high proportions of reconstructed embryos developed to advanced stages in KSOM for first 3 days and changed medium with KSOM supplemented with additional glucose (0.04%, 2.2 mM) at days 3 and 5, suggesting that this *in vitro* culture system may be a suitable culture medium for caprine interspecies SCNT embryos using bovine oocytes as the recipient cytoplasts and caprine foetal fibroblast cells as the donor karyoplasts.

In a nutshell, from the significant findings of this study, the optimised conditions for interspecies SCNT are suggested as following:

a) Enucleation method : Squeezing method
b) Nuclear transfer method : Sub-zonal injection together with electrofusion
c) *In vitro* culture medium : KSOM A on days 1-3; change medium with KSOM B on days 3 and 5

5.6.5 Future Directions

Limited information is available in the literature on the developmental competence of caprine interspecies SCNT embryos. In the present study, it is demonstrated that such embryos could be generated through goat-cattle interspecies SCNT with the production of blastocysts after culturing in modified KSOM medium. However, numerous extrinsic and intrinsic including technical factors influencing the efficiency of interspecies SCNT need to be refined and studied in details before this procedure could be used routinely for the production of cloned caprine embryos for biological research and industry application. It is well known that it is difficult to produce high percentage of caprine blastocysts in normal IVF and ICSI techniques. Hence, to produce such embryos using SCNT is more challenging particularly in interspecies SCNT. Since ABEL laboratory

obtained encouraging results from previous research, it is believed that a high percentage of viable blastocyst is achievable provided that all the constraints related to developmental competence of these interspecies embryos are solved. It should be appreciated that nuclear transfer is a complex protocol, which involves various steps and each step might affects the overall efficiency of its performance. The protocol involves the following steps such as the preparation of somatic cells, the preparation of enucleated oocytes, the injection or fusion of somatic cell nuclei into the enucleated oocyte, the reconstruction of the somatic chromosomes, oocyte activation and culture medium methods to produce a diploid cloned embryo. The subsequent challenge is to ensure the viability of the embryos after culturing *in vitro* using suitable culture medium. In order to obtain healthy and live born offspring, not only embryo quality but also the appropriate foster mothers physically and physiologically must be taken into consideration. In addition, proper embryo transfer skill must be acquired before venturing into embryo transfer programme. In a nutshell, besides the above-mentioned factors, the clarification and understanding of the genetic background, molecular biology and developmental biology of the interspecies SCNT should be given priority in future research in order to make increase caprine production using cloning as an alternative approach a reality in the near future.

Chapter 6

6.0 CONCLUSIONS

This study was carried out to investigate the effects of enucleation methods, nuclear transfer methods and *in vitro* culture systems for the *in vitro* development of cloned-caprine embryos produced through interspecies somatic cell nuclear transfer (interspecies SCNT) technique using caprine foetal fibroblast cells as donor karyoplast and bovine oocyte as recipient ccytoplast. Specific conclusions can be made based on the findings from this research as follows:

a) This is the first report of interspecies SCNT using caprine-foetal fibroblast cells as donor karyoplasts and bovine oocytes as recipient cytopalsts in the local setting at Animal Biotechnology-Embryo Laboratory, the University of Malaya, Malaysia.
b) Bovine cytoplast could be reprogrammed by caprine foetal fibroblast karyoplast with the cloned-caprine blastocyst formation cultured *in vitro* ranging from 5 to 12%.
c) Both enucleation methods (squeezing and aspiration) have the ability to produce viable cloned-caprine embryos up to blastocyst with no significant difference to each another.
d) Sub-zonal injection with electro-fusion was shown to be significantly higher in the production of cloned-caprine blastocyst compared to intracytoplasmic injection for the nuclear transfer method.
e) Supplementation of glucose level (0.04%, 2.2 mM) at the late stage embryo culture has significantly improved the blastocyst formation. It is believed that this is the first report of using KSOM at early stages development and KSOM

supplemented with additional glucose (0.04%, 2.2 mM) at later stages development that produces good cleavage rate and satisfactory blastocyst rate of cloned-caprine embryos following interspecies SCNT.

f) Production of *in vitro* caprine embryos through assisted reproduction was limited by the shortage of caprine oocytes source in Malaysia. It is suggested that, interspecies SCNT by substitution the recipient cytoplast to bovine oocyte will be a better alternative for the production of caprine embryos in Malaysia in near future. These embryos subsequently can be transferred into foster mother to produce live offspring in large number at a rapid rate for the industry.

g) In a nut-shell, successful production of cloned-caprine embryos *in vitro* until blastocyst stage was obtained from interspecies nuclear transfer using bovine cytoplast and caprine karyoplast.

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http://www.fao.org/nr/cgrfa/cthemes/animals/it/

APPENDICES

APPENDIX 1: LIST OF MATERIALS

Appendix Table 1.1: List of equipment/instrument

Equipment/instrument	Model no.	Manufacturer
Abrasive stone or oilstone	-	Hall's Arkansas Oilstones, USA
Autoclave	HA-300MII	Hirayama Hiclave, Japan
Centrifuge	D37520	Heraeus, Germany
CIDR applicator	-	Pharmaciaand Upjohn, New Zealand
CO_2 incubator	HeraCell 240	Heraeus, Germany
CO_2 insufflator system	PG001	Aesculap®, Germany
Digital analytical balance	AB104	Mettler Toledo, Switzerland
Digital camera (X-Cam-α)	-	microLAMDA Sdn Bhd, Malaysia
Dissecting microscope	SZH10	Olympus, Japan
Electrofusion machine	SUTF-1	Suranaree University of Technology, Thailand
Heating stage (Thermoplate)	HATS-U55R30	Tokai Hit, Japan
Impulse sealer	KF-300H	Khind, Taiwan
Inverted microscope	IX71	Olympus, Japan
Laminar flow cabinet	HLF-120	Gelman Sciences, Australia
Laparoscopic system:		Aesculap®, Germany
(a) Endoscopic camera system	PV431	
(b) CCD camera	PV430	
(c) Pediatric Storz laparoscope (7 mm)	PE688A	
(d) Light probe with fibre optic cable	PO913	
(e) Light system (300 W)	OP927	
Liquid nitrogen tank	SC2/1V	MVE, USA
Microforge	-	Technical Products Internationals, USA
Micropipette grinder	EG-4	Narashige, Japan
Micropipette dispenser	-	Eppendorf, Germany
Micropipette puller	P-97	Sutter Instrument Co., USA
Narishige hydraulic micromanipulators	ON3-99D	Narashige, Japan
Osmometer	Vapro 5520	WESCOR Inc., USA
Oven	40050-IP20	Memmert GmbH, Germany
pH meter	HI-122	Hanna Instruments, Singapore
Refrigerator and freezer	SR-21NME	Samsung Electronics, Korea

(continued)

(continued)

Equipment/instrument	Model no.	Manufacturer
Spirit burner	-	Shanghai Machinery
Stereomicroscope	SZH10	Olympus Optical, Japan
Surgical set	-	Aesculap®, Germany
Surgical table	-	Syarikat Copens Enterprise, Malaysia
Trocar and canula (5.5 mm & 7.0 mm)	EJ456, EJ457	Aesculap®, Germany
Ultrapure water purification system	Milli-Q PF Plus	Millipore, USA
Vapour pressure osmometer	5520	Vapro Wescor, USA
Equipment/instrument	Model no.	Manufacturer
Vortex mixer	VTX-3000L	LMS, Japan
Water bath	GMP-GC-19	Memmert GmbH, Germany

Appendix Table 1.2: List of chemicals, reagents and media

Chemicals, reagents and media	Catalogue no.	Manufacturer
6-dimethylaminopurine (6-DMAP)	D2629	Sigma-Aldrich, USA
70% ethanol	-	Prepared from absolute ethanol
α-MEM powder	M0644	Sigma-Aldrich, USA
BSA-FV	A7030	Sigma-Aldrich, USA
BSA	A6003	Sigma-Aldrich, USA
Ethyl alcohol 99.8% (absolute ethanol)	ET150-50	System ChemAR®, Poland
Hibiscrub (antiseptic)	HK-06770	SSL International Plc, UK
BME amino acids solution (50X)	B6766	Sigma-Aldrich, USA
Calcium chloride ($CaCl_2$)	C5670	Sigma-Aldrich, USA
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	C3881	Sigma-Aldrich, USA
Calcium ionophore (Ca^{2+} ionophore)	C7522	Sigma-Aldrich, USA
Cleaning solution 7X®-PF	-	FlowLab™, Australia
Cysteamine	M9768	Sigma-Aldrich, USA
Cytochalasin B	C6762	Sigma-Aldrich, USA
D-glucose	G6152	Sigma-Aldrich, USA
Dimethyl sulphoxide (DMSO)	D5879	Sigma-Aldrich, USA
Disinfectant Gigasept® FF	-	Schülke & Mary GmbH, Germany
EDTA	E4884	Sigma-Aldrich, USA
EDTA	E9884	Sigma-Aldrich, USA
Foetal bovine serum	10270	Gibco BRL, USA
FSH	Folltropin-V®	Intervet International, Holland
Gentamicin sulphate salt	G3632	Sigma-Aldrich, USA
Goat pellet feed	-	KMM Berhad, Malaysia
HEPES (free acid)	H3375	Sigma-Aldrich, USA
HEPES: $C_8H_{17}N_2O_4SNa$	H3784	Sigma-Aldrich, USA
HEPES: $C_8H_{18}N_2O_4S$	H6147	Sigma-Aldrich, USA

(continued)

(continued)

Chemicals, reagents and media	Catalogue no.	Manufacturer
Hoechst 33342	B2261	Sigma-Aldrich, USA
Hydrochloric acid	HY450-70	Systerm ChemAR®, Poland
Hyaluronidase (from bovine testes)	H4272	Sigma-Aldrich, USA
Intravaginal progesterone release devise	-	Pharmacia and Upjohn, New Zealand
Ketamil injection (ketamine hydrochloride)	L10077	Troy Laboratories, Australia
K-Y Lubricating Jelly	-	Pharmedica Lab, South Africa
L-glutamine	G3126	Sigma-Aldrich, USA
Liquid nitrogen	-	Mox Gases Berhad, Malaysia
Magnesium acetate tetrahydrate $[Mg(CH_3COO)_2]$	M0631	Sigma-Aldrich, USA
Magnesium chloride hezahydrate ($MgCl_2.6H_2O$)	M2393	Sigma-Aldrich, USA
Magnesium sulphate ($MgSO_4$)	M7506	Sigma-Aldrich, USA
MEM non-essential amino acids solution (100x)	M7145	Sigma-Aldrich, USA
Mineral oil	M8410	Sigma-Aldrich, USA
Oestradiol-17β	E8875	Sigma-Aldrich, USA
PBS Dulbecco A tablets	BR0014G	Oxoid, England
Penicillin-G	P7794	Sigma-Aldrich, USA
Phenol red powder	P3532	Sigma-Aldrich, USA
Potassium chloride (KCl)	P5405	Sigma-Aldrich, USA
Potassium phosphate monobasic (KH_2PO_4)	P5655	Sigma-Aldrich, USA
PVP	PVP360	Sigma-Aldrich, USA
Sodium bicarbonate ($NaHCO_3$)	S5761	Sigma-Aldrich, USA
Sodium chloride (NaCl)	S5886	Sigma-Aldrich, USA
Sodium DL-lactate (60% syrup)	L7900	Sigma-Aldrich, USA
Sodium phosphate dibasic (Na_2HPO_4)	S5136	Sigma-Aldrich, USA
Sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$)	S9638	Sigma-Aldrich, USA
Sodium pyruvate	P4562	Sigma-Aldrich, USA
Sorbitol	S3889	Sigma-Aldrich, USA
Streptomycin	S1277	Sigma-Aldrich, USA
Streptomycin	S9137	Sigma-Aldrich, USA
TCM-199	M4530	Sigma-Aldrich, USA
Trypsin	T4799	Sigma-Aldrich, USA
Weak iodine solution	-	ICN Biomedicals, USA
Xylazine hydrochloride (Ilium Xylazil-20)	L10600	Troy Laboratories, Australia

Appendix Table 1.3: List of labwares and disposables

Labwares and disposables	Manufacturer
Aluminium foil	Reynolds Consumer Products, USA
Autoclave disposable bag	Megalab supplies, Malaysia
Blades (Super Nacet)	Gillette, USA
Borosilicate glass tubing (Microcaps®)	Drummond Scientific Company, USA
Chromic catgut and other suture materials	Aesculap®, Germany
Culture dish	Nunc, Denmark
Disposable glass Pasteur pipette	Hirschmann® Laborgerete, Germany
Disposable hand tissues	Megalab supplies, Malaysia
Falcon™ conical tube	Becton Dickinson, USA
Falcon™ polystyrene round-bottom test tube	Becton Dickinson, USA
Glassware (beaker, flask, measuring cylinder etc.)	Pyrex®, Japan
Lens cleansing tissue (Kimswipe® EX-L)	Kimberly-Clark, USA
Microcentrifuge tube	Elkay, Costelloe
Micropipette tips without filter	Axygen Scientific, USA
Microscope slide	Sail Brand, China
Microscope glass cover slip	Hirschmann® Laborgerate, Germany
Millex®-GS syringe driven filter	Schleicher and Schuell, Germany
Needle	Terumo Corporation, Japan
Parafilm	Pechiney Plastic Packaging, USA
Schott bottle	Duran, Germany
Serogocal pipette	LP Italian SPA, Italy
Sterile glove	Ansell International, Malaysia
Syringe	Terumo Corporation, Japan
Tissue culture flask	Nunc, Denmark

APPENDIX 2: STATISTICAL DATA

Appendix Table 2.1: Successfully enucleated and injected rates, and duration needed to complete the enucleation together with injection in interspecies SCNT cloned-caprine embryos

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
enucleated	squeezing	6	88.0083	7.35186	3.00138	80.2930	95.7236	76.60	95.00
	aspiration	6	91.6750	4.71479	1.92480	86.7271	96.6229	84.62	96.88
	Total	12	89.8417	6.19185	1.78743	85.9076	93.7758	76.60	96.88
injected	squeezing	6	93.2583	6.49917	2.65328	86.4379	100.0788	83.33	100.00
	aspiration	6	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00
	Total	12	96.6292	5.62096	1.62263	93.0578	100.2006	83.33	100.00
timing	squeezing	6	83.8167	7.74515	3.16194	75.6886	91.9447	70.84	91.30
	aspiration	6	41.1800	6.77865	2.76737	34.0662	48.2938	30.00	47.73
	Total	12	62.4983	23.32253	6.73263	47.6799	77.3168	30.00	91.30

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Matured	Between Groups	55.815	1	55.815	3.633	.086
	Within Groups	153.621	10	15.362		
	Total	209.436	11			
Enucleated	Between Groups	40.333	1	40.333	1.058	.328
	Within Groups	381.395	10	38.140		
	Total	421.729	11			
Injected	Between Groups	136.350	1	136.350	6.456	.029
	Within Groups	211.196	10	21.120		
	Total	347.546	11			

Appendix Table 2.2: Percentages of reconstructed couplets and *in vitro* embryo development for different enucleation methods in interspecies SCNT cloned-caprine embryos

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
reconstructed	squeezing	6	87.5150	3.85099	1.57216	83.4736	91.5564	83.33	93.33
	aspiration	6	84.8383	6.98171	2.85027	77.5115	92.1652	75.00	92.00
	Total	12	86.1767	5.55441	1.60342	82.6476	89.7058	75.00	93.33
twocell	squeezing	6	60.1783	5.95601	2.43153	53.9279	66.4288	52.38	68.97
	aspiration	6	61.5450	10.27959	4.19663	50.7572	72.3328	50.00	74.07
	Total	12	60.8617	8.04151	2.32138	55.7523	65.9710	50.00	74.07
fourcell	squeezing	6	53.8017	6.95327	2.83866	46.5047	61.0987	42.86	62.07
	aspiration	6	49.8550	9.47305	3.86736	39.9136	59.7964	41.18	62.96
	Total	12	51.8283	8.18625	2.36317	46.6270	57.0296	41.18	62.96
eightcell	squeezing	6	37.7067	8.07377	3.29610	29.2338	46.1796	28.57	51.72
	aspiration	6	39.2217	10.43737	4.26104	28.2683	50.1750	29.41	55.56
	Total	12	38.4642	8.93160	2.57833	32.7893	44.1390	28.57	55.56
morula	squeezing	6	24.4517	6.63297	2.70790	17.4908	31.4125	18.18	34.48
	aspiration	6	21.0700	9.64728	3.93849	10.9458	31.1942	11.76	37.04
	Total	12	22.7608	8.08837	2.33491	17.6217	27.8999	11.76	37.04
blastocyst	squeezing	6	12.0783	7.22265	2.94864	4.4986	19.6580	4.76	20.69
	aspiration	6	10.9317	4.58586	1.87217	6.1191	15.7442	5.77	18.52
	Total	12	11.5050	5.79913	1.67406	7.8204	15.1896	4.76	20.69

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
reconstructed	Between Groups	21.494	1	21.494	.676	.430
	Within Groups	317.872	10	31.787		
	Total	339.366	11			
twocell	Between Groups	5.603	1	5.603	.079	.784
	Within Groups	705.721	10	70.572		
	Total	711.324	11			
fourcell	Between Groups	46.729	1	46.729	.677	.430
	Within Groups	690.434	10	69.043		
	Total	737.162	11			
eightcell	Between Groups	6.886	1	6.886	.079	.784
	Within Groups	870.623	10	87.062		
	Total	877.508	11			
morula	Between Groups	34.307	1	34.307	.501	.495
	Within Groups	685.331	10	68.533		
	Total	719.638	11			
blastocyst	Between Groups	3.945	1	3.945	.108	.749
	Within Groups	365.984	10	36.598		
	Total	369.929	11			

Appendix Table 2.3: Successfully enucleated, injected, reconstructed couplets and embryos cleaved rates for different injection methods in interspecies SCNT cloned-caprine embryos

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Enucleated	SUZI	6	88.0083	7.35186	3.00138	80.2930	95.7236	76.60	95.00
	ICI	8	92.0400	5.09540	1.80150	87.7801	96.2999	85.71	100.00
	Total	14	90.3121	6.24943	1.67023	86.7038	93.9205	76.60	100.00
Injected	SUZI	6	93.2583	6.49917	2.65328	86.4379	100.0788	83.33	100.00
	ICI	8	90.6975	6.51700	2.30411	85.2492	96.1458	79.17	100.00
	Total	14	91.7950	6.39098	1.70806	88.1050	95.4850	79.17	100.00
Reconstructed	SUZI	6	87.5150	3.85099	1.57216	83.4736	91.5564	83.33	93.33
	ICI	8	91.2775	5.97941	2.11404	86.2786	96.2764	83.78	100.00
	Total	14	89.6650	5.35623	1.43151	86.5724	92.7576	83.33	100.00
Cleaved	SUZI	6	60.1783	5.95601	2.43153	53.9279	66.4288	52.38	68.97
	ICI	8	54.1425	5.06303	1.79005	49.9097	58.3753	45.16	58.82
	Total	14	56.7293	6.08730	1.62690	53.2146	60.2440	45.16	68.97

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Enucleated	Between Groups	55.729	1	55.729	1.480	.247
	Within Groups	451.991	12	37.666		
	Total	507.720	13			
Injected	Between Groups	22.484	1	22.484	.531	.480
	Within Groups	508.495	12	42.375		
	Total	530.980	13			
Reconstructed	Between Groups	48.536	1	48.536	1.795	.205
	Within Groups	324.424	12	27.035		
	Total	372.960	13			
Cleaved	Between Groups	124.907	1	124.907	4.201	.063
	Within Groups	356.811	12	29.734		
	Total	481.718	13			

Appendix Table 2.4: Percentages of reconstructed couplets and *in vitro* embryo development for different injection methods in interspecies SCNT cloned-caprine embryos

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
reconstructed	SUZI	6	87.5150	3.85099	1.57216	83.4736	91.5564	83.33	93.33
	ICI	8	91.2775	5.97941	2.11404	86.2786	96.2764	83.78	100.00
	Total	14	89.6650	5.35623	1.43151	86.5724	92.7576	83.33	100.00
twocell	SUZI	6	60.1783	5.95601	2.43153	53.9279	66.4288	52.38	68.97
	ICI	8	54.1425	5.06303	1.79005	49.9097	58.3753	45.16	58.82
	Total	14	56.7293	6.08730	1.62690	53.2146	60.2440	45.16	68.97
fourcell	SUZI	6	53.8017	6.95327	2.83866	46.5047	61.0987	42.86	62.07
	ICI	8	38.6013	6.36451	2.25019	33.2804	43.9221	29.03	47.62
	Total	14	45.1157	10.06696	2.69051	39.3032	50.9282	29.03	62.07
eightcell	SUZI	6	37.7067	8.07377	3.29610	29.2338	46.1796	28.57	51.72
	ICI	8	31.3775	9.37741	3.31542	23.5378	39.2172	16.67	42.86
	Total	14	34.0900	9.10969	2.43467	28.8302	39.3498	16.67	51.72
morula	SUZI	6	24.4517	6.63297	2.70790	17.4908	31.4125	18.18	34.48
	ICI	8	16.0488	4.05301	1.43295	12.6604	19.4371	6.45	19.23
	Total	14	19.6500	6.66251	1.78063	15.8032	23.4968	6.45	34.48
blastocyst	SUZI	6	12.0783	7.22265	2.94864	4.4986	19.6580	4.76	20.69
	ICI	8	4.5075	4.09603	1.44816	1.0831	7.9319	.00	9.52
	Total	14	7.7521	6.64942	1.77713	3.9129	11.5914	.00	20.69

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
reconstructed	Between Groups	48.536	1	48.536	1.795	.205
	Within Groups	324.424	12	27.035		
	Total	372.960	13			
twocell	Between Groups	124.907	1	124.907	4.201	.063
	Within Groups	356.811	12	29.734		
	Total	481.718	13			
fourcell	Between Groups	792.181	1	792.181	18.097	.001
	Within Groups	525.288	12	43.774		
	Total	1317.469	13			
eightcell	Between Groups	137.343	1	137.343	1.751	.210
	Within Groups	941.480	12	78.457		
	Total	1078.823	13			
morula	Between Groups	242.088	1	242.088	8.673	.012
	Within Groups	334.969	12	27.914		
	Total	577.057	13			
blastocyst	Between Groups	196.517	1	196.517	6.234	.028
	Within Groups	378.276	12	31.523		
	Total	574.793	13			

Appendix Table 2.5: Successfully enucleated, injected and reconstructed couplets rates for different *in vitro* culture systems in interspecies SCNT cloned-caprine embryos

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Matured	Group A	6	57.8633	2.68018	1.09418	55.0507	60.6760	54.29	62.50
	Group B	6	55.8650	5.34522	2.18218	50.2555	61.4745	52.86	66.67
	Group C	6	56.7783	5.24326	2.14055	51.2759	62.2808	50.00	65.71
	Total	18	56.8356	4.39413	1.03571	54.6504	59.0207	50.00	66.67
Enucleated	Group A	6	91.4733	2.79839	1.14244	88.5366	94.4101	86.21	94.59
	Group B	6	93.3767	5.01378	2.04687	88.1150	98.6383	86.36	100.00
	Group C	6	88.0083	7.35186	3.00138	80.2930	95.7236	76.60	95.00
	Total	18	90.9528	5.55184	1.30858	88.1919	93.7136	76.60	100.00
Injected	Group A	6	90.9700	2.85733	1.16650	87.9714	93.9686	85.71	94.29
	Group B	6	91.6867	5.75225	2.34835	85.6501	97.7233	81.25	97.14
	Group C	6	93.2583	6.49917	2.65328	86.4379	100.0788	83.33	100.00
	Total	18	91.9717	5.05208	1.19079	89.4593	94.4840	81.25	100.00
Reconstructed	Group A	6	87.5650	3.70442	1.51232	83.6774	91.4526	83.33	92.86
	Group B	6	85.7983	4.91688	2.00731	80.6384	90.9583	76.92	91.67
	Group C	6	87.5150	3.85099	1.57216	83.4736	91.5564	83.33	93.33
	Total	18	86.9594	4.02773	.94934	84.9565	88.9624	76.92	93.33

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Matured	Between Groups	12.009	2	6.005	.285	.756
	Within Groups	316.233	15	21.082		
	Total	328.242	17			
Enucleated	Between Groups	88.896	2	44.448	1.532	.248
	Within Groups	435.094	15	29.006		
	Total	523.990	17			
Injected	Between Groups	16.440	2	8.220	.295	.748
	Within Groups	417.460	15	27.831		
	Total	433.900	17			
Reconstructed	Between Groups	12.141	2	6.071	.345	.713
	Within Groups	263.643	15	17.576		
	Total	275.784	17			

Post Hoc Tests

Matured

Duncan[a]

		Subset for alpha = 0.05
VAR00001	N	1
Group B	6	55.8650
Group C	6	56.7783
Group A	6	57.8633
Sig.		.486

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Enucleated

Duncan[a]

VAR00001	N	Subset for alpha = 0.05
		1
Group C	6	88.0083
Group A	6	91.4733
Group B	6	93.3767
Sig.		.121

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Injected

Duncan[a]

VAR00001	N	Subset for alpha = 0.05
		1
Group A	6	90.9700
Group B	6	91.6867
Group C	6	93.2583
Sig.		.488

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Reconstructed

Duncan[a]

VAR00001	N	Subset for alpha = 0.05
		1
Group B	6	85.7983
Group C	6	87.5150
Group A	6	87.5650
Sig.		.500

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Appendix Table 2.6: Percentages of reconstructed couplets and *in vitro* embryo development for different *in vitro* culture systems in interspecies SCNT cloned-caprine embryos

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
reconstructed	Group A	6	87.5650	3.70442	1.51232	83.6774	91.4526	83.33	92.86
	Group B	6	85.7983	4.91688	2.00731	80.6384	90.9583	76.92	91.67
	Group C	6	87.5150	3.85099	1.57216	83.4736	91.5564	83.33	93.33
	Total	18	86.9594	4.02773	.94934	84.9565	88.9624	76.92	93.33
twocell	Group A	6	49.0117	4.95857	2.02433	43.8080	54.2154	41.03	55.00
	Group B	6	49.8450	7.99828	3.26529	41.4513	58.2387	40.00	60.00
	Group C	6	60.1783	5.95601	2.43153	53.9279	66.4288	52.38	68.97
	Total	18	53.0117	7.98714	1.88259	49.0398	56.9836	40.00	68.97
fourcell	Group A	6	36.9167	7.40840	3.02447	29.1420	44.6913	28.57	45.16
	Group B	6	39.6800	6.66740	2.72196	32.6830	46.6770	33.33	51.72
	Group C	6	53.8017	6.95327	2.83866	46.5047	61.0987	42.86	62.07
	Total	18	43.4661	10.06670	2.37274	38.4601	48.4722	28.57	62.07
eightcell	Group A	6	26.4617	4.27364	1.74471	21.9768	30.9466	21.43	32.26
	Group B	6	29.3400	4.57170	1.86639	24.5423	34.1377	25.00	37.93
	Group C	6	37.7067	8.07377	3.29610	29.2338	46.1796	28.57	51.72
	Total	18	31.1694	7.40120	1.74448	27.4889	34.8500	21.43	51.72
morula	Group A	6	8.4183	1.14962	.46933	7.2119	9.6248	7.14	10.00
	Group B	6	10.2217	3.65592	1.49252	6.3850	14.0583	6.67	17.24
	Group C	6	24.4517	6.63297	2.70790	17.4908	31.4125	18.18	34.48
	Total	18	14.3639	8.46808	1.99595	10.1528	18.5750	6.67	34.48
blastocyst	Group A	6	.0000	.00000	.00000	.0000	.0000	.00	.00
	Group B	6	.0000	.00000	.00000	.0000	.0000	.00	.00
	Group C	6	12.0783	7.22265	2.94864	4.4986	19.6580	4.76	20.69
	Total	18	4.0261	7.04764	1.66115	.5214	7.5308	.00	20.69

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
reconstructed	Between Groups	12.141	2	6.071	.345	.713
	Within Groups	263.643	15	17.576		
	Total	275.784	17			
twocell	Between Groups	464.333	2	232.167	5.615	.015
	Within Groups	620.170	15	41.345		
	Total	1084.504	17			
fourcell	Between Groups	984.321	2	492.161	9.997	.002
	Within Groups	738.433	15	49.229		
	Total	1722.754	17			
eightcell	Between Groups	409.472	2	204.736	5.886	.013
	Within Groups	521.751	15	34.783		
	Total	931.223	17			
morula	Between Groups	925.625	2	462.813	23.660	.000
	Within Groups	293.418	15	19.561		
	Total	1219.043	17			
blastocyst	Between Groups	583.545	2	291.772	16.779	.000
	Within Groups	260.833	15	17.389		
	Total	844.378	17			

Post Hoc Tests

reconstructed

Duncan[a]

		Subset for alpha = 0.05
VAR00001	N	1
Group B	6	85.7983
Group C	6	87.5150
Group A	6	87.5650
Sig.		.500

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

twocell

Duncan[a]

VAR00001	N	Subset for alpha = 0.05	
		1	2
Group A	6	49.0117	
Group B	6	49.8450	
Group C	6		60.1783
Sig.		.825	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

fourcell

Duncan[a]

VAR00001	N	Subset for alpha = 0.05	
		1	2
Group A	6	36.9167	
Group B	6	39.6800	
Group C	6		53.8017
Sig.		.506	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

eightcell

Duncan[a]

VAR00001	N	Subset for alpha = 0.05	
		1	2
Group A	6	26.4617	
Group B	6	29.3400	
Group C	6		37.7067
Sig.		.411	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

morula

Duncan[a]

VAR00001	N	Subset for alpha = 0.05	
		1	2
Group A	6	8.4183	
Group B	6	10.2217	
Group C	6		24.4517
Sig.		.491	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

blastocyst

Duncan[a]

VAR00001	N	Subset for alpha = 0.05	
		1	2
Group A	6	.0000	
Group B	6	.0000	
Group C	6		12.0783
Sig.		1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

APPENDIX 3: SUPPLEMENTARY RESULTS FROM PRELIMINARY STUDIES

Appendix Table 3.1: Preliminary experiment to develop techniques on nuclear transfer using intraspecies caprine, bovine and porcine as model animals

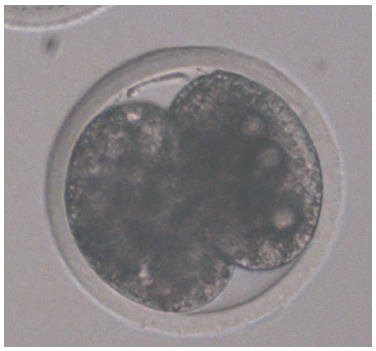
Model animals	Types of donor cell used	Total no. of matured oocytes	Percent oocyte successfully enucleated (n)	Percent oocytes successfully injected (n)	Percent reconstituted couplets (n)	Percent cleaved SCNT cloned-embryos at different cell stage (n)				
						Cleaved embryos	4 cell	8 cell	16 cell	Blastocyst
Caprine (Squeezing + WCICI)	Cumulus cell	43	90.70 (39/43)	94.87 (37/39)	100.00 (37/37)	13.51 (5/37)	2.70 (1/37)	0.00 (0/37)	-	-
Bovine (Squeezing + WCICI)	Cumulus cell	218	76.15 (166/218)	90.96 (151/166)	76.16 (115/151)	29.57 (34/115)	15.65 (18/115)	10.43 (12/115)	0.87 (1/115)	0.00 (0/115)
Porcine (Aspiration + Electrofusion)	Foetal fibroblast cell	1270	89.37 (1135/1270)	95.24 (1081/1135)	83.35 (901/1081)	77.47* (698/901)	NA	NA	NA	21.75 (196/901)

*For porcine SCNT, the total number of cleaved embryos was included from 2- to 4 cell stages due to the embryos were only observed on days 2 and 6.

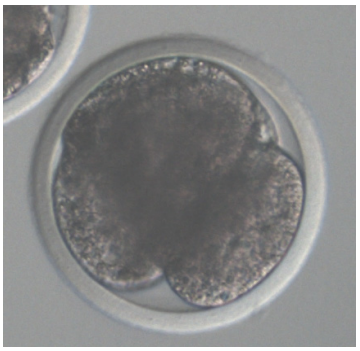
The preliminary experiments on caprine and bovine intraspecies SCNT were carried out in ABEL, Institute of Biological Sciences, Faculty of Science, University of Malaya (July 2009 – April 2010).

The experiment on porcine intraspecies SCNT was performed in YunNan Agricultural University (May – August 2010), China, on an agreement of "Memorandum on Academic Exchange between University of Malaya and YunNan Agricultural University, 2010-2015".

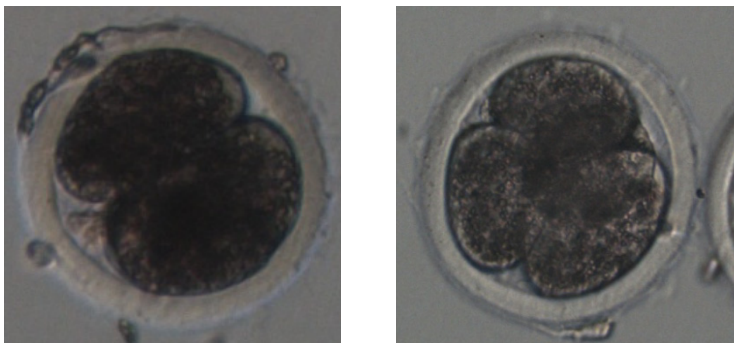
This table shows the summarised results of the actual experiments with aims to establish the SCNT methodology and to gain the nuclear transfer skill through learning curve from these preliminary studies.



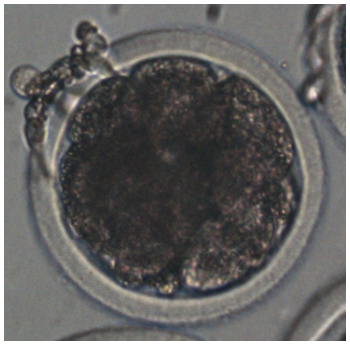
Appendix Figure 3.1: *In vitro* embryo development of intraspecies SCNT cloned-caprine embryos at day 1 (2 cell stage).



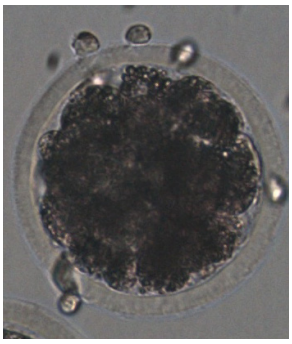
Appendix Figure 3.2: *In vitro* embryo development of intraspecies SCNT cloned-caprine embryos at day 2 (4 cell stage).



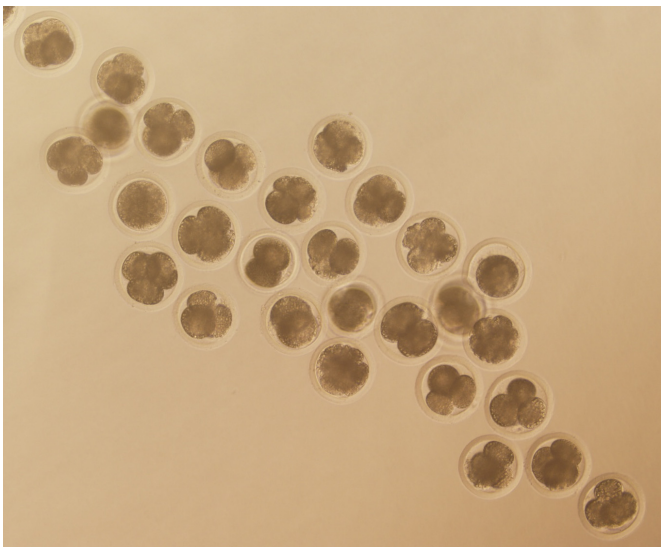
Appendix Figure 3.3: *In vitro* embryo development of intraspecies SCNT cloned-bovine embryos at day 1 (2 cell stage) and day 2 (4 cell stage).



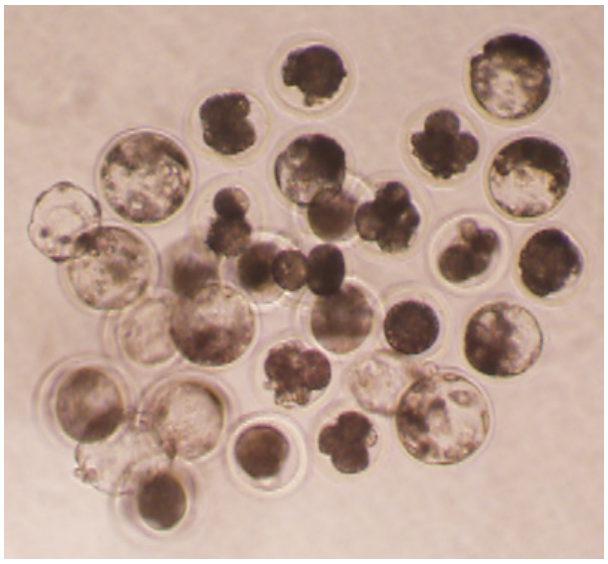
Appendix Figure 3.4: *In vitro* embryo development of intraspecies SCNT cloned-bovine embryos at day 3 (8 cell stages).



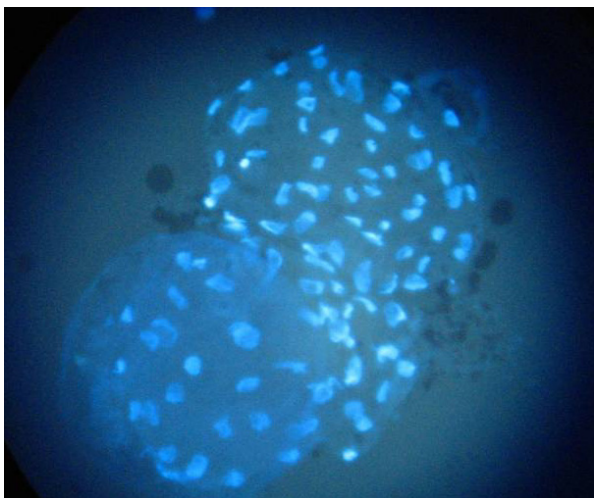
Appendix Figure 3.5: *In vitro* embryo development of intraspecies SCNT cloned-bovine embryos at day 4 (16 cell stage).



Appendix Figure 3.6: *In vitro* embryo development of intraspecies SCNT cloned-porcine embryos at day 2 (2- to 4 cell stages).



Appendix Figure 3.7: *In vitro* embryo development of intraspecies SCNT cloned-porcine embryos at day 6 (blastocyst stage).



Appendix Figure 3.8: Hoechst staining on blastocyst.

APPENDIX 4: LIST OF PUBLICATIONS AND PRESENTATIONS

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Sub-zonal *versus* intracytoplasmic injection produces a higher rate of cloned caprine-bovine interspecies blastocysts

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ABSTRACT

The production of cloned-caprine embryos using the intraspecies somatic cell nuclear transfer (SCNT) technique is limited by the low source of caprine oocytes as the recipient cytoplasts in certain countries. Therefore, using bovine oocytes as recipient cytoplasts in interspecies somatic cell nuclear transfer (iSCNT), is an alternative approach to produce a large number of cloned-caprine embryos and subsequently offspring at a rapid rate. The aim of this research was to compare the effect of nuclear transfer methods on iSCNT cloned embryos' developmental competence, involving: (a) an intracytoplasmic injection (ICI), and (b) the sub-zonal injection with electrofusion (SUZI), using fetal fibroblast cells as donor karyoplasts. The bovine ovaries were collected from local abattoir and transported to the laboratory within 2–3 h in NaCl (0.9%). The oocytes ($n = 725$) were recovered by checkerboard slicing of the entire surface of the ovary, inside a culture dish, using a razor blade. After slicing, the cumulus–oocyte complexes (COCs) were recovered and selected under a stereomicroscope. Oocytes with several compact layers of cumulus cells were selected and cultured in *in vitro* maturation (IVM) medium for 20–22 h. After maturation, COCs were denuded in hyaluronidase (0.1%) to remove the cumulus cells. The matured oocytes (with extrusion of first polar body) were selected for enucleation to remove the spindle. Caprine-fetal fibroblast cells (donor karyoplasts) were harvested and transferred to enucleated bovine oocytes, by using either an intracytoplasmic injection or sub-zonal injection, with electrofusion. The injected/fused oocytes were activated and the reconstructed couplets were cultured in KSOM medium for *in vitro* embryo development in a CO_2 (5%) incubator, at 38.5 °C in a humidified atmosphere for 8–9 days. The culture medium was changed every 2 days of IVC. The percentage of cleaved embryos and blastocyst formation following sub-zonal injection, with electrofusion was higher than for oocytes which underwent intracytoplasmic injection (60.2% *vs.* 54.1% and 12.1% *vs.* 4.5%, respectively). In summary, the nuclear transfer using both methods of sub-zonal injection and intracytoplasmic injection showed satisfactory results – with the former method being apparently higher in *in vitro* developmental competence in both cases. In conclusion, using caprine-bovine iSCNT to produce caprine embryos and offspring may offer a new approach to increase genetically superior goat populations at a rapid rate to meet the goat meat and milk demand for the industry – especially in the developing countries.

1. Introduction

The application of reproductive technologies in goats is still low, compared to other livestock species such as cattle, sheep and pigs. However, in the current trend of goat commercialization, there are vigorous efforts worldwide to

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incorporate developed, advanced reproductive technologies (ARTs), such as artificial insemination (AI), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), oocytes and embryo cryopreservation and embryo transfer into modern farm management practices. Other newer techniques such as somatic cell nuclear transfer (SCNT) and stem cell research are currently also being actively researched in the various laboratories across the globe. However, one main issue of goat research is the lack of source of ovaries to obtain the oocytes for the various reproductive techniques. This is due to the low goat populations slaughtered; and those slaughtered, are mainly male goats. Therefore, using bovine oocytes as recipient cytoplasts in interspecies somatic cell nuclear transfer (iSCNT) is an alternative approach to produce large number of cloned-caprine embryos and subsequently offspring at a rapid rate.

In iSCNT, a donor cell (karyoplast) is transplanted into a recipient enucleated oocyte (cytoplast) of a different species/family/order/class. The resulting embryo is then transplanted into the oviduct/uterus of a suitable foster mother for development to term (Loi et al., 2011). However, most of the successfully live offspring have been obtained by combining closely related species, such as Gaur-cattle (Lanza et al., 2000), domestic argali sheep-sheep (White et al., 1999), river buffalo-swamp buffalo (Yang et al., 2010) and wild cat-domestic cat (Gómez et al., 2004).

The first report of mammalian iSCNT experiments was on sheep, pigs, monkeys and rats by using ear skin fibroblasts as donor karyoplasts and cattle as the recipient cytoplast (Dominko et al., 1999). Even though there was no pregnancy reported from these studies, this approach has opened a window of opportunity for interspecies cloning using 2 different species to produce a large number of animals and to conserve endangered species. However, the efficiency of iSCNT is still low. Therefore, future studies should focus on factors, such as effect of donor cell type, manipulation methods (*e.g.* enucleation, nuclear transfer and oocyte activation) and embryo culture systems in order to overcome iSCNT technical problems (Srirattana et al., 2010).

Besides mitochondrial heteroplasmy, nuclear-cytoplasmic incompatibilities and appropriate nuclear reprogramming, the nuclear transfer method is also one of the factors affecting the developmental ability of iSCNT embryos. Two procedures are currently being used to produce cloned animals. The sub-zonal injection (SUZI) involves placing a donor cell in the perivitelline space of an enucleated recipient oocyte and subsequently fusing both the donor and the recipient cells with an electrical pulse. This technique has been used to produce cloned sheep (Wilmut et al., 1997), cattle (Cibelli et al., 1998; Kato et al., 1998; Kubota et al., 2000), goats (Baguisi et al., 1999) and pigs (Bondiolo et al., 2001; Dai et al., 2002; Lai et al., 2002). In another non-fusion method, an intracytoplasmic injection (ICI), the plasma membrane of the donor cell is ruptured by pipetting it in and out a few times, using a narrow injection micropipette to facilitate the nuclear reprogramming by the oocyte cytoplasm. This technique has been successfully applied in mice (Wakayama et al., 1998) and pigs (Onishi et al., 2000). However, the comparison between these 2 techniques has not been reported in iSCNT of cloned caprine-bovine. This study was conducted with the aim to compare the sub-zonal injection and intracytoplasmic injection methods on the developmental competency of interspecies cloned-caprine embryos, using fetal fibroblast cells as donor karyoplasts.

2. Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich Co. (USA), and the media used in preparation of donor cells obtained from Gibco (Grand Island, NY, USA). The Petri dishes for *in vitro* culture were purchased from Nunc (Denmark).

2.1. Preparation of donor cells

The caprine-fetal fibroblast cell was used as the donor cell, according to the method of Keefer et al. (2001), with minor modifications. Two caprine fetuses, produced by natural mating, were derived at days 28 and 35 of gestation, respectively. Briefly, fetuses were surgically removed and placed in a culture dish containing equilibrated PBS(−). In the PBS(−) medium, the head and internal organs of the fetuses were removed, using a pair of surgical forceps. The explants (remaining fetus' skin tissues) were mechanically dissociated in a culture dish containing α-modified Eagle medium (α-MEM), supplemented with fetal bovine serum (FBS; 20%) and penicillin–streptomycin (P–S) under a humidified atmosphere of CO_2 (5%), in air at 37 °C. While the explant cultures contained a mixed population of cells, fetal fibroblasts were considered predominant. When the cells from the explants reached 70% confluence, they were harvested using trypsin/EDTA (0.25%) and then sub-cultured (approximately 30% of donor cells were seeded on each plate) to passage 1 or 2. The fetal fibroblast cells at passage 1 or 2 were then cryopreserved using DMSO (10%), mixed in the tissue culture medium and stored in liquid nitrogen. The frozen cells were thawed and cultured up to 90% confluence (it is suggested that 90% confluence of donor cells can be used as karyoplasts to produce optimal interspecies cloned caprine-bovine embryos) (unpublished data, H.H. Soh, 2011), to use as donor cells. However, subsequent sub-cultured donor cells (approximately 30% of donor cells were seeded on each plate) were also cultivated to use as donor cells in nuclear transfer (passages 2–5).

2.2. Oocyte collection and in vitro maturation

Bovine ovaries were transported to the laboratory in sterile saline (0.9%), supplemented with penicillin-G (60 μg/ml) and streptomycin (50 μg/ml) at 30–35 °C. Cumulus-oocyte complexes (COCs) were recovered by checkerboard slicing the entire surface of the ovary inside a culture dish containing TL-HEPES medium, with the aid of a razor blade. The COCs surrounded with at least 3 compact layers of granulosa cells were selected for *in vitro* maturation under a stereomicroscope. The selected COCs were washed 2 times in TL-HEPES medium, followed by 3 times in *in vitro* maturation (IVM) medium before being cultured in microdroplets (80 μl) 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 under a humidified atmosphere of CO_2 (5%) in air, at 38.5 °C for 20–22 h.

2.3. Enucleation of matured oocytes

After maturation, the COCs were denuded in TL-HEPES medium containing 1 mg/ml hyaluronidase (0.1%) for 5 min and subsequently washed 5 times in TL-HEPES medium. All selected matured oocytes with the extrusion of a first polar body were transferred to a microdroplet containing TL-HEPES medium, supplemented with cytochalasin B (5 μg/ml) for 10 min, prior to enucleation. Briefly, a cut was made on the zona pellucida above the first polar body and 10% of the cytoplasm beneath the first polar body was gently squeezed out. The enucleated oocytes were then washed 3 times in TL-HEPES medium, 3 times in holding medium (TCM-199 medium supplemented with 10% FBS), and lastly kept in holding medium at 38.5 °C in the presence of CO_2 (5%) in air in a humidified atmosphere of a CO_2 incubator for 30–60 min, prior to nuclear transfer.

Fig. 1. First polar body together with cytoplasm (10%) after enucleation.

2.4. Nuclear transfer

For the sub-zonal injection, a single fetal fibroblast cell was injected into the perivitelline space of an enucleated oocyte. The injected oocyte was washed 3 times in TL-HEPES medium, 3 times in holding medium and incubated in the final microdroplet of holding medium for 30–45 min prior to fusion. The injected oocyte was fused in 50 μl fusion medium (0.25 M sorbitol, 500 μl magnesium acetate, 0.5 mM HEPES and 0.2% BSA), overlaid with mineral oil. The fusion machine used was a SUTF-1, manufactured by the Suranaree University of Technology, Thailand. The current of the fusion was 20 V, with 2 direct current (DC) pulses in 15 μs. After fusion, all the fused oocytes were washed 5 times in TL-HEPES medium and 3 times in holding medium – followed by the transfer into the final microdroplet of holding medium, and incubated for at least 30–45 min prior to activation.

For intracytoplasmic injection, a single fetal fibroblast cell was pipetted in and out few times in polyvinylpyrrolidone (PVP; 10%) medium, using a blunt 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 into the needle was observed. This confirmed the breakage of the cytoplasmic membrane, thereby facilitating donor cell injection. A single donor cell was then gently deposited into the cytoplasm. All the injected oocytes were washed 3 times in TL-HEPES medium, 3 times in holding medium and incubated in the final microdroplet of holding medium for 30–45 min prior to activation.

2.5. Activation

All the injected or fused oocytes were subjected to activation with a calcium ionophore (5 μM) for 5 min, followed by 6-dimethylaminopurine (6-DMAP; 1.9 μM) for 5 h.

2.6. In vitro culture

Following activation, the reconstructed oocytes were washed 5 times and then cultured in KSOM medium under a humidified atmosphere of CO_2 (5%) in air, at 38.5 °C. The development of cloned-caprine embryos were monitored every 2 days and recorded. At the same time, the replacement of culture medium was also made every 2 days of culture.

2.7. Statistical analyses

Effects of nuclear transfer methods (sub-zonal injection and intracytoplamic injection) on cloned-caprine embryo developmental competence following iSCNT were all analyzed by using the one-way analysis of variance (ANOVA). Using the SPSS statistical program, means were obtained and analyzed using the ANOVA and differences between the means were determined using Duncan multiple range test (DMRT) (Steel and Torrie, 1980).

3. Results

A total of 725 recovered bovine oocytes were matured *in vitro* for 20–22 h, with 400 (55.2%) of the matured oocytes showing the extrusion of a first polar body. The reconstructed and *in vitro* developmental rates are set out in Table 1 and Figs. 1–3. No significant differences were recorded in the injection and reconstruction rates for both nuclear transfer methods. As for the *in vitro* development, the percentage of cloned caprine-bovine embryos at the 2- and 8-cell stage using the SUZI and ICI methods did not differ significantly. However, the percentage of cloned caprine-bovine embryos at the morula and blastocyst stage derived from SUZI method, were significantly higher ($P < 0.05$), compared to the ICI method (24.5% *vs.* 16.1% and 12.1% *vs.* 4.5%, respectively). There were significant differences in embryonic development between the stages from the 2-cell up to the blastocyst stage, for both nuclear transfer methods ($P < 0.05$).

4. Discussion

Results demonstrate that using both nuclear transfer methods (SUZI and ICI), both have the ability to produce interspecies cloned caprine-bovine blastocysts (12.1 and 4.5%, respectively). It was also shown that the fetal

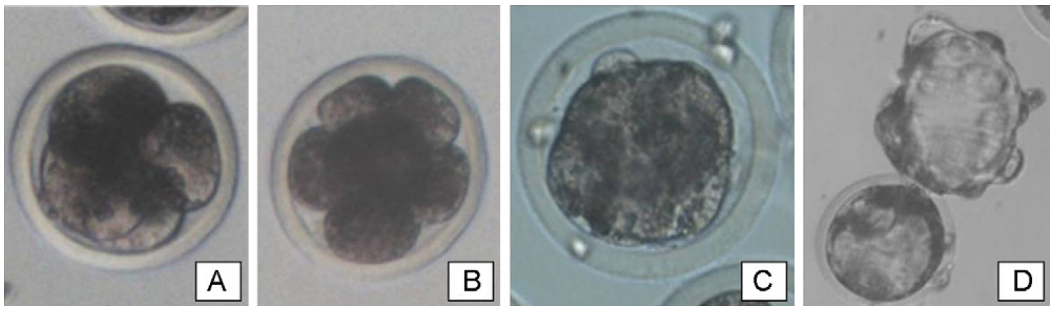

Fig. 2. Caprine iSCNT embryos at day-2 (4- to 8-cell stages) (A and B), caprine iSCNT cloned embryo at day-5 (morula stage) (C), caprine iSCNT cloned embryo at day-7 (blastocyst stage) (D) post-activation.

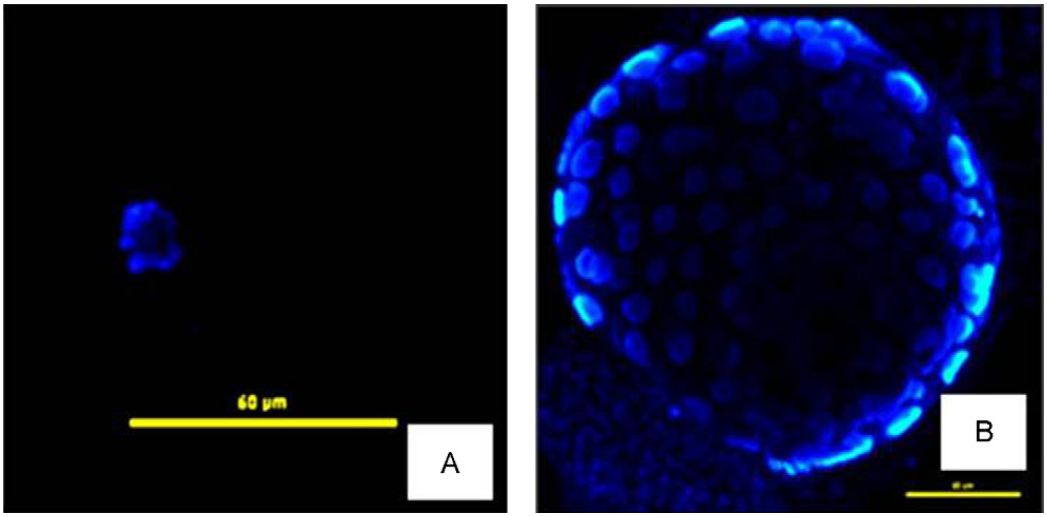

Fig. 3. Hoechst 33342 stained the DNA nucleus after enucleation corresponding to Fig. 1. (A), and day-7 blastocyst formed following iSCNT showing the nuclei (B).

fibroblast nucleus could be redifferentiated and reprogrammed in enucleated bovine oocytes. Similar results have been reported by Ma et al. (2008) in caprine-ovine interspecies cloning. In these studies, the caprine-fetal fibroblast nuclei were transferred into enucleated ovine oocytes and a 7.4% blastocyst formation rate obtained. In contrast, a previous study demonstrated that only 6.9% morula were obtained from interspecies caprine-bovine cloning using adult skin fibroblast cells (Abdullah et al., 2011). One factor that may cause the difference in results may be ascribed to the difference in donor animal age (fetal fibroblast cells *vs.* adult fibroblast cells) (Hua et al., 2008). As mentioned by Saikhun et al. (2002), more embryos are derived from fetal fibroblast cells after fusion with adult cumulus cells and fetal fibroblast cells with enucleated bovine oocytes. Therefore, the fetal fibroblast nucleus can be easily reprogrammed in the enucleated bovine oocyte, when compared with the adult fibroblast nucleus. According to Zhang et al. (2004), 90% of the cell cycle phases are found in the G0 + G1 stage, when the cells grow to nearly 90–100% confluence.

In this study, the nuclear transfer by the SUZI method gave higher percentages of 2-, 8-cell, morulae and blastocyst embryos, compared with nuclear transfer by the ICI method. However, only the percentage for morulae and blastocysts showed significant differences ($P < 0.05$) between these 2 nuclear transfer methods. The low efficiency of cloned-caprine embryonic development using the ICI method may be due to the damage induced in the isolated nucleus before injection (Lee et al., 2003; Chen et al., 2007). In the ICI method, the plasma membrane of the donor cell had to be ruptured by pipetting it in and out for a few times, in a narrow micro-injection needle. Therefore, it is suggested that the nucleus of the donor cells may be damaged during these micromanipulation procedures. As a result, the donor cell nucleus failed to be reprogrammed by the oocyte cytoplasm after injection. Similar findings have been reported in somatic cell nuclear transfer in porcine, with the blastocyst formation for SUZI and ICI being 18 and 13%, respectively (Kawano et al., 2004). Zou et al. (2001) on the other hand, demonstrated that the survival rate of cloned embryos derived from the direct injection method was higher than the fusion method (62.7 and 45.9%, respectively), following caprine nuclear transfer. This ICI method developed by Wakayama et al. (1998) was later modified by Lee et al. (2003), who used the whole cell intracytoplasmic injection (WCICI) method in porcine nuclear transfer – bypassing the donor nucleus isolation and electrofusion. This method has then been successfully used to produce panda-rabbit interspecies cloned embryos by Jiang et al. (2004). However, extra pressure and a large microneedle were needed to properly insert the entire donor cell content into the recipient cytoplasm (Hosseini et al., 2008). Thus, a lower efficiency of embryo reconstruction was found in the larger donor cells, such as skin fibroblast cells (15–20 μm) (Inoue et al., 2003). In this study, fetal fibroblast cells were used as donor karyoplasts, to produce interspecies cloned-caprine blastocysts.

There are very few reports on caprine iSCNT research, compared to other livestock species. The examples of iSCNT in caprine have been reported were caprine-bovine (Abdullah et al., 2011) with the morula formation rate of 6.9%; caprine-ovine (Ma et al., 2008) and caprine-bovine (Sansiñena, 2004) with a blastocyst formation rate of 7.4 and 15.0%, respectively. Present results on iSCNT using caprine-bovine gave 5 and 12% blastocyst rates for the ICI and SUZI methods, respectively. Using SCNT in caprine, Abdullah et al. (2011) demonstrated that 21% of the morula stages had been obtained after nuclear transfer – however, the embryos did not develop further to blastocysts. Tang et al. (2011) reported a higher percentage of blastocysts after nuclear transfer (20%), while Melican et al. (2005) produced cloned caprine offspring. The reason for the lower efficiency in iSCNT than in SCNT, is currently unclear. However, it may be due to the mitochondrial/genomic DNA incompatibility and/or embryonic genome activation of the donor nucleus by the recipient oocyte (Loi et al., 2011). In an interspecies experiment, 2 different species are involved and used to produce cloned embryos, as well as offspring. It always comes with mitochondrial heteroplasmy (mtDNA

Table 1
Mean (±SE) *in vitro* embryonic development rate for interspecies cloned caprine-bovine embryos.

Nuclear transfer methods	No. of oocytes	% of MII oocytes (*n*)	% of enucleated oocytes (*n*)	% of injected oocytes (*n*)	% of reconstructed oocytes[a] (*n*)	% of cleaved caprine-bovine embryos (*n*)			
						2-Cell	8-Cell	Morula	Blastocyst
SUZI	360	55.8 (201/360)	88.0 ± 3.0y (179/201)	93.3 ± 2.7y (166/179)	87.5 ± 1.6y (146/166)	60.2 ± 2.4d,y (88/146)	37.7 ± 3.3c,y (56/146)	24.5 ± 2.7b,z (36/146)	12.1 ± 3.0a,z (18/146)
ICI	365	54.5 (199/365)	92.0 ± 1.8y (186/199)	90.7 ± 2.3y (166/186)	91.3 ± 2.1y (150/166)	54.1 ± 1.8d,y (81/150)	31.4 ± 3.3c,y (47/150)	16.1 ± 1.4b,y (23/150)	4.5 ± 1.5a,y (6/150)
Total	725	55.2 (400/725)	91.3 (365/400)	91.0 (332/365)	89.2 (296/332)	57.1 (169/296)	34.8 (103/296)	19.9 (59/296)	8.1 (24/296)

Means with different letters (abcd) in a row were significantly different ($P < 0.05$). Means with different letters (yz) in a column were significantly different ($P < 0.05$).
[a] Percentage reconstructed oocytes after activation.

from the donor cell and the recipient oocyte being mixed in the cytoplasm of the reconstructed embryo), found in the interspecies cloned embryos. It has been suggested that mtDNA is responsible for the developmental arrest in interspecies reconstructed embryos (Thongphakdee et al., 2008), and it may be the insufficient mitochondrial respiration that hampers the survival of cloned embryos. Ma et al. (2008) reported that in caprine-ovine cloned embryos, the expression of mtDNA from the caprine fetal fibroblast cells (donor karyoplasts) was decreased from 1- to 2-cell stages, being undetectable from the 4-cell up to the blastocyst stage. However, the expression of mtDNA from recipient oocyte remains constant in the 1- to 8-cell stages, and gradually increases from 16-cell to the blastocyst stage. In contrast, in the closely related genetic interspecies, cloned animals such as the Gaur-mouflon, the mtDNA were found to be exclusively derived from the recipient oocytes (Lanza et al., 2000; Loi et al., 2011; Meirelles et al., 2001).

Embryonic genome activation of the donor nucleus by the recipient oocyte is one of the important factors causing the low efficiency in interspecies cloning of embryos. The transcription is often associated with a so-called critical or developmental block stage during *in vitro* culture of the pre-implantation embryos (Telford et al., 1990). The *in vitro* block has been shown to coincide with the time of transition from the maternal to embryonic genomic control. The new established genome starts to become transcriptionally active at different stages in pre-implantation embryos, according to the species [*e.g.* mice (2-cell) (Schultz, 1986; Telford et al., 1990); pigs (4-cell) (Prather et al., 1989); human and rabbits (8-cell) (Braude et al., 1979; Telford et al., 1990); sheep, cattle and goats (16-cell) (Camous et al., 1984; Bavister, 1988)]. In the case of intra-order SCNT the differences in the gene(s) products that trigger the first burst of transcription contained in the oocyte, probably fail to bind the corresponding sequences of the introduced nucleus – leading to early embryonic arrest (Loi et al., 2011). Therefore, it is essential to understand how embryonic genome activation occurs in the iSCNT, in an effort to increase the fundamental scientific information on early embryo life and the potential technological applications in medicine, agriculture and wildlife.

5. Conclusions

Cloned caprine embryos could be produced *in vitro* through iSCNT, using both nuclear transfer methods and fetal fibroblast cells as donor karyoplasts. The SUZI method shows a satisfactory rate of cleavage to the blastocyst stage, compared to the ICI method. With the establishment of iSCNT in the caprine and future refined related techniques, caprine-bovine interspecies cloning could be the best alternative. To produce a large number of superior caprine clones genetically, both for research, production as well as commercialization in the 21st century.

Acknowledgements

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PRODUCTION OF CLONED-PORCINE EMBRYOS THROUGH SOMATIC CELL NUCLEAR TRANSFER (SCNT)

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INTRODUCTION

Since 1997 the first cloned sheep (1) derived from somatic cell nuclear transfer (SCNT), numerous studies have been done to improve the efficiency of cloning, such as effect of donor cell type, manipulation methods (e.g. enucleation, cell injection, oocyte activation) and embryo culture systems (2).

OBJECTIVES

a) To produce cloned-porcine embryos *in vitro* through SCNT.
b) To compare the effect of timing of donor cell injection into the enucleated oocyte on cleavage rate of porcine embryos *in vitro*.

MATERIALS AND METHODS

The ovaries were collected from local slaughterhouse. Ovarian follicular fluid was aspirated by using a 20 ml disposable syringe attached with a 20-gauge needle and collect in a 15 ml centrifuge tube. After sedimentation, the cumulus-oocyte complexes (COCs) were recovered and selected under a stereo microscope. Oocytes with several compact layers of cumulus cells were selected and cultured in *in vitro* maturation (IVM) medium. After 44 hr of maturation, COCs were denuded in 0.1% hyaluronidase to remove the cumulus cells. The matured oocytes with polar bodies were selected for enucleation to remove the spindle. Fetal fibroblast cells (donor cells) were harvested and transferred into enucleated oocytes 1-2 hr after or injected simultaneously with enucleation. After nuclear transfer, both groups of the injected oocytes underwent electro-fusion between the donor cell and cytoplasm. The reconstructed oocytes were activated and cultured in PZM3 medium for *in vitro* embryo development until blastocyst.

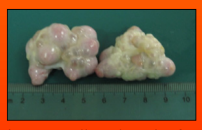

Figure 1: Collected ovaries from local slaughterhouse.

Figure 2: Aspiration of the ovarian follicular fluid.

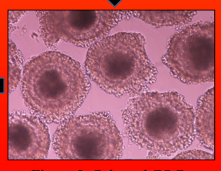

Figure 3: Selected COCs.

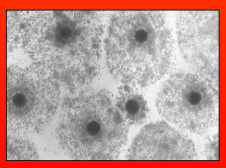

Figure 4: After IVM.

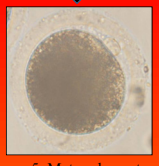

Figure 5: Matured oocyte with polar body.

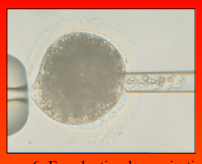

Figure 6: Enucleation by aspiration technique.

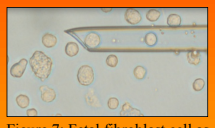

Figure 7: Fetal fibroblast cell as the donor cell.

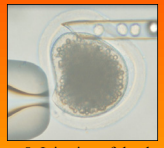

Figure 8: Injection of the donor cell in perivitelline space.

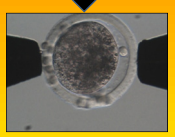

Figure 9: Electro-fusion by using 1 x 100 µs at 20 v/mm.

RESULTS

Table 1: The efficiency of porcine cloning on different times of donor cell injection

Time of donor cell injection	No. of matured oocyte	Successfully enucleated rate (n)	Successfully injected rate (n)	Successfully reconstituted rate (n)	Developmental competency rate of cloned embryos (n)		
					Cleaved embryo	Blastocyst	Hatching Blastocyst
Injection 1-2 hr after enucleation	610	92.30 (563/610)	90.41 (509/563)	87.62 (446/509)	67.49 (301/446)	17.94 (54/301)	9.97 (30/301)
Injection simultaneously with enucleation	660	86.67 (572/660)	100.00 (572/572)	79.55 (455/572)	87.25 (397/455)	35.77 (142/397)	22.67 (90/397)
Pathenogenesis activation	662	-	-	-	60.42 (400/662)	26.50 (106/400)	7.75 (31/400)

Table 1 shows the efficiency of porcine cloning on different times of donor cell injection to the enucleated oocyte. From the results obtained, the proportion of successfully reconstituted oocytes by incubating the enucleated oocyte 1-2 hr before injection method was higher than that injection simultaneously with enucleation (87.62% vs. 79.55%). However, the percentages for cleaved embryo, blastocyst and hatching blastocyst formation for injection simultaneously with enucleation were higher than the oocytes which have been injected 1-2 hr after enucleation (87.25% vs. 67.49%; 35.77% vs. 17.94% and 22.67% vs. 9.97%, respectively).

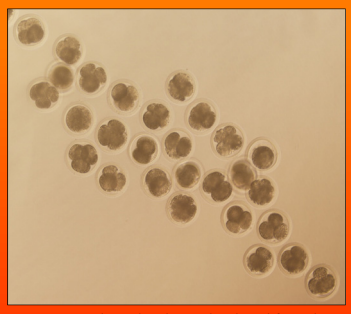

Figure 9: Day-2 cleaved embryos developed from the nuclear transfer embryos reconstructed with fetal fibroblasts cell.

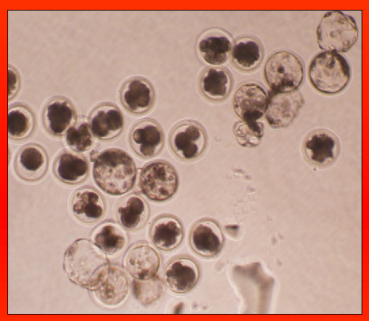

Figure 10: Day-7 blastocysts developed from the nuclear transfer embryos reconstructed with fetal fibroblasts cell.

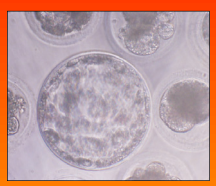

Figure 11: Expanded blastocyst.

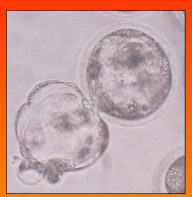

Figure 12: Hatching blastocyst.

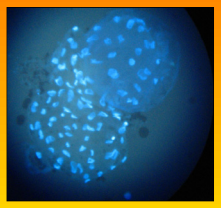

Figure 13: Hoechst 33342 stained 7-day blastocysts formed following SCNT showing the nuclei of ICM.

DISCUSSION

Injection of donor cell simultaneously with enucleation at the perivitelline space of enucleated oocyte gave higher percentages of cleaved, blastocyst and hatching blastocyst embryos compared to the enucleated oocyte which have been injected 1-2 hr after enucleation. This might due to the time exposure of oocyte to outside environment and manipulation medium is less in injection simultaneously with enucleation than the other method. Therefore, this method can reduce the oocyte injury during the cloning process.

Our results demonstrate that in terms of time, the injection simultaneously with enucleation method is more efficient than the injection 1-2 hr after enucleation in the production of a large number of SCNT embryos.

Furthermore, less time is needed for the injection simultaneously with enucleation:
a)100 oocytes: For injection simultaneously with enucleation, 1 hr 30 min needed to finish the enucleation and nuclear transfer (each batch of oocytes is 33).

b)100 oocytes: For injection 1-2 hr after enucleation, 3 hr needed to finish the enucleation and nuclear transfer (each batch of oocytes is 33).

Besides that, it is very difficult to find back the cutting point for the enucleated oocyte which have been injected 1-2 hr after enucleation. This is because the method we use to remove the DNA is aspiration technique. Therefore, the cutting point we made at the zona pellucida during the enucleation is much smaller and easier to re-close back (especially the oocytes have been injected 1-2 hr after enucleation) than using squeeze technique (3).

During the long manipulation time required to produce a large number of embryos by SCNT, embryos might be exposed to fluctuations in medium pH and temperature, which may be detrimental to subsequent viability of SCNT embryos. Therefore, a simple and efficient method of enucleation and cell injection is necessary in porcine to prepare a large number of SCNT embryos to transfer into surrogates (3).

CONCLUSIONS

Porcine embryos could be produced *in vitro* through SCNT using the fetal fibroblast. The injection of donor cell simultaneously with enucleation shows satisfactory rate of cleavage, however, further technical improvement is needed in order to produce maximum developmental competence cloned embryos *in vitro* as well as *in vivo*.

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PRODUCTION OF CLONED-GOAT EMBRYOS THROUGH INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER (iSCNT) USING TWO DIFFERENT NUCLEAR TRANSFER METHODS

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INTRODUCTION

Production of cloned-goat embryos using the intraSCNT is limited by low source of goat oocytes as the recipient cytoplast in Malaysia. Therefore, by using the cattle oocytes as recipient cytoplast in interSCNT (iSCNT) is the only alternative approach to produce large number of cloned-goat embryos and subsequently offspring at a rapid rate. The first report of mammalian iSCNT experiments was on sheep, pig, monkey and rat by using ear skin fibroblast as donor karyoplast and cattle as the recipient cytoplast (1). Even though there was no pregnancy reported from their studies, this approach has opened a window of opportunity for interspecies cloning using 2 different species to produce a large number of animals and to conserve endangered species. However, the efficiency of iSCNT is still low; therefore, future studies should focus on factors, such as effect of donor cell type, manipulation methods (e.g. enucleation, nuclear transfer and oocyte activation) and embryo culture systems in order to overcome the iSCNT technical problems (2).

OBJECTIVES

a) To produce cloned-goat embryos *in vitro* through iSCNT.
b) To compare the effect of 2 different nuclear transfer methods on iSCNT cloned-goat embryos developmental competence *in vitro*.

MATERIALS AND METHODS

The cattle ovaries were collected from local abattoir and transported to the laboratory within 2 to 3 hours in 0.9% NaCl. Cattle oocytes (n=586) were recovered by checkerboard slicing the entire surface of the ovary inside the culture dish by using the razor blade. After slicing, the cumulus-oocyte complexes (COC) were recovered and selected under a stereomicroscope. Oocytes with several compact layers of cumulus cells were selected and cultured in *in vitro* maturation (IVM) medium for 22 hours. After maturation, COC were denuded in 0.1% hyaluronidase to remove the cumulus cells. The matured oocytes (n=358) with polar bodies were selected for enucleation to remove the spindle. Goat-foetal fibroblast cells (donor cells) were harvested and transferred into enucleated cattle oocytes by using either intracytoplasmic injection (ICI) (n=157) or sub-zonal injection (SUZI) with electrofusion (n=201). The reconstructed oocytes were activated and cultured in KSOM medium for *in vitro* embryos development in CO_2 (5%) incubator at 38.5°C in humidified atmosphere for 8 to 9 days.

Establishment of goat-foetal fibroblast cells

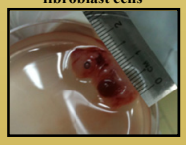

Figure 1: Collected goat foetus on day 35 of gestation.

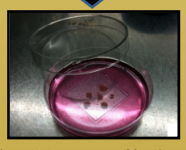

Figure 2: Arrangement of foetal tissue in culture dish.

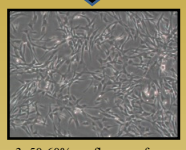

Figure 3: 50-60% confluency of foetal fibroblast cell line.

Establishment of cloned-goat embryos

Figure 4: Collected ovaries from local abattoir.

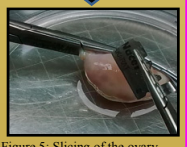

Figure 5: Slicing of the ovary.

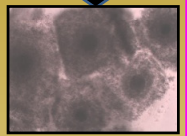

Figure 6: COC (sun-burst) after IVM.

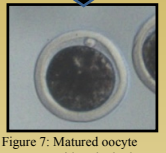

Figure 7: Matured oocyte with polar body.

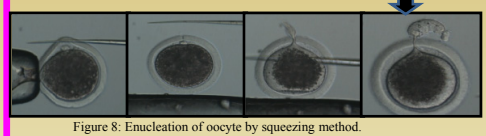

Figure 8: Enucleation of oocyte by squeezing method.

Figure 9: Foetal fibroblast cell as the donor karyoplast.

Figure 10: Nuclear transfer by SUZI method.

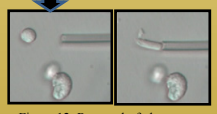

Figure 12: Removal of plasma membrane of donor karyoplast.

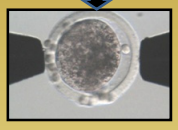

Figure 11: Electrofusion.

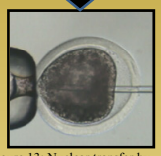

Figure 13: Nuclear transfer by ICI method.

Oocyte activation using calcium ionophore (5 min) and 6-DMAP (4-5 hr), subsequently, cultured in KSOM medium.

RESULTS

Table 1: The embryo development of cloned-goat iSCNT for different types of nuclear transfer method

Types of nuclear transfer insertion/ injection	No. of MII oocyte	Enucleation rate (n)	Insertion/ injection rate (n)	Reconstruction rate (n)	Developmental competence rate of cloned embryos (n)				
					2 cell	4 cell	8 cell	Morula	Blast.
SUZI	201	90.07±2.97^{a} (179/201)	93.26± 2.65^{a} (166/179)	87.51±1.57^{a} (146/166)	60.18± 2.43^{a,x} (88/146)	53.80± 2.84^{a,y} (79/146)	38.50± 2.93^{a,x} (56/146)	23.88± 2.31^{b,y} (36/146)	12.00± 2.49^{a,y} (18/146)
ICI	157	90.90±1.61^{a} (144/157)	91.07± 2.62^{a} (129/144)	92.35±2.10^{a} (119/129)	55.42± 1.44^{a,x} (67/119)	39.97± 2.06^{b,x} (48/119)	32.59± 3.52^{a,x} (40/119)	17.42± 0.48^{b,x} (23/119)	5.15± 1.50^{a,x} (7/119)

^{xy}: Means with different superscripts in a column were significantly different (P≤0.05).
^{abcd}: Means with different superscripts in a row were significantly different (P≤0.05).

Table 1 shows the efficiency of iSCNT cloned-goat embryos using different types of nuclear transfer methods. There were no significant differences in the reconstruction rate (P>0.05) for both nuclear transfer methods. As for the *in vitro* development of cloned-goat embryos, the percentages of cloned-goat embryos at 2- and 8 cell using SUZI and ICI methods did not differ significantly (P>0.05). However, the percentages of cloned-goat embryos at 4-cell, morula and blastocyst derived from SUZI method was significantly higher (P≤0.05) compared to the ICI method (53.80 vs. 39.97%; 23.88 vs. 17.42% and 12.00 vs. 5.15%, respectively). Conversely, there were no significant differences between 2- and 4 cell cloned-goat embryos for the SUZI method; however, there were significant differences in embryo development among the stages from 4-cell up to blastocyst. While in ICI method, there were significant differences in embryo development among stages from 2 cell to blastocyst.

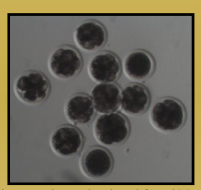

Figure 14: 4-8 cell cloned-goat embryos developed from iSCNT technique (day 3).

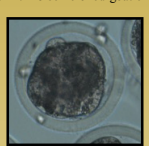

Figure 15: Compact morula (day 5).

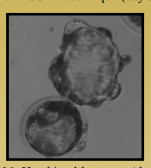

Figure 16: Hatching blastocyst (day 7).

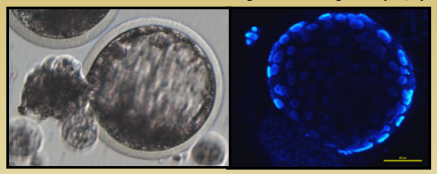

Figure 17: Hoechst 33342 stained 7 day blastocysts formed following iSCNT showing the nuclei.

DISCUSSION

Our results demonstrate that the nuclear transfer by SUZI method gave higher percentages for 2-, 4-, 8 cell, morula and blastocyst embryos compared to the nuclear transfer by ICI method. However, only the percentages for 4 cell, morula and blastocyst showed significant differences (P≤0.05) between these 2 nuclear transfer methods. Low efficiency of cloned-goat embryo development in the ICI method might be due to the damage to the isolated nucleus before injection (3). Similar finding was reported in SCNT on pig with the blastocyst formation for SUZI and ICI were 19% and 5%, respectively (4). In addition, our present study shows that no difference between electrofusion and intracytoplasmic injection on reconstruction of oocytes.

Also, there were significant differences for the embryo development from 8 cell to blastocyst (P≤0.05) for both nuclear transfer methods. This might be due to the maternal-embryo transition or maternal-zygotic transition is mainly occurring in the fourth cell cycle (8 cell to morula stage) in *in vitro* obtained embryos and the block moment is concurrent at this cycle (5).

To date, there is scarce report on the goat iSCNT research compared to other mammalian animals (e.g. cattle and pig). The examples of iSCNT on goat were goat-cattle (6), goat-sheep (7) and goat-cattle (8) with the blastocyst rate 1.7%, 7.4% and 15%, respectively. Our results on iSCNT using goat-cattle gives 12% and 5% blastocyst for SUZI and ICI methods respectively. In view of these, further studies are needed to obtain optimum results for the goat iSCNT.

*In our research, embryo transfer of cloned-goat embryos obtained from iSCNT is done and undergoing pregnancy. Pregnancy diagnosis will be done using ultrasound scanner and the delivery of the offspring as is expected in a few months time (unpublished data).

CONCLUSIONS

Cloned-goat embryos could be produced *in vitro* through iSCNT using both nuclear transfer methods. The SUZI method shows satisfactory rate of cleavage up to blastocyst than ICI methods. **To our knowledge, we believe this is the first report of producing cloned-goat embryos using goat foetal fibroblast cells as donor karyoplast and cattle as recipient cytoplasts in iSCNT in Malaysia.** With the establishment of iSCNT in goat and future refined of related techniques, the goat-cattle interspecies cloning could be the best alternative to produce a large number of superior goat genetically in Malaysia both for research, production as well as commercialisation in the 24th century.

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