PRODUCTION OF INTERSPECIES CLONED CAPRINE EMBRYOS AND MOUSE EMBRYONIC FIBROBLAST FEEDER CELL LAYER TO CULTURE EMBRYONIC STEM CELL

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ABSTRACT

The extreme variability in efficiency of the interspecies somatic cell nuclear transfer showed improvement is needed to both procedures and resources used to produce interspecies cloned caprine embryos blastocysts prior subjected for production of embryonic stem cell on mouse embryonic fibroblast feeder cell layer. The main objective of this study was to produce interspecies cloned caprine embryos and mouse embryonic feeder cell layer which was important for production of embryonic stem cell outgrowth. Briefly, caprine ear fibroblast cells of Boer and Katjang goats were cultured and cryopreserved as donor karyoplasts (Experiment 1). For recipient cytoplasts, bovine cumulus oocytes complexes were collected from abattoir-derived ovaries and subjected for *in vitro* maturation duration: a) 22-24 hours or b) 25-27 hours before subjected to interspecies somatic cell nuclear transfer using the caprine ear fibroblast karyoplasts. The embryos *in vitro* development was recorded (Experiment 2). The post-activated couplets and 8-cells were treated with trichostatin A (25 nM) before culturing. The morula and blastocyst rates of the treated embryos were recorded (Experiment 3). Blastocysts produced in Experiment 3 were used for whole blastocyst culture on mouse embryonic feeder cell layer in attempt to produce embryonic stem cell. The mouse embryonic fibroblasts were cultured from murine foetal ages of 14 and 15 days *post coitus* and cryopreserved using quick freezing technique (Experiment 4). In Experiment 1, generally, no significant effects of breed and gender were observed on viability rate of fresh early passages of the ear fibroblast culture. No significant different between the crypreserved and fresh passages. Based on the similar morphological characteristics and comparable viability rate of the ear fibroblast cell, suggesting stable production of ear fibroblast cell line could be produced using adult female and male Boer and Katjang up to three passages. In Experiment 2, combination 22-24 hour maturation duration of bovine cytoplasts and Boer and Katjang male ear fibroblast karyoplasts

gave significantly better (P<0.05) 2-cell and 4-cell rates (84.17% with 73.14% and 61.25% with 64.99%, respectively). Only combination of 22-24 hours maturation cytoplast with both female and male Boer ear fibroblast karyoplast successfully produced interspecies cloned caprine blastocyst (0.49% and 1.50%, respectively). In Experiment 3, no significant difference of morula and blastocyst rate of interspecies cloned caprine embryos regardless it was treated at post-activated couplets or 8-cell as well as non-treated cloned embryos. In Experiment 4, no significant difference on viability rate was observed between the 14 and 15 days *post coitum*. Meanwhile, the frozen-thawed passages of the mouse embryonic fibroblast cell cultures were significantly lower (P<0.05) compared with fresh passages between foetal ages. The current study also attempted to culture embryonic stem cell outgrowth on the mouse embryonic fibroblast feeder cell layer, however, no successful attachment of inner cell mass was observed. In conclusion, the current study elucidated several selected issues on the donor karyoplast, recipient cytoplast quality and also treatment of histone deacetylase inhibitor in *in vitro* culture of interspecies cloned caprine embryos. Interspecies somatic cell nuclear transfer in caprine is an alternative to the intraspecies, however, further optimisation on protocol is needed prior to be routinely used.

ABSTRAK

Kepelbagaian yang melampau dalam kecekapan teknik interspesies pemindahan nukleus sel somatik menunjukkan bahawa penambahbaikan diperlukan untuk kedua-dua prosedur dan sumber yang digunakan untuk menghasilkan blastosis klon kaprin interspesies, sebelum dapat digunakan untuk menghasilkan sel stem embrionik di atas lapisan sel sokongan fibroblas embrionik mencit. Objektif utama kajian ini untuk menghasilkan embrio klon kaprin interspesies dan lapisan sel sokongan fibroblas embrionik mencit yang penting dalam pertumbuhan lanjutan sel stem embrionik. Ringkasnya, sel fiboblas telinga kaprin kambing Boer dan Katjang telah dikultur dan dikrioawet sebagai penderma karyoplas (Eksperimen 1). Bagi sitoplas penerima, kompleks oosit kumulus bovin telah diperoleh daripada ovari abatoir dan dimatangkan secara *in vitro* berdurasi: a) 22- 24 jam atau b) 25- 27 jam sebelum oosit digunakan dalam interspesies pemindahan nuleus sel somatik menggunakan karyoplas fibroblast telinga kaprin. Kadar pertumbuhan embrio *in vitro* direkodkan (Eksperimen 2). Kuplet pasca-pengaktifan dan 8-sel dirawat trichostatin A (25 nM) sebelum dikultur. Kadar morula dan balstosis embrio yang terawat direkodkan (Eksperimen 3). Blastosis terhasil daripada Eksperimen 3 digunakan untuk kultur keseluruhan blastosis di atas lapisan sel sokongan embrionik mencit dalam usaha untuk menghasilkan sel stem embrionik. Sel embrionik fibroblas dikultur daripada fetus berumur 14 dan 15 hari selepas persenyawaan dan dikioawet menggunakan teknik penyejukan cepat sebelum dinyahaktif sebagai penyediaan lapisan sel sokongan (Eksperimen 4). Data projek ini dianalisis menggunakan ANOVA diikuti DMRT dan dikira siknifikan apabila $P<0.05$. Dalam Eksperimen 1, amnya, tiada kesan siknifikan antara baka dan jantina pada kadar keterushidup dalam pasaj awal segar sel fibroblas. Tiada perbezaan siknifikan dapat dilihat antara pasaj sejukbeku dan pasaj segar bagi sel fibroblas daripada jantan Boer dan Katjang. Berdasarkan persamaan ciri-ciri morfologi dan kadar hidup yang

setanding mencadangkan penghasilan sel fibrolas yang stabil dapat dihasilkan dengan menggunakan jantan dan betina Boer dan Katjang dewasa sehingga tiga pasaj. Dalam Eksperimen 2, kombinasi 22-24 jam tempoh matang sitoplas bovin mempunyai kadar 2-sel dan 4-sel yang lebih siknifikan ($P<0.05$) menggunakan karyoplas fibroblas telinga jantan Boer dan Katjang (84.17% dengan 73.14% dan 61.25% dengan 64.99%, masing-masing). Hanya kombinasi 22-24 jam kematangan sitoplas dengan karyoplas fibroblast telinga betina dan jantan Boer berjaya menghasilkan klon blastosis kaprin interspesies (0.49% dan 1.50%, masing-masing). Dalam Eksperimen 3, tiada perbezaan siknifikan dalam kadar morula dan blastosis (4.88% dan 2.50%, masing-masing) sama ada embrio klon kaprin interspesies pada tahap pasca-pengaktifan kuplet dan 8-sel diberikan ataupun embrio tidak terawat. Dalam Eksperimen 4, tiada perbezaan siknifikan dilihat pada kadar hidup antara fetus berumur 14 dan 15 hari selepas persenyewaan. Manakala, pasaj sejukbeku sel fibroblas embrionik mencit lebih siknifikan rendah ($P<0.05$) daripada pasaj segar. Kajian ini turut menyaksikan usaha mengkultur sel stem embrionik atas lapisan sel sokongan fibroblas embrionik mencit, namun, tiada kejayaan menempelkan sel jisim dalaman dilihat. Kesimpulannya, penyelidikan ini menjelaskan beberapa isu terpilih mengenai karyoplas penderma, kualiti sitoplas penerima dan juga rawatan *histone deacetylase inhibitor* dalam pengkulturan *in vitro* embrio klon kaprin interspesis. Pemindahan nukleus sel somatik kaprin interspesies merupakan alternatif kepada intraspesis, namun, memerlukan lebih pengotimuman dari segi protokol sebelum digunakan secara rutin.

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Life is a journey of improving yourself. Learn. Improve.

Sincerely,
Nurin

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
°C	degree Celcius
µl	microlitre
µm	micrometer
mm	millimeter
cm	centimeter
mg	milligram
l	litre
ml	millilitre
g	gramme
mOsm	millimosmole
O_2	oxygen
CO_2	carbon dioxide
pH	hydrogen potential
w/v	weight/volume
v/v	volume/volume
kV	Kilovolt
6-DMAP	6-dimethylaminopurine
ABEL	Animal Biotechnology-Embryo Laboratory
ART	assisted reproductive technologies
BSA-FV	bovine serum albumin-faction V

CaI	calcium ionophore
CC	cumulus cell
CHX	cycloheximide
CIDR	controlled internal drug release device
CL	corpus luteum
COC	cumulus-oocyte complexes
DC	direct current
DMSO	dimethyl sulfoxide
DNA	deoxyribo nucleic acid
DMEM	Dulbecco‟s Modified Eagle‟s Medium
d.p.c.	days *post coitum*
DPBS	Dulbecco‟s phosphate buffer saline
EFC	Ear fibroblast cell
EMiL	Embryo Micromanipulation Laboratory
ET	embryo transfer
EFC	ear fibroblast cell
ESC	embryonic stem cell
FBS	foetal bovine serum
FSH	follicular stimulating hormone
GV	germinal vesicle
GVBD	germinal vesicle breakdown
hCG	human chorionicgonadotrophin
hr	hour

ID	internal diameter
intraSCNT	intraspecies somatic cell nuclear transfer
iSCNT	interspecies somatic cell nuclear transfer
IVC	*in vitro* culture
IVD	*in vitro* development
IVEP	*in vitro* embryo production
IVM	*in vitro* maturation
IVP	*in vitro* production
KSOM	potassium simplex optimisation medium
LH	Luteinising hormone
LOPU	laparoscopic oocyte pick-up
MII	metaphase II
MEF	mouse embryonic fibroblast
MPF	maturation promoting factor
mtDNA	mitochondrial DNA
mRNA	messenger RNA
NaTuRe	Nuclear Transfer and Reprogramming Laboratory
NEBD	breakdown of the nuclear envelope
PA	parthenogenetic activation
PB1	first polar body
PBII	second polar body
PCC	premature chromatin condensation
PMSG	Pregnant mare‟s serum gonadotrophin
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid

RO	reverse osmosis
SCNT	somatic cell nuclear transfer
SUZI	sub-zonal injection
TSA	trichostatin A
UV	ultraviolet
WCICI	whole cell intracytoplasmic injection
XCI	X chromosome inactivation
XIST	X-inactive specific transport

Chapter 1

1.0 INTRODUCTION

1.1 BACKGROUND

Assisted reproduction techniques have been applied to solve reproductive issues in livestock animals, wildlife conservation and also human infertility. Over the past decades, the assisted reproductive techniques (ART) have come to forefront of applied reproductive research alongside with fundamental scientific reproductive research. These techniques included artificial insemination (AI), oestrus synchronisation, superovulation, *in vitro* matutation, fertilisation and culture (IVMFC), intracytoplasmic sperm injection (ICSI), embryo transfer (ET), gametes and embryos cryopreservation, nuclear transfer, gene transfer and stem cells production. Although many techniques such as AI and ET are now available that promise to enhance reproductive performance, additional refinement and optimisation allowing for subsequent embryo development and well being of the offspring are needed for these technologies to sustain the livestock industry. These techniques are powerful tools capable of providing significant improvement in solving reproductive problems in mammalian species. Some of these technologies are widely used in modern livestock management and production in many advance countries; however, Malaysia is still lagging behind in animal agriculture including caprine production, particularly, the used of technologies including reproductive techniques in the farm practices. By combining these technologies, it will provide significant changes in the next decade especially in animal reproduction area in Malaysia. In spite of this, the incorporation of some appropriate reproductive techniques tailored for local indigenous breeds and local natural resources are deemed necessary to ensure the sustainability and profitability of animal production including caprine farming in Malaysia.

Malaysia is generally self-sufficient in non-ruminant production, especially in swine and poultry, and nearly 20% of production was exported. However, ruminant production was in a slow growth. The self-sufficiency for local mutton accomodates only 13% of the demand and 87% were imported from Australia, Indonesia and Vietnam (Ministry of Agriculture and Agro-Based Industry Malaysia, 2014). The consumption of mutton in Malaysia in 2013 was 0.7 kg per capita and was expected to surge in the following years. Hence, the overall scenario in goat production in the country is showing strong demand exceeding domestic production. The application of biotechnology to animal reproduction has in itself furthered our understanding of the reproduction process.

Somatic cell nuclear transfer (SCNT) is a technique in cloning in which the nucleus of a somatic cell is transferred to the cytoplasm of an enucleated egg. Once somatic nuclei inside the oocytes, it is reprogrammed by the oocytes cytoplasmic factors to become a zygote nucleus. The fertilised egg is allowed to develop into blastocyst stage. The oocyte at blastocyst stage will be transferred into the surrogate mother where further development into a foetus occurred. The kid will resemble the somatic cell donor in its physical appearance and other qualities such as the ability to produce large quantities of milk.

The birth of Dolly the sheep in 1997 first demonstrated that fully differentiated adult somatic cells can be reprogrammed to produce live clones (Wilmut *et al*., 1997). Since then, a number of different species including mice (Wakayama *et al*., 1998), cattle (Cibelli *et al.*, 1998), caprines (Baguisi *et al.,* 1999), pigs (Polejaeva *et al.*, 2000) and rabbit (Chesne *et al.,* 2002) have been cloned using somatic cell nuclear transfer technique.

Animal cloning technology using adult somatic cells by nuclear transfer has several advantages, one of which is that the resulting animals are exactly the same as

the provider of the donor cells. Compared with nuclear transfer of germ line cells, somatic cells are easily collected and cultured without serious injury to animals. In addition, somatic cells do not undergo the essential modification of imprinted genes that occurs during the production of germ cells.

Baguisi *et al.* (1999) first reported of cloning in caprines by using foetal somatic cells as donor karyoplast. This work obtained 3 healthy identical female offspring. Based on literature available, scientists had conducted research on improving the somatic cell nuclear transfer technique in caprines. This including improvement on the somatic cell nuclear transfer protocols and the *in vitro* culture system so that it could better support the development of cloned caprine embryos and the blastocyst in order to support it to develop to term and give birth to a healthy kid in term.

Animal Biotechnology - Embryo Laboratory (ABEL), University of Malaya have conducted preliminary research in both in reproductive cloning and embryonic stem cells experiments involving mice and caprines. Their findings are encouraging and they are the pioneer in this research in our country as indicated by their recent publications (Abdullah *et al.*, 2011; Soh *et al.*, 2012; Goh *et al.* 2012, Kwong *et al.*, 2012) as listed in Table 1.1. However, more research is needed to increase understanding on fundamentals as well as application of cloning and embryonic stem cells technologies particularly in caprines under Malaysian condition.

Embryonic stem cell lines derived from somatic cell nuclear transfer can be manipulated to differentiate into various cell downstream lineages, either through the use of tissue culture or manipulation of cells during the normal progression of developing embryo. This provides endless possibilities for a treatment in wide range of medical conditions of degenerative diseases such as Parkinson disease, Alzheimer disease and cardiovascular disease.

Table 1.1: Timeline of significant finding of interspecies somatic cell nuclear transfer in caprine in ABEL

Year	Authors	Significant finding
2011	Abdullah *et al.*	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
2012	Goh *et al.*	Establishment of caprine embryonic stem cell from *in vivo* and *in vitro* poduced embryos
2012	Kwong *et al.*	Increasing glucose in KSOMaa basal medium on Day 2 improved efficiency of intra- and interspecies somatic cell nuclear transfer
2012	Soh *et al.*	Subzonal injection of caprine karyoplast into bovine cytoplast produce high interspecies blastocyst rate compared to intracytoplasmic injection
2014	Kwong *et al.*	By using mesenchymal stem cell as donor cell, the blastocyst rate was significantly higher compare to EFC as donor cell
2014	Asdiana Amri	Ear fibroblast cell significantly improved cleavage rate than fresh cumulus cell

1.2 STATEMENT OF PROBLEMS

The complexity of the technique of somatic cell nuclear transfer involving many intricate steps and processes poses many challenges to overcome the various factors affecting the viability of cloned embryos produced. The somatic cell nuclear transfer protocols involves: preparation of somatic cells as donor karyoplast, enucleation technique the oocytes as recipient cytoplast, reconstruction of the oocytes to be cloned, oocyte activation and culture system and environment to produce viable diploid cloned embryos. Over the years, researchers had already solved many technical issues in producing viable cloned embryos and offspring. However, the outcomes of the results were still variable and unpredictable due to biological variation in both donor karyoplast and recipient cytoplast.

Spikings *et al.* (2006) drew out the obvious problem faced by the somatic cell nuclear transfer researcher; especially the low efficiency of the cloned embryos developing to blastocyst. In addition, the low rate of implantation of the cloned embryos to the surrogate mother, the rate of survival of pregnancy and low viability of birth cloned offspring were also emphasised. Several factors were suggested, including incompatible genetic backgrounds of the donor and the recipient, synchronisation of the cell cycle of recipient and donor cells, the nuclear transfer procedure, or culture conditions (Dinnyés *et al*., 2002). The extreme variability in development of cloned embryos dictated that improvement was needed to both the procedures as well as the resources used to clone embryos (Miyoshi *et al.*, 2001).

To date, there is a significant research progress in the somatic cell nuclear transfer and embryonic stem cell of caprines. Nevertheless, there were still a lot of issues to be solved before these techniques could be widely applied in caprine production. Below are selected important questions to be answered:

a) Are differences in breed and gender of donor karyoplast give significant difference to establish ear fibroblast cell line?
b) Can quick freezing technique cryopreserve cell line without any detrimental effect?
c) Are differences in breed and gender of donor for karyoplasts give significant different to interspecies somatic cell nuclear transfer efficiency?
d) What is the optimum maturation rate for bovine oocytes to be efficiently use recipient cytoplast to reprogramme caprine karyoplast?
e) Does ear fibroblast cells at Passages 1 to 3 may give the best result for interspecies somatic cell nuclear transfer?

f) Is supplementation of histone deacetylator in *in vitro* culture medium can increase the interspecies cloned blastocyst rate?

g) Which stage(s) of blastocyst for both types of cloned embryos would give optimum embryonic stem cell line production?

h) Which is the best feeder cell layer for culturing the embryonic stem cells?

i) How many passage(s) of culture to establish embryonic stem cell line?

1.3 JUSTIFICATION

The main goal of this study was to produce interspecies cloned caprine embryos and mouse embryonic feeder cell layer which was important for production of embryonic stem cell outgrowth. To fulfill this main goal, four experiments were designed which will be further discussed in Chapter 3 (Materials and Methods).

In this study, ear fibroblast cells of male and female purebreed Katjang and Boer were cultured, sub-passaged to Passage 3 and subsequently cryopreserved using quick freezing technique. This experiment was important as these cells were used as donor karyoplasts for producing interspecies cloned caprine embryos. One of the specific objectives of this study was to evaluate the difference in viability rates between the breeds, gender and passages. In addition, the viability rates between the fresh and frozen-thawed passages were also compared to evaluate the efficiency of quick freezing technique. Purebreed Katjang was selected for conservation of germplasm as this indigenous caprine breed is endangered, whereas Boer breed was used as this breed is popular among the breeders and farmers for its meat in this country.

Secondly, the cloned caprine blastocysts were produced via interspecies somatic cell nuclear transfer in the present research. The interspecies cloned blastocysts were produced by using bovine oocytes derived from abattoir as recipient cytoplasts, with caprine ear fibroblast as donor karyoplasts. The caprine oocytes supply was obtained from abattoir was limited due to low number of caprine slaughtered in local abattoir, resulting in low production cloned blastocysts via intraspecies somatic cell nuclear transfer (caprine cytoplast-caprine karyoplast). Laparoscopic ovum pick-up (LOPU) could be conducted as an alternative method to retrieve caprine oocytes. However, the cost of caprine laparoscopic ovum pick-up was expensive as the hormones used to stimulate the does and the cost of caprine herd management were expensive. Due to high number of cows slaughtered in local abattoir, it was decided to

be the preferred technique to obtained abundant number of bovine ovaries and subsequent oocytes.

The current research was focusing in few selected issues in interspecies somatic cell nuclear transfer to improve the efficiency of interspecies somatic cell nuclear transfer in caprine. The protocol was adapted from previous studies in the laboratory, conducted by Kwong (2012) and Soh (2012). For improvement of the protocol, three aspects were selected which were a) *in vitro* maturation duration of bovine cytoplasts, b) effect of breed and gender of the ear fibroblast cell as donor karyoplast and c) supplementation of trichostatin A in the *in vitro* culture medium. *In vitro* maturation duration is important to determine the maturation age of the oocytes as the aging of the matured oocytes may lead to impairment in embryo development. Gender and breed karyoplasts were important factors as donor karyoplasts gave out the nuclear genetic content to the embryos. Since there were lack of the reports on role of these factors in interspecies cloned caprine embryos, it was interesting to find the association of these two factors in *in vitro* developmental competence of interspecies cloned caprine embryos. For trichostatin A, current study was modified from Wang *et al.* (2015) who achieved the significant improvement in *in vitro* developmet of intraspecies cloned caprine embryos when treated the donor cell (ear fibroblast cell) with 25 nM of trichostatin A for 12 hours. The treatment was expected to increase the cloned blastocyst rates to be used to subsequent embryonic stem cell experiment.

Initial research in our laboratory showed encouraging results on production of caprine embryonic stem cell outgrowth (Goh, 2012). In this study, the production of embryonic stem cell outgrowth is focused on the technical issues. As for preliminary experiment for embryonic stem cell study, the author produced mouse embryonic feeder cell layer derived from different foetal ages, as the mouse embryonic feeder cell layer was also one of the important factors to ensure optimum production of embryonic

stem cell line. Cryopreservation of mouse embryonic cells was important in order to preserve as it could be used as feeder cell layer for producing embryonic stem cell line. Quick freezing technique was used to cryopreserve the mouse embryonic cell and the viability rate between the fresh and frozen-thawed passages was compared to evaluate the cryopreservation efficiency.

While it had been established that somatic cell nuclear transfer was a powerful tool and that embryonic stem cell was superior, there was less attempt of research on production of cloned embryos as well as embryonic stem cell lines in Malaysia. Considering that it was new research field worldwide including in our country, therefore, it was timely to initiate this research so that Malaysia would be abreast with other countries venturing in this frontier animal biotechnological research,particularly, for the promotion of animal production for food security and food safety.

1.4 APPLICATION

The development of new methods of nuclear transfer in mammals is creating many new opportunities in research, medicine and agriculture. The method of cloning is repeatable and has been established in many laboratories worldwide. However, the present procedure is inefficient with fewer than 4% of embryos becoming viable offspring. A considerable improvement in efficiency is required before wide scale use for livestock improvement. The opportunity to introduce precise genetic changes to livestock is available for the first time through the use of gene targeting procedures in cultured cells that are used as nuclear donors. This has potential application in the production of organs for transplantation to humans, studies of human genetic disease and basic research in to the control of gene expression and function.

Somatic cell nuclear transfer is a biotechnological tool that beneficial to provide and augment agriculture industry produce in Malaysia. With this technique, it can mass propagate the caprine population. This will increase the income of the farmers and reduce the importation of caprine meat which is more than 92% annually. This is in line with the government agriculture policy in food security and food safety. The technology generated in this study has the potential to be utilised in the conservation of endangered wildlife population. The offspring can be produced through the use of SCNT and embryo transfer technique without undergoing adverse natural breeding process. This is especially important when considering that certain wildlife is presently facing extinction.

The potential for biopharming is enormous in that 10-100 caprines would be sufficient to satisfy current world demands for many pharmaceutical proteins. While the initial application of biopharming will be to produce pharmaceutical proteins, more wide scale applications could include the production of nutriceuticals or orally taken proteins having both nutritional and therapeutic value. Further applications include

specific antibody production in milk to confer increased immunity to newborn kids and the production of caprines with resistance to diseases.

Presently, only few research projects on the production of embryonic stem cells from cloned caprine blastocysts. Subsequently, caprine embryonic stemcells outgrowth can be produced after using proper culture system. This finding will open the window of opportunity to transform caprine farming to caprine pharming which is a novel approach in regenerative medicine for production of pharmaceutical drugs, proteins, cells, tissues, and organs for treatment of degenerative diseases.

1.5 OBJECTIVES

The objectives of this study are:

a) To culture and cryopreserve caprine ear fibroblast cell line of female and male purebreed indigenous Katjang and Boer as donor karyoplast to be subsequently used in interspecies somatic cell nuclear transfer;
b) To determine the most optimum maturation duration for bovine oocytes as cytoplast preparation for interspecies somatic cell nuclear transfer;
c) To produce cloned indigenous Katjang and purebred Boer embryos using ear fibroblast cells by using interspecies somatic cell nuclear transfer technique;
d) To evaluate the combination effect of maturation duration of cytoplast as well as breeds and genders of donor karyoplast in producing of interspecies cloned caprine embryos;
e) To supplement the TSA (25 nM) in the *in vitro* culture medium to optimise the production interspecies cloned caprine blastocysts;
f) To produce and cryopreserve mouse embryonic fibroblast derived from different foetal ages as preparation of feeder cell layer, to be used in attempt to produce caprine embryonic stem cells.

Chapter 2

2.0 REVIEW OF LITERATURE

2.1 *IN VIVO* VERSUS *IN VITRO* DEVELOPMENT OF EMBRYOS

The understanding of embryonic development is a vital key to optimise the somatic cell nuclear transfer and embryonic stem cells production techniques. This optimisation could be achieved through refinement by genetic modification and establishment of different stem cell tools, as well as for optimising the use of the embryo technologies for breeding and production (Hall *et al.,* 2013).

2.1.1 *In vivo* development of embryos

Proper maturation of the oocyte to metaphase II is a prerequisite for fertilisation and preimplantation development. In the domestic mammals, maturation occurred in the pre-ovulatory follicle within 24 to 42 hours of ovulation. After normal fertilisation, major embryonic genome activation occurred around the 8-cell stage (Buhi *et al.*, 1997) accompanied by changes in chromatin structure (Keus *et al.*, 2008). At the morula stage, compaction occurred followed by blastulation at Days 7 to 8.

The embryonic genomic activation paved the path for the first lineage segregation into trophoblast and inner cell mass (ICM). Inner cell mass underwent next lineage segregation to form pluriporent epiblast and the hypoblast, which developed into flat layer epithelium that gradually covered the inside of the epiblast and trophoblast (Brons *et al*., 2007). The flattened cell layer of trophoblast transformed to a more cuboidal cell. The trophoblast which covered the epiblast became increasingly thinner until the epiblast penetrate trophoblast and formed embryonic disc (Brons *et al*., 2007). At the same time, the gene expression changes of key pluripotency transcription factors took place such as *POU5F1*, *SOX2* and *NANOG* (Khan *et al.*, 2012).

The formation of endoderm, mesoderm and ectoderm happened during gastrulation process due to cell migration process (Mikawa *et al.,* 2004). The trophoblast gradually lined by extra-embryonic mesoderm and become trophectoderm, which engaged in placenta formation. The chorio-aminiotic folds consisting trophectoderm and mesoderm developed into amnion. The elongation of the conceptus until the implantation usually started at Days 20 to 21 of embryonic development (Mikawa *et al.,* 2004).

2.1.2 *In vitro* development of embryos

In vitro production (IVP) of embryos is the third generation of animal reproductive technologies (ART) techniques after artificial insemination (AI) and multiple ovulation embryo transfer (MOET) which aimed to control the animal production (Cognie *et al.*, 2004). The *in vitro* production consist of four major procedures: (1) Oocyte collection, (2) *In vitro* maturation (IVM) of the oocytes, (3) *in vitro* production of embryos and lastly, (4) *in vitro* culture of the *in vitro* production embryos for development. *In vitro* production embryos could be produced by *in vitro* fertilisation (IVF) technique, intracytoplasmic sperm injection (ICSI), parthenogenetic activation (PA) and somatic cell nuclear transfer. *In vitro* production has several advantages such as reliability (Baldassarre *et al.,* 2004), reproducibility (Stangl *et al.*, 1999) and the possibility to collect oocytes from variation of hormonal, health, breeds, age and pregnancy status of female donors.

After *in vitro* production of the embryos, the presumptive zygotes or reconstructed embryos are subjected for *in vitro* culture in an embryo culture medium that allows the development up to a stage that is compatible with its transfer to the recipient uterus. Although the *in vitro* production success rate relies on the oocyte‟s intrinsic quality and maturation conditions, the quality of the resulting blastocysts relies on the conditions encountered during earlier steps of development (Rizos *et al.*, 2002). *In vitro*–derived

embryos show significant differences compared with embryos produced *in vivo* in term of morphology, timing of development, resistance to low temperature (Massip *et al.*, 1995), embryo metabolism, and especially gene expression (Donnay *et al.,*1999). One of the most dramatic effect of *in vitro* environment is the modification of embryo lipid metabolism leading to increased storage of triglycerides and decreased phospholipid production, translating into altered membrane fluidity, and in turn, lower cryoresistance. *In vitro* conditions may also induce sex-ratio deviation because of metabolic advantage of male embryos in *in vitro* environment.

2.1.3 Comparison of ultrastructure of *in vivo* and *in vitro* produced embryos

The reduced competence of nuclear transfer embryos is associated with morphological deviations during bovine pre-hatching development (Hyttel *et al.*, 2001; Baran *et al.*, 2002; Laurincik *et al.*, 2002). The quality of *in vitro* produced embryos is compromised when compared with *in vivo* embryos living in a physiologically optimal microenvironment. The *in vitro* fertilised embryos differ ultrastructurally from those produced *in vivo* (Crosier *et al.*, 2001).

Bovine compacted morula which produced *in vitro* had greater volume density of lipid droplets, a reduced proportional volume of total mitochondria, large volume density of vacuoles and an increased cytoplasmic-to-nuclear ratio (Crosier *et al.*, 2000). Moreover, the blastomeres of *in vivo* embryos had a tendency to be rounder up to the 16-cell stage and to form a more compact mass at morula stage compared to *in vitro* fertilised morula. Along the development of *in vitro* fertilised bovine embryos, some structures (i.e. microvilli, lysosomes, intercellular junctions and intermediate filaments) appeared or reappeared, while others (i.e. liquid droplets, vesicles with flocculent materials, cortical granules, nuclear annulate lamellae, nuclear envelope blebs) decreased or disappeared (Crosier *et al.*, 2000). Vacuolated nucleoli in both *in vitro*

produced and *in vivo* embryos were observed from the 2-cell stage until blastocyst (Plante and King, 1994).

Compared with *in vivo* embryos, *in vitro* produced embryos had lower volume densities of cytoplasm, mitochondria and nuclei, and higher volume densities of mature mitochondria, nuclei, blastocoel, and apoptotic bodies, proportional volumes of nuclei and inclusion bodies in inner cell mass cells of blastocysts and proportional volume of vacuoles and the volume density of lipid (Crosier *et al.*, 2001). Bovine blastocysts produced *in vitro* possessed deviations in volume densities of organelles associated with cellular metabolism, as well as deviations associated with altered embryonic differentiation (Crosier *et al.*, 2001).

It is clear that *in vitro* fertilised embryos differ in term of ultrastructure of morula and blastocyst compared to *in vivo* morula and blastocyst, which might answered the difference efficiency in term of the development and the viability of *in vitro* produced embyros compared with *in vivo* embryos. How about the ultrastructure differences between the intraspecies and interspecies somatic cell nuclear transfer? There is no definite explanation regarding this. However, morphometric analysis may offer a more objective method of assessing differences in cellular ultrastructure that may occur in embryos because of nuclear transfer. Interspecies nuclear transfer is an invaluable tool for studying nucleus–cytoplasm interactions, and it provides a possible alternative to clone animals whose oocytes are difficult to obtain (Wen *et al.*, 2003). Few reports were available for understanding ultrastructural changes in interspecies cloned embryos (Hamilton *et al.*, 2004, Tao *et al.*, 2008). Nevertheless, it is an important aspect to be discussed.

According to Tao *et al.* (2008) who studied the ultrastructural changes in early embryos of intra- and interspecies cloned caprine, changes in intraspecies cloned caprine embryos were more similar to changes in *in vivo* produced embryos than were

in interspecies cloned goat embryos, with extreme mitochondrial deviation. The results indicated the effects of the cytoplast on mitochondria development. The zona pellucida in all three types of embryos became thinner and pores in both intra- and interspecies cloned embryos showed an increased rate of development, especially for interspecies embryos, while *in vivo* produced embryos had smooth ZP. The Golgi apparatus and rough endoplasmic reticulum of two reconstructed embryos became apparent at the 8-cell stage, as was found for *in vivo* embryos. Lipid droplets of intra- and interspecies cloned embryos became bigger and congregated. In *in vivo* produced embryos lipid droplet changed little in volume and dispersed gradually from the 4-cell period onward until blastocyst stage. The nucleolus of intra- and interspecies embryos changed from electron dense to a fibrillo-granular meshwork at the 16-cell stage, showing that nucleus function in the reconstructed embryos was activated. The broken nuclear envelope and multiple nucleoli in one blastomere illuminated that the nucleus function of reconstructed embryos was partly changed. In addition, at a later stage in intraspecies embryos, the nuclear envelope displayed infoldings and the chromatin was concentrated, implying that the blastomeres had an obvious trend towards apoptosis. The gap junctions of the three types of embryos changed differently and both cloned embryos had bigger perivitelline and intercellular spaces than did *in vivo* embryos. These findings indicated normal intercellular communication at an early stage of embryos in cloned embryos, but this became weaker in later stages. The interspecies cloned embryos showed more severe destruction (Tao *et al.* 2008).

2.2 SOMATIC CELL NUCLEAR TRANSFER (SCNT) IN CAPRINE

2.2.1 Historical Background of Somatic Cell Nuclear Transfer

Animal cloning is a potential biotechnology to produce superior genetic identical animals in rapid rate. Production of Dolly (Wilmut *et al.,* 1997), the successfully viable cloned sheep was a major breakthrough in animal cloning technology. Consequently, the production of large number of animals at a rapid rate, especially in mammals, had become interesting and attractive research field of animal reproductive technology.

Cloning using nuclear transfer, preferred by many researchers, is based on the unique ability of metaphase II arrested cytoplasts (recipient oocytes) to convert transplanted somatic karyoplasts (donor cell nuclei) to become capable of completely reversing from differentiated stages to totipotency embryos (Tachibana *et al.*, 2013). This process involves the removal of the genetic material from the cytoplast (enucleation) and subsequently transferring nuclear genetic material from donor karyoplast into the enucleated cytoplast. After going through the process of fusion, activation and cleavage, the resultant embryos produced are identical to the genetic of the donor karyoplast. There are two factors to determine the clonality of the resultant offspring. One is the recipient cytoplast which is the oocyte obtained from an unrelated animal. The second is the donor karyoplast, which can be obtained from widely variety of sources such as adult somatic cells, foetal somatic cells and stem cells (Loi *et al.*, 2011). Fundamentally, when cells from somatic karyoplasts are used, the procedure is termed as somatic cell nuclear transfer.

Animal nuclear transfer history were started back in 1928 when the Hans Spemann used 16-cell embryos as donor nuclei to the zygotic cytoplasts to produce cloned salamanders (Spemann, 1938). Briggs and King (1952) developed nuclear transfer procedure by transplating frog blastula into enucleated ocytes. In 1975, first nuclear transfer experiment on mammal was conducted on rabbit using morula blastomeres

transferred into the enucleated oocytes, however, the reconstructed embryos failed to develop into blastocysts (Bromhall, 1975)

McGrath and Solter (1983) showed that the nuclear transfer experiment was feasible in mammalian species by successfully transplanted zygotic karyoplasts into enucleated zygotic cytoplasts to produce mice developing to term. However, when the cleaved embryo blastomeres were used as karyoplasts, the nuclear transfers were unsuccessful (McGrath and Solter, 1984). Based on this finding, in choosing cytoplast for nuclear transfer experiments, subsequent research approaches in laboratories worldwide were carried out by using the enucleated oocytes as cytoplasts instead of enucleated zygotes.

One major breakthrough in nuclear transfer experiment was achieved by Campbell *et al.* (1996) who used cell cycle coordination between the karyoplast and cytoplast. Wilmut *et al.* (1997) was the pioneer to produce the first cloned Dolly the sheep by using adult somatic mammary cells as donor karyoplasts. This significant milestone in animal cloning using adult somatic cells as donor karyoplasts has revolutionised the approach of producing massive number of animals that has numerous applications, particularly in livestock production and wildlife conservation.

As reviewed by Mizutani *et al.* (2012), despite advances in the field, the development of mammalian embryos produced by somatic cell nuclear transfer remains low, with only 1–10% reaching term, depending on the species. The pace of progress of nuclear transfer in mammals has appeared slow, mainly because of extremely low success rates with regard to somatic cell nuclear transfer embryo production and pregnancy. Moreover, a number of cloned animals were born with abnormalities. This might due to inaccuracy in reprogramming the donor karyoplast or cytogenetic abnormalities during the early cleavage.

Although the efficiency of somatic cell nuclear transfer has improved throughout

these two decades, efforts to improve the success rate are continuing in an attempt to make this technology of increased commercial interest to livestock breeders or to produce the embryonic stem cell for medical purposes. Nuclear transfer is a technically demanding technology, with practitioners only developed and improved their skills continuously through experimentation and experience (Wakayama and Yanagimachi, 1999). Development of nuclear transfer had advanced through the availability of protocols including simplified manipulation of recipient oocytes by using handmade cloning methods and modification of the *in vitro* culture system. The pressing need to improve the efficiency of somatic cell nuclear transfer may require a modified or entirely novel methodology to be established (Do and Taylor-Robinson, 2014).

2.2.2 Intraspecies somatic cell nuclear transfer in caprine (intrasp SCNT)

Intraspecies somatic cell nuclear transfer is generally the somatic cell nuclear transfer approach of using the similar species of the cytoplast and karyoplast donors to developed cloned embryos. The phylogenetic distance between the cytoplast and karyoplast donors were closely related. The interaction between the karyoplast and cytoplast were vital in nuclear reprogramming. This interaction occured between the nucleus DNA and mitochondrial DNA (mtDNA) where mtDNA received signal from nucleus DNA to supply energy for normal cellular function (Lagutina *et al.*, 2004). This interaction will be further discussed in Section 2.3.4 in this thesis. Thus, the intraspecies somatic cell nuclear transfer had advantages in term of efficiency and successful rate compared to interspecies somatic cell nuclear transfer as the molecular and genetic interaction between the same species karyoplast and cytoplast were preserved (Lagutina *et al.*, 2005).

In caprine somatic cell nuclear transfer research, the first cloned kid was obtained in the year 1999 (Baguisi *et al.*, 1999) by using intraspecies somatic cell nuclear

transfer approach. Table 2.1 showed intraspecies somatic cell nuclear transfer studies on production cloned caprine by various laboratories, worldwide.

Table 2.1: Timeline of significant finding of intraspecies somatic cell nuclear transfer in caprine

Year	Author	Significant finding
1999	Baguisi et *al.*	First report on goat somatic cell nuclear transfer with obtaining of 3 healthy identical female offspring using foetal somatic cells as donor karyoplast
2001	Keefer *et al.*	Both *in vitro* transfected and nontransfected caprine foetal fibroblasts could direct full term development following nuclear transfer
2001	Reggio *et al.*	First report of cloned goats produced from nuclear transfer using cytoplast derived from abattoir ovaries
2001	Zou *et al.*	Injection method produced high survival rate compare to fusion method
2002	Guo *et al.*	Microsatellite DNA analysis confirmed 2 kids derived from an adult donor karyoplast were identical
2002	Zou *et al.*	Transgenic cell could be used as donor karyoplast to produce transgenic cloned animals
2003	Das *et al.*	300V used for electrofusion produced higher cleavage rate and cytochalasin B could be used to stop synchronisation of donor karyoplast
2004	Zhang *et al.*	Stage G0/G1 donor karyoplast improved developmental rate of cloned embryos compared to G2/M stage donor karyoplast
2006	Lan *et al.*	Passages 20 to 25 of foetal fibroblast cells produced lower fusion and IVD rate compare to cell from earlier passages (Passages 3 to 5)
2007	Chen *et al.*	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

Year	Author	Significant finding
2008	Daniel *et al.*	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.
2009	Yuan *et al.*	Live goats were generated by somatic cell nuclear transfer from caprine mammary gland epithelial cells using long term cultured cell lines (25 to 27 passages).
2010	Dalman *et al.*	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	Liu *et al.*	Cytochalasin B treatment for 2 to 3 hours between fusion and activation was an efficient method for generating cloned goats by somatic cell nuclear transfer. In addition, increasing the number of embryos transferred to each recipient resulted in more live offspring from fewer recipients.
2012	Wan *et al.*	Serum starvation effectively synchronised donor karyoplasts for production of transgenic cloned goats.
2012	Soh *et al.*	SUZI method give higher morula and blastocyst rate compared to WCICI method in interspecies somatic cell nuclear transfer by using foetal fibroblast cell as donor karyoplast.
2012	Kwong *et al.*	Increasing glucose in KSOMaa basal medium on Day 2 improved efficiency of intra- and interspecies somatic cell nuclear transfer.
2013	Liu *et al.*	Less differentiated cell (SSEA3+ cell from goat skin fibroblast cell) could increase the clone efficiency.
2013	Zhang *et al.*	Supplementation of 25 uM a-lipoic acid in *in vitro* maturation medium increased the blastocyst rate of cloned goats.
2014	Kwong *et al.*	By using mesenchymal stem cell as donor karyoplast, the blastocyst rate was significantly higher compare to EFC as donor karyoplast.
2015	Wang *et al.*	Treatment of TSA to donor karyoplasts (goat ear fibroblast skin) improved the development rate of cloned goat embryos.

2.2.3 Interspecies somatic cell nuclear transfer in caprine (intersp SCNT)

The availability of the oocytes and the *in vitro* maturation and culture technology are vital factors in the nuclear reprogramming in somatic cell nuclear transfer. Livestock species were usually abundant due high number of slaughtering animals, especially in bovine and porcine (Galli and Lazari, 2003). However, this fact may varies depend on the species and the country as the number of slaughtering is depending on the availability of the animals in the country and the demand of the country citizen on the livestock product (meat and milk). The idea of using livestock or domestic species oocytes across other species has been conceived of since the early days of somatic cell nuclear transfer (Byrne *et al.*, 2007).

The ability of using the other species oocytes in somatic cell nuclear transfer has significant value in human and animal reproductive technology and embryonic stem cell development. Interspecies somatic cell nuclear transfer involved several complex molecular events for reprogramming. It takes a lot of stringency for the interspecies somatic cell nuclear transfer embryos to develop from the activation process to full term development (Oback, 2009). Interspecies somatic cell nuclear transfer approach could be used to clone endangered species or restore extinct animal species (Ma *et al.*, 2011; Loi *et al.*, 2011). Interspecies somatic cell nuclear transfer also helped to counter the problem of limited supply of oocytes in cloned desired animal production. This was based on interspecies somatic cell nuclear transfer success in producing cloned embryos (Jiang *et al.*, 2005; Li *et al.,* 2006; Sugawara *et al.*, 2009) and offsprings (Lanza *et al.*, 2000; Loi *et al.*, 2001).

The success rate of interspecies somatic cell nuclear transfer in mammals is related to the the species relationship between the karyoplast and cytoplast donor. Higher successful rates were obtained from the closely related species between the karyoplast and cytoplast donor (Mastromonaco *et al.*, 2007). This is also showed in nature as the

natural production of living hybrid offspring shows that a certain nuclear–cytoplasmic compatibility exists between the two species (Mastromonaco *et al.*, 2007). Inter-subspecies somatic cell nuclear transfer has produced healthy offspring of Boer goat (Jian-Quan *et al.*, 2007) and and grey wolf (Kim *et al.*, 2007). Interspecies somatic cell nuclear transfer embryos derived from mouflon nucleus donor karyoplasts and sheep oocytes (Loi *et al.*, 2001) can also develop to term. A gaur–bovine offspring was born (Srirattana *et al.*, 2012) through this technique.

The most successful progress of interspecies somatic cell nuclear transfer in mammals were achieved when using donor karyoplast and recipient cytoplast of very closely related species. As the species divergence increases, there is decreasing of ability embryo to sustain the development to full incompatibility. Lagutina *et al.*, (2013) discussed that the observed aberrant degradation of maternally inherited ooplasmic mRNA that should occur soon after oocyte activation and before EGA, the inability of maternally inherited factors to activate the embryonic genome, improper demethylation of the donor genome, and the nuclear–mitochondrial incompatibilities all contribute to the early death of iSCNT embryos.

In caprine, the first interspecies somatic cell nuclear transfer reported by Song *et al.* (2008) as they used the bovine oocyte as cytoplast donor and obtained 7.9% of blastocyst rate. This followed by Ma *et al.* at the same year which used ovine oocytes and produced 7.4% of caprine-ovine cloned blastocyst rate. Table 2.2 summarised the history of interspecies somatic cell nuclear transfer in caprine with its significant finding.

Table 2.2: Timeline of significant finding of interspecies somatic cell nuclear transfer in caprine

Year	Authors	Significant finding
2007	Jian-Quan *et al.*	Produce healthy Boer goat offspring by using inter sub-species cloning to produce cloned goat embryos.
2008	Ma *et al.*	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.
2008	Song *et al.*	First report of producing interspecies cloned caprine blastocyst by using bovine oocytes as donor cytoplast.
2008	Tao *et al.*	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.
2009	Folch *et al.*	First birth of extinct goat using subspecies donor cytoplast.
2010	Sansinena *et al.*	Foreign mitichondria which introduced during ooplasm transfer tend to remain as distinct clusters which subsequently caused heteroplasmy during nuclear-ooplasmic.
2011	Abdullah *et al.*	Cloned-caprine embryos could be produced *in vitro* via both intraspecies and interspecies SCNT approaches with comparable efficiency.
2011	Selokar *et al.*	Producing 2.2% of blastocyst by using handmade cloning to reprogramme caprine fibroblast in the buffalo cytoplast.
2012	Kwong *et al.*	Increasing glucose in KSOMaa basal medium on Day 2 improved efficiency of intra- and interspecies somatic cell nuclear transfer.
2012	Soh *et al.*	Subzonal injection of caprine karyoplast into bovine cytoplast produce high interspecies blastocyst rate compared to intracytoplasmic injection.

When the oocyte rest at metaphase II stage, maturation promoting factor activity are high which induced the donor karyoplast to undergo nuclear envelope breakdown

and premature chromosome condensation. These events are important in gene expression reprogramming and cloned embryo developmental ability (De Sousa *et al*, 2014). However, after several hours of activation, the high maturation promoting factor level decrease. Therefore, the reprogramming of the donor karyoplast could only be done before the oocyte activation. In interspecies somatic cell nuclear transfer cloned embryo production, some researchers delay the activation of the reconstructed oocyte and showed positive results (Cibelli *et al.*, 2003; Lee *et al.*, 2005; Shen *et al.,* 2008). Ma *et al.,* (2011) tested whether longtime exposure of donor karyoplast to xenogenic ooplasm improved the reprogramming of the donor karyoplast and subsequently increase the developmental ability of interspecies somatic cell nuclear transfer-derived embryos of caprine. From their finding, the blastocyst rate increased as the activation time of reconstructed oocyte delayed for 6 hours. However, the longer delay would cause the oocyte aging and lower the developmental rate. Their research group also developed 2 stage cell nuclear transfer procedure by using ovine donor cytoplast and caprine foetal fibroblast. They found that the procedure enhanced the blastocyst rate compares to conventional procedure. The two stage nuclear transfer procedure has two advantages, (1) the donor nucleus can be exposed to the ooplasm for a long time, (2) the problem of oocyte aging can be solved (Ma *et al.,*2011).

Due to limited information on interspecies somatic cell nuclear transfer in caprine, more research should be conducted to optimise the protocol for interspecies somatic cell nuclear transfer caprine production. Therefore, the present study adapted interspecies caprine somatic cell nuclear transfer protocol from Kwong (2012) and Soh (2012), with focused on the ability of the matured bovine cytoplast to reprogramme different breeds and genders of the ear fibroblast karyoplasts to produce higher rate of blastocyst, subsequently been used for production of embryonic stem cell attempt.

2.3 FACTORS AFFECTING THE EFFICIENCY OF SOMATIC CELL NUCLEAR TRANSFER EMBRYOS DEVELOPMENT

Somatic cell nuclear transfer has been applied in a range of species (Campbell *et al.*, 2007), however, the reconstructed cloned embryos had low developmental competency and had many abnormalities in the course of development for both *in vivo* and *in vitro.* Thus, this situation provides an ample opportunity for optimisation of nuclear transfer technology. Major factors affecting the efficiency of reconstructed embryos development including the quality of donor cytoplast, the selection of the donor karyoplast, the somatic cell nuclear transfer techniques, the *in vitro* culture systems as well as the nuclear and mitochondrial genomic compatibility.

2.3.1 Cytoplast source and quality

Excellent quality of oocytes were important in somatic cell nuclear transfer experiments as the recipient cytoplast source. An oocyte must undergo cytoplasmic and nuclear maturation to be developmentally competent. Cytoplasmic maturation involves mRNA synthesis, translation into protein, and post translational modifications. The maturation promoting factors (MPF) is one of the important protein produced during the cytoplasmic maturation. This protein is essential for meiotic progression during nuclear maturation (Krisher *et al.,* 1994). Oocytes could matured *in vivo* and *in vitro.* However, due to convenience, most livestock somatic cell nuclear transfer research used *in vitro* matured oocytes as cytoplast source (Rizos *et al*., 2002).

In livestock *in vitro* produced embryos experiments, oocytes could be obtained from either both alive or slaughtered/dead female. From alive does, oocytes could be recovered via laparoscopic ovum pick-up , ovariectomy and laparotomy techniques. On the other hand, oocytes also can derived from slaughtered female from slaughterhouse or during post-mortem (Goh *et al.*, 2012). For this study, bovine oocytes were collected

mainly from ovaries of slaughtered cows from slaughterhouse for interspecies somatic cell nuclear transfer.

Slaughterhouse source oocytes were easiest and cheapest to be collected for subsequently be used in *in vitro* production embryos (Rajikin, 1996; Amir, 2007; Asdiana, 2014). Large number of oocytes could be recovered from the slaughterhouse ovaries (Agrawal *et al.*, 1995). The non-hormone-stimulated ovaries could obtain various grades of cumulus oocytes complexes (COC) (Rajikin, 1996); thus, selection of excellent grades of cumulus oocytes complexes were needed. Ovary slicing was the most favourable technique to retrieve the embryos compared to follicle aspiration as it is simpler and oocytes could be collected in larger quantity (Martino *et al.,* 1994; Pawshe e*t al.,* 1994; Ongeri *et al*., 2001; Rho *et al*., 2001).

In order to obtain maximum number of high quality oocytes from ovaries of animals, as normally obtained in advanced countries, excellent females are needed to be slaughtered. However, in Malaysia, the slaughtered animals were usually old, sterile or unproductive and no history record were available. Consequently, the quality of the oocytes obtained were questionable (Goh *et al.*, 2012). Furthermore, limited number of slaughtered does was further contributing to the challenges to obtain the desired good quality caprine oocytes. Laparoscopic ovum pick-up is a good alternative technique to obtain the caprine oocytes for further reproductive techniques including somatic cell nuckear transfer.

Laparoscopic ovum pick-up is less invasive surgery technique to obtain the oocytes from viable animals, usually, the female will be superstimulated so that high number of oocytes could be retrieved. This technique requires high skill of handling the procedures as it involves surgery, anesthetic and sophisticated equipment (Flores-Foxworth, 1997). The number of oocytes retrieved from this procedure was more than 5 oocytes per donor (Baldassarre and Karatzas, 2004). Through this procedure, good

quality and quantity of oocytes could be retrieved from prepubertal, pubertal or aging animals (Koeman *et al.,* 2003; Baldassarre *et al.,* 2007). With the known age, health status and breed of the donor, the quality of the oocytes retrieved are better compared to unknown source of abattoir derived oocytes. Laparoscopic ovum pick-up also helps the researcher to control the parameter of the experiment especially when focusing on the effect of the donor age and breed (Baldassarre *et al.,* 2007). Laparoscopic ovum pick-up procedure is less traumatic and results in fewer surgical adhesion than laparotomy, thus, this procedure could be repeated for several times without ovarian damage or decrease in the donor fertility (Stangl *et al.,* 1999; Alberio *et al.,* 2002; Baldassarre and Karatzas, 2004). Hormonally stimulated does that used in laparoscopic ovum pick-up could produced high maturation rate of oocyte obtained in laparoscopic ovum pick-up compared to abattoir. Therefore, laparoscopic ovum pick-up technology is an excellent method to retrieved good quality oocytes as it allows harvesting of oocytes from defined individual caprine (De Roover *et al*., 2008).

Sugimura *et al*. (2012) also reported no difference in the blastocyst formation rates of cloned embryos between oocytes from a slaughterhouse and laparoscopic ovum pick-up, but follicle-stimulating hormone pre-treatment of laparoscopic ovum pick-up donor bovine improved oxygen consumption and *OCT4* and *IFN-τ* expression of cloned embryos to levels similar to *in vitro* fertilised embryos (Rizos *et al.,* 2002), suggesting that FSH pre-treatment of laparoscopic ovum pick-up donor bovine has a positive effect on oocyte quality. Furthermore, *in vivo* matured oocytes could be collected by laparoscopic ovum pick-up from hormone-treated females (van de Leemput *et al.,* 1999; Rizos *et al.*, 2002). *In vivo* matured oocytes are more developmentally competent after *in vitro* fertilisation than *in vitro* matured oocytes (van de Leemput *et al.,* 1999; Rizos *et al*., 2002). In contrast, *in vitro* maturation involves retrieving immature oocytes from the ovaries of slaughtered animals and placing them on favourable culture conditions to

complete the maturation process in the incubator.

2.3.2 Selection of donor karyoplast

There are several factors to be considered to choose the donor karyoplast for the somatic cell nuclear transfer experiment. The factors included types of donor karyoplast, passages of donor karyoplast as well as cell cycle of donor karyoplast.

2.3.2.1 Types of donor karyoplast

Donor karyoplasts of different ages including blastomeres (Campbell *et al*, 1993), embryonic stem cells (Eggan *et al*, 2001), foetal, and adult cells have been used for nuclear transfer. However, it is still unclear which type(s) of donor karyoplast is(are) the most suitable for nuclear transfer into enucleated oocytes, especially in interspecies nuclear transfer (Soh, 2011).

The birth of Dolly is a break through in the nuclear transfer and cloning technology history as the finding strongly suggested that the age of an adult somatic donor karyoplast could be transmitted to enucleated oocytes to produce reconstructed embryos and subsequently the viable offspring. Subsequently, numerous types of adult somatic cell were used in the somatic cell nuclear transfer research such as skin 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), mural granulosa cells (Wells *et al.,* 1999). Cumulus cells and ear fibroblast cells were mostly used for producing nuclear transfer embryos. This is due to their availability, easy to obtain and inflict no injuries to the donor (Yaun *et al*., 2012). Ear fibroblast cells have advantage over the cumulus cell, as it can provide the superior genetic male karyoplast compared to cumulus cell which could only provide only the female karyoplast. Obtaining ear fibroblast cell also were relatively cheaper as no superstimulation hormones or major surgery were required.

Compared to adult somatic cell, foetal cells are believed to have less genetic damage. With better ability in proliferation and less time required to achieve 80% confluency, the foetal fibroblast cells have been chosen to be used as donor karyoplast in ruminant somatic cell nuclear transfer (Hill *et al.*, 2000). However, more complex steps involving the molecular work to determine the genetic merit, including the gender, of the foetal cell used as this type of cell could not exhibit the genetic merit prior the nuclear transfer.

In caprine, Baguisi *et al.* (1999) used foetal fibroblast cell used as donor karyoplast and successfully produced 3 healthy cloned offspring. Following this work, a lot of literature reported the use of adult somatic cell and foetal somatic cell as donor karyoplasts with variation of success on obtaining viable cloned blastocyst, or, even producing viable cloned kids (Youisungern and Paul, 2014).

There are several studies reported the of different donor karyoplast types on the developmental rate of cloned embryos and production of cloned kids in caprine (Keefer *et al.,* 2002; Dutta e*t al.*, 2011; Kwong *et al*., 2014; Asdiana, 2014) . Most studies reported that there were no significant differences were found in caprine and bovine embryo developmental rates to the blastocyst stage by using either adult, newborn or foetal karyoplast (Kato *et al.*, 2000). However, more foetal losses after transfer into recipients were noted with embryos reconstructed with adult bovine donor karyoplasts (Hill *et al.,* 2000; Soh *et al.*, 2012).

2.3.2.2 Passages of donor karyoplast

Most somatic cell nuclear transfer studies preferred to use short term culture cells. This is because culturing somatic cells especially for prolonged periods is known to alter ploidy, genomic stability and post-translation histone modifications, factors which are known to reduce cloning efficiency (Jang *et al*., 2004, Kwong *et al.,* 2012). Long-term cultured somatic cells prompt to senescence as well as numerous mutations or allelic

loss of gene accumulated through many rounds of cell divisions, resulting to improper genetic reprogramming after somatic cell nuclear transfer and consequently abnormal development of the embryos (Cibelli *et al.,* 1998; De Sousa *et al.,* 1999; Kuhholzer *et al.,* 2000). In addition, sub-passaging to high number passages is associated with exposing of the cell cultures to excessive trypsinisation procedure. Excessive trypsin digestion could adversely affect the hereditary characteristics of cell cultures as well as other biological characteristics (Li *et al.*, 2009a). Therefore, fresh or short-term cultured (<10 sub-passages) donor karyoplasts have been the cell type of choice for the production of cloned embryos. Most researchers used Passages 3 to 7 as donor karyoplast (Kwong *et al.*, 2012).

However, some studies reported that donor karyoplasts in somatic cell nuclear transfer experiments are normally subjected to serially passaging (Kubota *et al.*, 2000; Kato *et al.*, 2000). According to Kubota *et al.* (2000), the developmental competence of reconstructed embryos derived from cells after long-term culture (10-15 passages) was better than those from cells of short term (<5 passages). Arat *et al.* (2003) also reported that the blastocyst development rates of cloned embryos for donor karyoplasts at Passage 15 were higher than that of early passages. Fibroblast cells from later passages produced high *in vitro* developmeal rate indicated that the cells of higher passages were receptive to nuclear reprogramming (Roh *et al.*, 2000). Enright *et al.* (2003) showed that cells of later passages contained less epigenetic modifications, i.e., their histones were more acetylated than earlier passages. *In vitro* culture of cells could induce expression of genes that were not expressing before culture (Kasinathan *et al.*, 2001). Therefore, it can be summarised that both short-term and long-term culture cells could be used as the donor karyoplast.

2.3.2.3 Donor karyoplast cell cycle stage

Cell cycle coordination is the most important factor in ensuring high efficiency of

somatic cell nuclear transfer (Campbell *et al.*, 1996). Most researchers used G0 and G1 karyoplasts as in G0 phase, karyoplasts exit the normal cell division cycle and enter a quiescent state, whereas, G1 phase is a transient stage between M-phase and S-phase in proliferating cells (Sansinena *et al.,* 2005).

The karyoplasts in G1 and G0 condense normally and could maintain the ploidy of the cloned embryos when transferred to high levels mitotic promoting factor cytoplasts (Campbell *et al.*, 1996). This initiate nuclear envelope breakdown and kayoplasts‟ chromatin condensation (Collas *et al.*, 1993). The exposure of chromosomes to licensing factors in cytoplast leads to replication of DNA following decreasing of mitotic promoting factor level and subsequently trigger the reformation of nuclear membrane (Campbell *et al.*, 1996). However, there is a concern on the synchronised timing between the nuclear envelope breakdown and chromatin condensation If DNA synthesis occur before nuclear envelope breakdown followed by DNA replication, resulting in abnormal ploidy and subsequently producing a defective, abnormal embryo. Furthermore, the progression of DNA synthesis in the donor nucleus is not compatible with normal chromatin condensation. Thus, it was hypothesised that only G0 and G1 nuclei (donor karyoplasts) should be used when transferring to metaphase-II cytoplasts. Several studies reported that both quiescent and proliferating somatic donor karyoplasts can be fully reprogrammed after nuclear transfer and result in viable offspring (Kasinathan *et al.*, 2001; Wells *et al.*, 2003).

There are several techniques for synchronising karyoplasts in different cell cycle phases. To arrest the karyoplasts in the G0 phase several techniques which were serum starvation, contact inhibition and reversible cycle inhibitors (Kues *et al.,* 2000; Gibbons *et al.,* 2002; Gomez *et al.,* 2004) could be adapted. For synchronising cells at the G1 phase, three methods could be used which were culturing cells to early confluence (Cibelli *et al.,* 1998), physically shaking or vortexing a sub-confluent cell culture to

obtain newly divided cells which cytoplasmic bridges at the beginning of G1 phase (Kasinathan *et al.,* 2001) and by serum deprivation to force the cells to enter G0 phase followed by stimulating G1 phase by culturing cells medium containing serum (Memili *et al.,* 2004).

2.3.3 Somatic cell nuclear transfer (SCNT) techniques

Somatic cell nuclear transfer techniques involves complex procedures. These involve with the enucleation of the cytoplast, donor karyoplast transfer, reconstructed embryo activation and *in vitro* culture. The enucleation and the karyoplast transfer were difficult and most of the laboratories including our research group used micromanipulator. Handmade cloning is also an alternative to somatic cell nuclear transfer procedure which eliminated the use of the micromanipulator. Somatic cell nuclear transfer techniques required skillful researcher which could only obtain through experience as lack of skill would affect the somatic cell nuclear transfer success rate.

2.3.3.1 Enucleation

Chromatin aspiration is the most favourable method for enucleation. In this method, the oocyte is held steady at one side with the holding pipette while the enucleation pipette is inserted from the opposite side to remove, depending on the maturation state, the extruding spindle or first polar body together with the metaphase plate. After enucleation, the confirmation can be done by visualised the chromosomal material. In livestock somatic cell nuclear transfer, the blind enucleation is used due to high lipid content. Generally, in blind enucleation, the polar body is used as the guidance point. The enucleation is done by removal 10% cytoplasm with the chromatin out from the oocytes (McGrath *et al.,* 1983; Kwong, 2012; Soh, 2012; Asdiana, 2015). The enucleated cytoplasm then is stained with Hoechst staining and confirmed under UV microscope (Tsunoda *et al.*, 1988).

Another enucleation technique could be utilised is direct enucleation, which was chemically assisted by cytoskeleton-relaxing agents such as Cytochalasin B. This technique produces a protrusion cone containing the oocyte‟s chromosomes. Drugs used for this purpose include demecolcine and nocodazole, both interfering with microtubule polymerisation. This method is applied in handmade cloning procedure. In handmade cloning, the zona pellucida is digested, and one third of the oocyte containing the extruding polar body and metaphase plate is manually bisected with a microblade under a stereomicroscope (Vatja *et al.*, 2001). Other enucleation methods that have been attempted were centrifugation and laser assisted squeezing method (Goh, 2012).

2.3.3.2 Injection of donor karyoplast

In livestock species, the most common method is sub-zonal injection (SUZI). This method is done by inserting the whole donor karyoplast into the perivitelline space to achieve contact between the membranes of both donor karyoplast and the cytoplasm of enucleated cytoplast. The donor chromatin is then incorporated into the enucleated oocyte by cell fusion which is commonly achieved by electrofusion. It has been successfully applied to produce cloned bovine (Wells *et al.,* 1999) and caprine (Baguisi *et al.,* 1999).

Another method is directly insertion the nucleus (nuclear microinjection) into the enucleated oocyte. This method is also known as whole cell intracytoplasmic injection (WCICI) method. For this method, the nucleus of the donor karyoplast is isolated using a micropipette with an inner diameter smaller than the donor karyoplast. The cell is pipetted in and out in order to break karyoplast membrane and nucleus is released. The nucleus then is transferred into the cytoplasm of the cytoplast. This method bypasses the need for fusion thus making it favourable as it is less technical labour intensive (Lee *et al.*, 2003).

However, is there any significant difference between these two injection methods to the somatic cell nuclear transfer efficiency? Comparing the direct injection method with the cell fusion technique on production of cloned porcine, Kawano *et al.* (2004) reported that reconstruction rate of porcine oocyte using direct injection was significantly higher than cell fusion technique. However, blastocyst rate was significantly lower for whole cell intracytoplasmic injection. No significant difference in total cell number, inner cell mass and trophectoderm cell number in blastocyst, thus suggesting that the injection method did not affect the cloned blastocyst quality. In caprine, Zou *et al.* (2001) reported that injection method showed high cleavage rate than fusion method by using the cumulus cell as donor karyoplast. However, this finding were contradicting with Soh *et al.* (2012) who reported high cleavage rate and blastocyst rate were obtained by using subzonal injection (SUZI) method compared to whole cell intracytoplasmic injection technique using caprine foetal fibroblast cell as donor karyoplast.

2.3.3.3 Activation

During normal fertilisation, egg activation is achieved when the summation of intracellular Ca^{2+} reach minimum threshold. This oscillation is triggered by a sperm specific isoform (Ross *et al.*, 2009). Mitotic promoting factor (MPF) also played role in the Ca^{2+} oscillation. The oocyte activation is enhanced when the mitotic promoting factor is inhibited and meiotic progression is induced. This can be done protein synthesis inhibition of phosphatases, and this could be achieved by using chemicals such as cyclohexamide (CHX) and 6-dimethylaminopurine (6-DMAP), respectively (Kishikawa *et al.*, 1999).

Several artificial techniques were developed to trigger activation of reconstructed somatic cell nuclear transfer embryos, including electrical pulses, ethanol, calcium ionophore A23187, ionomycin, strontium, and thimerosal (Thi) ⁄ dithiothreitol (DTT)

(Ross *et al.,* 2008; Cerevra *et al.*, 2010). With the exceptions of strontium and Thi/DTT, the other treatments induce a single Ca^{2+} oscillation. Another factor that can be controlled in activation protocols is the timing of activation following embryo reconstruction (Sparman *et al.*, 2010). It is common practice to delay activation for 1 to 3 hours to extend the time for nuclear remodeling after nuclear envelope breakdown.

2.3.4 *In vitro* culture

The *in vitro* culture (IVC) is a important period for the *in vitro* produced embryos development which has a great impact on the blastocyst quality (Rizos *et al.*, 2002). There are several factors that affect the success rate of the *in vitro* culture, such as osmolarity and ionic composition, temperature, pH and carbon dioxide, oxygen, carbohydrates, amino acids, lipids and fatty acids, proteins, growth factors and cytokines. Any variation from the optimum environment could lead to the embryonic development arrested at any stages.

For *in vivo* embryonic development, the composition of the mammalian changes from early to late cleavage stages. Embryonic genetic activation which occurs at 8-cell to 16-cell stage in mammals, is directly proportional with increasing in metabolic activity, oxygen uptake and carbohydrates consumption to the blastocyst stage (Rieger *et al.,* 1992; Thompson *et al.*, 1996). In earlier research in *in vitro* produced embryos development, the embryos failed to develop past 8-cell and 16-cell stages which was associated with genomic activation time (Song *et al.*, 2009).

Different culture media have been successfully used for embryo development, such as TCM 199 (Wani *et al.,* 2012), G1/G2 (Ongeri *et al.*, 2001; Koeman *et al.*, 2003) and potassium simplex optimum medium (KSOM) (Abdullah *et al.,* 2011). The most widely used medium for caprine *in vitro* culture embryo is synthetic oviductal fluid (SOF) medium (de Souza-Fabjan *et al.*, 2014). Subsequently, some laboratories made some modification to the original composition of the media. This including

supplementation of 5-10% foetal bovine serum (Catala *et al.*, 2012; Hammami *et al.,*2013) or even increasing of the glucose level in *in vitro* culture medium on Day 2 (Kwong *et al.,*2012; Soh *et al.*, 2012).

The progress in understanding of the requirements of the developing embryo resulted in the improvement of the media whereby modifications were made according to the embryo requirement. These media would mimic the *in vivo* environment, enabling the biochemical and morphological changes of embryos. Thus, physiological media are formulated to reflect the carbohydrate levels of the reproductive tract and to reduce cellular stress on the embryo (Lane *et al.,* 2003).

In *in vitro* embryo production, the culture system strongly influenced embryonic development (Lonergan *et al*., 1999). The early embryogenesis is a complex process that use maternal proteins and transcripts to support the development of the embryo until its genome activation (embryonic genome activation), leading to synthesis of new transcripts and proteins at the right amount and stage of development. Physiology and biochemistry of embryo from the early stage to blastocysts is different along with the morphological differences. Although the mammalian embryo has great plasticity, which allows it to survive *in vitro*, it usually shows lower quality and viability compared to *in vivo* cultured embryos (Lane, 2001).

Poor rate of successful cloned blastocysts produced and the *in vitro* developmental block in interspecies cloned embryos might be related to developmental cell block and mitochondrial incompability between the donor karyoplast and recipient cytoplast (Thongpakdee *et al.*, 2008), incomplete donor karyoplast reprogramming and abnormal epigenetic reprogramming, including DNA methylation and histone modification (Chen *et al.*, 2006; Lee *et al.*, 2010). To overcome this issues, the supplementation of histone deacytelase inhibitor, such as suberoylanilide hydroxamic acid, scriptaid, and the most widely used, trichostatin A, had been can improve *in vitro* blastocyst production

(Kishigami *et al.*, 2006; Srirattana *et al.*, 2012; Wittarayat *et al.*, 2013). The treatment of cloned embryos with trichostatin A also results in significant improvements in embryo development in murine (Kishigami *et al.*, 2006; Rybouchkin *et al.*, 2006), porcine (Zhang *et al.*, 2007; Chawalit *et al.*, 2012) and bovine (Sawai *et al.*, 2012; Wittarayat *et al.*, 2013). However, no reports are available for treatment of caprine embryos either in *in vitro* fertilisation studies or somatic cell nuclear transfer studies.

2.3.5 Compatibility of genomic-mitochondrial DNA

The somatic cell nuclear transfer technology progress has been appeared slow, as the efficiency of the somatic cell nuclear transfer is still low in term of cloned blastocyst production, pregnancy and viable cloned offspring production. In addition, many abnormalities occurs during the development of the embryos. Many research activities on somatic cell nuclear transfer embryos revealed that gene expression patterns in the cloned embryo, foetus and placenta were abnormal (reviewed by Jaenisch *et al.*, 2002). Low efficiency of somatic cell nuclear transfer, especially interspecies somatic cell nuclear transfer might be associated with two factors which are incomplete reprogramming of nuclear as well as incompatibility in mitochondrial physiology between the karyoplast nucleus and cytoplast mitochondrial (Beyhan *et al.,* 2007).

Mitochondrion is the organelle with its own DNA (mtDNA) which is maternally inherited. The vital function of this special double membrane organelle is to produce energy in the ATP form (Anderson *et al.,* 1981). mtDNA encodes some of the subunits of the electron transfer chain which is responsible for ATP production. Replication, transcription and expression of mtDNA are regulated by nuclear DNA, through the expression of transcription and replication factors that translocate into mitochondria in the cytoplast (Anderson *et al.,* 1981). Hence, coordinated mt-nuclear DNA interaction is essential for normal embryo 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 to ensure mitochondrial homoplasmy, which is important to the maintenance of proper mitochondrial function (Beyhan *et al.,* 2007). However, in somatic cell nuclear transfer, the donor karyoplast mitochondria can persisted and contributed to the reconstructed embryo, thus, resulting in heteroplasmy (two different populations of mtDNA) (Sansinena *et al.,* 2011). Donor mitochondrial contribution is quite variable, ranging from 0% to 59% (St. John *et al.*, 2004). This suggested that the preferential amplification of donor mitochondria resulted in high heteroplasmy of the cloned embryos.

The interaction between the donor karyoplast and recipient cytoplast influenced most of important biological functions during nuclear reprogramming and embryo reconstruction. mtDNA is mainly contributed by the recipient oocyte during somatic cell nuclear transfer, but is regulated by genes in the donor karyoplast. mtDNA has high rates of heritable polymorphism and *de novo* mutation which can result in many haplotypes (Bruggerhoff *et al.*, 2002) under high oxygen level condition and limited ability for DNA repairing. In bovine, oocytes with various mtDNA haplotypes, usually have different ATP contents, and this may affect the efficiency of *in vitro* development of cloned embryos (Tamassia *et al.*, 2003). However the relationship and the underlying mechanisms between mtDNA haplotypes and somatic cell nuclear transfer efficiency have not been fully investigated.

Research on production of cloned murine, ovine and bovine embryos revealed that high degree of variability in mitochondrial distribution, with some animals displaying complete homoplasmy (Evans *et al.,* 1999; Hua *et al.,* 2008), and others displaying heteroplasmy to different degrees (Han *et al.,* 2003; Hiendleder *et al.,* 2003; Inoue *et al.,* 2004). St John *et al.* (2004) reported that the degree of heteroplasmy increases

when interspecies somatic cell nuclear transfer is performed. Therefore, the low efficiency of the both intra- and interspecies somatic cell nuclear transfer was suggested to be the effect heteroplasmy introduced in these animals.

2.4 POTENTIAL APPLICATION OF INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER

Three major potential applications of interspecies somatic cell nuclear transfer will be discussed in this section: a) conservation of endangered and extinct animals, b) establishment of embryonic stem cells and c) improvement of goat production at a rapid rate.

2.4.1 Conservation of endangered and extinct animals

Since 1998, when the cloned sheep Dolly (Wilmut *et al.,* 1998) was produced from a differentiated cell line by somatic cell nuclear transfer, many other species have been successfully cloned. Increasingly, interspecies somatic cell nuclear transfer technique has been widely utilised to potentially rescue the endangered as well as extinct species. Unfortunately, most of these efforts were unsuccessful and less efficient, especially the problem of keeping the pregnancy to the term.

Despite increasing interest in using somatic cell nuclear transfer technique to rescue endangered species, reports on successful interspecies somatic cell nuclear transfer and *in vitro* blastocysts production are limited. One of the major concern on the interspecies cloned embryos production is whether one species cytoplast could reprogramme the highly differentiated somatic cells of the other species (Dominko *et al.*, 1999). One of the earliest attempts on interspecies somatic cell nuclear transfer was reported by Dominko *et al.* (1999), who used the enucleated bovine oocytes as recipient cytoplasts and skin fibroblast cells from bovine, ovine, porcine, monkeys and murine as karyoplast donors. Although interspecies cloned embryos were successfully produced, no pregnancies were carried to term after the embryos were transferred into surrogate animals (Dominko *et al.*, 1999).

It was reported that the interspecies cloned argali embryos were succesfully constructed from argali donor karyoplasts and domestic sheep cytoplasts. However,

only one pregnancy was obtained and it terminated on Day 59 of pregnancy (White *et al.*, 1999). Subsequently, Lanza *et al.* (2000) reported the pregnancies from interspecies cloned Gaur bull embryos lasted until Day 200 of gestation. Similar outcome was also reported when embryos were reconstructed with adult Banteng donor karyoplast and domestic bovine cytoplasts, but two pregnancies were terminated between Days 30 and 90 (Sansinena *et al.*, 2005). Wen *et al.* (2004) reported that although they successfully obtained interspecies cloned male gaur offpring, unfortunately, the calf succumbed to common dysentery within 48 hours after his birth.

The first report of a viable offspring by subspecies somatic cell nuclear transfer (a type of interspecies cloning) was reported on 2001 as the cross-species cloned embryos were produced from the post-mortem karyoplast of a mouflon. The karyoplasts were injected into enucleated ovine cytoplasts, a closely related domesticated species. Subsequently, embryos were transferred into four surrogate sheep, which resulted in two pregnancies. One surrogate mother aborted between Days 40 and 50 while the other pregnancy successfully went to term and produce viable offspring (Loi *et al.*, 2001).

Utilising interspecies somatic cell nuclear transfer technique to produce viable offspring, a goal of some of the earliest interspecies somatic cell nuclear transfer experiments, focuses primarily on applications involving the preservation rescue of endangered species (Dominko e*t al.*, 1999). Although the main procedures to construct cloned embryos for any purpose are essentially the same, embryos cloned to produce live offspring require a more comprehensive and complete reprogramming of the somatic genome, since they need to progress through all developmental milestones and survive a rigorous *in vivo* selection process (Beyhan *et al*., 2007). Table 2.3 shows the timeline of selected interspecies somatic cell nuclear transfer which focusing on conservation of desired species.

Table 2.3: Timeline of interspecies somatic cell nuclear transfer which focusing on conservation of desired species

Year	Author	Recipient cytoplast	Donor Karyplast	Blastocyst	Implantati-on	Live offspring
2000	Lanza *et al.*	Cow	Gaur	Yes	Yes	No
2001	Loi *et al.*	Sheep	Muflon	Yes	Yes	Yes
2002	Chen *et al.*	Rabbit	Panda	Yes	Yes	No
2004	Gomez *et al.*	Domestic cat	Wild cat	Yes	Yes	Yes
2005	Jiang *et al.*	Goat	Ibex	Yes	No	No
2005	Sansinena *et al.*	Cow	Banteng	Yes	Yes	No
2006	Li *et al.*	Cow	Yak	Yes	Yes	Yes
2006	Yin *et al.*	Wild cat	Leopard cat	Yes	Yes	No
2007	Mastromonaco *et al*.	Cow	Gaur	Yes	Yes	Yes
2007	Zhou *et al.*	Goat	Tibetan antelope	Yes	N.A.	N.A.
2009	Tao *et al.*	Rabbit	Red Panda	Yes	N.A.	N.A.
2010	Techakumphu *et al.*	Rabbit	Asian Elephant	Yes	N.A.	N.A.
2011	Gomez *et al.*	Cat	Black-Footed cat	Yes	N.A.	N.A.
2013	Seaby *et al.*	Cow	Bison	Yes	N.A.	N.A.
2013	Hwang *et al.*	Dog	Coyote	Yes	N.A.	N.A.
2014	Pan *et al.*	Sheep	Argali	Yes	N.A.	N.A.
2015	Moro *et al.*	Cat	Cheetah	Yes	N.A.	N.A.

Most are the problems connected with cloning endangered species are related with general characters of the animal, as well as strictly connected to the limited or impossible, access to rare animals kept in captive breeding and *in situ* conservation in their natural niches (Loi *et al.,* 2007). Among the general factors, the main one is represented by the limited knowledge of the reproductive anatomy and physiology of endangered species. Reproductive technologies are, in fact, easily transferred to wild animals that are genetically close to their domestic counterparts; it is in domestic animals where such procedures were originally mastered (Loi *et al.,* 2001).

One of the constraints of interspecies cloning in endangered animals is the availability of the cytoplast (Loi *et al.,* 2007). Oocytes should be retrieved from females that are phylogenetically close to the endangered animals and available in large numbers, for examples from domestic cows, pigs and goats. The unavoidable side effect of this approach is the production of chimaeric animals bearing mitochondrial DNA (mtDNA) from the host oocyte and genomic DNA from the nucleus donor. The incompatibility between the nuclear and genetic systems might be partially responsible for the developmental arrest in cross-species, reconstructed embryos, resulting, perhaps, from altered respiration in the mitochondria.

The availability of surrogate mothers for the cloned embryos is a real limitation, not only for the transfer of cloned embryos but also of ones produced *in vivo* or *in vitro* (Andarbi *et al.*, 2007). The genetic background of surrogate mothers and the embryos might be more important than the genomic and/or mitochondrial DNA compatibility (Loi *et al.*, 2007). The undesirable side effect of producing the chimeric offspring bearing mitochondrial DNA from the donor cytoplast and genomic DNA of donor karyoplast might be unavoidable if the compatibility is neglected (Li *et al.*, 2007). Therefore, the removal of species-specific barriers for embryo transfer is a fundamental requisite for successful multiplication of endangered genotypes through cloning or

related *in vitro* technologies.

The limited but promising achievement in cloning endangered animals bring optimism of the success of using interspecies somatic cell nuclear transfer as an important tool to conserve the endangered species.The understanding of the molecular clues underlying nuclear reprogramming also might help to increase understanding in interspecies somatic cell nuclear transfer technique. If it is possible to achieve a controlled reversal of the differentiated state of a cell, then it is probable that other issues that impair the cloning of endangered animals, such as the interspecies oocyte or womb donor, will be overcame.

2.4.2 Establishment of embryonic stem cells (ESC)

The utilisation of human cytoplast for research purposes poses serious ethical issues in society. Consequently, the development and advancement of promising research areas including somatic cell nuclear transfer and embryonic stem cells in human are limited. Therefore, the approach of to counter this challenge is to utilise the cytoplast from another species which presented by interspecies somatic cell nculear transfer technology. Interspecies somatic cell nuclear transfer had successfully produced viable blastocyst and live offspring have been obtained by combining closely related species, such as cattle/gaur (Bos taurus/Bos grunensis) (Lanza *et al.*, 2000) and domestic sheep/argali sheep (O. aries/O. musimon) (White *et al.*, 1999). Hypothetically, the ability of different species cytoplast to reprogramme donor karyoplast holds tremendous promise (Beyhan *et al.*, 2007).

The embryonic stem cells are pluripotent; these cells able to differentiate into almost all tissues of the 3 embryonic germ layers (ectoderm, mesoderm and endoderm) after *in vitro* induced differentiation. Embryonic stem cells are also capable to form teratocarcinomas (Cavaleri and Scholer, 2004). The general characteristics of embryonic stem cells were: a) embryonic stem cells derived from pre-implantation embryos, b) pluripotent (Suda *et al*., 1987), c) immortal (Amit *et al*., 2000), d) maintaining normal karyotype after prolonged culture, e) ability to contribute to all embryonic germ layers including the germ line after injection into blastocyst, f) expressing unique pattern of embryonic stem cells markers such as Oct 4, Sox 2, Nanog and cell surface markers such as stage specific embryonic antigen SSEA 1, SSEA 2, SSEA 3, SSEA 4, tumor resistant antigen TRA-1-60 and TRA-1-81 and g) clonogenic.

An attempt to create embryonic stem cell lines of the porcine, caprine, ovine and equine have most often used *in vivo* blastocysts acquired from the reproductive tract at various stages, but generally from the early blastocyst stage to the later elongated or

filamentous stage (Goh *et al.,* 2012). Embryonic stem cells can be derived from numerous techniques through *in vitro* produced embryos, incuding intra- and interspecies somatic cell nuclear transfer as well as *in vivo* produced embryos. Efficient and cost effective *in vitro* produced embryo culture systems are commonly used for bovine and caprine which early blastocyst stage embryos are the most frequent starting material for attempts at making embryonic stem cell lines (Goh, 2012).

In vitro produced blastocysts may be altered in terms of cell metabolism, epigenetic status and constituent cell numbers, but it is probably that some will prove competent for the establishment of bovine embryonic stem cell lines. This seems reasonable since human embryonic stem cell lines have been derived from *in vitro* fertilisation or *in vitro* culture embryos as well as the culture of *in vitro* produced derived bovine epiblast tissue displays normal differentiation and pluripotency *in vitro* (Talbot *et al*., 1995).

Is not necessary for cloned embryos for embryonic stem cell establishment to develop through all developmental stages to term. Instead, a few functional equivalents of inner cell mass cells or single blastomeres in cloned embryo is adequate to establish an embryonic stem cell line (Klimanskaya *et al.*, 2006;). This concept could be applied to interspecies somatic cell nuclear transfer technique, assuming that similar pattern of gene expression at the various stages of development was observed for interspecies cloned embryos, *in vivo* produced embryos*, in vitro* fertilised embryos as well as intraspecies cloned embryos.

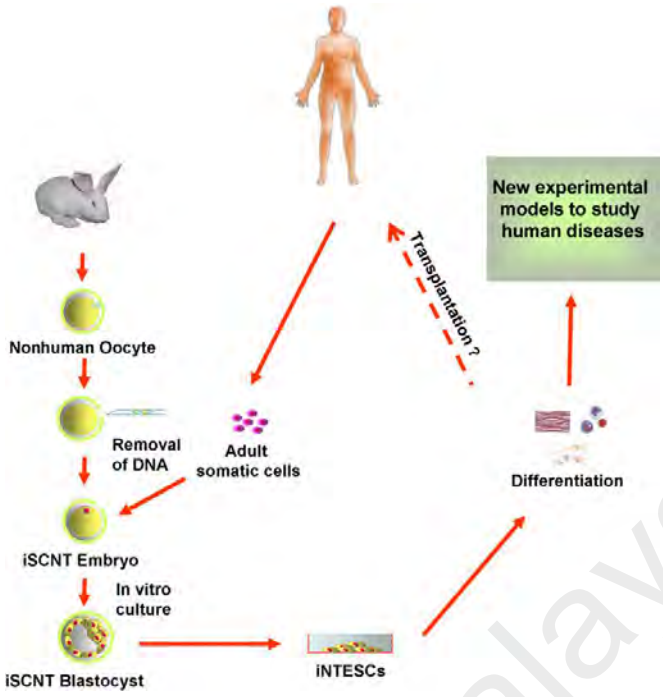

Figure 2.1: Schematic diagram of embryonic stem cell derived from interspecies somatic cell nuclear transfer.
(retrieved from Beyhan *et al.,* 2007.Interspecies Nuclear Transfer: Implications for Embryonic Stem Cell Biology. Cell Stem Review. 1: 505.)

As discussed earlier, interspecies somatic cell nuclear transfer has been proposed for creating human embryonic stem cell lines in an effort to avoid the ethically issues of utilisation of human cytoplasts for research purposes. While this alternative may alleviate some ethical and practical concerns, the use of such cells for therapeutic purposes is in doubt, due to the potential risks of transmission of infectious diseases (Beyhan *et al.*, 2007). Furthermore, embryonic stem cell isolated from cytoplasmic hybrids may maintain mitochondria derived from the non-human recipient cytoplast, a result that is likely to have deleterious long-term physiological and immunological consequences to human recipients (Hall *et al.*, 2006). Nevertheless, the ability to produce embryonic stem cell from interspecies cloned embryos could help the creation of new cellular models of human disease and could significantly advance our understanding of basic nuclear-cytoplasmic interactions between a somatic cell and an oocyte.

While many intraspecies cloned blastocysts failed to develop into live offspring, a substantial number of embryonic stem-like cells have been derived from intraspecies cloned embryos in bovine and murine models (Cibelli *et al.*, 1998; Wakayama *et al.*, 2006; Wang *et al.*, 2005). The embryonic stem cells derived from intraspecies cloned embryos comparable to their *in vivo* derived counterparts in terms of their differentiation capacity, pluripotency marker expression profile, global gene expression profile, and select methylation characteristics as reported in bovine, rabbit as well as caprine (Fang *et al.*, 2006; Wakayama *et al.*, 2006; Goh *et al.*, 2012). Chen *et al.* (2003) reported the first embryonic stem cell line established from interspecies cloned blastocysts (rabbit-human) with efficiency of 13 to 14%.

2.4.3 Improving caprine production at rapid rate

Caprine has a huge potential to serve as bioreactors to produce milk containing recombinant proteins suitable for pharmacological reasons. Caprine is suitable as a candidate as transgenesis animal as goat has a short generation interval compared to bovine. With the advantage of higher volume of milk production compared with ovine, dairy caprine is ideal for the production of therapeutic recombinant proteins because their high concentrations of recombinant protein of 1–5 g/L in milk allows for herds of transgenic goats of manageable size which could easily yield 1–300 kg of purified product per year (Paramio and Izquierdo, 2014). Caprine meat is an important food worldwide and it has high-quality protein and low cholesterol. This would provide high opportunity to utilise somatic cell nuclear transfer caprine not only as biopharming reactor, but to improve the caprine meat production.

Recently, transgenic caprine have been produced by somatic cell nuclear transfer technique as the genetically modified somatic karyoplasts were inserted into enucleated cytoplasts. Several reports reported on transgenic caprine producing malaria antigen (Behboodi *et al.*, 2005) and human acid beta-glucosidase (Zhang *et al*., 2010) have been successfully obtained. Yekta *et al.* (2013) reported great success in cloned transgenic caprine using foetal fibroblasts with which designed to secret the recombinant protein in caprine milk. Increased expression of recombinant human alpha-lactalbumin derived from transgenic goat using somatic cell nuclear transfer was observed in caprine milk (Yuan *et al.*, 2014). Zhou *et al.* (2013) used myostatin-targeted 2-month-old goat fibroblasts as donor karyoplast for producing myostatin-targeted goats for meat purpose. Apart from gene pharming, production traits such as growth rate, quantity and composition of milk, fibre production or disease resistance have been obtained by transgenic goats.

Mostly success cloning research in caprine were using the intraspecies somatic cell nuclear transfer. However, scarce information on interspecies somatic cell nuclear transfer in caprine is available in literature. The only successful production of interspecies cloned caprine offspring were reported by Jian-Quan *et al.* (2007) who obtained healthy offspring of Boer. One of the significant finding in interspecies somatic cell nuclear transfer is the first birth of extinct goat using subspecies donor cytoplast by Folch *et al.* (2009) which not only a significant milestone in caprine cloning research but also in conservation of extinct species as this is the first exticnt animal brought to life. However, after these two significant finding, no reports on the success of production cloned kids by interspecies somatic cell nuclear transfer. Most of the focus on interspecies somatic cell nuclear transfer were on improvement of technique (Kwong *et al.*, 2012, 2014 and Soh *et al.*, 2012).

Somatic cell nuclear transfer has exceptional potential to improve production caprine as it allows for the propagation of caprine with known phenotypes to serve as breeding caprine, especially the high genetic merit and elite breeds of caprine. This is critically important in breeding programs, as it also allows the propagation of animals whose reproductive function may be impaired and allows the propagation of valuable deceased animals from which tissue samples have been appropriately collected or preserved. In addition, somatic cell nuclear transfer also allows for the careful study of the "nature-nurture" interactions that influence breeding programs by allowing a large enough sample of genetically identical animals to be raised in different environments, or with different diets. This is of particular importance to the developing world and rural areas, subsequently improves the well-being of the farmers involved in caprine-based agriculture industry.

Chapter 3

3.0 MATERIALS AND METHODS

3.1 INTRODUCTION

The main objective of this research is to produce interspecies cloned embryos and mouse embryonic feeder cell layer for attempt to produce embryonic stem cell (ESC). In order to produce of interspecies cloned caprine blastocyst, the bovine oocytes were collected from bovine ovaries as recipient cytoplast and the donor karyoplast used were frozen-thawed ear fibroblast karyoplast of different breed and gender. Bovine ovaries were collected from the Abattoir Complex of Department of Veterinary Services (DVS), Shah Alam (1 hour journey) and Abattoir Complex of DVS, Banting (2 hours journey). Bovine oocytes were collected from these ovaries and subjected for *in vitro* maturation, subsequently for parthenotes and cloned embryos. For murine experiments, murine was used to produce mouse embryonic fibroblast (MEF) cell layers for feeder cell layer in order to culture the caprine embryonic stem cell.

Murine, bovine and caprine experiments were conducted in Animal Biotechnology-Embryo Laboratory (ABEL) and Embryo Micromanipulation Laboratory (EMiL), Institute of Biological Sciences, Faculty of Science, University of Malaya and Nuclear Transfer and Reprogramming (NaTuRe) Laboratory, Institute of Research Management and Monitoring (IPPP), University of Malaya. This study was conducted from September 2013 to April 2016.

3.2 EXPERIMENTAL ANIMALS

In this study, murine, bovine and caprine species were used. All animals care and experiment were accordance in University of Malaya guidelines.

3.2.1 Murine

For this study, murine were used as model animal for learning curvature and production of mouse embryonic fibroblast. The murine were bred and housed in the Animal House of ISB Mini Farm, University of Malaya. The murine were kept in the clean animal cages in the room temperature (25^{o}C) with natural light: dark cycle (12 hours:12 hours). Every cages were layered with dry sawdust purchased from local sawmill. For ensuring the cleanliness, the sawdust were changed every week and dirty cages were changed with clean cages. The murine were fed with commercial pellet and clean drinking water with *ad libitum* supply.

Prior of superovulation regime, the female murine need to be separated from the male at least a month. This to ensure that the female murine were not pregnant before the superovulation. Meanwhile, for obtaining the mouse embryonic fibroblast, after the superovulation, the female were mated in mating cages (1 female: 1 male). After the copulation, the female were transferred into pregnancy cages. The pregnant female were then euthanised for harvesting the foetus according to the experimental design. Only adult female (8 to 12 weeks old) were subjected for superovulation regime for subsequent experiments.

3.2.2 Bovine

A total of 1714 of oocytes were collected from 165 bovine ovaries. Bovine ovaries were collected from Abattoir Complex, Department of Veterinary Services of Shah Alam and Banting, Selangor according to availability of the bovine ovaries samples.

All the ovaries were transported from the abattoir to the laboratory by using thermal flask. The normal saline supplemented with 0.9% of $NaCl_2$ (Sigma Aldrich Co., USA) with penicillin and streptomycin (Sigma Aldrich Co., USA) were used to provide the good environment for the ovaries to prevent ischemic. The temperature were maintained around 30 to 35^{o}C. The record of breed, origin, health status, oestrus cycle phases of the ovaries donor were unavailable.

3.2.3 Caprine

In this study, caprine were used for establishing ear fibroblast cell line to be used as donor karyoplast. For this experiment, the ear fibroblast cells were collected from the live goats in the ISB Mini Farm (Livestock) in University of Malaya, Kuala Lumpur and Rumpun Asia Sdn. Bhd. (RASB) Farm in Taman Kekal Pengeluaran Makanan (TKPM) Hulu Tamu, Selangor. Ear tissues were collected from females and males of adult purebreed Katjang and purebreed Boer (3 to 4 years old). Samples for each male and female of each breed (Katjang and Boer) were collected, cultured, subpassaged, cryopreserved and stored in liquid nitrogen (-196°C) prior used.

3.3 MATERIALS

In this study, the materials used included: equipment, chemicals, media and reagents, labwares as well as disposables are briefly described as the following:

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 equipment included autoclave, centrifuge, CO_2 incubator, inverted microscope with micromanipulator, electrofusion machine, laminar air flow work station, liquid nitrogen tank, microforge, microgrinder, micropuller, reverse osmosis (RO) water machine, pH meter, oven, stage warmer, stereomicroscope, surgical set and water bath.

3.3.2 Chemicals, media and reagents

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 Labwares and disposables

A list labwares and disposables with manufacturer‟s name used in this study is presented in Appendix Table 1.3.

3.4 METHODOLOGY

This part will be discussing several parts; a) standard maintenance of embryo and tissue culture laboratory, b) preparation of media, c) preparation of microtools; and d) experimental procedures.

3.4.1 Standard maintenance of embryo and tissue culture laboratory

All laboratory activities and equipment adhered to strict cleanliness regimes and sterile techniques in order to maintaining the optimum embryo and tissue culture condition. Cleanliness and hygienic environment is important for optimising the results of the experiments. It is very important to minimise the contamination while handling and culturing the oocytes, embryos and cell lines. Below are the general procedures practised in our laboratory in order to achieve good embryo and tissue culture environment.

3.4.1.1 The researcher

Washing hand with the disinfectant before and after any experiments was a must to minimise the adherence of microorganism on the skin, which can bring diseases to human and contamination to the cell culture. Besides, wearing a clean laboratory coat, face mask and gloves must be practised in the laboratory to keep the environment of embryo clean and sterile. The researcher also must avoid using lotion or perfume which could be toxic to the embryos.

3.4.1.2 The glassware and apparatus

The washable glassware and non-disposable items were rinsed vigorously with tap water to remove any debris. Then, the glasswares were soaked in a diluted detergent (7x®-PF) for a few minutes. After that, they were cleaned using sponge after which

were rinsed vigorously with tap water followed by autoclaved reverse osmosis (RO) water. After that, they were either wrapped with aluminum foil or packed in autoclavable disposable bags. All the items were sterilised by autoclaving them at 121°C for 20 minutes. Later, all items were dried in an oven at 60°C.

3.4.1.3 The surface of work station

Before starting any experiment, the inside surfaces of the laminar air flow work station, microscope stages and other equipment such as micropipettes were sterilised with ethanol (70%). The residual traces of ethanol were allowed to evaporate for at least 20 minutes before experiment. Any spillage was wiped immediately with dry tissue. and if necessary, the UV radiation were applied for 10 to 20 minutes. When work finished, the inside surfaces of the laminar flow work station and all the equipment were wiped again with ethanol (70%). The water bath was needed to be cleaned frequently. The autoclaved RO water need to be used to fill the water bath and need to be changed at least fortnightly.

3.4.1.4 The CO_2 incubator

Most of the embryo works were carried out using the CO_2 incubator (5%) in maximum humidified air to maintain the correct physiological pH (pH range 7.3 to 7.4) with the temperature of 38.5°C (for bovine and caprine samples) or 37°C (for murine samples). In order to maintain the sterility and ideal environment for the embryos, oocytes or cell lines, the incubator was cleaned monthly. The cleaning process involved wiping the inside wall, doors and racks with sterile RO water using the sterile towels or tissues. The trays and the RO water contained in it which was to provide humidity were sterilised and changed according to the scheduled time. The CO_2 incubator (5%) should be monitored regularly and the LED display of temperature was checked with

independent reading. For better precaution steps, the repeated opening and closing of the CO_2 incubator (5%) must be kept to minimum because it may affect the optimum of the oocyte or embryo environment as well reducing the possibility for contaminants entering the incubator.

3.4.2 Preparation of media

All the media that were used for this study were prepared prior the experiments. It is advised to aliquot the chemical substances from the stock bottles into the autoclaved centrifuge tubes (15 ml or 50 ml) to ensure no contamination or damaged for the chemicals substances in the stock bottle. The spatula, conical flasks, magnetic stirrer, measuring cylinder and any apparatus utilised in the medium preparation should be cleaned and sterilised. Preparation of media must be carried out under laminar flow hood for aseptic purpose and the weighing balance should be wiped ethanol (70%) before and after the weighing of the chemical to ensure cleanliness. For medium preparation, autoclaved purified milli-Q water (Millipore, Ireland) was used as base solution. The pH and osmolarity were measured by using pH meter (7.2 to 7.4) and osmometer (280 to 300 mOsm), respectively. The prepared media were filtered into Scott bottle or centrifuge tubes (according the media volume) by using sterile disposable filter (0.22 μm). The media were labeled before stored in refrigerator (3 to 5°C) or freezer (-20°C). After reaching the shelf life, the expired media were discarded according to the waste disposal protocol in the laboratory.

3.4.2.1 Preparation of phosphate buffer saline (PBS-) without calcium chloride (Ca^{2+}) and magnesium chloride (Mg^{2+})

The phosphate buffer saline (PBS-) solution was used as a base medium of several stock solution as well as a form of balanced salt solution. It is also used for transporting, washing and suspending tissues and cells. This solution was prepared without including calcium chloride (Ca^{2+}) and magnesium chloride (Mg^{2+}). Table 3.1 listed chemical compositions for preparing phosphate buffer saline (PBS-). The stock solution was filtered by filter-sterilised and store in the refrigerator (3 to 5^{o}C) with shelf life of 3 months.

Table 3.1: Composition of phosphate buffer saline (PBS-)

Chemical component	Catalogue number	Final concentration	Quantity
NaCl	S5886	171 mM	5.0 g
KCl	P5405	3.35 mM	0.125 g
Na_2HPO_4	S5136	10.1 mM	0.72 g
KH_2PO_4	S5655	1.84 mM	0.125 g
Milli-Q water	-	-	500 ml

(Stored at 3-5^{o}C for 3 months only)

3.4.2.2 Preparation of penicillin G sodium salt and streptomycin sulphate salt stock solution [100x]

Chemical composition for preparing the penicillin G sodium salt and streptomycin sulphate salt stock solution with 100x concentration (PS) shown on Table 3.2. The stock solution was filtered by filter-sterilised and aliquot in microcentrifuge tube (1.5 ml). The aliquot stock stored in the freezer (-20°C) with shelf life of 6 months.

Table 3.2: Composition of penicillin G sodium salt and streptomycin sulphate salt stock solution [100x]

Chemical component	Catalogue number	Final concentration	Quantity
Phosphate buffer saline (PBS-)	-	1x	10 ml
Penicillin G sodium salt	P3032	168.4 mM	0.6 g
Streptomycin sulphate salt	S1277	137.2 mM	1.0 g

Stored at -20^{o}C for 6 months.

3.4.2.3 Heat-inactivation foetal bovine serum (FBS)

Foetal bovine serum (FBS) was used in this study as a nutrient supplement in the cell culture medium. FBS was purchased from Gibco® which was stored frozen at -20^{o}C freezer and not heat-inactivated. Before heat-inactivation, the frozen foetal bovine serum was thawed in refrigerator (3-5^{o}C) to avoid precipitation. The thawed foetal bovine serum was heated in water bath (56^{o}C) for 45 minutes to diminish the heat-sensitive complement protein which cause cell lysis from an immune reaction between cell and the serum proteins. The temperature of water bath was monitored accurately as higher temperature could cause protein denaturation.

3.4.2.4 Preparation of tissue culture medium for ear fibroblast cell (EFC) and mouse embryonic fibroblast cell (MEF) culture

For primary culturing of the ear fibroblast cell explant, Dulbecco‟s Modified Eagle‟s Medium (DMEM) +10% foetal bovine serum (FBS) + 3 penicillin-streptomycin (PS) were used as the culture medium. This high concentration of penicillin-streptomycin were needed to eliminate the contamination that still could be present on the ear tissue explant. Same medium was used for primary culture of the mouse embryonic fibroblast. For subpassaging, DMEM+10% FBS+1PS medium was used for both ear fibroblast cell and mouse embryonic fibroblast. The lower concentration of the penicillin-

streptomycin was important to prevent cell death due to high concentration of antibiotics. The mixed solution was filter-sterilised and stored in the refrigerator at 3 to 5^{o}C with shelf life of 1 month. Table 3.3 showed the composition of the tissue culture medium used in this study.

Table 3.3: Composition of tissue culture medium

Chemical component	Catalogue number	Final concentration	Quantity
Dulbecco's Modified Eagle Medium (DMEM)	D5796	-	90 ml
Foetal bovine serum	16000-044	-	10 ml
Penicillin-streptomycin (PS stock)	-	-	300 µl [3X] or 100 µl [1X]

Stored at 3-5°C for 1 month.

3.4.2.5 Preparation of Trypsin EDTA (0.25%) solution

Trypsin EDTA is widely used for detachment of adherent cells on the culture dishes. For subpassaging, Trypsin (0.25%) supplemented with ethylenediaminetetraacetic acid (EDTA) mixed in the PBS- as base medium as listed in Table 3.4. The solution was mixed well using a magnetic stirrer and filter-sterilised before stored in the refrigerator at 3-5^{o}C with shelf life of 3 months.

Table 3.4: Composition of Trypsin EDTA (0.25%) solution

Chemical component	Catalogue number	Final concentration	Quantity (100 ml)
Trypsin	T4799	0.25%	0.25 g
EDTA	E9884	1.37 mM	0.04 g
Phosphate buffer saline (PBS-)	-	-	100 ml
PS stock [100x]	-	1x	100 µl

Stored at 3-5^{o}C for 3 months.

3.4.2.6 Preparation of freezing medium

The EFC and MEF were cryopreserved in liquid nitrogen (-196°C) for storage. The freezing medium was prepared by adding foetal bovine serum (FBS, 20%) and dimethyl sulfoxide (DMSO, 20%) as cryoprotectant into DMEM. The composition of freezing medium is listed in Table 3.5. The solution was filter-sterilised before stored in the refrigerator at 3 to 5^{o}C with shelf life of 1 week.

Table 3.5: Composition of freezing medium for tissue culture

Chemical component	Catalogue number	Final concentration	Quantity (100 ml)
Dulbecco's Modified Eagle Medium (DMEM)	D5796	60%	60 ml
Foetal bovine serum (FBS)	16000-044	20%	20 ml
Dimethyl sulfoxide (DMSO)	5879	20%	20 ml

Stored at 3-5^{o}C for 1 week.

3.4.2.7 Preparation of ovary collection medium

Ovary collection medium consisted of normal saline consisted of sodium chloride (NaCl, 0.9%(w/v)) supplemented with penicillin G sodium salt (60 μg/ml) and streptomycin salt (50 μg/ml). Normal saline was important maintain the tonicity of ovary and antibiotics to prevent contamination, thus, the medium was important for collecting the ovaries from the slaughterhouse as well as washing the embryos prior ovary slicing. However, the normal saline should be autoclaved before adding the antibiotics to ensure sterility of the medium. The composition of ovary collection medium are listed in Table 3.6. The medium was kept at room temperature (27^{o}C) with shelf life of 1 month. Prior to ovary collection, the medium was warmed in water bath at 37^{o}C to maintain the temperature.

Table 3.6: Composition of ovary collection medium

Chemical component	Catalogue number	Final concentration	Quantity (1000 ml)
Normal saline	-	1x	1000 ml
Penicillin G sodium salt	P3032	0.17 mM	0.06 g
Streptomycin sulphate salt	S1277	0.07 mM	0.05 g

Stored at room temperature for 1 week.

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

For obtaining matured caprine and bovine oocytes as cytoplast donors, the cumulus oocytes complexes collected ovary slicing must be cultured in *in vitro* maturation medium for maturation. *In vitro* maturation medium must contain optimum concentration of nutrients, serum and hormone to stimulate the optimum maturation of the oocytes.

3.4.2.8 (a) Preparation of stock solution for *in vitro* maturation (IVM) medium

Stock solution of TCM-pyruvate, follicle stimulating hormone (FSH), oestradiol-17β and gentamicin sulphate salt must be prepared before preparing the *in vitro* maturation working medium. Table 3.7 listed the chemical components, final concentration, preparation and storage methods as well as the shelf life of the stocks for the *in vitro* maturation medium. After preparation, each stock was aliquot into microcentrifuge tubes (1.5 ml), sealed with parafilm and wrapped with aluminium foil. Stock solutions should be avoided from being exposed to the light to prevent the oxidation.

Table 3.7: List of stock solution for *in vitro* maturation medium

Stock solution	Final concentration	Preparation method	Storage; shelf life
TCM-pyruvate	2.2 mg/ml	2.2 mg of sodium pyruvate was dissolved in 1 ml of TCM-199 and sterile filtered.	3-5^{o}C; 2-3 days
Gentamicin sulphate salt (G3632)	50 mg/ml	1 g of gentamicin sulphate salt was dissolved in 20 ml of DPBS	3-5^{o}C; 12 months
Follicle Stimulating Hormone, FSH (Folltropin®-V)	5 mg/ml	5 mg of Folltropin®-V powder was dissolved in 1 ml solvent in microcentrifuge tube	-20^{o}C; 12 months
Oestradiol-17β (E8875)	1 mg/ ml	1 mg of oestradiol-17β was dissolved in 1 ml of filtered 95% ethanol in microcentrifuge tube	3-5^{o}C; 6 months

3.4.2.8 (b) Preparation of *in vitro* maturation (IVM) working medium

The *in vitro* maturation working medium was freshly prepared as shown in Table 3.8. First, L-cystein hydrochloride (0.0009 g) was added to TCM-199 medium (8.9 ml). Next, all the remaining stocks except the oestradiol stock were added into the medium. The medium, then, were filter-sterilised into sterile centrifuge tube. Lastly, the oestradiol-17β stock was added to the filter-sterilised medium. The medium to be used was aliquot into centrifuge tube (1.5 ml) to prevent contamination or spoilage of the remaining medium. Seven droplets (100 µl) overlaid with silicone oil were prepared in Petri dish. The Petri dish containing *in vitro* maturation droplets with the remaining aliquoted medium in microcentrifuge tube were incubated in CO_2 incubator (5%) with maximum humidified at 38.5^{o}C at least 4 hours before used. The remaining prepared *in vitro* maturation medium were stored in refrigerator with shelf life of 1 week.

Table 3.8: Composition of *in vitro* maturation working medium

Chemical composition	Final concentration	Quantity (1 ml)
TCM-199 (11150-059)	-	8.9 ml
L-Cystein hydrochloride (C7477)	0.57 mM	0.0009 g
FBS (16000-044)	10 %	1 ml
TCM-pyruvate stock solution	22 µg/ml	100 µl
FSH stock solution	5 µg/ml	10 µl
Gentamicin sulphate salt stock solution	25 µg/ml	5 µl
*Oestradiol-17β stock	1 µl/ml	9.5 µl

* Add oestradiol-17β (1.0 µl/ml) of the final volume after filter.
Stored at 3-5^{o}C for 1 week.

3.4.2.9 Preparation of media for somatic cell nuclear transfer procedure

The following media will be discussed in this theses involved in micromanipulation of oocytes to produce interspecies cloned caprine embryos. The media involved were (a) TL Hepes medium, (b) hyaluronidase solution, (c) cytochalasin B (CB) solution for enucleation and injection medium, and (d) fusion medium.

3.4.2.9(a) Preparation of TL Hepes medium

TL Hepes medium was used in this study for oocyte seaching and collection, oocytes and embryo washing and to incubate micromanipulated oocytes. With the Hepes presence in the medium, it helps to maintain the pH 7.2 to 7.4, thus making it suitable to be as basal medium to other micromanipulation media such as hyaluronidase solution and cytochalasin B (CB) solution.

(i) Preparation of TL Hepes stock solution

TL Hepes stock solution was prepared by dispensing all the chemicals (except sodium lactate solution) listed in Table 3.9 into Milli-Q water (500 ml) in conical flask. The mixture then stirred gently with sterile magnetic stirrer on hot plate cum stirrer. While stirring, the sodium lactate solution was added drop by drop into the mixture. The

resulting TL Hepes the filter sterilised and stored in the refrigerator (4°C) with the shelf life of 3 months.

Table 3.9: Chemical composition of TL Hepes stock solution

Chemical component	Catalogue number	Final concentration	Quantity (500 ml)
NaCl	S5886	114 mM	3.330 g
KCl	P5405	3.2 mM	0.120 g
$NaHCHO_3$	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
Na lactate, 60% syrup	L7900	10 mM	0.93 ml
Milli-Q water	-	-	500 ml

Stored at 3-5^{o}C for 3 months.

(ii) Preparation of TL Hepes working solution

TL-Hepes working solution was prepared by supplementing TL Hepes stock with gentamicin stock solution, sodium pyruvate and bovine serum albumin- fraction V (BSA-FV). Table 3.10 listed the chemical composition of the TL Hepes working solution. The resulting TL Hepes the filter sterilised and stored in the refrigerator (4°C) with the shelf life of 3 to 4 days.

Table 3.10: Chemical composition of TL Hepes working solution

Chemical component	Catalogue number	Final concentration	Quantity (100 ml)
TL-Hepes stock medium	-	-	99.95 ml
Gentamicin stock solution	-	-	25 µl
BSA-FV	A7030	1 g/ml	100 mg
Sodium pyruvate	P4562	0.022 mg/ml	2.2 mg

Stored at 3-5^{o}C for 1 week.

3.4.2.9(b) Preparation of hyaluronidase solution

Hyaluronidase solution was used for removing the cumulus cell from cumulus oocytes complexes after maturation. Hyaluronidase from bovine testes was used to prepare hyaluronidase solution. Hyaluronidase solution was prepared by dissolving hyaluronidase powder in TL-Hepes working solution (Table 3.11). The prepared solution was filter-sterilised, aliquot for 100 µl each in microcentrifuge tubes, sealed with parafilm, wrapped in aluminium foil and stored in the freezer (-20°C) with a shelf life of 6 months.

Table 3.11: Chemical composition of hyaluronidase solution

Chemical component	Catalogue number	Final concentration	Quantity (10 ml)
Hyaluronidase	H4272	0.01 g/ml	0.1 g
TL Hepes working solution	-	-	10 ml

Stored at -20^{o}C for 6 months.

3.4.2.9(c) Preparation of cytochalasin B (CB) solution

The function of cytochalasin B solution was to depolymerise microfilaments of matured oocytes and subsequently enhanced the enucleation process (Soh, 2012). It is a cell-permeable mycotoxin as it prevent the contractile microfilament contraction and

induces the nuclear extrusion. In somatic cell nuclear transfer procedure, it functioned as a relaxant agent so that rigidity of oocyte zona pellucida decreased and easier to pierce and squeeze the nucleus during enucleation.

(i) Preparation of cytochalasin B stock solution

Cytochalasin B stock solution was prepared by dissolving cytochalasin B powder in DMSO (Table 3.12). The prepared solution was filter sterilised, aliquot for 10 µl each in 100µl microcentrifuge tubes, sealed with parafilm, wrapped in aluminium foil and stored in the freezer (-20^{o}C) with a shelf life of 6 months.

Table 3.12: Composition of cytochalasin B stock solution

Chemical component	Catalogue number	Final concentration	Quantity (1 ml)
Cytochalasin B	C6762	1 mg/ml	0.001 g
Dimethyl sulfoxde (DMSO)	D5879	-	1 ml

Stored at -20^{o}C for 6 months.

(ii) Preparation of cytochalasin B working solution

On the day of experiment, cytochalasin B stock solution (10 µl) was diluted with TL-Hepes working solution (990 µl) to make a final volume of 1 ml (Table 3.13). The prepared solution can be stored in the refrigerator (4^{o}C) with a shelf life of 6 months.

Table 3.13: Composition of cytochalasin B working solution

Chemical component	Catalogue number	Final concentration	Quantity (1 ml)
Cytochalasin B stock	-	1 mg/ml	10 µl
TL Hepes working solution	-	-	990 µl

Stored at -20^{o}C for 6 months.

3.4.2.9(d) Preparation of fusion medium

The function of fusion medium is to promote the disintegration of the donor karyoplast plasma membrane and facilitate the fusion between the donor karyoplast with cytoplasm membrane of the recipient cytoplast.

(i) Preparation of fusion stock solution

Fusion stock solution was prepared by dissolving all chemicals listed in Table 3.14 in Milli-Q water (20 ml). The prepared solution was filter sterilised, sealed with parafilm, and stored in the refrigerator (4^{o}C) with a shelf life of 3 months.

Table 3.14: Composition of fusion stock solution

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

Stored at 3-5^{o}C for 6 months.

(ii) Preparation of fusion working solution

Fusion working solution was prepared by adding BSA-FV to fusion stock solution (Table 3.15). The prepared solution was filter-sterilised, sealed with parafin film and stored in the refrigerator (4^{o}C) with a shelf life of 3 months.

Table 3.15: Composition of fusion working solution

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

Stored at 3-5^{o}C for 3 months.

3.4.2.10 Preparation of activation medium

Activation procedure was not only use in the somatic cell nuclear transfer protocols, it was also used to produce parthenotes embryos. In the present study, two chemicals were used for activation, calcium ionophore and 6-dimethylaminopurine.

3.4.2.10(a) Preparation of calcium ionophore (CaI) solution

Calcium ionophore is vital in activation protocol as it increase the concentration of free calcium in the cytosol, which mimicking the physiological cell-signaling mechanism and form of calcium oscillations (Ozil *et al*., 2006).

(i) Preparation of calcium ionophore stock solution

Table 3.16 listed the chemical composition of calcium ionophore stock solution. The prepared solution was filter sterilised, aliquot for 10 µl each in microcentrifuge tubes (100µl), sealed with parafilm, wrapped in aluminium foil and stored in the freezer (-20^{o}C) with a shelf life of 6 months.

Table 3.16: Composition of calcium ionophore stock solution

Chemical component	Catalogue number	Final concentration	Quantity (5 ml)
Calcium ionophore (CaI)	C7522	500 µM	0.001 g
Dimethyl sulfoxide (DMSO	D5879	-	3.82 ml

Stored at -20^{o}C for 6 months.

(ii) Preparation of calcium ionophore working solution

On the day experiment, the calcium ionophore working solution was freshly prepared by diluting the calcium ionophore stock solution with KSOM A working solution with final concentration 5 µM (Table 3.17). Seven 100 µl droplets overlaid with silicone oil were prepared in Petri dish. The Petri dish containing calcium ionophore droplets with the remaining medium in microcentrifuge tube were incubated CO_2 incubator (5%) with maximum humidified air at 38.5^{0}C at least 4 hours before used. The remaining solution can be stored in the freezer (-20^{o}C) with a shelf life of 6 months.

Table 3.17: Composition of calcium ionophore working solution

Chemical component	Final concentration	Quantity (1 ml)
Calcium ionophore stock	500 µM	10 µl
TL Hepes working solution	-	990 µl

Stored at -20^{o}C for 6 months.

3.4.2.10(b) Preparation of 6-dimethylaminopurine (6-DMAP) solution

After activation with calcium ionophore, the reconstructed oocyte was subsequently activated with 6-dimethylaminopurine for 4 hours, which prolonged the effect of calcium ionophore as activating agent.

(i) Preparation of 6-dimethylaminopurine stock solution

Table 3.18 listed the chemical composition of 6-dimethylaminopurine stock solution. The prepared solution was filter sterilised, aliquot for 10 µl each in microcentrifuge tubes (100µl), sealed with parafilm, wrapped in aluminium foil and stored in the freezer (-20^{o}C) with a shelf life of 6 months.

Table 3.18: Composition of 6-dimethylaminopurine stock solution

Chemical component	Catalogue number	Final concentration	Quantity
6-dimethylaminopurine (6-DMAP)	D2629	0.2 M	0.1 g
Milli-Q water	-	-	3.08 ml

Stored at -20^{o}C for 6 months.

(ii) Preparation of 6-dimethylaminopurine working solution

On the day experiment, the 6-dimethylaminopurine working solution was freshly prepared by diluting the 6-dimethylaminopurine stock solution with KSOM A working solution with final concentration 5 µM (Table 3.19). Seven 100 µl droplets overlaid with silcone oil were prepared in Petri dish. The Petri dish containing 6-dimethylaminopurine droplets with the remaining medium in microcentrifuge tube were incubated in CO_2 incubator (5%) with maximum humidified air at 38.5°C at least 4 hours before used.

Table 3.19: Composition of 6-dimethylaminopurine working solution

Chemical component	Final concentration	Quantity (1 ml)
6-dimethylaminopurine (6-DMAP) stock	0.2 M	10 µl
TL Hepes working solution	-	990 µl

Stored at -20°C for 6 months.

3.4.2.11 Preparation of *in vitro* culture (IVC) medium

In the present study, two culture systems of KSOM medium were used as introduced by Kwong *et al.*(2012) which not only increased the *in vitro* developmental rate but increasing the efficiency of somatic cell nuclear transfer in caprine. First culture system is to culture the Day 0 - Day 1 cloned embryos in KSOM A which formulated by Lawitts and Biggers (1993). However, on Day 2 of *in vitro* culture, the KSOM A changed into KSOM B, which is KSOM A supplemented with glucose (2.78 mM) for later embryonic developmental stage.

3.4.2.11(a) Preparation of KSOM stock solution

All the chemicals, except for sodium lactate syrup, were dissolved in Milli-Q water (200 ml). The chemicals were slowly stirred by a sterilised magnetic stirrer. The sodium lactate syrup was added drop by drop to the stirring solution. The prepared KSOM stock solution was filter-sterilised and stored in the refrigerator (4°C) with a shelf life of 1 month. The chemical compositions for preparing the KSOM stock solution were listed in Table 3.20.

Table 3.20: Chemical composition of KSOM stock solution

Chemical component	Catalogue number	Final concentration	Quantity (200 ml)
NaCl	S5886	95.0 mM	1.1106 g
KCl	P5405	2.50 mM	0.0372 g
KH_2PO_4	P5655	0.35 mM	0.0096 g
$MgSO_4$	M7506	0.20 mM	0.0048 g
Na lactate, 60% syrup	L7900	10.0 mM	0.372 ml
Sodium pyruvate	P4562	0.20 mM	0.0044 g
D-Glucose	G6152	0.20 mM	0.0072 g
$NaHCO_3$	S5761	25.0 mM	0.4202 g
$CaCl_2$	C5670	1.71 mM	0.0384 g
L-Glutamine	G3126	1.0 mM	0.0292 g
EDTA	E9884	0.01 mM	0.0008 g
Milli-Q water	-	-	199.63 ml

Stored at 3-5^{o}C for 1 month.

3.4.2.11(b) Preparation of KSOM working solution

KSOM A not only used as *in vitro* culture medium on the early stages of embryo development, but as base medium for diluting calcium ionophore and 6-dimethylaminopurine. KSOM A was prepared as listed in Table 3.21 and filter-sterilised. Seven 100 µl droplets overlaid with silicone oil were prepared in Petri dish. The Petri dish containing KSOM A droplets with the remaining medium in microcentrifuge tube were incubated in CO_2 incubator (5%, 38.5^{0}C) at least 4 hours before used.

Table 3.21: Chemical composition of KSOM A working solution

Chemical component	Catalogue number	Final concentration	Quantity (10 ml)
KSOM stock solution	-	1x	9.85 ml
BSA-V	A6003	0.4%	0.04 g
MEM non-essential amino acids solution	M7145	-	50 µl
BME amino acid solution	B6766	-	100 µl

Stored at 3-5^{o}C for 1 week.

KSOM B was prepared as listed in Table 3.22 and filter-sterilised and aliquot into microcentrifuge tube (1.5 ml). The medium in microcentrifuge tube were incubated in CO_2 incubator (5%) with maximum humidified at 38.5°C at least 4 hours before used. The remaining solution solution can be stored in the refrigerator (3-5^{o}C) with a shelf life of 1 week.

Table 3.22: Chemical composition of KSOM B working solution

Chemical component	Catalogue number	Final concentration	Quantity (10 ml)
KSOM A stock solution	-	1x	10 ml
BSA-V	C6152	2.78mM	0.0046 g

Stored at 3-5^{o}C for 1 week.

3.4.2.12 Preparation of trichostatin A (TSA) solution

To improve the *in vitro* culture system of the interspecies cloned caprine embryos, histone deacetylase inhibitors, namely trichostatin A (TSA) were added into the *in vitro* culture medium. Trichostatin A (TSA) was obtained from Sigma-Aldrich Co.

3.4.2.12(a) Preparation of trichostatin A stock solution

Trichostatin A was dissolved in 99.5% ethanol as showed in Table 3.23. Stock solutions of were prepared at 2 mg/ml. The prepared stock was aliquoted in the microcentrifuge tubes (100 µl), sealed, wrapped with aluminium foil and stored at -20°C for 6 months.

Table 3.23: Chemical composition of trichostatin A stock solution

Chemical component	Catalogue number	Final concentration	Quantity (1 ml)
Trichostatin A	58880-19-6	-	2 mg
99.5% ethanol		-	1 ml

Stored at -20^{o}C for 6 months

3.4.2.12(b)Preparation of trichostatin A working solution

In this study, two stages of cloned caprine embryos which were a) activated couplets and b) 8-cell stage embryos were treated with trichostatin A (25nM) for 12 hours. Therefore, the prepared trichostatin A stock solution (2 mg/ml) needed to be diluted to fonal concentration of 25 nM in the *in vitro* culture medium i.e KSOM A medium or KSOM B (according to the stages of cloned embryos treated) as showed in Table 3.24. The trichostatin A working solution was prepared and filter-sterilised into microcentrifuge tube. Seven 100 µl droplets overlaid with silicone oil were prepared in Petri dish. The Petri dish containing trichostatin A working solution droplets with the remaining medium in microcentrifuge tube were incubated in CO_2 incubator (5%) with maximum humidified air at 38.5^{0}C at least 4 hours before used. The remaining solution solution can be stored in the refrigerator (3-5°C) with a shelf life of 1 week.

Table 3.24: Chemical composition of trichostatin A working solution

Chemical component	Catalogue number	Final concentration	Quantity (4 ml)
KSOM A / KSOM B working solution	-	-	4 ml
Trichostatin A stock solution	-	2 mg/ml	15µl

Stored at 3-5°C for 1 week.

3.4.3 Preparation of microtools

Before the oocytes and embryos manipulation, it is important to prepare the microtools which are a) the mouth pipette set, b) the mouth pipette needle, and c) the microneedles. All the microtools in this study were produced in-house in the laboratory with the aid of the instruments. The most important aspect in microtools preparation is the size of the internal and external diameter, as it needs to suit the used and the size of the oocytes and embryos, according to the experiments.

3.4.3.1 Capillary cleaning and sterilisation

It is important to use sterilised equipment, especially the microtools utilised in manipulation of the oocytes and embryos. This section will discuss the steps in cleaning and sterilisation of the capillaries, which are Pastuer pipette and Borosillicate glass. The capillary tubes were soaked overnight in 7x (FlowLabTM, Australia). Then, they were washed thoroughly and rinsed vigorously 5 times first with tap water followed by RO water. The washed capillary tubes (Borosillicate glass) for making holding pipette, enucleation needle, and injection needle were washed 20 times in autoclaved Milli-Q water. Lastly, the capillary for mouth pipette needle were sterilised by autoclaving at 121°C for 20 minutes and were dried in the oven at 60°C. The capillaries for holding pipettes and other injection needles were kept properly in a sterile container followed by drying in the oven. Before used, capillary was exposed with UV light for 20-30 minutes for sterilisation purposes.

3.4.3.2 Preparation of mouth pipette set

Mouth pipette set is important in manipulation procedure of oocytes and embryos. The mouth pipette was hand-made using 2 pipette tips (1000 µl) as an aspirator mouthpiece and as Pasteur pipette holder, silicone tube (5 mm diameter), a syringe filter (0.22 µm

pore size) and a narrow opening pulled Pasteur pipette.

During handling any oocytes and embryos, a small volume of clean medium was aspirated into the capillary of Pasteur pipette (ID: 200-300 μm) before picking up oocytes and embryos, followed by very low air pressure was given into the mouthpiece when releasing the oocytes and embryos. This precaution was taken to avoid bubbles in the medium which may cause oocyte and embryo losses.

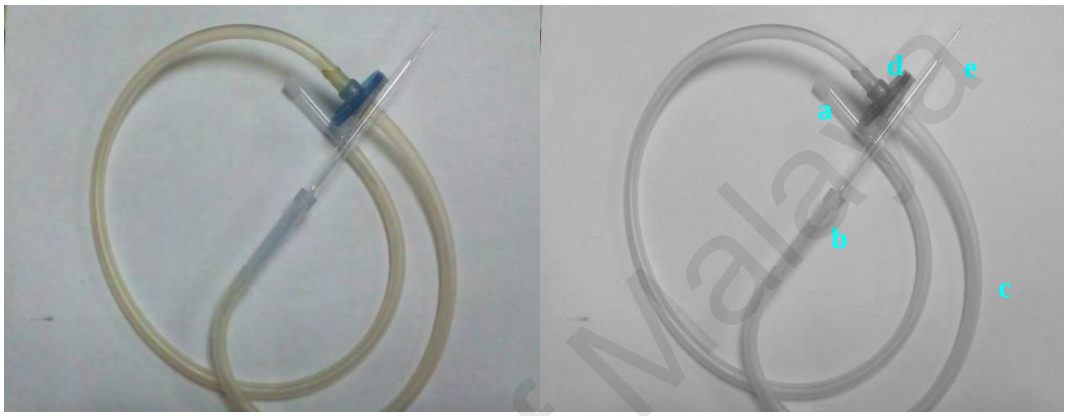

Figure 3.1: Original photograph (left) and labelled photograph (right) of mouth pipette assembly which consist of (a) aspirator mouthpiece, (b) Pasteur pipette holder, (c) silicone tube, (d) syringe filter (0.22 μm pore size) and (e) narrow opening pulled Pasteur pipette.

3.4.3.3 Preparation of mouth pipette needle

The mouth pipette needle is important part of the mouthpiece-pipette set and usually prepared exactly before the experiment was conducted. It was prepared by hand-pulling a sterilised glass Pasteur pipette in a flame of Bunsen burner into the appropriate internal diameter (200-300 μm) so that it could fit the oocytes and embryos for experimental manipulation. The internal diameter of the capillary tube was very important to transfer the oocytes and embryos safely without injury. The thinned part of the Pasteur pipette was scribed with a diamond stone and snapped gently. The resulting mouthpiece-controlled pipette was examined under the microscope for ensuring the

desire diameter and blunt tip were achieved. The tip of the mouthpiece-controlled pipette was fire-polished by a quick touch of the tip to the flame.

Figure 3.2: Resulting pulled needle.

3.4.3.4 Preparation of microneedles for somatic cell nuclear transfer procedures

Microneedles, which are holding pipette, enucleation needle and injection pipette, were prepared in-house for somatic cell nuclear transfer manipulation. The microtools were prepared using thin-walled borosilicate glass capillaries (inner diameter: 0.69 µm; outer diameter: 0.97 µm; length 10 cm) with the aid of three instruments; horizontal micropipette puller (P-97, Sutter Instrument, USA), microforge (Technical Products Internationals, USA) and microgrinder (EG-4, Narashige Co. Ltd., Japan).

Figure 3.3: Micropuller.

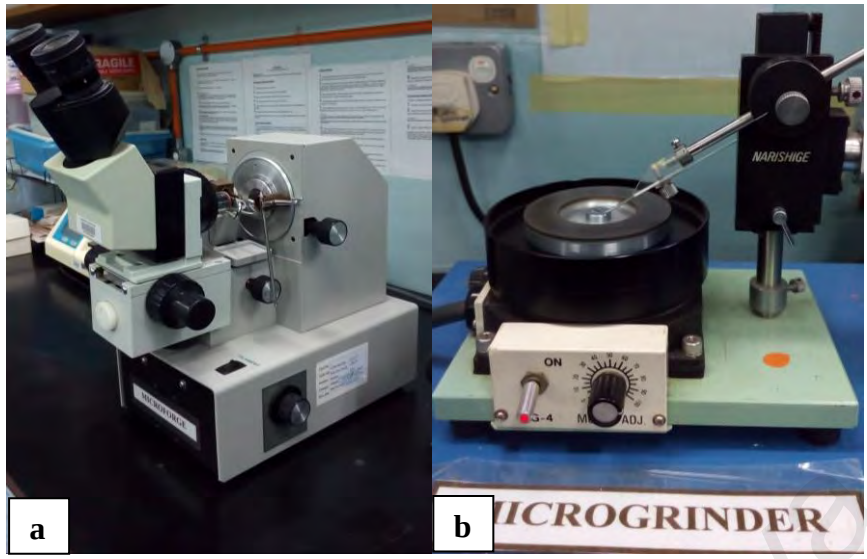

Figure 3.4: (a) Microforge and (b) microgrinder.

Before preparing the microneedles according to their functions, the capillary tubes were need to be pulled for producing two uniform sizes needle. This result could be done by aid of horizontal micropipette puller. Capillary tube were placed and fixed into the micropuller. According to the need, the parameters were set to a programme. For this study, the parameters were set at programme 0 (heat: 780, pull=80, velocity=130 and time=20). The pull button (green) was switched on. The filament is gradually heated up, became red hot and the needle was pulled to two opposite sides. As a result, two uniform sized pulled needles were produced.

3.4.3.4 (a) Preparation of holding pipette

The pulled micropipette was placed into the holder of the microforge horizontal to the glass bead. The pipette was squared and broke at a diameter between 120 to 130 µm. After squaring, the pipette placed vertically; the opening of the pipette should be on the top of the glass bead. The filament was fired polished in such a way that the inner diameter of the opening reduced to size of 25 - 30 µm. The pipette was placed again horizontal to the glass bead. The pipette was bent to an angle of 30°C. After preparing

holding pipettes, holding pipettes were stored in sterilised recycled needle and pipette holders. Before the SCNT procedures, the holding pipette was exposed with UV light for 20-30 minutes for sterilisation purposes.

3.4.3.4 (b) Preparation enucleation needle

The pulled microneedle was placed into the holder of the microforge horizontally to glass bead . The needle was squared and broke and placed vertically; the opening of the needle should be on the top of the glass bead. The filament was switched on and brought towards the needle. The filament was pulled it up and down to make a sharp end without a hole. The cutting needle was placed again horizontal to the glass bead. The enucleation needle was bent to an angle of 30°C. The dust accumulated in the needle was flushed away with 70% alcohol for 5 seconds and rinsed thoroughly with Milli-Q water for another 5 seconds. After preparation, enucleation needle were stored in sterilised recycled needle and pipette holders. Before the somatic cell nuclear transfer procedures, the enucleation needle was exposed with UV light for 20-30 minutes for sterilisation purposes.

3.4.3.4 (c) Preparation of injection pipette

The injection needle was prepared by cutting the tip of a pulled capillary on a heated glass bead of the microforge at an inner and outer diameters were approximately 16 to 18 μm and 18 to 20 μm, respectively. Next, the needle tip was ground to produce a bevelled edge with a microgrinder at 45° desired angle for 3 minutes. The injection pipette was then bent at 30° for easier manipulation when doing cloning on the microscope stage. The dust accumulated in the needle was flushed away with 70% alcohol for 5 seconds and rinsed thoroughly with Milli-Q water for another 5 seconds. After preparation, injection pipette was stored in sterilised recycled needle and pipette

holders. Before the somatic cell nuclear transfer procedures, the injection pipette was exposed with UV light for 20-30 minutes for sterilisation purposes.

Figure 3.5: Steps in preparing the microtools for somatic cell nuclear transfer (a) pulling needle by micropuller, (b) squaring and bending the needle tip with microforge and (c) grinding the needle tip with microgrinder.

3.4.4 Experimental procedures

3.4.4.1 Preparation of caprine ear fibroblast cell (EFC) as donor karyoplast

Ear fibroblast cell (EFC) was chosen as donor karyoplast. In the present study, breed and gender were been focus to study the efficiency of interspecies somatic cell nuclear transfer in caprine. Therefore, preparation of ear fibroblast cell is important procedure which involved a) collection of caprine ear tissue, b) primary culture of ear fibroblast cell, c) harvesting and subpassaging of ear fibroblast cell, d) freezing of ear fibroblast cell and e) thawing of ear fibroblast cell.

3.4.4.1 (a) Collection of caprine ear tissue

For collecting the ear tissue, the goat were pinned by 2 people to side of the pen to avoid the goat to struggle. Anaesthetic (ilium and xylezene) with appropriate amount written in the instruction given by the manufacturer. After that, the ear of the goat were held and the area for biopsied were shaved and sprayed with 70% ethanol for sterilisation. The area with less vein were chose for biopsied. By using sterilised surgical scissors, 1-1.5 cm^2 was cut and immediately rinsed with saline before placing it in the test tube containing phosphate buffered saline solution. The test tube were placed in polystyrene box with ice pack and were carried to the laboratory for culturing.

The area of the biopsied were sprayed with iodine to stop the blood flowing from the area and disinfection. After the blood stop, the area were sprayed with Woundsarex for disinfection. The biopsied ear tissues were washed with phosphate buffered saline thrice before placed into test tube. The test tubes were placed in polystyrene box with ice pack and were carried to the laboratory for culturing.

Figure 3.6: a) Katjang male, b) Katjang female, c) Boer female and d) Boer male as donor of ear fibroblast cells for preparation of donor karyoplasts.

3.4.4.1 (b) Primary culture of ear fibroblast cell

In the laboratory, the primary culture of ear fibroblast cell procedure under laminar flow with utilisation of sterile apparatus. For this procedure, the biopsied ear tissues were rinsed again with phosphate buffer saline before removing any remaining hair and blood clot on the ear tissue by using a sterile razor blade. The ear tissue was sprayed with ethanol (70%) and washed in DMEM + 10% FBS + 3PS thrice before removing the cartilage from the dermal layer. The dissection was conducted using a surgical blade clamped with a pair of haemostat. This step required an assistant to hold the 2 layers of dermal with a pair of sterile fine forceps. The dermal layer then were minced with a pair of sterile surgical scissors. The minced dermal tissues were rinsed once with the medium and subsequently arranged at the centre of the tissue culture dish (60 mm, Nunc) with the white surface facing the bottom of the tissue culture dish. A sterile, clean glass cover slip was placed onto the minced dermal tissues. The tissue culture medium (DMEM + 10% FBS + 3PS) was then slowly dispensed into the tissue culture dish until the glass cover slip was fully immersed. This explant culture was incubated under maximum humidified atmosphere of CO_2 (5%, 38.5°C) for 8 to 10 days. The culture medium was replenished with medium containing lower concentration of penicillin-streptomycin (DMEM + 10% FBS + 1PS) at Day 3 of culture.

3.4.4.1 (c) Harvesting and subpassaging of ear fibroblast cell

After the cell cultures reach 80% confluency, the cells were harvested for subculturing and cryopreservation. The cells were washed with phosphate buffer saline solution twice for complete media removal. Subsequently, 2-3 ml trypsin EDTA (0.25%) was dispensed into the culture dish and placed in the CO_2 incubator for 2-3 minutes to allow the enzymatic reaction. The dishes then were returned into the laminar flow and by

using the Pastuer pipette, the medium were suck in and out while rotating the culture dish for physicall detach the cell layer. The peeling off the cell layer from the bottom of the culture dish could be observed in this steps. The cell suspension was transferred into 15 ml centrifuge tubes. The DMEM + 10% FBS + 1PS were added into the mixture with ratio of 1:1.5 medium. The tubes were centrifuged at 1500 rpm for 5 minutes. After the centrifugation steps, cell pellet would be formed at the bottom side of the centrifuge tube. Subsequently, the supernatant was removed and the DMEM + 10% FBS + 1PS were added to make up 3 ml. The mixture then were pipette gently to dissociate the clump of the cell pellet which producing the suspension the EFC for subpassaging.

It is important to seed the cell with an optimum and fix seeding density to ensure the optimum production of EFC. Before subpassaging, the cell counting and viability testing was performed with 10 µl of EFC suspension mixed with 10 µl of 0.4% Tryphan blue. Counting of the viable and non-viable cells was done by using haemacytometer. The number of viable cells was determined by using the following formula:

$$c = \frac{d\,(n \times 10^4)}{5}$$

Where,
c is the number of cells
d is dilution factor
n is number of unstained cells

The number of viable cells determined the seeding density and the number of tissue culture dishes used for subpassaging. For this research, the seeding density of 5.0×10^5 were used. Ear fibroblast cell were seeded by dispersing droplets of ear fibroblast cell using micropipettor (10-100 µl). The cells were grown until confluent and the media were refreshed every two days by replacing half of the media. This procedure were repeated for Passages 2 and 3 of the ear fibroblast cell.

3.4.4.1 (d) Freezing of ear fibroblast cell

Passages 1 to 3 of the ear fibroblast cell cultures were cryopreserved by using quick freezing technique as adapted from Abas Mazni *et al.* (1989). The cells were harvested and cell counting as well as viability test were conducted as discussed in section 3.4.4.1 (c). For each cryovial, ear fibroblast cell mixture (0.5 ml) were placed into the cryovials. The freezing medium (60% DMEM medium + 20% FBS + 20% DMSO) were added into the cell mixture to give final volume of 2.0 ml and subsequently were transferred into cryovials and inserted into the cryocanes. Quick freezing technique involved of two steps of freezing, first step was freezing at -80°C by using liquid nitrogen gaseous for 15 minutes for equilibration. The second step was directly plunged into the liquid nitrogen (-196°C) for 15 minutes. This cryopreservation procedure were conducted by using the insulated ice box. The frozen ear fibroblast cell were stored in the liquid nitrogen tank minimally for 2 days.

3.4.4.1 (e) Thawing of ear fibroblast cell for preparation donor karyoplast for somatic cell nuclear transfer procedure

Three days prior of somatic cell nuclear transfer procedure, thawing of the frozen ear fibroblast cell need to be carried out to ensure that the ear fibroblast cell culture reached 80% confluency and for synchronisation of the cell cycle of the ear fibroblast cell as donor karyoplast.

Cryovials were removed from cryocanes in liquid nitrogen tank without exposing the cryocanes for too long outside liquid nitrogen tanks. The cryovials were then immediately thawed by placing the lower portion of the vials into water bath. Avoid contact between the cap and the water to prevent contamination. After the mixture has thawed for about 2 minutes, the vials were removed from the water bath and sprayed with ethanol (70%). The cap was flamed and removed in the laminar flow.

The contents were transferred into 15 ml centrifuge tubes containing DMEM + 10% FBS + 1PS medium with ratio of 1:1.5. The tubes were then centrifuged at 1500 rpm for 5 minutes. After completed centrifuged, a cell pellet was formed at the bottom of the centrifuge tube. Subsequently, the supernatant was removed and the DMEM + 10% FBS + 1PS were added to make up 3 ml. The mixture then were pipette gently to dissociate the clump of the cell pellet which producing the suspension the ear fibroblast cell for seeding the ear fibroblast cell. The cell counting and viability testing as well as seeding of the ear fibroblast cell were performed with similar procedure discussed in section 3.4.4.1 (c). The ear fibroblast cell were allowed to confluence to 100% confluency for cell cycle synchronisation. Prior to injection of ear fibroblast cell into recipient cytoplast, the synchronised cells were harvested and suspended into TL Hepes medium.

3.4.4.2 Preparation of bovine matured oocytes as recipient cytoplast

In this study, bovine oocytes were used in the interspecies somatic cell nuclear transfer protocols as donor cytoplasts. The procedure of recipient cytoplast preparation as followed:

3.4.4.2 (a) Collection of bovine oocytes

The ovaries were collected within 3 hours after the females were slaughtered. The collected ovaries were washed and kept in a flask containing ovary collection medium. The temperature of the medium were maintained around 30 to 35^{o}C. In the laboratory, the fatty and excessive tissue layer surrounding the ovaries were trimmed and the ovaries were washed in medium thrice to remove the blood and other contaminants. For oocyte retrieval, the ovary slicing technique were performed as this technique more efficient to retrieved the more oocytes from the ovaries compared to follicle aspiration.

The ovary was held with a sterile curved haemostat on a Petri dish containing TL Hepes medium (37^{o}C) and sliced using a quarter section of sterile stainless razor blade clamped to a heamostat. The ovaries were sliced in checkerboard incisions to the entire surface of the ovaries. If the ovaries have a numerous of big size of follicles, it is advised to puncture the follicles with 18 G needle prior slicing. The sliced ovaries were then rinsed in a beaker (50 ml) containing TL Hepes medium. In embryo room, the beaker was left for 5 minutes on a hot plate with the temperature maintained at 37°C in order to allow the tiny pieces of tissues and cumulus oocytes complexes in the beaker to settle down. While sedimentation, the cumulus oocytes complexes searching dish was prepared by drawing checkerboard lines on the bottom of Petri dish (60 mm diameter). This dish is important as guidance for researcher during cumulus oocytes complexes searching.

For oocyte searching, TL Hepes containing cumulus oocytes complexes and other tissues (2-3 ml) were transferred into the searching dish and cumulus oocytes complexes were searched grid by grid under stereomicroscope. The cumulus oocytes complexes were collected using mouthpiece-controlled pipette and were transferred into a Petri dish (35 mm) containing TL-Hepes microdroplets (200 µl).

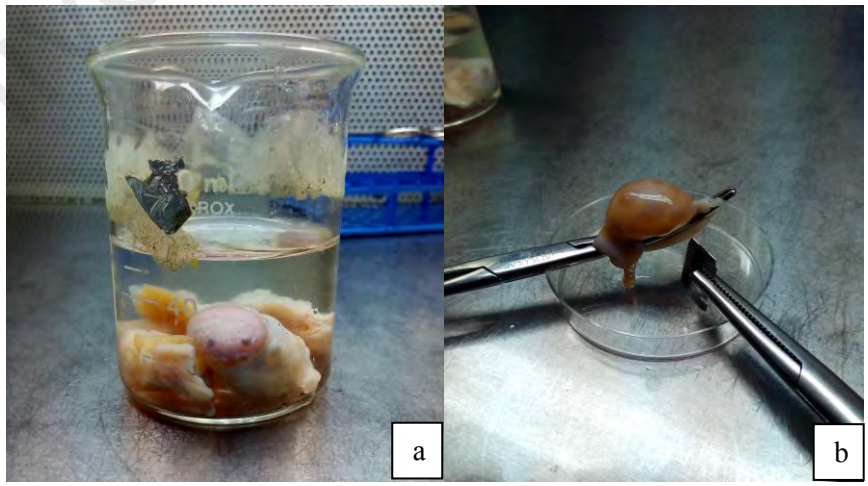

Figure 3.7: (a) Bovine ovaries in ovary collection medium and (b) bovine ovaries with obvious follicles on the surface.

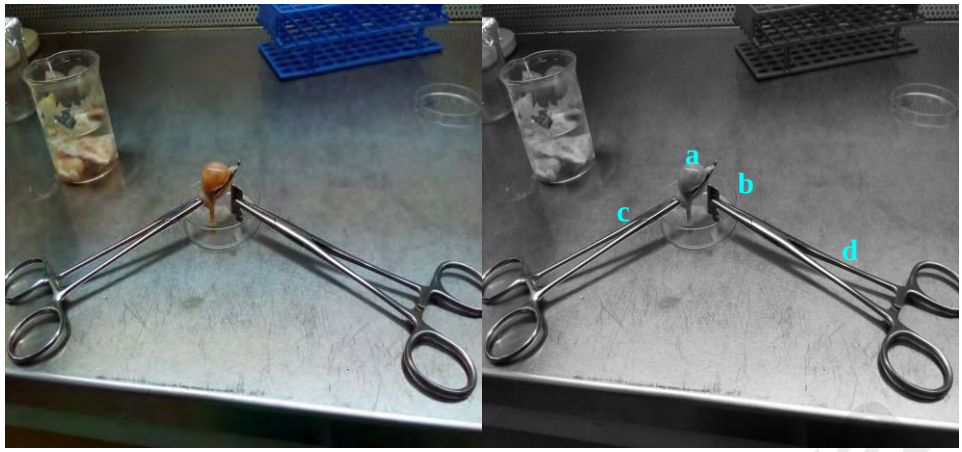

Figure 3.8: Original photograph (left) and labelled photograph (right) of preparation for bovine ovary slicing which consist of (a) bovine ovary, (b) sterile blade, (c) curved haemostat and (d) haemostat.

3.4.4.2 (b) Grading of bovine oocytes

Bovine cumulus oocytes complexes collected from abattoir-derived ovaries were washed twice in 200 µl TL-Hepes microdroplets before visually assessed under the stereomicroscope to classify in Grades A to E. The grading system is according to cumulus cell layers surrounding the oocytes, ooplasm structure and morphology of the oocytes, which based based on the criteria described by Rahman *et al*. (2008) as presented in Table 3.25. Generally, only Grades A to C cumulus oocytes complexes were subjected for *in vitro* maturation for cytoplast preparation in the interspecies somatic cell nuclear transfer procedure.

Table 3.25: Classification of the cumulus oocytes complexes according to grade

Grade	No. of cumulus layer	Ooplasm structure	Morphological features
A	> 5 (completely surrounding the oocytes)	Finely granulated homogeneous	Normal
B	3-5 (completely surrounding the oocytes)	Finely granulated homogeneous	Normal
C	1-2 layer of cumulus cells completely surrounding the oocytes or 3-5 layers partially surrounding the occytes	Finely granulated homogeneous	Normal
D	Incomplete cumulus layer or naked oocytes	Finely granulated homogeneous	Normal
E	Jelly like cumulus cell	Heterogenous	Abnormal size and shape, apoptotic

3.4.4.2 (c) *In vitro* maturation (IVM) of bovine oocytes

Seven 100 µl microdroplets of *in vitro* maturation medium were prepared and overlaid with silicone oil in a small Petri dish. The Petri dish with 1 ml of extra *in vitro* maturation medium was kept in the incubator 3 hours prior *in vitro* maturation of the cumulus oocytes complexes for equilibration.

After grading of the cumulus oocytes complexes, cumulus oocytes complexes were washed with the equilibrated extra *in vitro* maturation medium thrice before culturing in the *in vitro* maturation microdroplets. The cumulus oocytes complexes were cultured according to grade in different microdroplets of *in vitro* maturation medium with 13-15 cumulus oocytes complexes per microdroplet. The cumulus oocytes complexes were cultured under a maximum humidified atmosphere of CO_2 (5%) in air, 38.5°C. The bovine cumulus oocytes complexes collected in this study were cultured in *in vitro* maturation medium for two ranges of *in vitro* maturation duration; a) 22-24 hour and b) 25-27 hours of (A comparative study in Experiment 2).

After *in vitro* maturation, the oocytes were transferred into a droplet of hyaluronidase (100 µl) for denuding process to remove the cumulus cell surrounded the oocytes. Denuding was performed less than 5 minutes by repeated vigorous pipetting (40-50 times) using micropipette. The naked oocytes were rotated by using a mouthpiece-controlled pipette and assessed under stereomicroscope by presence protrusion of first polar body (PB1). Only matured and meiotic competent oocytes, with clear protrusion of first polar body were selected and transferred into TL Hepes microdoplets before used for oocyte manipulation in subsequent interspecies somatic cell nuclear transfer experiment.

3.4.4.3 Interspecies somatic cell nuclear transfer in caprine

In this study, interspecies somatic cell nuclear transfer used Roslin Technique. This technique involved three important steps; enucleation of oocyte using squeezing technique, injection of donor karyoplast using subzonal injection (SUZI) technique and electrofusion.

Interspecies somatic cell nuclear transfer technique requires a skillful researcher as this technique involving the using of micromanipulation system that incorporated an inverted microscope fitted with hydraulic micromanipulators and connected to a computer system (Figure 3.9). Apart from that, one should skillful enough for handling embryos in the environment outside of incubator as it is important to eliminate any chance of contamination and cell death. Thus, understanding the protocols and a lot of practice to improve the researcher skill in interspecies somatic cell nuclear transfer is very vital before conduction the experiment.

Figure 3.9: Micromanipulator system connected with computer system.

3.4.4.3 (a) Preparation of micromanipulation dishes

There are three micromanipulation dishes were prepared to be used micromanipulation of embryos; enucleation dish, injection dish and electrofusion dish. The preparation of dishes were done on the day of the experiment. Micromanipulation dish was equilibrated on the stage warmer (38.5^{o}C).

For enucleation dishes, one droplet of CB solution (15 µl) was dispensed on the the top centre of the dish. This first droplet was for alignment of holding pipette and enucleation needle purpose. Next, three droplets of CB solution (15 µl) were dispensed at the centre of the dish. These droplets were subjected for enucleation of the matured oocytes.

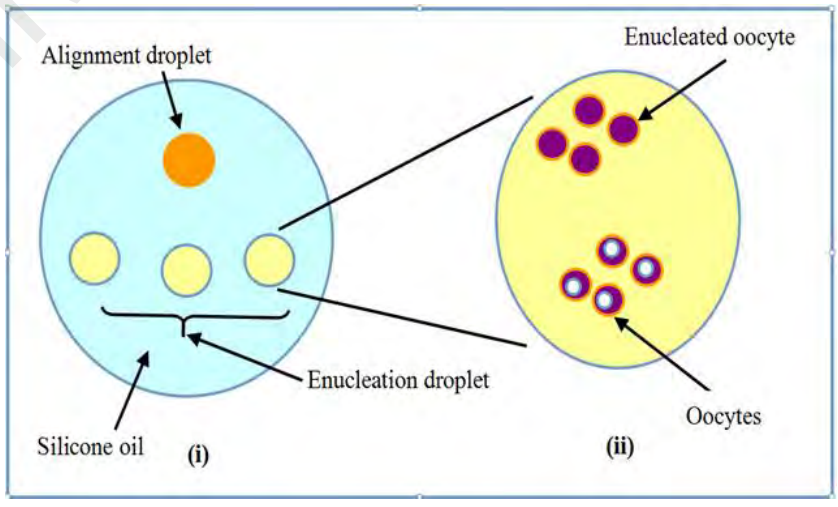

Figure 3.10: Enucleation dish. (i) Arrangement of alignment and enucleation droplets; (ii) Placement of samples in the microdroplet for donor karyoplast injection.

For injection dishes, similarly, one droplet of CB solution (15 µl) was dispensed on the the top centre of the dish. This first droplet was for alignment of holding pipette and enucleation needle purpose. Next, 3 droplets of CB solution (15 µl) were dispensed at the centre of the dish. These droplets were subjected for injection of donor karyoplast. 3 droplets of TL Hepes (10 µl) were dispensed below the droplets of injection for donor karyoplast placement.

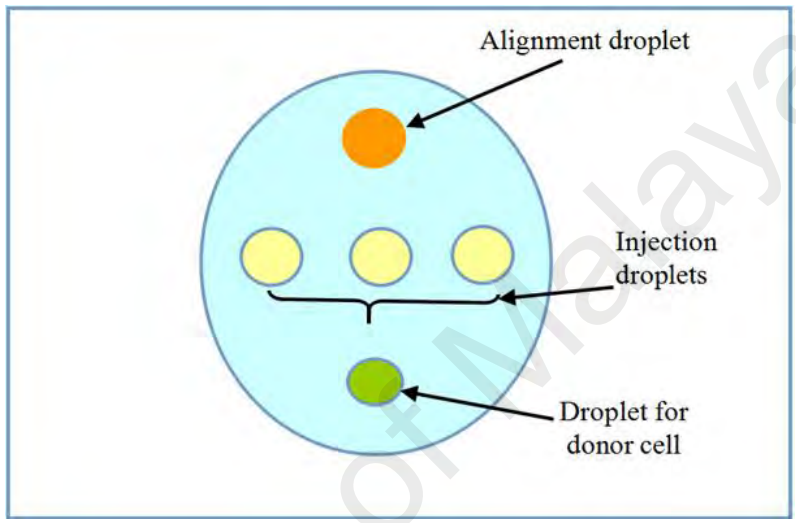

Figure 3.11: Injection dish.

The electrofusion dish was prepared for the fusion between the sub-zonal injected donor kayoplast with the recipient cytoplast using electrical pulse. The dish was prepared by drawing two parallel lines and another line inter-cross the centre point of Petri dish (60 mm diameter) bottom with a labelling marker (Figure 3.12). Subsequently, fusion medium (400 µl), was dispensed at the centre of the marked the Petri dish and the microdroplet was covered with silicone oil.

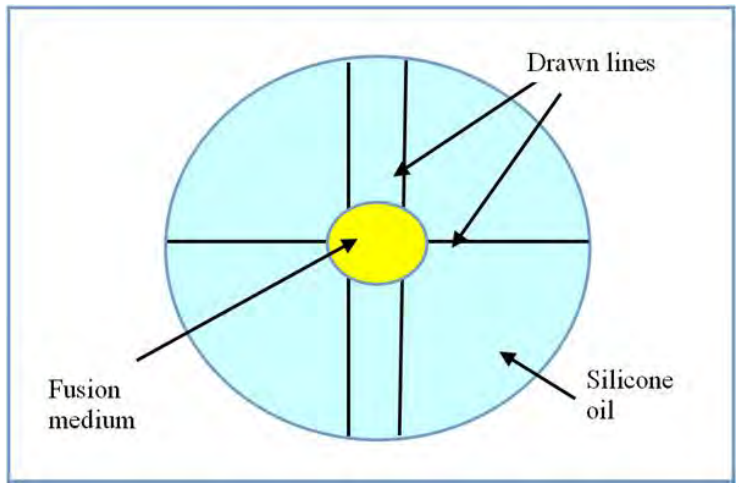

Figure 3.12: Preparation of electrofusion dish.

3.4.4.3 (b) Alignment of microtools on micromanipulator

Alignment of microtools in the micromanipulator dishes on micromanipulator is a crucial prelimenary step for current study. Perfect and accurate alignment of the microtools will ensure the smoothness and efficiency of the SCNT performance. Therefore, it is important to the researcher to achieve technical skill on alignment of microtools.

First, all the knobs of X-, Y- and Z-controls and the 3 ml syringes were adjusted to the centre of the scale. The enucleation dish was placed on the heated stage warmer with 38.5^{o}C of the micromanipulator. From the microscope, the alignment droplet was focused using lens under 4x objective. The holding pipette was inserted to the needle holder (left micromanipulator), tightened well and placed above the alignment droplet. 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 silicone oil by capillary action. The edge of the alignment droplet was sharply focused, subsequently the needle was brought inside the droplet near the edge and aligned properly.

After that, the enucleation needle was inserted to the needle holder (right micromanipulator) and tightened well. The enucleation needle was focused with the

holding pipette and tips of both microtools were touched for alignment. The tips also should parallel to the microscope stage. Finally, both the holding pipette and enucleation needle were checked under high magnification to ensure the accurate alignment (sharply in focus) and parallel.

For alignment of the injection pipette with the holding pipette, the injection pipette was inserted to the needle holder (right micromanipulator) and tightened well. 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 silicone oil by capillary action. The injection pipette was focused with the holding pipette and tips of both microtools were touched for alignment. The tips also should parallel to the microscope stage. Finally, both the holding pipette and injection pipette were checked under high magnification to ensure the accurate alignment (sharply in focus) and parallel.

Figure 3.13: Alignment of microtools.

3.4.4.3 (c) Enucleation using squeezing technique

Enucleation is the process of removal genomic DNA content from matured oocytes. There are several types of enucleation techniques such as squeezing technique, aspiration technique and laser shoot technique. For current study, squeezing technique was choose as enucleation technique.

The selected matured oocytes were treated with CB solution for 5 minutes for destabilising actin of oocytes cytoskeleton prior enucleation. After that, matured oocytes treated with cytochalasin B were transferred to the enucleation droplets on the

micromanipulation dish for enucleation. Firstly, the first polar body of the matured oocyte was placed at 12 o"clock position by rotating the oocyte with tips of the holding needle and 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. Next, 10% of the cytoplasm beneath the first polar body together with the first polar body was gently squeezed out. All the steps in the process were repeated until all the oocytes were enucleated. It is important to ensure that exposure of the oocytes to CB solution for the entire enucleation step was not exceeding 30 minutes. The enucleated oocytes were then washed 5 times in TL Hepes medium, lastly kept in TL Hepes medium at 38.5^{o}C in presence of CO_2 (5%) in a maximum humidified atmosphere of a CO_2 incubator for 30 to 45 minutes prior to nuclear transfer.

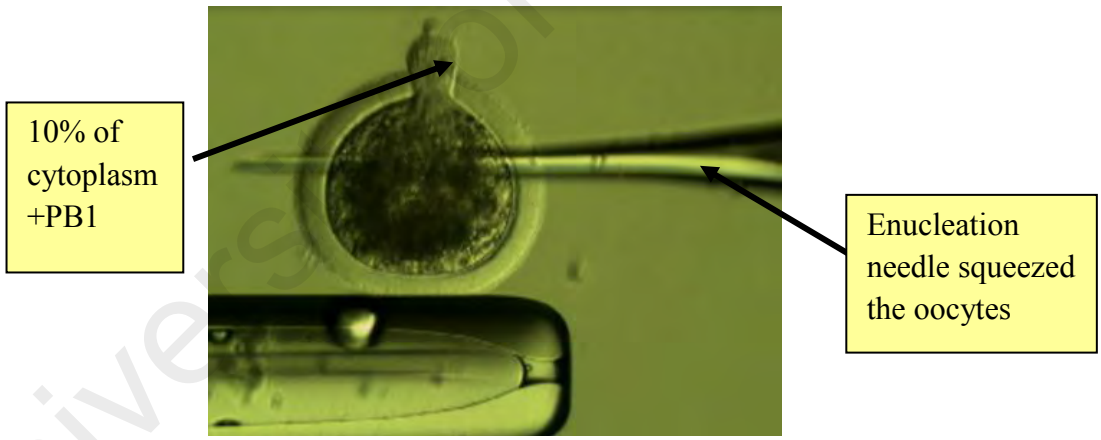

Figure 3.14: Enucleation of bovine oocytes using squeezing technique. (retrieved from Kwong, P.J,. 2012. Development of intra- and interspecies somatic cell nuclear transfer using ear fibroblast cells as donor karyoplasts for production of cloned caprine embryos. PhD Thesis, University of Malaya, Kuala Lumpur, Malaysia. Pp. 143)

3.4.4.3 (d) Subzonal injection (SUZI) of donor karyoplast for nuclear transfer

The synchronised ear fibroblast karyoplast were harvested and prepared as discussed in Section 3.4.4.1. After that, the cells were placed in donor cell droplets (10 µl TL Hepes) on the injection dish. After alignment of the holding and injection pipettes, the injection pipette was brought to the donor cell droplets. A cell of the donor karyoplast was sucked into the injection pipette with small amount of the TL Hepes medium.

The incubated enucleated oocytes were transferred and placed in injection droplets of the injection dish. An injection needle with the donor cell was brought to the droplet containing oocytes. The cutting point that was made during enucleation was placed at 1 or 2 o"clock. While the oocyte was held firmly by the holding pipette, the injection pipette was brought near to the oocyte, gently passed through the cutting point and transferred the donor cell at the perivitelline space at the point in which the donor cell could adhere closely to the recipient cytoplast. The injected oocytes were then washed 5 times in TL Hepes medium, lastly kept in TL Hepes medium at 38.5^{o}C in presence of CO_2 (5%) in a maximum humidified atmosphere of a CO_2 incubator for 30 to 45 minutes prior to fusion.

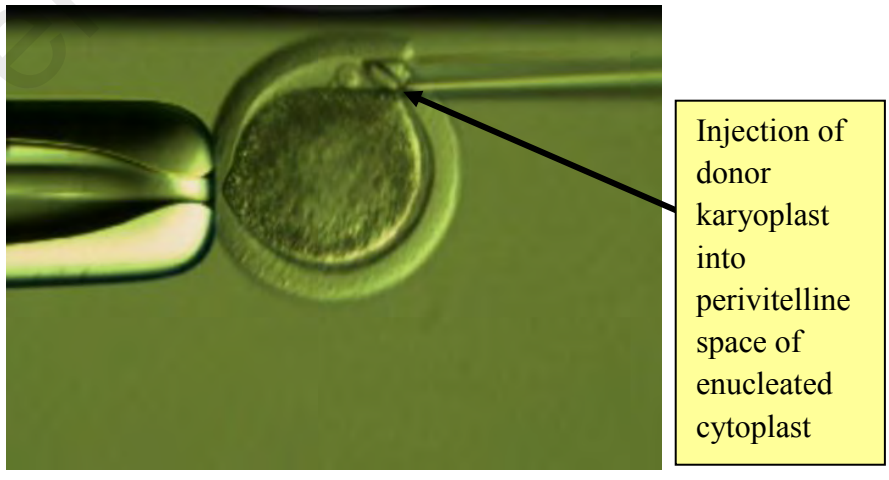

Figure 3.15: Injection of donor karyoplast using SUZI technique.
(retrieved from Kwong, P.J, 2012. Development of intra- and interspecies somatic cell nuclear transfer using ear fibroblast cells as donor karyoplasts for production of cloned caprine embryos. PhD Thesis, University of Malaya, Kuala Lumpur, Malaysia. Pp.144)

3.4.4.3 (e) Fusion of the reconstructed couplet

The electrofusion was conducted using a fusion machine (SUT F-1, Suranaree University of Technology, Thailand) connected to a pair of electrodes. The electrodes were fixed on the left and right universal joint of the micromanipulator and aligned in the fusion dish. Before fusion, the reconstructed couplets were washed thrice in the fusion medium. The reconstructed couplets were transferred into fusion droplet between two vertical lines marked on the fusion dish. The couplet was sandwiched between the two electrodes with donor karyoplast positioned at 3 o"clock in the cytoplast. Two direct current (2 DC) pulses (20 V, 15 µsec) were applied to fuse the couplets. The couplets were washed 5 times in TL Hepes medium in presence of CO_2 (5%) in a maximum humidified atmosphere of a CO_2 incubator at 38.5°C for 30 to 45 minutes prior to fusion assessment. Fusion was considered successful when the donor karyoplast membrane fused with the cytoplast membrane. The successfully fused couplets were subjected for subsequent activation treatment.

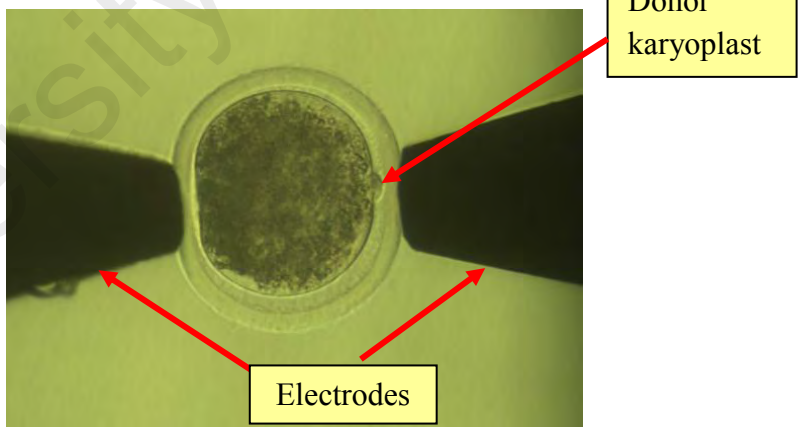

Figure 3.16: Fusion of reconstructed couplet.
(retrieved from Kwong, P.J, 2012. Development of intra- and interspecies somatic cell nuclear transfer using ear fibroblast cells as donor karyoplasts for production of cloned caprine embryos. PhD Thesis, University of Malaya, Kuala Lumpur, Malaysia. Pp. 145)

3.4.4.3 (f) Activation of the fused couplets

For activation treatment, the successfully fused couplets were washed 5 times on calcium ionophore (CaI) microdroplets. Fused oocytes were incubated in calcium ionophore droplets in presence of CO_2 (5%) in a maximum humidified atmosphere of a CO_2 incubator at 38.5^oC for 5 minutes. After that, the oocytes were washed 5 times in 6-dimethylaminopurine (6-DMAP) droplets to wash off the calcium ionophore medium prior to culture in 6-dimethylaminopurine in presence of CO_2 (5%) in a maximum humidified atmosphere of a CO_2 incubator at 38.5^oC for 4 hours.

3.4.4.3 (g) *In vitro* culture of the cloned embryos

After activation treatment, the activated couplets were washed 5 times in KSOM A medium to wash off the activation medium that might disturb the *in vitro* development of embryos. The couplets were then incubated in the KSOM A medium in presence of CO_2 (5%) in a maximum humidified atmosphere of a CO_2 incubator at 38.5^oC. At Day 2 post-activation, KSOM A changed to KSOM B medium. Subsequently, the medium replenished with KSOM B at Days 4,6,7 and 8 post-activation. The development of cloned embryos *in vitro* was observed and recorded.

3.4.4.3 (h) Treatment of trichostatin A on cloned embryos

For improvement of in *in vitro* culture system in order to obtain high rate of interspecies cloned embryos, cloned embryos were treated with trichostatin A (25 nM) for 12 hours. Prior treatment, the trichostatin A working solution were prepared in 7 microdroplets (100 µl) overlaid silicone oil in the Petri dish and incubated in CO_2 incubator. In the present study, two stages of embryos were treated with trichostatin A as comparative study which were post-activated couplets and 8-cell stage embryos.

For post-activated couplets, after activation with combination of calcium

ionophore and 6-dimethylaminopurine, the post-activated couplets were washed 2 times with KSOM A to wash off the activation medium and subsequently washed 3 times with trichostatin A working droplets for equilibration. Then, the post-activated couplets were incubated in the equilibrated trichostatin A working solution in presence of CO_2 (5%) in a maximum humidified atmosphere of a CO_2 incubator at 38.5°C. After 12 hour of treatment, the treated embryos were culture as discussed in Section 3.4.4.3 (g). The development of cloned embryos *in vitro* was observed under inverted microscope, and the embryo numbers were recorded.

For 8-cell stage embryos, the cloned embryos were washed 5 times with trichostatin A working droplets for equilibration. Then, the embryos were incubated in the equilibrated trichostatin A working solution in presence of CO_2 (5%) in a maximum humidified atmosphere of a CO_2 incubator at 38.5^{o}C. After 12 hours of treatment, the treated embryos were subjected for future culture in KSOM B medium. The development of cloned embryos *in vitro* was observed under inverted microscope, and the embryo numbers were recorded.

3.4.4.4 Preparation of mouse embryonic fibroblast (MEF) as feeder cell layer for embryonic stem cell (ESC) production

The present study included mouse embryonic fibroblast (MEF) cell culture preparation as preliminary step for embryonic stem cell (ESC) study. Mouse embryonic fibroblast usually will be inactivated and used as feeder cell layer for embryonic stem cell culture. Preparation of mouse embryonic fibroblast as feeder cell layer involved five steps; a) superovulation and mating, b) isolation and primary culture of mouse embryonic fibroblast, c) harvesting and subpassaging of mouse embryonic fibroblast , d) freezing and thawing of mouse embryonic fibroblast and e) inactivation of mouse embryonic fibroblast for feeder cell layer preparation.

3.4.4.4 (a) Superovulation and mating

For obtaining optimum number of foetus for mouse embryonic fibroblast culture, superovulation and timed-mating is important in order to obtain specific age of foetus. Pregnant mare serum gonadotrophin (PMSG; 10 IU) were injected intraperitoneally at Day 1 (suggested time: 1600 hour) into female mice. After 48 hours (Day 3), human chorionic gonadotrophin (hCG; 10 IU) were injected intraperitoneally. The male mice were introduced to the female mice for mating, at a ratio of 1 male to 1 female. The females were checked for vaginal plugs for the next morning (0700 hour). The plugged females were noted as successful mated.

3.4.4.4 (b) Isolation and primary culture of mouse embryonic fibroblast

Foetus at the age of 14 and 15 days *post coitum* were used to culture the mouse embryonic fibroblast in this current study. Firstly, the whole uterus was surgical removed from the murine and kept in phosphate buffer saline. The uterus was surgical open with a pair of scissors then each foetal sac was removed out of the uterus. The foetuses were washed in buffer saline, head limbs and internal organ was removed from the foetuses. The processed foetuses were minced into the small pieces by using a sterile blade and the mincing tissues were taken to a beaker. The trypsin-EDTA (0.25%; 20 ml) was added in the beaker and stir on the magnetic stirrer for 20 minutes. After that, it was filtered through nylon mesh into the conical tube (50 ml). DMEM + 10% FBS + 3PS was added to the supernatant (1.5 fold of supernatant volume) and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed, the cell pellet were resuspended with DMEM + 10% FBS + 3PS and seeded to the culture dish (60 mm). The primary mouse embryonic fibroblast were cultured in presence of CO_2 (5%) in a maximum humidified atmosphere of a CO_2 incubator at 37°C, until the cell reaching 80% confluency.

3.4.4.4 (c) Harvesting and subpassaging of mouse embryonic fibroblast

After the cell cultures reach 80% confluency, the cells were harvested for subculturing and cryopreservation. The cells were washed with phosphate buffer saline solution twice for complete media removal. Subsequently, trypsin EDTA (0.25%; 2-3 ml) was dispensed into the culture dish and placed in the CO_2 incubator for 2-3 minutes to allow the enzymatic reaction. The dishes then were returned into the laminar flow and by using the Pastuer pipette, the medium were aspirated in and out while rotating the culture dish for physically detach the cell layer. The peeling off the cell layer from the bottom of the culture dish could be observed in this steps. The cell suspension was transferred into 15 ml centrifuge tubes. The DMEM + 10% FBS + 1PS were added into the mixture with ratio of 1:1.5 medium. The tubes were centrifuged at 1500 rpm for 5 minutes. After the centrifugation steps, cell pellet would be formed at the bottom side of the centrifuge tube. Subsequently, the supernatant was removed and the DMEM + 10% FBS + 1PS were added to make up 3 ml. The mixture then were pipette gently to dissociate the clump of the cell pellet which producing the suspension the mouse embryonic fibroblast for subpassaging.

It is important to seed the cell with an optimum and fix seeding density to ensure the optimum production of mouse embryonic fibroblast. Before subpassaging, the cell counting and viability testing was performed with mouse embryonic fibroblast suspension (10 µl) mixed with Tryphan blue (0.4%; 10 µl). Counting of the viable and non-viable cells was done by using haemacytometer. The number of viable cells was determined by using the following formula:

$$c = \frac{d\,(n \times 10^4)}{5}$$

Where,
c is the number of cells
d is dilution factor
n is number of unstained cells

The number of viable cells determined the seeding density and the number of tissue culture dishes used for subpassaging. For this research, the seeding density of $5.0x10^5$ were used. Mouse embryonic fibroblast were seeded by dispersing droplets of mouse embryonic fibroblast using micropipettor (10-100 µl). The cells were grown until confluent and the media were refreshed every two days by replacing half of the media. This procedure were repeated for Passage 2 of the mouse embryonic fibroblast.

3.4.4.4 (d) Freezing and thawing of mouse embryonic fibroblast

Passages 1 and 2 of the mouse embryonic fibroblast cultures were cryopreserved by using quick freezing technique as adapted from Abas Mazni *et al.* (1989). The cells were harvested and cell counting as well as viability test were conducted as discussed in section 3.4.4.1 (c). For each cryovial, 0.5 ml of the mouse embryonic fibroblast mixture were placed into the cryovials. The freezing medium (60% DMEM medium + 20% FBS + 20% DMSO) were added into the cell mixture to give final volume of 2.0 ml and subsequently were transferred into cryovials and inserted into the cryocanes. Quick freezing technique involved of two steps of freezing, first step was freezing at -80°C by using liquid nitrogen gaseous for 15 minutes for equilibration. The second step was directly plunged into the liquid nitrogen (-196°C) for 15 minutes. This cryopreservation procedure were conducted by using the insulated ice box. The frozen mouse embryonic fibroblast were stored in the liquid nitrogen tank minimally for 2 days.

For thawing, cryovials were removed from cryocanes in liquid nitrogen tank without exposing the cryocanes for too long outside liquid nitrogen tanks. The cryovials were then immediately thawed by placing the lower portion of the vials into water bath. Avoid contact between the cap and the water to prevent contamination. After the mixture has thawed for about 2 minutes, the vials were removed from the

water bath and sprayed with ethanol (70%). The cap was flamed and removed in the laminar flow. The contents were transferred into centrifuge tubes (15 ml) containing DMEM + 10% FBS + 1PS medium with ratio of 1:1.5. The tubes were then centrifuged at 1500 rpm for 5 minutes. After completed centrifuged, a cell pellet was formed at the bottom of the centrifuge tube. Subsequently, the supernatant was removed and the DMEM + 10% FBS + 1PS were added to make up 3 ml. The mixture then were pipette gently to dissociate the clump of the cell pellet which producing the suspension the mouse embryonic fibroblast for seeding the mouse embryonic fibroblast. The cell counting and viability testing as well as seeding of the mouse embryonic fibroblast were performed with similar procedure discussed in section 3.4.4.4 (c).

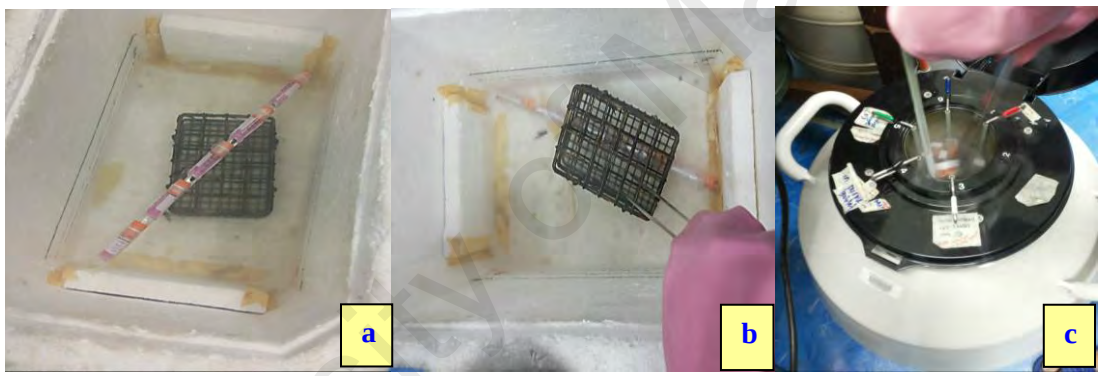

Figure 3.17: Steps in cryopreservation using quick freezing technique.

3.4.4.4 (e) Inactivation of mouse embryonic fibroblast for feeder cell layer preparation

The culture media was removed from the confluent culture dish. Mitomycin C (50 µl) was added to 5 ml of DMEM+10% FBS for a final concentration (10µg/ml) and incubated for 3 hours in the CO_2 incubator (5%,37°C). After 3 hours, the mitomycin C was removed and washed thrice with phosphate buffer saline. Trypsin EDTA was added just to cover the cells and leaved it for 3 minutes. Next, the cell was pipetted to break the cell into single cell and all the cell in trypsin were taken to conical tube. DMEM+10% FBS (1.5 fold of trypsin EDTA volume) was added and centrifuged at 1500 rpm for 5

minutes. The cell was seeded and cultured in the CO_2 incubator (5%,37°C). The culture dish was shaken well in the direction shown below to make sure the cell will distributed cover all the bottom area of the culture dish. The feeder cell should be prepared 1 day prior to use as the feeder layer for embryonic stem cell. The feeder layers were prepared by expanding mouse embryonic fibroblast cultures on culture dishes. After the mouse embryonic fibroblasts culture reaches 80% confluency, the media was removed. 2 ml of mitomycin C (10 µg/ml) solution was added and the cells were incubated for 3 hours. The solution was then aspirated, and the cells were washed twice times with phosphate buffer saline. Around 2 ml of culture media were added to the culture dishes and incubated for at least 3 hours.

3.4.4.4 (f) Culturing inner cell mass on inactivated mouse embryonic fibroblast feeder cell layer

In order to culture embryonic stem cell, the inner cell mass need to be isolated prior culturing on inactivated mouse embryonic fibroblast feeder cell layer. In this study, whole blastocyst culture technique were used to isolate the inner cell mas from the blastocyst. Blastocysts were removed from the micro-droplet cultures using a drawn-out pipette into a 20 µl droplet of pronase (0.5%) until the zona pellucida has degraded. The blastocysts were then washed in droplets of 30 µl culture media. Washed blastocysts were directly transferred onto feeder layers of inactivated mouse embryonic fibroblast on a culture dish (35 mm). Sufficient gap was left between each blastocyst. The blastocysts were left for 10-15 seconds prior carefully carried and placed back into the incubator. They were left for 2 days and the media were refreshed on Day 2 and the culture was observed daily and media were refreshed on every alternate day.

3.5 EXPERIMENTAL DESIGN

Several experiments had been designed for this study to study the effect of breeds and genders of the donor karyoplast on production of the interspecies cloned caprine embryos. With special focus on the somatic cell culture (ear fibroblast cell) and cryopreservation, this study also included production of mouse embryonic fibroblast cell culture as preliminary study for embryonic stem cell production. The present study was divided into four experiments as the following:

3.5.1 Effect of breed, gender and cryopreservation on viability rate of early passages of caprine ear fibroblast cell culture (Experiment 1)

The main objective of this study to investigate the effect of breeds and gender in production of ear fibroblast culture which subsequently were cryopreserved to determine the effect of quick freezing technique as cryopreservation technique to ear fibroblast culture. In this experiment, the early passages (Passages 1 to 3) of ear fibroblast cell cultures were developed from adult female and male (3 to 4 years old) from two breeds, purebreed Katjang and purebreed Boer. The experiments were replicated 6 times with 2-3 samples for each replicate. The parameters measured were the viability rate for each passages and the mortility rate. The morphology of the culture also was observed. The viability rate of the ear fibroblast cell were calculated and compared between the gender, passages as well as fresh and frozen-thawed ear fibroblast cell.

Briefly, ear tissues (1 to 1.5 cm^2) were collected from adult (3 to 4 years old) female and male purebreed Boer and Katjang goats and placed into test tube containing phosphate buffered saline (PBS-). The test tubes were placed in polystyrene box with ice pack and were carried to the laboratory for for primary culture. This explant culture was incubated under maximum humidified atmosphere of CO_2 (5%) in air at 38.5°C for 8 to 10 days. The medium was replenished with the culture medium with lower

concentration of penicillin-streptomycin (1x). The cells were harvested at 80% confluence by using trypsin-EDTA (0.25%). The ear fibroblast cell in pellet form were suspended in the culture medium and seeded in a tissue culture dish (25 mm^2). The cell cultures were subcultured until Passage 3. Passages 1 to 3 of the EFC cultures were cryopreserved by using quick freezing technique (Abas Mazni *et al.*, 1989). Cell count was carried out before cryopreservation and after thawing of ear fibroblast cell to evaluate the viability of the ear fibroblast cell cell cultures before and after cryopreservation.

3.5.2 Effect of *in vitro* maturation duration of bovine oocytes as well as breed and gender of donor karyoplast on *in vitro* development competency of interspecies cloned caprine embryos (Experiment 2)

The present experiment was designed to evaluate relationship of cytoplast factor (*in vitro* maturation duration) and karyoplast factor (breeds and genders) with interspecies somatic cell nuclear transfer efficiency in caprine. This experiment consists of two sub-experiments; 1) effect of maturation duration to maturation rate of bovine cytoplasts and 2) effect of maturation duration of bovine cytoplast combine with different breeds and genders of donor karyoplast on *in vitro* developmental rate of interspecies cloned caprine embryos. Briefly, the matured bovine oocytes derived from two different *in vitro* maturation duration ranges were enucleated and injected with single ear fibroblast cell donor karyoplast from two different breeds and genders. The couplets then were fused by electrofusion technique and subsequently activated in calcium ionophore and 6-dimethylaminopurine. The activated oocytes then were cultured *in vitro* to blastocyst stage.

For sub-experiment 1, two ranges of maturation rate were used a) 22 - 24 hour and b) 25 - 27 hour, to evaluate its effect on maturation rate of bovine oocytes. A total of 1714 bovine oocytes were collected from 165 ovaries. Only Grades A, B, and C oocytes were selected for this sub-experiment. Comparison was made on the

maturation rate of the bovine oocytes between the two ranges if maturation duration. Oocytes with protruding first polar body were denoted as matured oocytes.

For sub-experiment 2, ear fibroblast cells of two breeds, purebreed Katjang and purebreed Boer for both female and male from Experiment 1 were used as donor karyoplasts. The donor karyoplast was injected into mature bovine cytoplast matured from two ranges of maturation duration, a) 22 - 24 hour and b) 25 - 27 hour. This 2x2x2 factorial experiment was designed to evaluate the effects of maturation duration of cytoplasts as well as breeds and genders of donor karyoplast on the *in vitro* developmental competency of interspecies cloned caprine embryos. A total of 1085 matured bovine oocytes were used as cytoplast for constructing the interspecies cloned embryos. Comparison was made between the combination of the maturation durations, breeds and genders on successful enucleation and fusion rates as well as *in vitro* developmental rate of the interspecies cloned caprine embryos.

3.5.3 Effect of trichostatin A (25 nM) supplementation in *in vitro* culture medium on interspecies cloned caprine embryos development competency (Experiment 3)

The aim of this experiment to use trichostatin A (TSA), a potent histone deacetylase inhibitor, in the *in vitro* culture medium to improve the efficiency of interspecies somatic cell nuclear transfer in caprine. Therefore, two stages of embryos which were a) post-activated couplets and b) 8-cell stage embryos were treated with trichostatin A (25 nM). Stage of post-activated interspecies cloned caprine embryos were selected as to determine whether trichostatin A treatment of interspecies cloned caprine embryos in their early stage of embryos affected the frequency of the blastocyst development. Meanwhile, 8-cell stage was chosen as at this stage, *de novo* mRNA transcription followed by the embryonic genomic activation in ruminant usually occured at this stage. The gene encoding proteins required for transcriptional regulation, cell adhesion, signal transduction, and metabolism are activated. Therefore, it was hypothesised that

treatment of TSA at 8-cell stages might increase the development of cloned blastocyst.

Briefly, interspecies cloned caprine embryos (activated couplets and 8-cell) were treated with trichostatin A (25nM) for 12 hours. The treated embryos were then cultured in *in vitro* culture medium for subsequent development. The experiment were replicated 10 times. Comparison were made between the interspecies cloned embryos between treated and non-treated (as control) to evaluate the efficiency of trichostatin A (25nM) treatment on production of interspecies cloned morula and blastocyst rates.

3.5.4 Using quick freezing technique to cryopreserve the mouse embryonic fibroblast (MEF) derived from different foetal ages for whole blastocyst culture (Preliminary caprine ESC culture) (Experiment 4)

The objective of this study to investigate the production of mouse embryonic fibroblast from different foetal ages and efficiency of quick freezing technique to cryopreserve the mouse embryonic fibroblast . In this experiment, mouse embryonic fibroblast were produced by using two different foetal ages, 14 and 15 days *post coitum* (d.p.c). A total of 12 samples of mouse embryonic fibroblast derived from 14 d.p.c. and 6 samples of mouse embryonic fibroblast derived from 15 d.p.c. were produced in this study. The foetuses were isolated from the pregnant female mice of ICR and albino strains. After isolation, suspension and seeding of the mouse embryonic fibroblast cells from the foetus, the mouse embryonic fibroblast cells were cultured up to 80% confluency. The confluenced cells were harvested and subpassaged until Passage 2. The viability rate of the mouse embryonic fibroblast derived from different foetal ages were compared between the foetal age and passages.

The efficiency of quick freezing technique was evaluated by evaluating the viability rate of the frozen-thawed mouse embryonic fibroblast with fresh mouse embryonic fibroblast. Passages 1 and 2 of the mouse embryonic fibroblast cultures were cryopreserved by using quick freezing techniques. Briefly, the confluence cell

culture were trypsinised. The freezing medium were added into the cell mixture and subsequently were transferred into cryovials and inserted into the cyocanes. Quick freezing technique was adapted by Abas Mazni *et al.* (1989). Quick freezing technique involved of two steps of freezing, first step was freezing at by using liquid nitrogen gaseous (-80°C) for 15 minutes for equilibration. The second step was directly plunged into the liquid nitrogen (-196°C) for 15 minutes. The viability rates of frozen-thawed passages were compared with the fresh passages for all mouse embryonic fibroblast derived from two age of foetuses.

3.6 STATISTICAL ANALYSES

Data were analysed using SPSS (Statistical Packages for Social Sciences) for Windows (version 23.0, IBM, USA). Significance was determined when $P<0.05$.

For Experiment 1, the difference of the viability rates between the genders were analysed using unpaired t-test. The same statistical method was used to analyse the viability differences between fresh and frozen-thawed for Passages 1 to 3. The differences of viability rate between the three passages were analysed using one way ANOVA followed by Duncan Multiple Range Test.

For Experiment 2, the maturation rate (in Experiment 2a) between two ranges of maturation duration were analysed using Students unpaired t-test. For Experiment 2b, comparison was made between the combination of the maturation durations, breeds and genders on successful enucleation and fusion rates as well as *in vitro* developmental rate of the interspecies cloned caprine embryos and these data were analysed using one way ANOVA followed by Duncan Multiple Range Test.

For Experiment 3, the morula and blastocyst rate between the two stages of cloned embryos treated with trichostatin A as well non-treated embryos for interspecies cloned caprine embryos were analysed using one way ANOVA followed by Duncan Multiple Range Test.

Lastly, for Experiment 4, the effect of foetal ages on viability rate of each passages and the effect of cryopreservation, the comparison between viability rates of fresh and frozen-thawed passages were analysed using unpaired t-test.

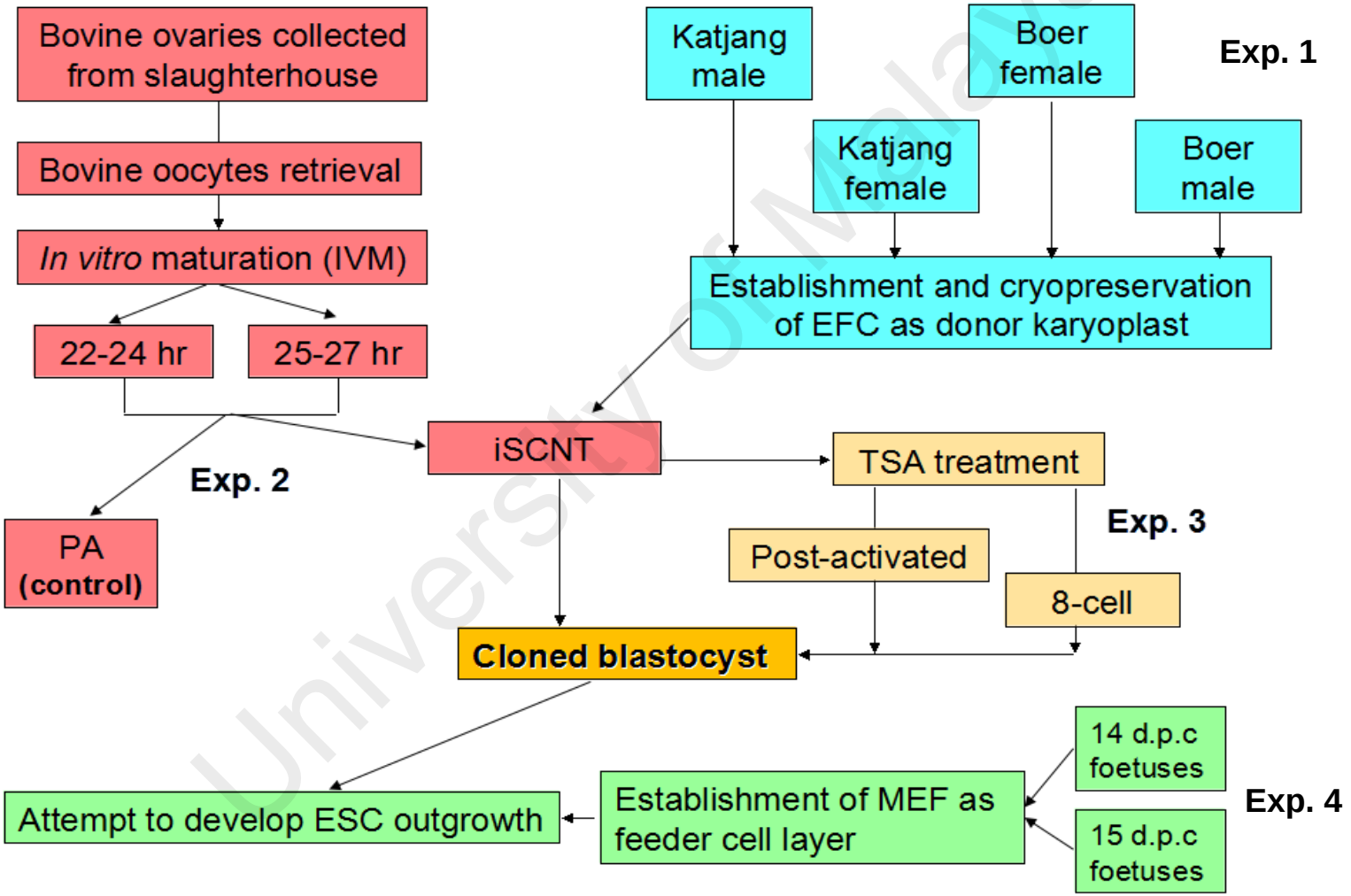

Figure 3.18: Flow chart of methodology.

Effect of breed, gender and cryopreservation on viability rate of early passages caprine ear fibroblast cell culture (Experiment 1)

Treatments:
a) Breeds and genders (Female and male of Boer and Katjang).
b) Number of passages (Passage 1 vs. Passage 2 vs. Passage 3).
c) Fresh passages vs. frozen-thawed passages.

Factor:

a) Viability rate of each passage.

Analysis:
a) Unpaired t-test.
b) One way ANOVA followed by Duncan Multiple Range Test.

Effect of *in vitro* maturation duration of bovine oocytes as well as breed and gender of donor karyoplast on *in vitro* development competency of interspecies cloned caprine embryos (Experiment 2)

Treatments:
a) *In vitro* maturation duration bovine oocytes (22-24 hr vs. 25-27 hr).
b) Breed and gender karyoplasts (Female and male of Boer and Katjang).

Factors:
a) Maturation rate.
b) Cleavage rate (2-cell).
c) *In vitro* developmental rate.

Analysis:
a) One way ANOVA followed by Duncan Multiple Range Test.

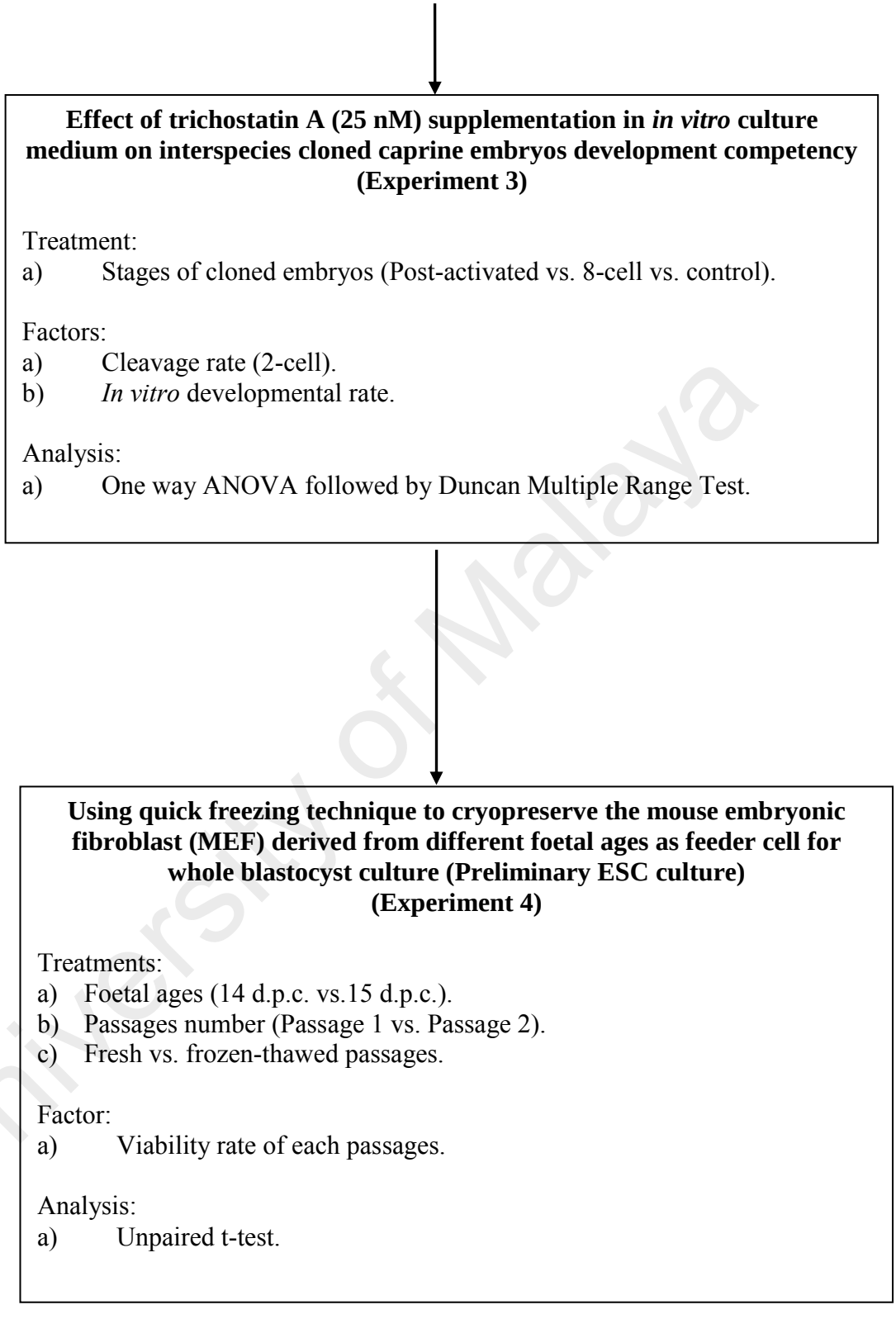

Figure 3.19: Flow chart of experimental design

Chapter 4

4.0 RESULTS

4.1 EFFECT OF BREED, GENDER AND CRYOPRESERVATION ON VIABILITY RATE OF EARLY PASSAGES OF CAPRINE EAR FIBROBLAST CELL CULTURE (EXPERIMENT 1)

This experiment was carried out to evaluate the effect of 2 caprine breeds and gender of the donor ear fibroblast cell as donor karyoplast on viability rate of 3 early passages of the culture. This experiment was also designed to evaluate the effect of quick freezing technique to cryopreserve the different breed and gender of donor of ear fibroblast cell on the viability rate on frozen thawed early passages of the culture.

4.1.1 Effect of breed and gender on viability rate of early fresh passages of caprine ear fibroblast cell culture

A total of 18 ear skin samples from both female and male purebred Boer as well as 17 samples from both female and male purebred Katjang were collected subsequently cultured and sub-passaged to Passage 3. The viability rates of Passages 1 to 3 of female and male Boer and Katjang were calculated and compared. The result obtained depicted in Table 4.1.

Between the 3 passages, for female Boer, Passage 2 was significantly higher ($P<0.05$) compared to Passages 1 and 3 (92.39% vs. 85.77% vs. 88.20%, respectively). However, for female Katjang, Passage 1 showed significantly lower viability rate ($P<0.05$) compared to Passages 2 and 3 (76.40% vs. 87.28% vs. 87.30%, respectively). No significant different on the viability rate of the 3 passages of both male Boer and Katjang ($P>0.05$).

Comparing the viability rates between the gender and breed of the donor, female Katjang at Passage 1 showed the most lower viability rate (76.40% vs. 85.77% vs 82.16% vs. 87.10% at $P<0.05$). However, for Passages 2 and 3, no significant effect of breed and gender of the donor on the viability rate of the culture ($P>0.05$). Therefore, generally, no significant effect of breed and gender on viability rate of fresh early passages of the ear fibroblast culture.

Table 4.1: Effect of breed and gender of the ear fibroblast donor on viability rate of fresh Passages 1 to 3 of ear fibroblast cell culture (mean±SEM)

Donor		Percentage of viability (%)		
Breed	Gender	Passage 1	Passage 2	Passage 3
Boer	Female	85.77 ± 2.08^{ay} (18)	92.39 ± 1.92^{bx} (18)	88.20 ± 2.26^{ax} (18)
	Male	82.16 ± 3.40^{ay} (18)	88.81 ± 3.05^{ax} (18)	87.90 ± 3.36^{ax} (18)
Katjang	Female	76.40 ± 2.26^{ax} (17)	87.28 ± 2.21^{bx} (17)	87.30 ± 0.55^{bx} (17)
	Male	87.10 ± 2.89^{ay} (17)	89.60 ± 3.56^{ax} (17)	81.37 ± 2.52^{ax} (17)

^{a,b} Means with different superscripts in a row were significantly different ($P<0.05$).
^{x,y} Means with different superscripts in a column were significantly different ($P<0.05$).
(n) No of samples

4.1.2 Effect of cryopreservation on early passages of female and male Boer and Katjang ear fibroblast cell

Similar to Section 4.1.1, a total of 18 ear skin samples from both female and male purebred Boer as well as 17 samples from both female and male purebred Katjang were collected subsequently cultured and sub-passaged to Passage 3. All the passages then were cryopreserved using quick freezing technique. The result is shown in Table 4.2.

No significant differences ($P>0.05$) were observed between frozen-thawed and fresh passages of ear fibroblast cells derved from male Boer and Katjang. However, the viability rates of frozen-thawed Passages 2 and 3 of female Boer were significantly lower ($P<0.05$) than fresh (81.70% vs. 92.39% and 79.53% vs. 88.20%, respectively). Similarly, viability rate of frozen-thawed Passage 3 was significantly lower ($P<0.05$) than fresh Passage 3 for Katjang female (78.52% vs. 87.30%, respectively).

Table 4.2: Viability rate of fresh and frozen-thawed of Passages 1 to 3 of female and male Boer and Katjang ear fibroblast cell after cryopreservation (mean±SEM)

Donor	Percentage of viability (%)					
	Passage 1		Passage 2		Passage 3	
	Fresh*	Frozen-thawed	Fresh*	Frozen-thawed	Fresh*	Frozen-thawed
Boer Female	85.77 ± 2.08^{ay} (18)	83.26 ± 6.33^{ay} (18)	92.39 ± 1.92^{by} (18)	81.70 ± 1.01^{ax} (18)	88.20 ± 2.26^{bx} (18)	79.53 ± 1.48^{ax} (18)
Boer Male	82.16 ± 3.40^{ay} (18)	80.63 ± 0.77^{ay} (18)	88.81 ± 3.05^{ax} (18)	81.91 ± 2.18^{ax} (18)	87.90 ± 3.36^{ax} (18)	83.22 ± 1.47^{ay} (18)
Katjang Female	76.40 ± 2.26^{ax} (17)	78.30 ± 1.33^{ax} (17)	87.28 ± 2.21^{ax} (17)	87.57 ± 2.23^{ax} (17)	87.30 ± 0.55^{bx} (17)	78.52 ± 3.55^{ax} (17)
Katjang Male	87.10 ± 2.89^{ay} (17)	86.12 ± 1.87^{ay} (17)	89.60 ± 3.56^{ax} (17)	85.50 ± 3.14^{ax} (17)	81.37 ± 2.52^{ax} (17)	80.57 ± 1.07^{axy} (17)

[a,b] Means with different superscripts in a row within group were significantly different ($P<0.05$).

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

(n) No. of samples

* Control (Data from Table 4.1)

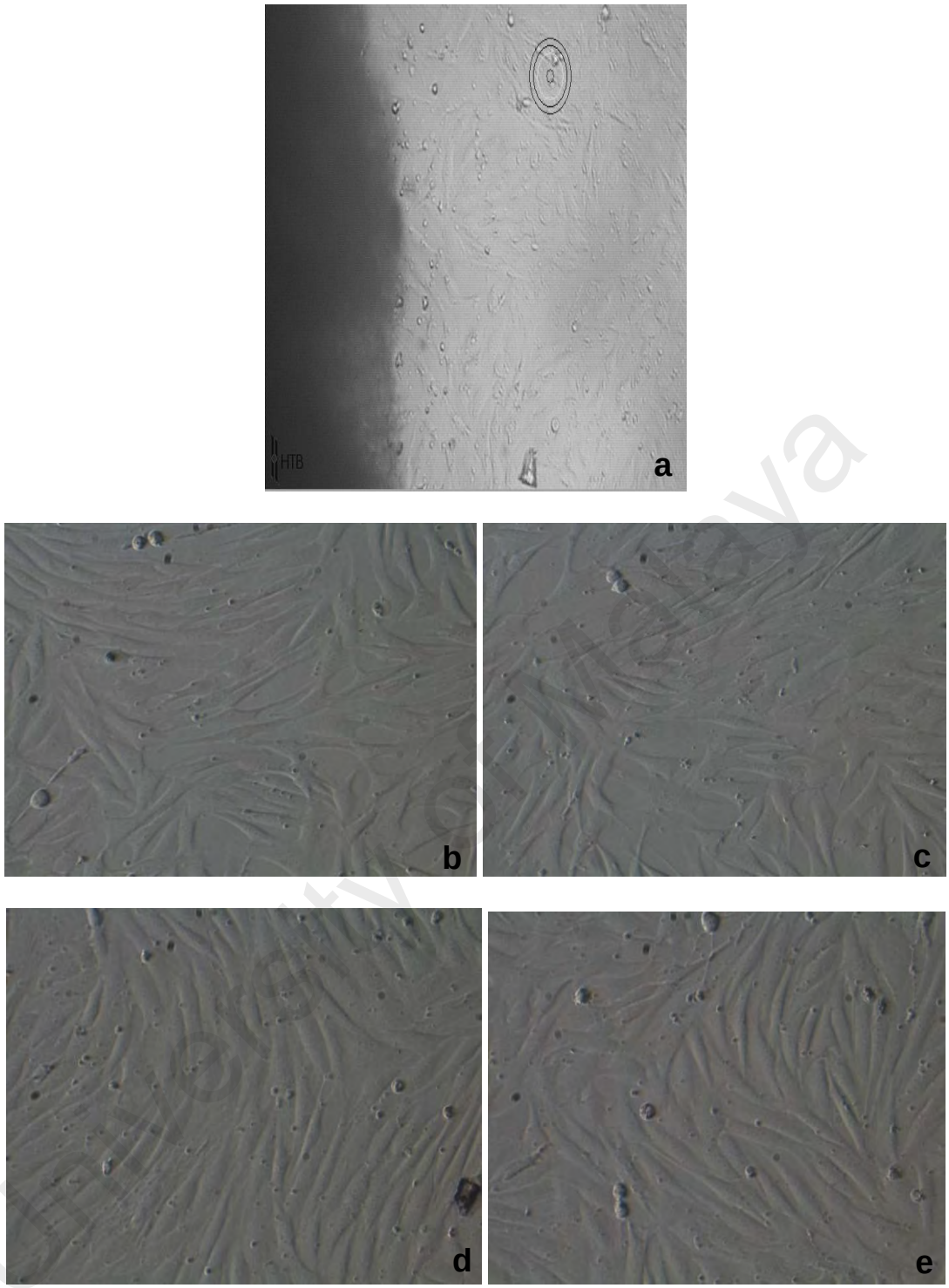

Figure 4.1: Confluency of caprine ear fibroblast cell reach more than 80% for a) primary explant culture (10x magnification), b) Boer female passage, c) Boer male passage, d) Katjang female passage and e) Katjang male passage (b-e: 20x magnification) viewed under inverted microscope.

4.2 EFFECT OF *IN VITRO* MATURATION DURATION OF BOVINE OOCYTES AS WELL AS BREED AND GENDER OF DONOR KARYOPLAST ON *IN VITRO* DEVELOPMENT COMPETENCY OF INTERSPECIES CLONED CAPRINE EMBRYOS (EXPERIMENT 2)

This experiment was designed to evaluate the cytoplast factor and karyoplast factor in *in vitro* developmental competency of interspecies cloned caprine embryos. For the cytoplast factor, the maturation duration of the bovine oocytes effect was evaluated on maturation rate of the bovine oocytes. The results of this experiment were discussed in sub-experiments 1 of Section 4.2.1.

For sub-experiment 2, the effect of maturation duration of bovine oocytes was determined on the *in vitro* development of interspecies cloned caprine oocytes. The karyoplast factors which were breeds and gender were also evaluated on the competency of *in vitro* development of interspecies cloned caprine embryos. Lastly, the combination effects of maturation duration with the breeds and gender of karyoplast on the *in vitro* development of interspecies cloned caprine embryos were determined. All of these results were discussed in Section 4.2.2.

4.2.1 Effect of maturation duration on maturation rate of bovine oocytes (Sub-experiment 1)

A total of 1714 bovine oocytes of Grades A to C were successfully retrieved from 165 bovine ovaries collected from local slaughterhouse. The oocytes were subjected for *in vitro* maturation at 2 different ranges of maturation duration: a) 22-24 hours and b) 25-27 hours with 1194 oocytes and 520 oocytes for each maturation duration, respectively. No significant difference of maturation rate of bovine oocytes (P>0.05) was found between the two maturation duration (85.13% vs. 83.53%, respectively). The data were depicted in Table 4.3.

Table 4.3 : Maturation rate of bovine oocytes according to maturation duration (mean±SEM)

Maturation duration (hr)	No of ovaries	No of oocytes retrieved*	No of oocytes/ ovaries*	No of matured oocytes	Maturation rate (%)
22-24	107	1194	12.84±0.12	990	85.13±2.44[x]
25-27	58	520	8.89±0.20	437	83.53±3.72[x]

*Grades A, B and C oocytes were only selected

[x]Maturation rate with similar superscript in a column were not significantly different at P>0.05.

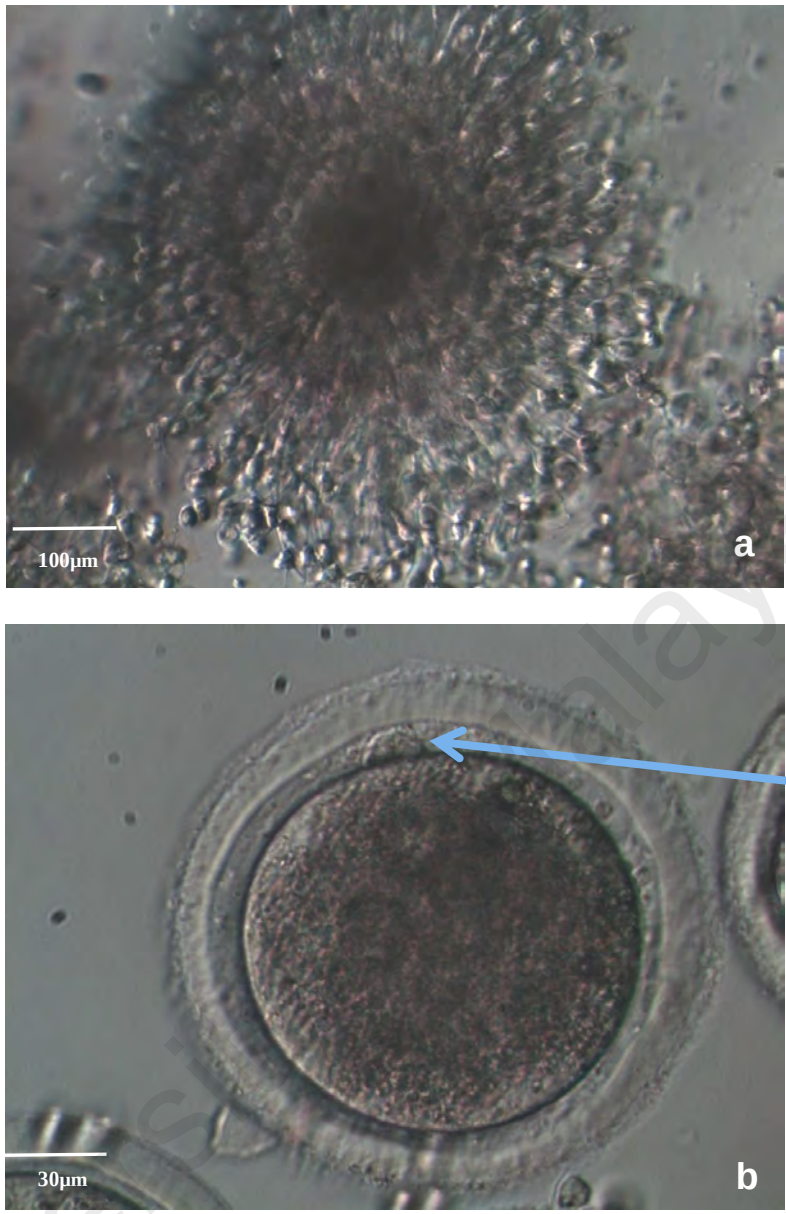

Figure 4.2: After *in vitro* maturation of bovine cytoplast, a) expanded cumulus oocytes complexes after maturation (10x magnificaion) and b) matured denuded bovine oocytes (20x magnification) viewed under inverted microscope.

4.2.2 Effect of maturation duration of bovine cytoplast on *in vitro* developmental rate of interspecies cloned caprine embryos (Sub-experiment 2: combined data of breed and gender of donor karyoplast)

In this sub-experiment, the result discussed as follows: a) the effect of maturation duration on *in vitro* development of interspecies cloned caprine embryos as well as bovine parthenotes, b) effect of breeds an gender of ear fibroblast donor karyoplast on *in vitro* development of interspecies cloned caprine embryos, and c) effect of maturation duration of bovine cytoplast on *in vitro* development of interspecies cloned caprine embryos (combined data with breeds and gender of ear fibroblast donor karyoplast).

4.2.2.1 Effect of maturation duration on *in vitro* development of interspecies cloned caprine embryos as well as bovine parthenotes

Table 4.4 shows comparison of *in vitro* development rate of interspecies cloned caprine embryos with bovine parthenotes when using bovine oocytes that subjected for different maturation durations. A total of 1029 of activated interspecies cloned caprine embryos and 344 parthenotes bovine embryos from 22-24 hours and 25-27 hours maturation duration were cultured *in vitro* and the developments were analysed. For 22-24 hours maturation duration, both interspecies cloned caprine embryos and parthenotes embryos successfully developed up to blastocyst stage. However, only parthenotes embryos of 25-27 hours maturation were successfully developed up to blastocyst, but the interspecies cloned caprine embryos of 25-27 hours maturation only developed up to morula stage.

For 22-24 hours maturation duration, both interspecies cloned caprine and bovine parthenotes embryos had significantly higher cleavage rate ($P<0.05$) compared to 25-27 hours maturation duration of bovine parthenotes embryos (72.08% and 64.19% vs.

25.24%, respectively). Similar results were obtained for 4-cell stage (47.37% and 42.99% vs. 16.71% at $P<0.05$, respectively). However, no significant of effect of maturation duration for interspecies cloned caprine embryos and bovine parthenotes embryos at 8-cell and morula stage ($P>0.05$). For blastocyst rate production, bovine parthenotes from 22-24 hours maturation duration was significantly higher ($P<0.05$) than the interspecies cloned caprine embryos (4.62% vs. 0.48% and 0%, respectively).

Table 4.4: Effect of different maturation duration of bovine cytoplast on *in vitro* development rate of interspecies cloned caprine embryos compared to bovine parthenotes (mean±SEM)

Maturation duration (hr)	Types of embryos	No of activated oocytes	*In vitro* developmental rate of manipulated embryos*, % (n)				
			2-cell	4-cell	8-cell	Morula	Blastocyst
22-24	iSCNT	762	72.08 ± 3.92^{y} (571)	47.37 ± 4.28^{y} (375)	33.52 ± 4.11^{x} (265)	19.05 ± 2.86^{x} (161)	0.48 ± 0.35^{x} (4)
	PA	252	64.19 ± 9.08^{y} (156)	42.99 ± 8.29^{y} (112)	28.44 ± 6.42^{x} (73)	16.81 ± 4.58^{x} (44)	4.62 ± 2.04^{y} (13)
25-27	iSCNT	267	48.85 ± 8.90^{xy} (134)	33.94 ± 8.68^{xy} (86)	30.14 ± 8.79^{x} (75)	13.01 ± 3.98^{x} (33)	0^{x} (0)
	PA	92	25.24 ± 13.15^{x} (39)	16.71 ± 10.86^{x} (21)	15.54 ± 9.92^{x} (20)	9.00 ± 5.92^{a} (11)	1.67 ± 1.14^{xy} (2)

*Based on number of matured oocytes
(n) No of oocytes
iSCNT interspecies cloned caprine embryos
PA Parthenogenetic embryos
^{xy} Mean with different superscripts in a column is significantly different at $P<0.05$

4.2.2.2 Effect of breed and gender of ear fibroblast donor karyoplast on *in vitro* development of interspecies cloned caprine embryos

The *in vitro* developmental competencies of the interspecies cloned caprine embryos were compared between the donor karyoplast of female and male Boer and Katjang as depicted in Table 4.5. A total of 1029 successfully fused oocytes were used with 407 of Boer female, 178 of Boer male, 200 of Katjang female and 244 of Katjang male.

When using both female and male Boer ear fibroblast cell as donor karyoplast, the interspecies cloned caprine embryos were successfully developed up to blastocyst stage with no significant difference ($P>0.05$) between female and male (0.38% vs. 1.50%, respectively). However, the interspecies cloned caprine embryos were only developed up to morula stage when using female and male Katjang ear fibroblast.

For cleavage rate (2-cell), no significant difference was observed between the breed and gender ($P>0.05$). For 4-cell stage, the males of Boer and Katjang were significantly higher ($P<0.05$) than the females (58.03% and 56.33% vs. 35.74% and 29.43%, respectively). Similar result was also obtained for 8-cell stage (43.41% and 41.52% vs. 27.15% and 19.85%, respectively). However, in morula stage, no significant difference was obtained between the breeds and gender ($P>0.05$) with male Boer and Katjang showed apparently higher morula rate compared to female.

Table 4.5: Effects of breed and gender of the donor karyoplast on *in vitro* developmental rate of interspecies cloned caprine embryos (mean±SEM)

Breed	Gender	No. of fused oocytes	*In vitro* developmental rate of cloned embryos, % (n)*				
			2-cell	4-cell	8-cell	Morula	Blastocyst
Boer	Female	407	62.40± 6.21^{x} (261)	35.74± 5.68^{xy} (153)	27.15± 5.67^{xy} (111)	13.25± 3.79^{x} (60)	0.38^{x} (2)
	Male	178	76.36± 9.09^{x} (138)	58.03± 9.36^{y} (109)	43.41± 8.83^{y} (82)	23.57± 5.34^{x} (48)	1.05^{x} (2)
Katjang	Female	200	66.56± 10.50^{x} (147)	29.43± 7.08^{x} (62)	19.85± 8.82^{x} (45)	12.63± 5.41^{x} (29)	0^{x} (0)
	Male	244	64.80 ±7.34^{x} (159)	56.33± 7.25^{y} (137)	41.52± 6.59^{y} (102)	23.05± 4.11^{x} (57)	0^{x} (0)

^{xy} Means with different superscript in a column were significantly different (P<0.05).
***** *In vitro* developmental rate were based on number of fused oocytes
(n) No. of samples

4.2.2.3 Effect of maturation duration of bovine cytoplast as well as breed and gender of ear fibroblast donor karyoplast on *in vitro* development of interspecies cloned caprine embryos

Table 4.6 shows that the cleavage rates (2-cell) are significantly higher when using 22-24 hours of maturation cytoplast with Boer male karyoplast and both female and male Katjang karyoplast (84.17%, 70.32% and 73.14% respectively, $P<0.05$). Although both Boer and Katjang male karyoplast with 22-24 hours of maturation cytoplast gives significantly higher 4-cell rates (61.25% and 64.99%, $P<0.05$), only Katjang male karyoplast remain significantly higher for subsequent embryonic developmental rate of 8-cell and morula (49.78% and 28.18%, $P<0.05$). The combination of 25-27 hour maturation cytoplast with Katjang male karyoplast gave significantly lowest developmental rate ($P<0.05$). Only combination of 22-24 hours maturation cytoplast with both female and male Boer karyoplast successfully produced interspecies cloned caprine blastocyst (0.49% and 1.50%, $P<0.05$).

Table 4.6: Effect of maturation durations of bovine cytoplast combined with breeds and genders of donor karyoplast on the developmental competency of interspecies cloned caprine embryos (mean±SEM)

Maturation duration (hr)	Breed	Gender	No. of fused oocytes	*In vitro* developmental rate of cloned embryos, % (n)*				
				2-cell	4-cell	8-cell	Morula	Blastocyst
22-24	Boer	Female	302	66.10±6.81^{xy} (209)	36.76±6.70^{xy} (122)	25.95±6.72^{xy} (87)	12.22±4.56^{xy} (45)	0.49^{x} (2)
		Male	125	84.17±6.25^{y} (106)	61.25±8.81^{y} (81)	41.42±7.93^{xy} (56)	25.61±6.70^{xy} (36)	1.50^{x} (2)
	Katjang	Female	132	70.32±13.87^{y} (109)	29.50±9.26^{xy} (43)	17.60±11.37^{xy} (28)	13.63±8.52^{xy} (23)	0^{x} (0)
		Male	203	73.14±5.22^{y} (147)	64.99±4.52^{y} (129)	49.78±4.35^{y} (100)	28.18±2.79^{y} (57)	0^{x} (0)
25-27	Boer	Female	105	49.46±14.4^{xy} (52)	32.15±11.71^{xy} (31)	31.37±11.19^{xy} (30)	16.85±6.72^{xy} (15)	0^{x} (0)
		Male	53	58.12±26.99^{xy} (32)	50.52±27.00^{xy} (28)	48.05±26.72^{y} (26)	18.80±9.86^{xy} (12)	0^{x} (0)
	Katjang	Female	68	55.27± 5.27^{xy} (38)	29.21±10.79^{xy} (19)	26.58±13.42^{xy} (17)	9.65±7.02^{xy} (6)	0^{x} (0)
		Male	41	27.30±16.19^{x} (12)	17.39±17.39^{x} (8)	4.35±4.35^{x} (2)	0^{x} (0)	0^{x} (0)

^{xy} Means with different superscript in a column were significantly different ($P<0.05$).
* *In vitro* developmental rate were based on number of fused oocytes
(n) No. of samples

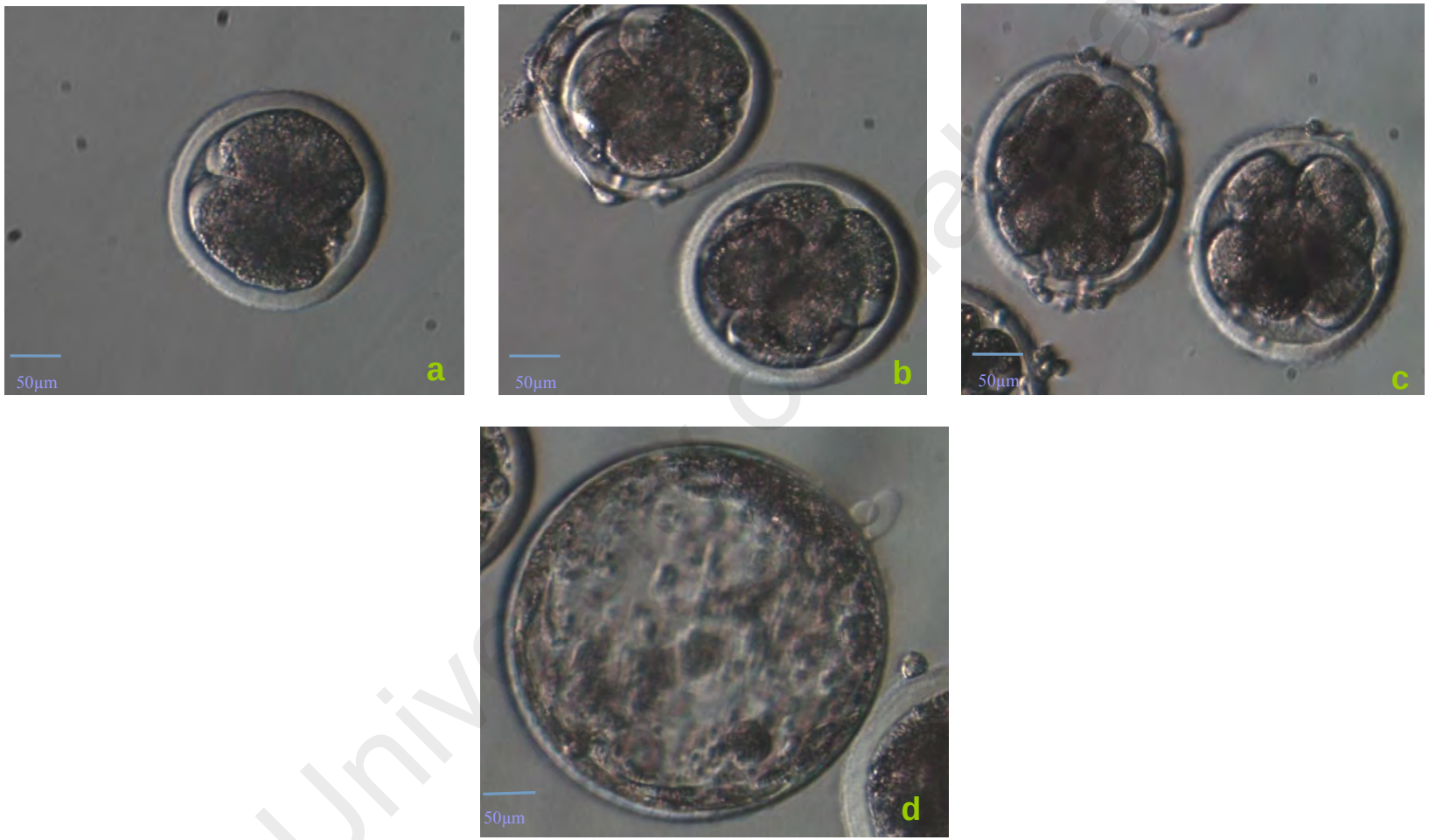

Figure 4.3: *In vitro* development of interspecies cloned caprine embryos at a) 2-cell, b) 4-cell, c) 8-cell and d) blastocyst (10x magnification) stages viewed under inverted microscope.

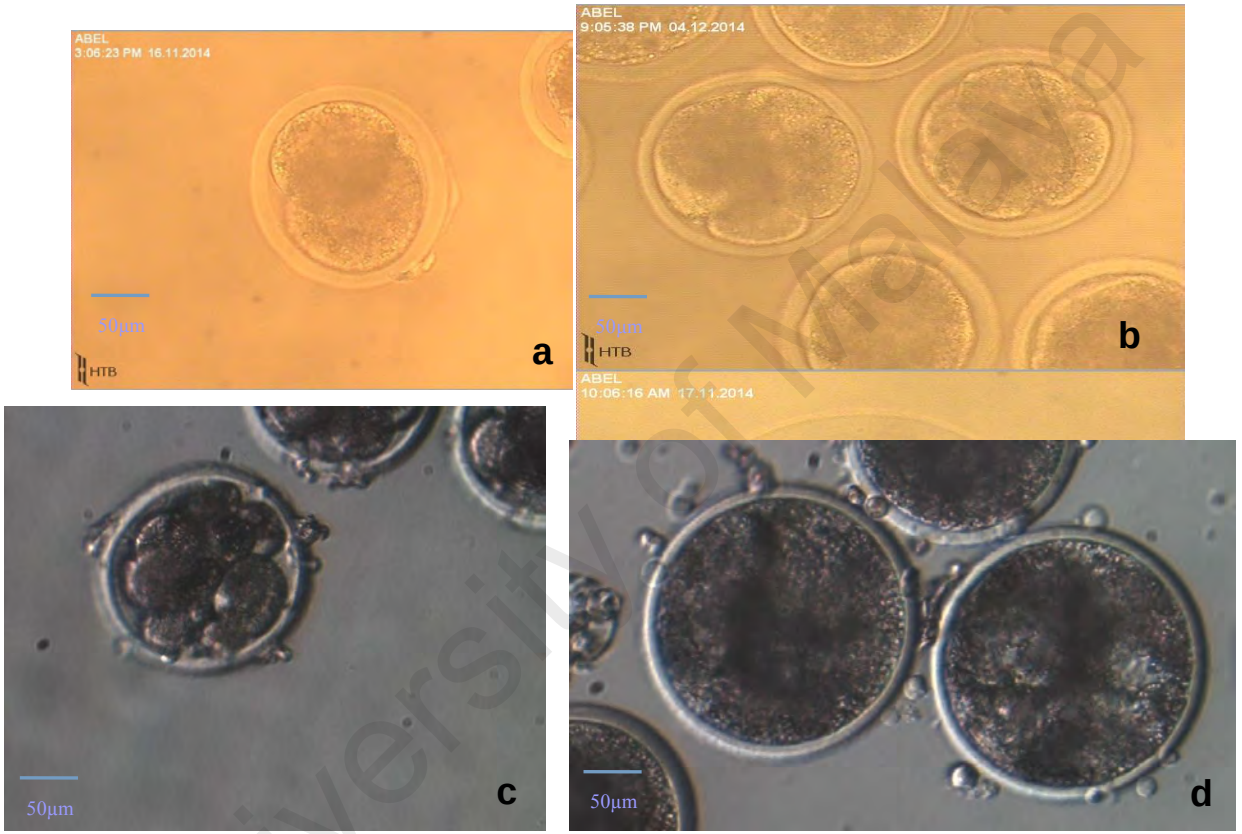

Figure 4.4: *In vitro* development of bovine parthenotes embryos at a) 2-cell, b) 4-cell, c) 8-cell and d) early blastocysts (10x magnification) stages viewed under inverted microscope.

4.3 EFFECT OF TRICHOSTATIN A (25 nM) SUPPLEMENTATION IN *IN VITRO* CULTURE MEDIUM ON INTERSPECIES CLONED CAPRINE EMBRYOS DEVELOPMENT COMPETENCY (EXPERIMENT 3)

In order to improve the blastocyst rate of the interspecies cloned caprine embryos, histone deacetylase inhibitor, trichostatin A was supplemented into the *in vitro* culture medium to enhance the expression of the transcriptionally silent alleles of imprinted genes (Pedone *et al.*, 1999). Therefore, 25 nM of TSA was supplemented in KSOM A for treating post-activated cloned embryos and in KSOM B for treating 8-cell cloned embryos, for 12 hours.

4.3.1 Effect of trichostatin A (25 nM) supplemented at post-activated cloned embryos on *in vitro* development of interspecies cloned caprine embryos

A total of 227 post-activated cloned caprine embryos were treated with trichostatin A (25nM). No significant different was observed in the *in vitro* developmental rate of trichostatin A-treated interspecies cloned caprine embryos compared with the non-treated interspecies cloned caprine embryos ($P>0.05$).

Table 4.7: *In vitro* development of interspecies cloned caprine embryos when TSA (25 nM) was supplemented at at post-activated cloned embryos compared with control (mean±SEM)

TSA treatment	No. of fused oocytes	*In vitro* developmental rate of cloned embryos, % (n)*				
		2-cell	4-cell	8-cell	Morula	Blastocyst
Post- activated cloned embryos	227	64.81± 6.93^{x} (160)	46.63± 5.97^{x} (111)	39.10± 7.42^{x} (95)	21.77± 2.96^{x} (53)	4.88±1.23^{x} (13)
Non-treated (Control)	125	84.17± 6.25^{x} (106)	61.25± 8.81^{x} (81)	41.42± 7.93^{x} (56)	25.61± 6.70^{x} (36)	1.50^{x} (2)

Means with superscript „x" in a column were not significantly different (P>0.05).
* *In vitro* developmental rate were based on number of fused oocytes
(Control) Control experiment using *in vitro* development rate of Boer male interspecies cloned embryos
(n) No. of samples

4.3.2 Effect of trichostatin A (25 nM) supplemented at post-activated cloned embryos and 8-cell stage on morula and blastocyst rate of interspecies cloned caprine embryos

In this experiment, trichostatin A (25 nM)was supplemented at 8-cell stage. The development of morula and blastocyst were compared with the morula and blastocyst rate which supplement at post-activated cloned embryos and control experiment from Table 4.7.

As shown on Table 4.8, no significant different of morula and blastocyst rate of interspecies cloned caprine embryos whether it was treated at post-activated cloned or 8-cell as well as non treated cloned embryos. At morula stage, the rate of non-treated is insignificantly highest and Trichostatin A treated at post-activated cloned embryos showed insignificantly highest number and rate of blastocyst (P>0.05).

Table 4.8: *In vitro* development of interspecies cloned caprine embryos compared with when trichostatin A (25 nM) supplemented at different stages of embryos development compared with control experiment (mean±SEM)

TSA treatment	*In vitro* development*, % (n)	
	Morula	Blastocyst
Post-activated cloned embryos	21.77±2.96^{x} (53)	4.88±1.23^{x} (13)
8-cell	15.15±4.09^{x} (37)	2.50±1.11^{x} (6)
Non treated (Control)	25.61±6.70^{x} (36)	1.50^{x} (2)

Means with superscript „x" in a column were not significantly different (P>0.05).
* *In vitro* developmental rate were based on number of fused oocytes
(Control) Control experiment using *in vitro* development rate of Boer male interspecies cloned embryos
(n) No. of samples

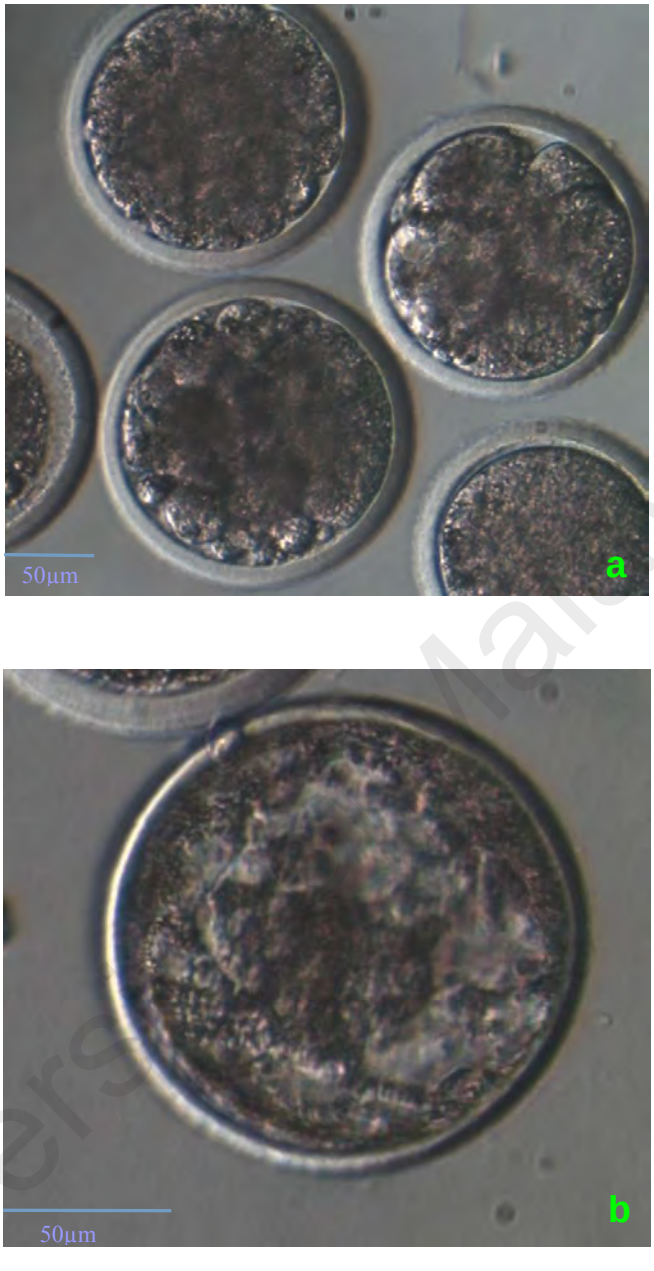

Figure 4.5: *In vitro* developmet of interspecies cloned caprine embryos at a) 8-cell and morula (10x magnification) and b) early blastocyst (20x magnification) stages after trichostatin A treatment viewed under inverted microscope.

4.4 USING QUICK FREEZING TECHNIQUE TO CRYOPRESERVE THE MOUSE EMBRYONIC FIBROBLAST (MEF) DERIVED FROM DIFFERENT FOETAL AGES AS FEEDER CELL FOR WHOLE BLASTOCYST CULTURE (PRELIMINARY CAPRINE ESC CULTURE) (EXPERIMENT 4)

The different foetal ages of murine were used to determine the quality of mouse embryonic fibroblast cell layer to be subsequently used culturing the caprine embryonic stem cell (ESC) culture. This experiment was also designed to determine the effect of cryopreserving mouse embryonic fibroblast derived from different foetal ages using quick freezing technique. Briefly, the murine females were superovulated and mated. The 14 and 15 days *post coitum* mouse foetuses were collected and sacrificed and subsequently the mouse embryonic fibroblasts were cultured up to Passage 2 and cryopreserved using quick freezing technique. The mouse embryonic fibroblasts were then inactivated prior culturing the caprine blastocysts onto the mouse embryonic fibroblasts.

4.4.1 Effect murine foetal age on viability rate of mouse embryonic fibroblast culture

Mouse embryonic fibroblast cell line at Passages 1 and 2 were successfully established using foetuses at 14 and 15 days *post coitum* (d.p.c). Table 4.9 shows the viability rate of Passages 1 and 2 of mouse embryonic fibroblast derived from different foetal ages. No significant difference on viability rate was observed between the 14 and 15 days *post coitum* ($P>0.05$). No significant differences was observed between the Passages 1 and 2 of both foetal ages ($P>0.05$).

Table 4.9: Viability rate of Passages 1 and 2 mouse embryonic fibroblast when using different foetal ages (mean±SEM)

Foetal ages (d.p.c)	No. of replicates	Percentage of viability, %	
		Passage 1	Passage 2
14	6	95.68±1.2^{ax}	91.99±1.2^{ax}
15	12	95.40±0.7^{ax}	91.10±1.9^{ax}

Means with superscript „a" in row were not significantly different (P>0.05).
Means with superscript „x" in a column were not significantly different (P>0.05).

4.3.2 Effect of quick freezing technique on viability of mouse embryonic fibroblast derived from different foetal ages

Quick freezing technique was used to cryopreserve the mouse embryonic fibroblast culture. As shown in Table 4.10, the frozen-thawed passages of the mouse embryonic fibroblast cell cultures were significantly lower compared with fresh passages ($P<0.05$), for between the passages and foetal ages.

Between the passage numbers, the frozen-thawed Passage 1 of the mouse embryonic fibroblast derived from foetal age of 15 days *post coitum* was significatly lower ($P<0.05$) compared with foetal age of 14 days *post coitum* (79.40% vs. 87.95%, respectively). However, for frozen-thawed Passage 2, no significant difference was observed between the foetal ages ($P>0.05$).

4.3.3 Attempt to culture the embryonic stem cell on the inactivated of mouse embryonic fibroblast feeder cell layer

In order to produce embryonic stem cell like culture, inner cell mass of blastocysts derived from interspecies cloned caprine, caprine *in vivo* fertilised and bovine parthenotes were cultured on the inactivated frozen-thawed mouse embryonic fibroblast feeder cell layer using whole blastocyst culture technique. However, no attachment was recorded for all the attempt (Table 4.11). Based on the observation, the blastocysts floated away from feeder cell alyer for more than 7 days and was considered failed to attached.

Table 4.10: Viability rate of fresh and frozen-thawed mouse embryonic fibroblast culture derived from different foetal ages (mean±SEM)

Foetal ages (d.p.c.)	No. of replicates	Percentage of viability (%)			
		Passage 1		Passage 2	
		Fresh	Frozen-thawed	Fresh	Frozen-thawed
14	6	95.68 ± 1.2^{bx}	87.95 ± 0.9^{ay}	91.99 ± 1.2^{bx}	83.33 ± 1.1^{ax}
15	12	95.40 ± 0.7^{bx}	79.40 ± 2.7^{ax}	91.10 ± 1.9^{bx}	81.80 ± 0.9^{ax}

^{a,b}Means with different superscripts in row were significantly different ($P<0.05$).
^{x,y}Means with different superscripts in a column within a group were significantly different ($P<0.05$).
* Viability rate

Table 4.11: Attempt to produce of embryonic stem cell on inactivated mouse embryonic feeder cell layer (MEF) using caprine and bovine (as model) blastocysts

No	Mouse embryonic feeder cell layer properties				Blastocyst properties			Attachment of blastocyst on MEF
	Fresh/ frozen/thawed	Foetal age	Passage number	Inactivation technique	Types of embryos	Blastocyst stage	Number of blastocyst	
1	Frozen-thawed	14	1	With mitomycin C	*In vivo*	Mid-blastocyst	3	-
2	Frozen-thawed	14	2	With mitomycin C	Interspecies cloned	Mid-blastocyst	2	-
3	Frozen-thawed	15	2	With mitomycin C	Parthenotes (bovine)	Mid-blastocyst	5	-
4	Frozen-thawed	15	1	With mitomycin C	Interspecies cloned	Mid-blastocyst	1	-
5	Frozen-thawed	15	2	With mitomycin C	Parthenotes (bovine)	Early blastocyst	3	-
6	Frozen-thawed	14	2	With mitomycin C	Interspecies cloned	Mid-blastocyst	1	-

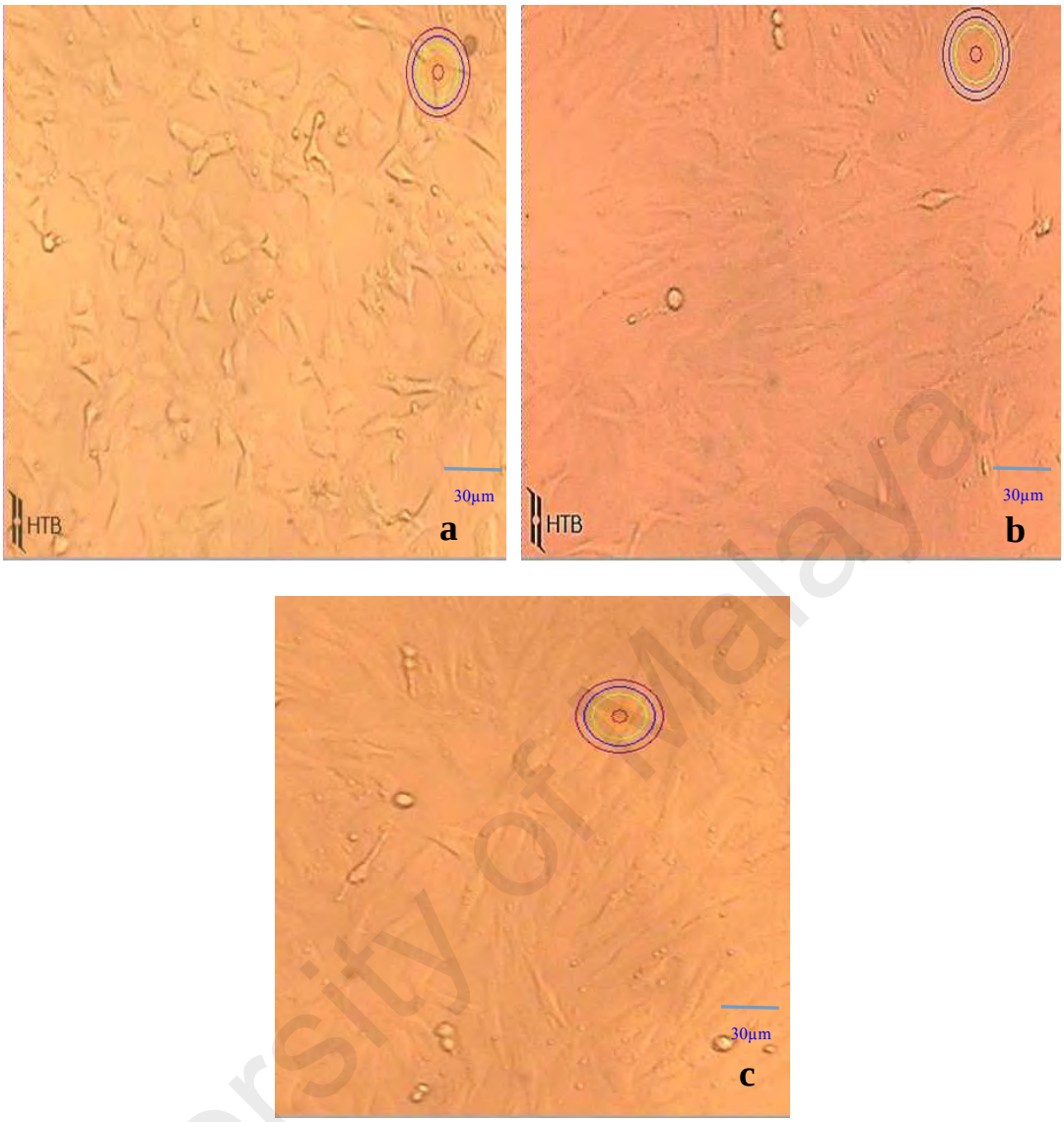

Figure 4.6: Confluency of mouse embryonic fibroblast culture of a) fresh passages at <80% confluency, b) fresh passages at >80% confluency and c) frozen-thawed passages at >80% confluency viewed under inverted microscope (magnification 20x).

Chapter 5

5.0 DISCUSSION

5.1 INTRODUCTION

Interspecies somatic cell nuclear transfer involved several complex molecular events for reprogramming. It takes a lot of stringency for the interspecies somatic cell nuclear transfer embryos to develop from the activation process to full term development (Oback *et al*., 2009). Successful interspecies cloned blastocysts were reported with a range of 5% to 33% of blastocyst rate, which included buffalo-cattle (Kitiyanant *et al*., 2001), giant panda-rabbit (Chen *et al*., 2002), banteng-cattle (Sansinena *et al*., 2005), yak-cattle (Li *et al*., 2006), goat-sheep (Ma *et al*., 2008), marbled cat-domestic cat (Thongphakdee *et al*., 2010) and goat-cattle (Song *et al*., 2008; Kwong *et al*., 2012, 2014; Soh *et al*., 2012). Although the efficiency of somatic cell nuclear transfer has improved throughout these two decades, efforts to improve the success rate are continuing in an attempt to make this technology of increased commercial interest to livestock breeders or to produce the embryonic stem cell for medical purposes. The pressing need to improve the efficiency of somatic cell nuclear transfer may require a modified or entirely novel methodology to be established (Do and Taylor-Robinson, 2014). Due to limited information on interspecies somatic cell nuclear transfer, especially in caprine, more research should be conducted to optimise the protocol for interspecies somatic cell nuclear transfer caprine production. In our laboratory, Animal Biotechnology-Embryo Laboratory (ABEL), University of Malaya, several studies were conducted in interspecies somatic cell nuclear transfer in caprine and caprine embryonic stem cell studies, which are listed in Table 1.1 in Chapter 1 (Introduction).

In the present study, an attempt to increase the efficiency of the interspecies somatic cell nuclear transfer in caprine, in a goal to produce more blastocysts which subsequently used for culturing the embryonic stem cell outgrowth on mouse embryonic fibroblast feeder cell layer. This attempt includes in determining the effect of *in vitro* maturation duration of the bovine cytoplasts, the effect of breed and gender of ear fibroblast as donor karyoplast and supplementation of trichostatin A in the *in vitro* culture medium. The present study also includes the culture of cryopreservation of ear fibroblast which was used as donor karyoplast in this study. As preliminary experiment for caprine embryonic stem cell study, mouse embryonic fibroblast were produced from different foetal ages of murine which subsequently be used as feeder cell layer for attempt to culture embryonic stem cell from *in vivo* and interspecies cloned caprine and bovine parthenotes blastocysts.

5.2 EFFECT OF BREED, GENDER AND CRYOPRESERVATION ON VIABILITY RATE OF EARLY PASSAGES OF CAPRINE EAR FIBROBLAST CELL CULTURE (EXPERIMENT 1)

In this study, Boer and Katjang ear fibroblast cell cultures from both female and male donors were successfully developed up to 3 passages. Generally, no significant effect of breed and gender on viability rate of fresh early passages of the ear fibroblast culture. However, the Boer female at Passage 2 showed significantly higher viability rate whereas Katjang female at Passage 1 showed significantly lower viability rate. The reason for this different is unknown, since molecular investigation to clarify this result was not carried out. Significant variation of the several passages of fresh ear fibroblast cell could be due to inconsistent of skill efficiency of the researcher, rather than effect of molecular or genetic of the genders and breeds on the viability rate of the ear fibroblast cell culture.

The reports on the effect of breed and and gender of the establishment of ear fibroblast cell line,especially in caprine are scarce. Most establishment of the caprine ear fibroblast cell line focused on one specific breed without comparison between female and male effect (Gupta *et al.*, 2007; Li *et al.,* 2009b; Bai *et al.*, 2012). Singh and Sharma (2011) established caprine ear fibroblast cell line from two different breeds, Saanen and Kiko. They reported that there were no significant differences on viability rate between the two breeds. The result is in agreement with the current finding which generally showed no significant different between the breed on the viability rate of the ear fibroblast cell culture.

Morphological observation is one of the most important qualitative parameters of epidermal tissue reconstitution which is included in this study to evaluate the efficiency of ear fibroblast production as donor karyoplast for interspecies cloned caprine embryos.

From the morphological observation through inverted microscope, the cells from both female and male Boer and Katjang were in radiating, elongated, fibrous and thin distinctive shape (Figure 4.1). This typical morphology of the ear fibroblast cell with fibrous characteristics was also observed by on Gaddi, Jining Black and Grey caprine ear fibroblast cell (Gupta *et al.*, 2007; Li *et al.,* 2009b; Bai *et al.*, 2012) as well as on Mongolian horse, Siemman cattle and Ujumqin sheep ear fibroblast cell (Li *et al.,* 2009a; Li *et al.*, 2009c; Na *et al.*, 2010). No morphological difference was observed between Boer and Katjang ear fibroblast cell throughout all three passages.

There were many reports on the presence of the epithelial cells which contaminated the purity of the ear fibroblast cell line which derived from the ear tissues (Li *et al.,* 2009a; Li *et al.,* 2009b; Li *et al.*, 2009c; Na *et al.*, 2010; Bai *et al.*, 2012). Therefore, it was suggested that after 2-3 passages, a pure ear fibroblast cell line could be obtained (Bai *et al.*, 2012; Zhou *et al.*, 2013). Interestingly, the caprine ear fibroblast culture in this study did not experienced the contamination of the epithelial cells in Passages 1 to 3 for both Boer and Katjang. Although there was presence of the epithelial cells was observed during the primary culture of the ear fibroblast cell, however, the pure ear fibroblast culture was obtained in the Passage 1. This is because, fibroblast adhere more easily onto the culture dish surface and trypsinised more readily than epithelial cells (Li *et al.*, 2009b). Epithelial cells, on the other hand, do not adhere onto cell surface in short duration and could be easily shed using gentle mechanical agitation and trypsinisation (Ren *et al.*, 2002). Therefore, the fibroblast will quickly outgrow the epithelial cells.

In this experiment, the cell line was established until Passage 3 only. This is because culturing somatic cells especially for prolonged periods is known to alter ploidy, genomic stability and post-translation histone modifications, factors which are known to reduce

cloning efficiency (Jang *et al*., 2004; Kwong *et al.,* 2012). In addition, subpassaging to high number passages is synonym to the exposure of the cell line to excessive trypsinisation process. Excess trypsin digestion could adversely affect the heriditary characteristics of cell line as well as other biological characteristics (Li *et al.,* 2009a). Therefore, a minimum number of passages is suggested for preserving cell line.

Quick freezing technique was used in this experiment for cryopreservation of ear fibroblast cell. Results obtained showed that generally, no significant effect of the cryopreservation as the the viability rate between the frozen-thawed ear fibroblast cell culture was similar with the fresh ear fibroblast cell for Katjang male and Boer male. However, female Boer ear fibroblast frozen-thawed Passages 2 and 3 were significanlty lower viability rate compared to fresh passages. One of the finding in this experiment is frozen-thawed Katjang female at Passage 1 and Passage 3 had significantly lower viability rate compared to other breeds and gender. This suggests that Boer and Katjang female ear fibroblast cell is not suitable to be cryopreserved by quick freezing technique especially at higher passages number. This finding also suggested that quick freezing technique is not reliable to cryopreserve female ear fibroblast cell. However, these hypothesis should be tested to higher passages number (Passage 3 and above) to prove it true. Further analysis on the genetic and molecular level could be conducted to study the correlation of the gender of the fibroblast and the viability of the frozen-thawed cells.

In quick freezing technique, the ear fibroblast cells were cryopreserved at two temperatures, started with 15 minutes of equilibration at -80°C followed by direct plunging into -196°C of liquid nitrogen for 15 minutes. It has short equilibration duration with fast cooling rate, which was -5°C/minutes. Mazur *et al.* (1972) stated that each biological cells has specific optimal cooling rate, with decreased survival at cooling rates that are too low

(slow-cooling damage) or too high (fast-cooling damage). When cells are cooled at slow rate, the intracellular water left in the cell is minimal, resulting in less risk of intracellular ice formation. However, it maximise the dehydration of the cells, which is not desired. On the other hand, when cooling rates are increased too much, the dehydration may not be fast enough to prevent intracellular ice nucleation (Mazur *et al.,* 1972). Quick freezing damage could be also be caused by rapid water flow through membrane pores which lead to an uneven distribution of pressure on the membrane (Muldrew *et al.,* 2004) and resulting in sudden changes in size, shape and ultrastructure, caused by the rapid efflux of water (Woelders *et al.,* 1997). In the current study, the viability rate of frozen-thawed ear fibroblast cell ranges at 78% to 88%, which was lower compared to other studies which used conventional freezing (-80^{0}C for 12 hours) which viability rate at range of 90% (Li *et al.,* 2009a; Li *et al.;* 2009b; Li *et al.*, 2009c; Na *et al.*, 2010; Bai *et al.*, 2012).

Despite of the disadvantages of the quick freezing technique listed above, it has several advantages to be used as a reliable method for cryopreserving the ear fibroblast cell. Prolonged exposure to hypertonic freezing medium induced osmotic stress which resulted in irreversible damage to the cell integrity (Fuller and Paynter, 2004). This finding was also supported by Higgins *et al.* (2011) who obtained higher post-thawed viability rate on rat neural cell when freezing at faster cooling rate. Moreover, the cells are exposed to the unfavourable conditions for a shorter period of time and caused less intracellular dehydration, intracellular solute concentration and shrinkage of the cells. By using quick freezing technique, the Boer and Katjang fibroblast cell could be cryopreserved in the cheapest and simplest way. The cell line could be used and stored for more than 3 years for subsequent experiment of somatic cell nuclear transfer.

There are many factors that affect the cryopreservation of the ear fibroblast cell

including the types of medium, serum percentage as well as type of cyoprotecting agent. Usually, the freezing medium consists of similar constituent of the culture medium but with different concentrations of serum and without the presence of antibiotics. Li *et al.* (2009a) used 10% DMSO+50% foetal bovine serum+40% DMEM placed in a −80°C freezer overnight prior transferred to a liquid nitrogen storage system successfully obtained 93% viability rate after thawed. Li *et al.* (2009b) reported 10% dimethyl sulfoxide (DMSO), 30% FBS and 60% DMEM and the tubes were placed at 4°C for 20–30 minutes to enable the DMSO to permeate efficiently prior placed in liquid nitrogen for long-term storage obtained 92% of viability rate after thawed.

The similar morphological characteristic and comparable viability rate of the ear fibroblast suggesting that stable production of ear fibroblast cell line could be produced using adult female and male Boer and Katjang up to three passages. These cell lines subsequently could be used as donor karyoplast for interspecies cloned embryos. Based on these finding, it was concluded that the effect of the passages number (Passages 1 to 3) will be insignificant to cloning efficiency. Experiment conducted by not taking consideration of different passages in cloning experiment.

5.3 EFFECT OF *IN VITRO* MATURATION DURATION OF BOVINE OOCYTES AS WELL AS BREED AND GENDER OF DONOR KARYOPLAST ON *IN VITRO* DEVELOPMENT COMPETENCY OF INTERSPECIES CLONED CAPRINE EMBRYOS (EXPERIMENT 2)

5.3.1 Effect of maturation duration on maturation rate of bovine oocytes (Sub-experiment 1)

No significant difference of maturation rate of bovine oocytes was found between maturation duration of 22-24 hours and 25-27 hours. High maturation rate up to 85% of maturation rate was successfully obtained when using both maturation duration. Therefore, both maturation duration could be used for *in vitro* maturation of the bovine oocytes obtained from the slaughterhouse.

Large numbers of growing follicles in bovine ovaries offer a large pool of oocytes for manipulation to produce embryos of genetically valuable animals. To utilise these oocytes efficiently, it is important to develop culture systems for *in vitro* develoment that permit oocytes to acquire the maturation in term of nuclear, cyptolasmic and molecular. *In vitro* maturation of bovine oocytes is affected by several factors, including transport time and temperature from the slaughterhouse to the laboratory, follicle size, developmental stage of oocyte, oocyte size and composition of media (Pavlok *et al.*, 1992; Hyttel *et al.*, 1997; Izquierdo *et al.*, 2002; Habsah *et al.*, 2009).

Duration of maturation is one of the important factors to ensure the optimal accomplishment of oocytes maturation. Literature reported different optimum *in vitro* maturation duration in bovine between 18 hours (Park *et al.*, 2005) and 24 hours maturation duration (Ward *et al.*, 2002) as it is known metaphase II stage occur 18 to 24 hours after the

beginning of *in vitro* maturation. However, these results disagreed with Semple *et al.* (1993) who reported higher bovine blastocyst production from oocytes matured for only 14 hours. These conflicting findings may be accounted for by the presence of many factors affecting. It took between 19 and 25 hours *in vivo* to extrude the first polar body after the luitenising hormone (LH) surge (Kruip *et al.*, 1997), but it varied between 12 and 24 hours *in vitro* (van der Westerlaken *et al.*, 1994). In *in vitro*-matured bovine oocytes, the changes in level of oxidative stress (Morado *et al.*, 2009), mitochondrial activity (Tarazona *et al.*, 2006; Nabenishi *et al.,* 2012) and ATP content (Nagano *et al.*, 2006) during *in vitro* maturation culture for less than 24 hours.

There are three levels of maturation during *in vitro* and *in vivo* maturation of oocytes which are meiotic, cytoplasmic and molecular maturation. Meiotic maturation is the fall of nuclear events triggered by the LH surge or by the removal of the oocyte from follicular environment. These events are programmed to occur in the oocyte upon the removal of a still unidentified inhibitory substance. Once allowed to proceed, maturation promoting factor is synthesised and activated. Subsequently, the cell cycle machinery activated and the oocyte underwent the metaphase I and extrusion of the first polar body before arresting after the formation of the metaphase II. The timing of meiotic maturation is quite precise and defined (Sirard *et al.*, 2006). Cytoplasmic maturation could only be detected based on ultrastructural observations during the few days before the LH surge. The oocyte stopped at preparation phase (RNA and protein synthesis) by modifying the transcription and translation by ribosome (Krisher, 2004). A second series of changes occur close to the LH surge which result in a redistribution of organelles such as the mitochondria and the cortical granules along with the changes occurring with the cell progression to metaphase (Rizos *et al.*, 2004).

Most of fully grown oocytes undergo normal meiotic and cytoplasmic maturation although not all of them will be successfully developed to the blastocyst stage. The difference between a developmentally capable oocyte and an incompetent one was the differentiation state of the follicle of origin and these differences could not be detected at the ultrastructural level (Sirard *et al.*, 2006). Hypothetically, specific mRNA and some proteins are produced and added to the oocyte in the last few days before ovulation which resulting in altering the developmental ability of the gamete produced. Since no clear discrepancies associated with the first two types of oocyte maturation have been identified to be responsible for developmental competence, it was believed that molecular maturation represents the closest association with the intrinsic capacity of an oocyte to reach the blastocyst stage and probably beyond (Sirard, 2001).

However, optimising the maturation duration based on the maturation rate alone was not enough to evaluate the overall efficiency of the matured oocytes (derived from the maturation duration) to reprogramme the donor karyoplast to produce the interspecies cloned caprine embryos. Thus, in order to find the optimum maturation duration for bovine cytoplasts to be used in interspecies cloned caprine embryos, evaluation on the in vitro developmental competency is necessary.

5.3.2 Effect of *in vitro* maturation duration of cytoplast as well as breeds and genders of donor karyoplast on *in vitro* developmental rate of interspecies cloned caprine embryos (Sub-experiment 2)

In order to evaluate the maturation duration efficiency, the *in vitro* development of bovine parthenotes and interspecies cloned caprine embryos were compared between two range of maturation duration. The current result showed that bovine blastocyst could be produced by parthenogenetic activation of bovine oocytes (control) matured at both maturation duration (22-24 hours and 25-27 hours). However, only bovine oocytes matured at 22-24 hours could be used to successfully produced interspecies cloned caprine blastocysts.

The current result was in agreement with bovine, murine and ovine somatic cell nuclear transfer and parthenogenetic activation studies which obtained high cleavage and blastocyst rates when using metaphase II oocytes (Takano *et al.*, 1993, Li *et al.*, 2007, Shirazi *et al.*, 2009). The aged oocytes (long maturation duration) had higher activation rates but lower developmental potential than young oocytes (Shirazi *et al.*, 2007). However, Shen *et al.* (2008) reported no differences in bovine parthenotes cleavage and blastocyst rates were observed between the oocytes matured for 20 and 24 hours. In contrast, previous studies in bovine and murine reported the low response of young oocytes to parthenogenetic activation (Nagai, 1992; Tsunoda and Kato, 1993) and aged oocytes were often used as recipient oocytes for nuclear transfer in murine and rabbit has higher blastocyst rate (as reviewed by Tanaka and Kanagawa, 1997).

As LH surge occurred and nuclear maturation took place, the oocyte continued meiosis to progress from the arrested prophase I to metaphase I, anaphase I, telophase I, and cytokinesis with unequal cytoplasmic distribution, when half of the chromosomes are

discarded in the first polar body. The oocyte progresses to meiosis II and is arrested at metaphase II. Following fertilisation or artificial activation, the metaphase II–arrested oocyte resumes meiotic progression once more to complete metaphase II, anaphase II, and telophase II, discarding again half the number of chromosomes in a second polar body, achieving a haploid number of chromosomes. Enucleation for somatic cell nuclear transfer technique could be conducted at any stage, however, mostly conducted when oocytes at metaphase II. Racedo *et al.* (2012) reported that at 24 hour of maturation the formation of mini-Golgi apparatus that were exclusively located in the cortical area which suggesting that fragmentation is a prerequisite for distribution throughout the cytoplasm after germinal vesicle break down stage.

For somatic cell nuclear transfer, it is most important that the oocyte undergoes cytoplasmic maturation to construct a developmentally competent embryo, while nuclear maturation is less important because the oocyte DNA is eliminated at enucleation and does not form part of the embryo.As the cumulus oocyte complexes is left undisturbed during the entire *in vitro* maturation period, thus increasing the probability of cytoplasmic maturation. In order to obtain high *in vitro* developmental competency post-somatic cell nuclear transfer, young matured oocyte at metaphase II are required (22 to 24 hour of maturation).

One of important factors that need to be considered is the time taken for researcher to complete the whole protocol of somatic cell nuclear transfer. In current study, the complex and tedious protocol could last for more than 3 hours for 30 matured oocytes. Therefore, using the aged oocytes is not advisable as the time-consuming cloning procedure can increase the age of the oocytes prior to *in vitro* culture. Inappropriate timing of maturation could lead to oocyte aging (Hunter, 1989; Hunter and Greve, 1997), the formation of

abnormal chromatin (Dominko and First, 1997) and impairment in embryo development (Marston and Chang, 1964). As the age of the oocytes at the time of MII arrest increased, a decrease in the ability of bovine oocytes to cleave and develop to the blastocyst stage (Dominko and First, 1997).

The *in vitro* developmental rate of male embryos for both Katjang and Boer at 4-cell and 8-cell was higher than female embryos. However, no significant effect of gender of ear fibroblast karyoplast on cleavage and morula rates of the cloned interspecies Katjang and Boer embryos. Present finding was in agreement with Sansinena *et al.* (2005) who obtained higher *in vitro* developmental rate of cloned male banteng using interspecies approach. Several intraspecies studies also reported higher *in vitro* developmental rate were obtained in male on bovine, canine and caprine (Chen *et al.*, 2003; Kim *et al.*, 2009; Yaun *et al.*, 2012). However, the present result contradicted Yin *et al.* (2006) who worked intergenus somatic cell nuclear transfer in leopard cat-domestic cat who found no significant effect of the gender. No significant effect of gender on the *in vitro* development of cloned embryos using intraspesies somatic cell nuclear transfer studies of bovine and ovine (Kato *et al.*, 2000; Hosseini, 2004).

Despite their phenotypic similarities, male and female embryos present some major differences regarding their preimplantation development pattern. Male embryos develop faster (Avery *et al.,* 1992) and reach the blastocyst stage more frequently (Xu *et al.,* 1992), compared to female embryos during *in vitro* culture (Edwards *et al.*, 2001). Female bovine blastocysts derived from *in vitro* fertilisation displayed lower cell numbers and increased apoptosis rates than male embryos (Oliveira *et al.*, 2010).

The main molecular differences between male and female embryos during *in vitro* development is the double expression levels of X-linked genes. Female embryos had

present two active X chromosomes at the beginning. Therefore, one of them must be inactivated at appropriate duration for further *in vitro* development. The inactivation or better known as dosage compensation, took place in the inner cell mass at the blastocyst stage (Augui *et al.,* 2011). At the onset of this process, one X chromosome is coated by the upregulated XIST transcript, followed by asynchronous replication timing, and finally chromatin modifications set the stable inactive and condensed chromatin state (Augui *et al.,* 2011). Silencing of X chromosome might be initiated in the absent of XIST, but XIST RNA is required for imprinted X chromosome inactivation (XCI) and stabilisation of silencing (Kalantry *et al.,* 2009). XIST dependence for XCI appeared to be stronger at genes that had acquired dosage compensation for longer time during evolution.

XCI in bovine occurred between Days 7 and 14 *in vivo* (Bermejo-Alvarez *et al.*, 2011). However, XCI-related mechanisms could be initiated *in vitro* as early as at the 2-cell stage, when low XIST expression can be detected, even though late replication could only be detected at early blastocyst stage (De La Fuente *et al.,* 1999). Higher levels of XIST expression are detected on *in vitro* derived embryos (Wrenzycki *et al.,* 2002,; Nino-Soto *et al.,* 2007). This will make the reprogramming process become more difficult and inappropriate which lead to embryonic block (Oback and Wells, 2007). Over expression of the X-inactive specific transport may decrease the cloning efficiency observed in female (Wrenzicky *et al.,* 2002).

The current finding also study the capability of cytoplast derived from different maturation duration to reprogramme ear fibroblast karyoplast from different breeds and gender. However, only 22-24 hour matured oocytes could reprogramme the female and male Boer embryos up to blastocyst stage. Whereas, the male Katjang embryos showed lowest *in vitro* developmental rate and using 25-27 hours matured oocytes and only

developed up to 8-cell stage. Interestingly, female and male embryos of Boer and Katjang showed no significant effect on their *in vitro* developmental rate when using bovine cytoplast matured at 22-24 hours and 25-27 hours. However, the reduction of *in vitro* developmental rate when using 25-27 hours between embryos stages was apparent. This showed that, there was a systematic embryonic block when using bovine cytoplast matured at later maturation duration as the age of matured oocytes increased.

Up to date, there is no report on relationship between *in vitro* maturation of cytoplast with breed and gender of karyoplast on production of interspecies cloned goat embryos. Present result on *in vitro* developmental rate of the interspecies cloned Boer and Katjang embryos gave higher cleavage rate (2-cell) and morula rate than Abdullah *et al.* (2011) who worked with Jermasia breed and 22-24 hour maturation duration. The cleavage rate obtained when using female karyoplast was comparable with Kwong *et al.* (2012). However, male-derived karyoplast showed slightly better result in 4-cell and 8-cell than Abdullah *et al.* (2010) and Soh *et al.* (2012). In current study, the interspecies cloned Katjang embryos could not develop beyond morula stage even though the same *in vitro* culture procedure was implemented with Kwong *et al.* (2012, 2014) and Soh *et al.* (2012) who obtained blastocyst. Asdiana (2014) reported that interspecies cloned caprine embryos stopped at morula stage when using 24-27 hour maturation duration.

5.4 EFFECT OF TRICHOSTATIN A (25 nM) SUPPLEMENTATION IN *IN VITRO* CULTURE MEDIUM ON INTERSPECIES CLONED CAPRINE EMBRYOS DEVELOPMENT COMPETENCY (EXPERIMENT 3)

As discussed in the Section 5.3, bovine cytoplasts were proven to be capable to reprogramme the caprine cell for constructing the interspecies cloned caprine embryos. However, low blastocyst rate and the developmental block in morula state (using Katjang ear fibroblast karyoplast) might be associated with a developmental cell block and mitochondrial incompability between the donor karyoplast and recipient cytoplast (Thongpakdee *et al.*, 2008). Incomplete donor karyoplast reprogramming and abnormal epigenetic reprogramming, including DNA methylation and histone modification, were thought to be related to low efficiency in interspecies cloned embryos (Chen *et al.*, 2006; Lee *et al.*, 2010).

Histone acetylation provides the great potential for unfolding chromatin to recruit different transcriptional factors. Removal of acetylated groups by histone deacetylases caused chromatin compaction and gene silencing (Johnstone, 2002; Shi *et al.*, 2008). Numerous and diverse types of histone deacetylases have been identified, including short-chain fatty acids such as valporic acid and phenyl butyrate and hydroxamic acid derivatives such as suberoylanilide hydroxamic acid, scriptaid, and the most commonly used, trichostatin A (TSA). Trichostatin A is an effective inhibitor of histone deacetylase, which resulting in increasing of acetylated histones pool (Yoshida *et al.*, 1990) and enhancing the expression of the transcriptionally silent alleles of imprinted genes (Pedone *et al.*, 1999).

In the present study, trichostatin A (25 nM) was supplemented in the *in vitro* culture medium at two stages of cloned embryos development, post-activated embryos and 8-cell stages. Trichostatin A was supplemented in KSOM A for the former and in KSOM B for the

latter. However, no significant difference was observed in the *in vitro* developmental rate of trichostatin A-treated interspecies cloned caprine embryos compared with the non-treated interspecies cloned caprine embryos. The rates of cloned morula and blastocyst showed no significant difference between the treated (both post-activated and 8-cell) and non-treated embryos. The current finding is similar in development of interspecies cat embryos and bovine parthenotes which showed no significant difference in morula and blastocyst rates between the trichostatin A treated (25 nM) and non-treated embryos (Wittayarat *et al.*, 2013). In contrast, treatment of caprine donor karyoplast with similar trichostatin A concentration and duration had significantly increased the blastocyst rate as well as the the total cell number of the blastocyst (Wang *et al.*, 2015). Although the treatment did not significantly increase the histone acetylation, the moderate increment in histone acetylation might play an important role in the improved reprogramming ability of the transferred chromatin.

Several studies on intraspecies somatic cell nuclear transfer used 50 nM trichostatin A as treatment to significantly increase the blastocyst rate of the interspecies cloned embryos in porcine (Zhao *et al.*, 2010), ovine (Hu *et al.*, 2012), and bovine (Sawai *et al.*, 2012; Wittayarat *et al.*, 2013). However, Maalouf *et al.* (2009) reported 5 nM trichostatin A enhanced the development of intraspecies cloned murine embryos with increasing the numbers of inner cell mass and live offspring compared to control. In contrast, no differences between the development rates of bovine interspecies cloned bovine embryos treated with 5 nM and 500nM of trichostatin A have been reported (Akagi *et al.*, 2011; Sawai *et al.*, 2012).

Similar to current results, trichostatin A treatment has been shown to have no effects on the embryonic development of interspecies cloned embryos in human–rabbit (Shi *et al.*,

2008), sei whale– cow (Bhuiyan *et al.*, 2010) and guar–cow (Srirattana *et al.*, 2012). In contrast, Wittayarat *et al.* (2013) reported that interspecies cat blastocyst rate significantly increased when treated with 50 nM of trichostatin A.

The difference in results might be associated with the trichostatin A applications (concentration, timing, and the onset of treatment), species-specific effects, and phylogenetic distance between the oocyte and somatic cell donor (Wittarayat *et al.,* 2013). The selection of optimised trichostatin A applications for different species might be an important key to improve the success rate in animal somatic cell nuclear transfer technique (Wang *et al.*, 2011), especially for interspecies somatic cell nuclear transfer, for which the genetic distance between the donor cell and recipient cytoplast is important.

In *in vitro* embryo production, the culture system strongly influenced embryonic development (Lonergan *et al*., 1999). The early embryogenesis is a complex process that uses maternal proteins and transcripts to support the development of the embryo until its genome activation (embryonic genome activation), leading to synthesis of new transcripts and proteins at the right amount and stage of development (Memili and First, 1999). Physiology and biochemistry of embryos from the early stage to blastocysts is different along with the morphological differences. Although the mammalian embryo has great plasticity, which allows it to survive *in vitro*, it usually shows lower quality and viability compared to *in vivo* cultured embryos (Lane, 2001).

One of the key parameters governing which genes are available for transcription and when those genes become available is the temporal control of chromatin structure (Latham and Schultz, 2001). A major determinant of chromatin structure is histones which package DNA into nucleosomes and are responsible for condensing the chromatin (Landsberger and Wolffe, 1995). The tight association of DNA with core histones and the linker histones

could render the DNA effectively inaccessible to transcription factors. During development, different linker histones associate with the chromatin of oocytes, early embryos, and later somatic cells (Dimitrov and Wolffe, 1996). These differences in linker histones affect chromatin structure because the different linker histones differ in their overall basicity and tightness of association with the DNA. The binding of core histones to DNA can be modulated by phosphorylation and acetylation (Poccia, 1986). Of these, acetylation of the lysine residues in the histone tails reduce the contact of the DNA with the core histones and provide access to the DNA by other DNA binding proteins (Dimitrov and Wolffe, 1996). The acetylation of histones is related to an increase in gene expression by permitting the access of transcription factors to DNA (Schubeler *et al.*, 2004).

Changes in histone protein expression occur at earlier stages. Histone synthesis in the early stage embryo is largely uncoupled from S phase and is regulated at the level of maternal mRNA recruitment. This reliance on maternally encoded mRNAs provides the necessary mechanism to support histone transitions during the early period of transcriptional silence. Changes in the post-translational modifications of histones are very striking during early cleavage stages. DNA replication appears to be facilitated by increased histone acetylation labeling reveals enhanced replication at the nuclear periphery that can be accelerated by inhibitors of histone deacetylase (Aoki and Schultz, 1999). These observations indicate that DNA replication facilitates the creation of the specialised domain of enhanced histone acetylation at the nuclear periphery, and that the enhanced degree of histone acetylation in turn enhances DNA replication. This functional link between a domain of increased histone deacetylation and DNA replication at the 2-cell stage provides a potentially important mechanism by which DNA replication and the widespread activation of gene transcription may be coordinated (Latham and Schultz, 2001).

Mechanism of how trichostatin A increased the expression of pluripotent genes in cloned embryos has not been clearly elucidated. Previous studies had indicated that inhibitors of deacetylases in somatic cells increased histone acetylation levels in amino acid residues, resulting in decondensation of dense chromatin regions (Tóth *et al.*, 2004). In cloned embryos, trichostatin A remodeled the constitutive heterochromatin into a zygotic-like organisation (Maalouf *et al*., 2009). As a result, the chromatin structure which relaxed after somatic cell nuclear transfer was exposed to oocyte proteins such RNA polymerases and transcription factors (Shogren-Knaak *et al*., 2006), promote transcription of genes associated with pluripotency (Li e*t al.*, 2008). Gomez *et al.* (2011) in their interspecies cloned cat study reported that histone acetylation modulated structural changes in the chromatin, allowing certain proteins to bind to the DNA and induced upregulation of proto-oncogene and pluripotent genes. It was clear that the analysis of an individual lysine in a specific histone did not confirm that gene upregulation was a consequence of histone hyperacetylation.

Our previous studies on interspecies cloned caprine embryos achieved ~8% blastocyst rate without trichostatin A treatment (Kwong , 2012; Soh, 2012). However, the embryonic block in morula stage (Asdiana, 2014) and low blastocyst rate in this study needed to be improved with the trichostatin A. Nevertheless, optimisation is needed for treatment of trichostatin A in term of concentration, duration or the stage of embryos to study the effect of trichostatin A in improving the *in vitro* development of the interspecies cloned caprine embryos. Here, it was confirmed that 25 nM of trichostatin A treated post-activated embryos and 8-cell stages did not improved the morula and blastocyst rates of interspecied cloned caprine embryos. . It was noted that this the first study to treat interspecies cloned caprine embryos with trichostatin A.

5.5 USING QUICK FREEZING TECHNIQUE TO CRYOPRESERVE THE MOUSE EMBRYONIC FIBROBLAST (MEF) DERIVED FROM DIFFERENT FOETAL AGES FOR FEEDER CELL FOR WHOLE BLASTOCYST CULTURE (PRELIMINARY CAPRINE ESC CULTURE) (EXPERIMENT 4)

Maintaining the embryonic stem cell in undifferentiated state is the most critical challenge in producing embryonic stem cell. Embryonic stem cells are derived from totipotent cells of inner cell mass isolated from the mammalian blastocyst and are capable of unlimited, undifferentiated proliferation *in vitro* (Morrison *et al*., 1997). Undifferentiated embryonic stem cells can be maintained for unlimited time in culturing media containing the cytokine, leukaemia inhibitory factor or mouse embyonic fibroblast (Smith *et al*., 1988). However, not all embryonic stem cell could be maintained undifferentiated with cytokine or leukimia inhibitory factor alone. For example, human embryonic stem cells cultures required mouse fibroblasts as feeder cells and cannot be maintained with leukaemia inhibitory factor for self-renewal (Thomson *et al.*, 1998).

Mouse embryonic fibroblast functions as feeder cell layer in embryonic stem cell culture (Evans and Kaufman, 1981; Smith, 2001; Turksen, 2002) as it forms intracellular junctions such as gap junctions, adherens junctions and tight junctions with embryonic stem cell (Ehmann *et al.*, 1998). Mouse embryonic fibroblast provides a suitable environment for the interplay of signaling networks that regulate the fate of embryonic stem cell (Xu *et al.,* 2001). Apart from that, this feeder cells function to provide adequate nutrition or biological signals, such as collagens, laminins and heparan sulfate proteoglycans (Hongisto *et al.*, 2012). Mouse embryonic feeder cell also secret various cytokines, such as fibroblast growth factor, bone morphogenetic protein-4, activin A, transforming growth factor-b1

(Brons *et al.*, 2007; Eiselleova *et al.*, 2009) for ensuring the optimum growth of embryonic stem cells. In brief, feeder layers can mimic the real environment *in vivo*, to some degree, for the embryonic stem cells to be cultured *in vitro*.

In the current study, no significant difference on viability rate between the foetal ages of 14 and 15 days *post coitum* was observed with 95% of viability rate of Passage 1 and 91% of viability rate of Passage 2. This finding was comparable than previous related research by Chung (2011) who obtained 88% of viability rate using foetal age of 12.5 to 14.5 days *post coitum*. Garfield (2011) found that when using foetal age of 14.5 days *post coitum* could generated high quality of mouse embryonic fibroblast cell line. However, the earlier age was found to yield lower quality of mouse embryonic feeder cell line. Jiang *et al.* (2015) reported that the best optimal age is in range of 12.5-13.5 days *post coitum,* however, they agreed that foetus at 15.5 days *post coitum* could be used to produce high yield of mouse embryonic fibroblasts.

Most mouse embryonic feeder cell layer used 12.5-13.5 days *post coitum* (Eisellova *et al.*, 2008; Diekmann *et al.*, 2011; Jozefczuk *et al.*, 2012). This is because shorter gestational age is associated with purity of mouse embryonic fibroblast line. Longer gestational age may lead to decreasing the quality in cell morphology, growth rate as well as the capacity of the mouse embryonic fibroblast to support culture of embryonic stem cell (Jiang *et al.*, 2015). However, the present result showed that foetus at 14 and 15 days *post coitum* also could be used and produced high viability of mouse embryonic fibroblast culture.

Large amount of mouse embryonic fibroblast is a prerequisite factor for culturing embryonic stem cell to remain in the undifferentiated state (Piedrahita *et al.*, 1990). For each subsequent passage of embryonic stem cell, a fresh confluence feeder cell layer of mouse embryonic fibroblast is required. The dependency on the primary mouse embryonic

fibroblast alone is not enough, consequently, mouse embryonic fibroblast should be passaged and cryopreserved to ensure production of sufficient stock. Thus, cryopreservation and subsequent thawing are important protocols in mouse embryonic fibroblast production that need to be focused in order to produce high quality feeder cell layer (Diekmann *et al.*, 2011). This is in turn enables the successful consecutive culture of embryonic stem cell.

There are a lot of factors that need to be considered before choosing the optimum and reliable mouse embryonic fibroblast cryopreservation protocol such as types of cryoprotecting agent (McGann, 1978), molarity of cryoprotecting agent (Fahy *et al.*, 2004), techniques of cryopreservation (Saragusty and Arav, 2011), freezing cell density (Freshney, 1994) as well as equilibration duration (Kuleshova *et al.,* 2007). An effective cryopreservation protocol must be able to maintain the quality of the mouse embryonic fibroblast and ensure high viability rate after thawing. According to Diekmann *et al.* (2011) inconsistent production of embryonic stem cell is related to differences in mouse embryonic fibroblast preparation, including the cryopreservation as it greatly influences the cellular quality.

Similar to cryopreservation protocol of ear fibroblast cell, quick freezing technique was used to cryopreserved mouse embryonic fibrobast cell. In quick freezing technique, the ear fibroblast cells were cryopreserved at two temperatures, started with 15 minutes of equilibration at -80^0C followed by direct plunging into -196^0C of liquid nitrogen for 15 minutes. It has short equilibration duration with fast cooling rate, which was -5^0C/minutes. From the result, both frozen-thawed of Passages 1 and 2 of mouse embryonic fibroblast derived from both foetal ages showed significantly lower viability rate compared to the fresh passages. This is because the short duration of equilibration disallowed the water

diffusion out of the cell which resulting in formation of intracellular ice crystal (Show and Jones, 2003). This causes damage to cell organelles and lead to cell apoptosis.

It is interesting to note that, the current finding was compared with the previous research in our laboratory (Chung, 2011) to evaluate the equilibration duration differences to freeze mouse embryonic fibroblast. It is interesting to note that 15 minutes equilibration duration (current finding) was superior to 20 hours duration in term of the viability rate the frozen-thawed. This finding was contradicted with the results obtained from previous mammalian cell freezing studies (Freshney, 1994; Li *et al.,* 2009; Yokumuro *et al.,* 2010, Jiang *et al.,* 2015). Long equilibration duration, which have low cooling rate, permits the water diffusing out of cells which minimise the risk of cell injury and prevent apoptosis. However, Mazur *et al.* (1972) proposed on „2 factor" hypothesis on cell injury during cryopreservation, as the optimum equilibration duration or cooling rate varies among cell types. Prolonged exposure to hypertonic freezing medium induced osmotic stress which resulted in irreversible damage to the cell integrity (Fuller and Paynter, 2004). This finding was also supported by Higgins *et al.* (2011) who obtained higher post-thawed viability rate on rat neural cell when freezing at faster cooling rate. Therefore, based on this finding, quick freezing technique could be used to cryopreserve mouse embryonic fibroblast cell layer without any detrimental effect.

In attempt to produce embryonic stem cell on the feeder cell layer, the blastocyst obtained in this study were subjected for whole blastocyst culture. However, the inner cell mass failed to attach onto the feeder cell layer in the culture dish.In whole blastocyst culture, the whole embryo culture tends to manifest an abundance of both trophectoderm and differentiated cells, and the isolation of murine embryonic stem cell-like colonies from differentiated cells requires a great deal of care and caution (Goh, 2012). However, the poor

quality of the blastocysts often results in failures in the attachment of the inner cell mass to the dish. Therefore, this procedure runs a much greater risk of trophectoderm overgrowth than other methods because the entire trophectoderm is cultured along with the inner cell mass.

5.6 GENERAL DISCUSSION

It is crucial to develop reliable protocols of karyoplast cultures and cryopreservation as well as somatic cell nuclear transfer protocols in order to mass produce healthy offspring at rapid rate. The development of somatic cell nuclear transfer protocols should take into consideration the availability of oocytes sources to be utilised as cytoplast. Due to low goat population and number of caprine slaughtering in Malaysia abattoirs (Ministry of Agriculture and Agro-Based Industry Malaysia, 2014), caprine oocytes source is scarce in order to produce intraspecies cloned caprine embryos. The used of superstimulated does for oocytes collection is possible, but requires high expertise in goat surgery, expensive yet difficult to purchase stimulation hormones and high cost of goat feed and health management. Therefore, with approach of using bovine oocytes as cytoplast, interspecies somatic cell nuclear transfer protocol as adapted from Kwong (2012) and Soh (2012) was applied for producing cloned caprine embryos which is feasible to be applied in Malaysia situation.

In order to produce the caprine embryonic stem cell, large number of blastocysts is required for inner cell mass isolation. Embryonic stem cells can be derived from numerous techniques through *in vitro* produced blastocysts, such as *in vitro* fertilisation, intracytoplasmic sperm injection, parthenogenesis and somatic cell nuclear transfer and *in vivo* produced blastocysts (Goh, 2012). In literature, attempts to produce embryonic stem

cell lines of the porcine, caprine, ovine and equine mostly using *in vivo* blastocysts, Efficient and cost effective *in vitro* produced embryo culture systems are frequently used for bovine at early blastocyst stage embryos. In our laboratory, caprine embryonic stem cell growth was successfully cultured from inner cell mass of both *in vivo* and *in vitro*, which including cloned and parthenotes blastocysts (Goh, 2012).

The present research attempted to produce caprine embryonic stem cell-like outgrowth from the interspecies cloned caprine blastocyst. However, prior to deepen the study in producing the embryonic stem cell, optimum production of interspecies caprine cloned blastocysts is utmost important aspect in this study. Although the protocol for interspecies somatic cell nuclear transfer using bovine cytoplasts and caprine karyoplasts had been successfully produced with satisfactory blastocyst rates (Kwong, 2012; Soh, 2012), the present study which adapted the protocols could not produce high number of blastocysts. Therefore, in this study, the focus is on improvement of efficiency of interspecies cloned caprine embryos production. The author selected a) *in vitro* maturation duration of bovine cytoplast, b) breed and gender of donor karyoplasts and c) supplementation of trichostatin A in *in vitro* culture medium as important factors to be studied in the interspecies somatic cell nuclear transfer protocols. Apart from that, the author established the early passages of Katjang and Boer ear fibroblast culture for germplasm conservation and karyoplast source. As for preliminary experiment for embryonic stem cell study, the author produced mouse embryonic feeder cell layer derived from different foetal ages, as the mouse embryonic feeder cell layer is also one of the important factors to ensure optimum production of embryonic stem cell line.

5.6.1 Summary of significant findings

In summary, 6 significant findings were successfully achieved in the present study. Firstly, the germplasms of female and male purebred Katjang and Boer at early passages derived from ear fibroblast cell were successfully conserved through culturing and cryopreservation technique. This is important especially for purebred Katjang is indigenous breed and at the risk of extinction. Another importance of this finding is as source of donor karyoplast for somatic cell nuclear transfer technique. Subsequently, the achievement of the first significant finding lead to second significant finding, which is successful production of interspecies cloned Katjang and Boer embryos (morula and blastocyst, respectively). Thirdly, *in vitro* maturation duration at 22 to 24 hours is the most optimum duration for bovine cytoplast to be used for producing both bovine parthenotes embryos and interspecies cloned caprine embryos. Fourthly, male donor karyopasts significantly improved the interspecies cloned caprine embryos. Fifthly, quick freezing technique successfully cryopreserved caprine ear fibroblast cell culture and mouse embryonic feeder cell culture without any detrimental effect. Lastly, 14 and 15 days *post coitum* murine foetuses could be used to establish mouse embryonic feeder cell layer.

5.6.2 Constraints of the study

i) Skill acquisition

Due to lack of skills and facilities which were a prerequisite for successful somatic cell nuclear transfer and embryonic stem cell experiments, intensive learning skills were planned and eventually attained during the first year of candidature for preparing the microtools and media, handling, culturing and cryopreserving the cells, oocytes and embryos, as well as optimisation the micromanipulation technique for somatic cell nuclear transfer protocol. After optimisation of the skills, the data collected were more stable and optimum.

ii) Oocyte supply

The availability of the bovine and caprine ovaries from the local abattoir was irregular and inadequate. This condition worsen as the local abattoir only slaughtered the imported cows which already been injected with chemicals to regress the ovaries structure. To tackle this issue, bovine ovaries were collected in abattoir located 70 km away from the laboratories and could be only collected thrice a week at maximum. However, the number of ovaries obtained were low due to low number of slaughtering and had to be shared with the other laboratories. In Malaysia, the slaughtered animals were usually old, sterile or unproductive and no history record were available. Consequently, the quality of the oocytes obtained were questionable. Therefore, after the author improved and optimised her skills in the somatic cell nuclear transfer technique, the experiments were conducted extensively and fully utilised the oocytes obtained.

iii) Low blastocyst rate

In this experiment, the challenge was to overcome the low blastocyst rate of parthenotes and interspecies cloned caprine embryos. This problem subsequently affected the experiment on producing the caprine embryonic stem cell. To improve this, the author tried to supplement trichostatin A in the *in vitro* culture medium as attempt to improve the blastocyst rate. However, the blastocyst rate did not significantly improve although higher number of blastocyst obtained. Thus, the obtained blastocysts were used to produce embryonic stem cell on the mouse embryonic feeder cell layer.

5.6.3 Improvement for the present study

Due to time constraint, several issues in current study could not be improved and perfected. In this section, the author will discuss several aspects that could be improved in current experimental design which could be conducted for future studies.

i) Passages of ear fibroblast karyoplast

The current research is focusing on the 3 early passages of ear fibroblast for culturing, passaging, cryopreserving and subsequently utilised as donor karyoplast. Due to no significant difference on viability rate between the 3 passages, the effect of passage number of the donor karyoplast was ignored in the production of interspecies cloned caprine embryos. Although interspecies cloned blastocyst is successfully produced in this study, the blastocyst rate was lower compared with Kwong *et al.* (2012) which used Passages 3 to 7 of ear fibroblast cell. Did the earlier passages (Passages 1 to 3) of donor karyoplast (ear fibroblast) negatively affect the development of interspecies cloned caprine blastocyst and how? One of the findings in this experiment was frozen-thawed Boer and Katjang female at Passage 1 and Passage 3 had significantly lower viability rate compared to other breeds and gender. This suggested that Boer and Katjang female ear fibroblast cell was not suitable to be cryopreserved by quick freezing technique especially at higher passages number. This finding also suggested that quick freezing technique was not reliable to cryopreserve female ear fibroblast cell. Therefore, the author suggested that the number of passages of ear fibroblast cell should be increased to observe whether the quick freezing technique was not reliable to cryopreserve female ear fibroblast cell. The author also suggested to study the different passage numbers of ear fibroblast karyoplast used as it was more interesting to know whether each passage number really affected the development.

ii) Determination of polar body 1 extrusion of the oocytes prior enucleation

The current study was focusing on selecting the maturation duration based on suggested maturation of Kwong (2012). It was found that 22-24 hours was the optimum maturation duration of bovine oocytes to be used as recipient cytoplasts in interspecies cloned caprine embryos production. However, only 2-cell and 4-cell stages showed significant improvement, whereas, no significant differences between the maturation duration on the subsequent stages of embryos. The number if the interspecies cloned caprine blastocysts in this experiment was also low. As discussed earlier, literature reported varies range of maturation duration of the bovine oocytes. Therefore, it was suggested that frequent checking of extrusion of the first polar body could be detected after 18 hours of *in vitro* maturation with interval 15 minutes, so that the early matured oocytes could be used as recipient cytoplast resulting the improvement of the efficiency.

iii) Optimisation of trichostatin A treatment in in vitro culture medium

As mentioned earlier, current study was modified from Wang *et al.* (2015) which achieved the significant improvement in *in vitro* developmet of intraspecies cloned caprine embryos when treated the donor cell (ear fibroblast cell) with 25 nM of trichostatin A for 12 hours. However, in the present study, no significant difference was obtained when treating the post-activated cloned embryos and 8-cells with similar concentration and duration. No report was available for treatment of trichostatin A in caprine studies, especially for interspecies cloned caprine embryos. Therefore, it was suggested to optimise the concentration of trichostatin A and the treatment duration for the interspecies cloned embryos. The current experiment was consider novel as the author try to compare the trichostatin A in interspecies cloned caprine embryos at different stages of embryos. In

current study, the *in vitro* culture applies two ways of culture system, KSOM A followed by KSOM B on Day 2. Therefore, it was interesting to know if the trichostatin A might have negative or positive effect in the KSOM A and KSOM B as the diluent of trichostatin A to the embryos. In addition, the outcome of this suggested study should evaluate the blastocyst quality based on blastocyst cell number and histone acetylation of immuno-detection analysis.

iv) Using manual cut technique or laser isolation technique to isolate inner cell mass

Inner cell mass isolation technique is an important step to ensure the success of caprine embryonic stem cell establishment (Goh *et al.*, 2012). In the present study, the whole blastocyst culture was applied for culturing the embryonic stem cell resulting in zero attachment rate of the blastocyst on the mouse embryonic feeder cell layer. This might be due to trophectoderm cells were cultured along with the inner cell mass which often disturb the inner cell mass growth. Therefore, complete removal of trophoblastic cells provided more advantageous to inner cell mass attachment to become embryonic stem cell which could be conducted by using either mechanical or laser dissection technique. This had been proved that mechanical isolation of the inner cell mass has previously been successfully used in the derivation of 2 cell lines and successful derivation by using laser dissection on murine and caprine embryonic stem cell (Goh *et al.,* 2012).

5.6.4 Future directions

The results obtained from the current study provide additional knowledge to existing information in production of interspecies cloned caprine embryos by using bovine cytoplast and caprine ear fibroblast as donor karyoplast. The present study further elucidated selected issues on the donor karyoplast, recipient cytoplast quality and also treatment of histone deacetylase inhibitor in *in vitro* culture of interspecies cloned caprine embryos. As discussed in detail in Section 5.6.2, more improvement could be conducted in the future such as:

a) Detailed analysis on transcriptional profile of interspecies cloned caprine embryos with a complete embryo RNA seq-derived microarray.
b) Investigate the potential meaning of the alteration of specific genes or categories of genes, to associate specific mechanisms or events that occur in somatic cells, reconstituted oocytes and developing embryos with the observed alterations after treatment of trichostatin A.
c) Using at early stage (8-cell or morula) to produce the caprine embryonic stem cell culture.
d) Use species specific feeder cell layer, for example caprine foetal fibroblast for culturing the caprine embryonic feeder cell layer.
e) Transferring the embryos produced from Boer and Katjang to surrogate mother to produce cloned Boer and Katjang kids.
f) Using different cytoplast from different species (such as rabbit) and different sources of karyoplast (such as foetal fibroblast cell).

g) Improvement and optimisation of somatic cell nuclear transfer protocol as well as embryonic stem cell protocol to go further on reproductive cloning and therapeutic cloning research.

h) Application of therapeutic cloning in solving human medicine especially in degenerative diseases such as Parkinson‟s disease and Alzheimer.

i) Application in reproductive cloning combined with embryo transfer for conservation of wildlife and increase livestock production at rapid rate.

Chapter 6

6.0 CONCLUSIONS

The main goal of this study was to produce interspecies cloned caprine embryos and mouse embryonic feeder cell layer which was important for production of embryonic stem cell outgrowth. The current research was focusing in few selected issues in interspecies somatic cell nuclear transfer to improve the efficiency of interspecies somatic cell nuclear transfer in caprine as well as preparation of feeder cell layer for embryonic stem cell culture. From the findings, it could be concluded as follows:

a) Ear fibroblast cell line of female and male purebreed indigenous Katjang and Boer were successfully cultured and cryopreserved as donor karyoplasts to be subsequently used in interspecies somatic cell nuclear transfer (Objective a);
b) The most optimum maturation duration for bovine oocytes as cytoplast for interspecies somatic cell nuclear transfer was 22-24 hours (Objective b);
c) Cloned indigenous Katjang and purebred Boer embryos using ear fibroblast cells was successfully produced by interspecies somatic cell nuclear transfer technique up to morula and blastocyst stage, respectively (Objective c);
d) Interspecies cloned caprine blastocysts were only successfully produced by using 22-24 hour matured bovine cytoplast with female and male Boer ear fibroblast karyoplasts (Objective d);
e) Treatment of trichostatin A (25 nM) on interspecies cloned caprine embryos did not significantly improve morula and blastocyst rates (Objective e);

f) Mouse embryonic fibroblast could be produced using both foetal ages of 14 and 15 days *post coitum* and cryopreserved using quick freezing technique (Objective f).

In a nutshell, interspecies somatic cell nuclear transfer is an alternative protocol to produce cloned caprine embryos, to intraspecies somatic cell nuclear transfer, particularly limited availability of caprine oocytes. However, more research is needed to optimise the intrinsic and extrinsic factors affecting the efficiency of interspecies somatic cell nuclear transfer protocol before it could be integrally incorporated with other reproductive technologies.

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