PRODUCTION OF CAPRINE CLONED EMBRYOS USING SOMATIC CELL NUCLEAR TRANSFER

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PRODUCTION OF CAPRINE CLONED EMBRYOS USING SOMATIC CELL NUCLEAR TRANSFER

ABSTRACT

Production of cloned goat embryos using interspecies somatic cell nuclear transfer (SCNT) technique could be applied using cattle oocyte as recipient cytoplast since the source of goat oocytes is limited in our country. The main aim of this study was to produce goat and cattle cloned embryos using intra- and interspecies SCNT technique. Cattle oocytes were retrieved from abattoir-derived ovaries to be used as recipient cytoplasts for subsequent SCNT experiment. Cattle oocytes were then matured *in vitro* by culturing in *in vitro* maturation (IVM) medium and denuded in hyaluronidase (0.1%) to remove the cumulus cells on following day. The matured cattle oocytes were selected based on extrusion of first polar body and subjected to enucleation using laser-shooting technique afterwards. Donor karyoplasts derived from goat (Rumpun Asia Sdn Bhd Farm, Selangor) and cattle (Banting abattoir) were harvested and injected into enucleated cattle oocytes. Reconstructed oocytes were then activated and the reconstructed couplets were cultured *in vitro* in potassium simplex optimisation medium (KSOM) in CO_2 incubator (5%, 38.5°C). Cloned embryos development were monitored and recorded for data analysis. All data were presented as mean±SEM and were analysed using one-way ANOVA. The significant differences among treatments were further analysed by Duncan's Multiple Range Test (DMRT) and $P<0.05$ was considered significant. In Experiment 1, two IVM durations of cattle oocytes were compared, i.e. Group 1 (22-24 hr) and Group 2 (25-27 hr). The maturation rate of Group 1 (22-24 hr) was significantly higher ($P<0.05$) than Group 2 (25-27 hr). Group 1 showed significantly higher ($P<0.05$) compared with Group 2 with the cleavage rates of 69.18% vs. 55.27% (2-cell); 53.09% vs. 38.32% (4-cell); 41.40% vs. 26.92% (8-cell) and 21.26% vs. 9.31% (morula), respectively, except for 2- and 4-cell stages in Group 1. In experiment 2, effect

of different types of donor karyoplasts (goat ear fibroblast cells; EFC and goat foetal fibroblast cells; FFC) as well as comparison between fresh and frozen-thawed samples on viability of cells were studied. There were no significant differences in percentages of cells viability between two breeds of ear fibroblast cells (EFC) (Jermasia vs. Boer) in all passages, except for P1 that Boer was significantly higher in percent viability than Jermasia (86.72% vs. 80.44%), respectively. However, there were significant differences of cell viability between fresh and frozen-thawed samples in all passages of EFC and foetal fibroblast cells (FFC). The FFC also showed higher in term of cells viability as oppose to EFC. In experiment 3, intraspecies SCNT was conducted to produce cloned cattle embryos using cumulus cells from cattle as donor karyoplast. The values of cleavage rates in cattle intraspecies SCNT were 53.57% (2-cell), 33.17% (4-cell), 22.15% (8-cell) and 11.90% (morula). There were significant decreases in the cleavage rate at different stages (2-cell until morula) of cloned cattle embryo development ($P<0.05$). In experiment 4, the efficiency rate of interspecies SCNT of goat-cattle cloned embryos was evaluated using two different sources of donor karyoplast, which were goat EFC and FFC. The results showed that FFC gave significantly higher cleavage rate compared with EFC ($P<0.05$), 2-cell (64.40% vs. 38.43%), 4-cell (54.24% vs. 24.60%), 8-cell (36.82% vs. 14.54%) and morula (22.10% vs. 7.90%), respectively. In conclusion, cattle and goat cloned embryos could be produced using advanced reproductive techniques such as pathenogenetic activation, intra- and interspecies SCNT techniques. However, using goat FFC was more efficient than EFC as donor karyoplast to produce cloned goat embryos.

Keywords: Cattle, goat, donor karyoplast, recipient cytoplast, Intraspecies SCNT, Interspecies SCNT

PENGHASILAN KLON EMBRIO KAPRIN DENGAN MENGGUNAKAN PEMINDAHAN NUKLEUS SEL SOMATIK

ABSTRAK

Penghasilan klon embrio kambing menggunakan teknik pemindahan nukleus sel somatik interspesies boleh dilakukan dengan menggunakan oosit lembu sebagai sitoplas penerima kerana sumber oosit kambing yang terhad. Oosit lembu diperolehi daripada ovari yang dikumpul dari rumah sembelihan digunakan sebagai sitoplas penerima untuk eksperimen SCNT. Oosit lembu kemudiannya dikultur dalam medium kematangan *in vitro* dan sel kumulus ditanggalkan dengan menggunakan enzim hialuronidase (0.1%) pada keesokan harinya. Oosit yang matang dipilih berdasarkan kemunculan jasad kutub pertamanya dan dinyahkeluar dengan menggunakan kaedah tembakan laser. Karioplas penderma daripada kambing dan lembu dikutip dan dipindahkan ke dalam oosit yang telah dienukleasi. Oosit yang telah direkonstruksi dikulturkan di dalam medium *potassium simplex optimisation* (KSOM) untuk perkembangan embrio secara *in vitro* di dalam incubator CO_2 (5%, 38.5°C). Perkembangan embrio diperhati dan direkodkan untuk analisis data. Data dikemukakan sebagai min±SEM dan dianalisis dengan menggunakan ANOVA sehala. Perbezaan yang signifikan di antara perlakuan telah dianalisis selanjutnya dengan menggunakan Duncan's Multiple Range Test (DMRT) dan $P<0.05$ dianggap sebagai signifikan. Dalam eksperimen 1, dua masa kematangan oosit lembu dibandingkan, i.e. Kumpulan 1 (22-24 jam) dan Kumpulan 2 (25-27 jam). Kumpulan 1 menunjukkan lebih signifikan rendah ($P<0.05$) berbanding Kumpulan 2. Kumpulan 1 juga menunjukkan signifikan rendah pada kadar perkembangan embrio partenot dengan kadar perkembangan 69.18% vs. 55.27% (2-sel); 53.09% vs. 38.32% (4-sel); 41.40% vs. 26.92% (8-sel) dan 21.26% vs. 9.31% (morula), kecuali peringkat 2-sel dan 4-sel dalam Kumpulan 1. Dalam eksperimen 2, kesan daripada jenis karioplas penderma yang berbeza (sel fibroblas telinga kambing; EFC dan sel fibroblas fetus; FFC) dan juga perbandingan

di antara sampel sel segar dan dinyahbeku pada kadar hidup sel dikaji. Tiada perbezaan signifikan peratus kadar hidup sel di antara dua baka sel fibroblast telinga kambing (EFC) (Jermasia vs. Boer) pada semua pasaj, kecuali P1 di mana baka Boer mempunyai peratus kadar hidup sel yang tinggi berbanding Jermasia (86.72% vs. 80.44%). Walaubagaimanapun, terdapat perbezaan signifikan pada kadar hidup sel di antara sampel sel segar dan dinyahbeku pada semua pasaj EFC dan sel fibroblast fetus (FFC). FFC juga menunjukkan kadar hidup sel yang lebih tinggi berbanding EFC. Dalam eksperimen 3, SCNT intraspesies dijalankan untuk menghasilkan embrio klon lembu menggunakan sel kumulus lembu sebagai sitoplas penerima. Nilai kadar pembelahan embryo pada peringkat berbeza adalah 53.57% (2-sel), 33.17% (4-sel), 22.15% (8-sel) dan 11.90% (morula). Terdapat kadar penurunan yang signifikan pada kadar pembelahan embrio dalam peringkat yang berbeza (2-sel hingga morula) bagi perkembangan embrio klon lembu ($P<0.05$). Dalam eksperimen 4, kadar efisiensi SCNT interspesis embrio kambing-lembu dikaji dengan menggunakan dua sumber karioplas penderma yang berbeza iaitu, EFC dan FFC. Data menunjukkan FFC memberikan kadar signifikan tinggi dalam kadar pembelahan embrio berbanding EFC iaitu, 2-sel (64.40% vs. 38.43%), 4-sel (54.24% vs. 24.60%), 8-sel (36.82% vs. 14.54%) dan morula (22.10% vs. 7.90%). %). Kesimpulannya, embrio klon lembu dan kambing boleh dihasilkan dengan menggunakan teknik reproduksi termaju seperti pengaktifan partenogenetik serta teknik SCNT intra- dan interspesies. Walau bagaimanapun, penggunaan FFC sebagai karioplas adalah lebih efisien daripada EFC untuk menghasilkan embrio kambing klon.

Kata kunci: Lembu, kambing, kariolas penderma, karioplas penerima, SCNT intraspesis, SCNT interspesis

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Sincerely,

Siti Haslinda Mohd Sharif

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%	percentage
°C	degree Celcius
cm	centimeter
CO_2	carbon dioxide
g	gramme
l	litre
mg	milligram
ml	millilitre
mm	millimeter
mOsm	millimosmole
O_2	oxygen
pH	hydrogen potential
v/v	volume/volume
w/v	weight/volume
µl	microlitre
µm	micrometer
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
DMSO	dimethyl sulfoxide
DNA	deoxyribo nucleic acid
DMEM	Dulbecco's Modified Eagle's medium
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
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

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Ruminants are very important in livestock industry to provide sources of animal protein in the diet of Malaysian population. Unlike cattle, the small ruminants such as goats and sheep are traditionally reared by small-holders to complement the socio-economic livelihood of the rural population. For example, Malaysia only produced 4,000 metric tonnes of mutton while the demand was around 28,000 metric tonnes level (Fadhilah, 2015). The author suggested that Malaysia needs to increase at least until 50% of local market needs to ensure sustainability of goat production in this country. The government has implemented various strategies and policies to improve the livestock industry and positive progress has been perceived in recent years but still yet to achieve self-sufficiency standard (Amna & Hifzan, 2015). Hence, to cater for the high demand, Malaysia imported annually most of the mutton from abroad such as New Zealand and Australia (Mohamed, 2007).

Goat is one of livestock species that provides meat and milk for human consumption, particularly for rural population in under-developed countries. However, in these countries goat production is still very low both in quantity and quality. In order to increase the goat production at a rapid rate, it is imperative to use appropriate technologies and good animal husbandry practices. For example, application of assisted reproduction technologies (ART) allows animals of high genetic merit to produce more offspring than natural breeding. These technologies include artificial insemination (AI), multiple ovulation and embryo transfer (MOET), *in vitro* fertilisation (IVF), intracytoplasmic sperm injection (ICSI), embryo sexing, cryopreservation of gametes and embryos, transgenesis, embryonic stem cell and somatic cell nuclear transfer (SCNT). Conventionally, the most commonly used ART in domestic species is artificial

insemination (AI) due to relatively simple and low cost method (Leboeuf *et al*., 2000; Gillan *et al*., 2004). For example, at the University of Malaya, Jermasia goat was first developed by researchers through artificial insemination (AI) more than 20 years ago. AI has been used for the development of synthetic breeds incorporating both exotic and indigenous species. Jermasia which is a dual purpose Malaysian goat breed was stabilised by crossing artificially between German Fawn (superior in meat and milk production) and Katjang goat (adaptability and resistance to diseases) (Abdullah, personal communication, 2016). Katjang goats are small in size, well-adapted and widely distributed in small herds owned by local farmers in Malaysia.

Application of ART in the goats is still low when compared with other domestic species such as cattle, pig and sheep. With the increase in the demand of red meat globally including Malaysia, the governments of various countries place a high priority to increase a small ruminant and large ruminant production through application of reproductive technologies.

Various efforts have been made by scientists worldwide to understand underlying mechanisms to increase the success rate of somatic cell nuclear transfer (SCNT) in animals. However, there are still many issues and technical constraints that need to be solved before this technology could be transferred in general livestock production (Niemann & Peterson, 2016). Especially in interspecies SCNT, in which involves the transfer of somatic cells from different species into recipient cytoplast of other species. The rate of efficiency is still low compared to intraspecies SCNT. One of the major causes is the lack of capacity to trigger embryonic genome activation to support proper embryo development up to blastocyst stage (Canovas & Cibelli, 2014) due to incompatibility between mitochondrial DNA (mtDNA) genes and the nuclear genes encoding mitochondrial proteins (Beyhan *et al*., 2007).

1.2 STATEMENT OF PROBLEMS

SCNT is a complicated protocol involves major technical steps such as collection and removal of maternal nucleus of recipient cytoplast, preparation of donor karyoplast, injection of donor karyoplast into recipient cytoplast via sub-zonal intracytoplasmic injection (SUZI) or whole cell intracytoplasmic injection (WCICI), activation of the reconstructed oocytes and subsequently transfers to recipient doe. Various strategies have been made by researchers worldwide to improve the success of SCNT, for example, by treating donor cells with chemical treatments [e.g. trichostatin A (TSA)] (Wee *et al*., 2007). However, the results are still unpredictable due to biological variation between donor karyoplast and recipient cytoplast.

Below are some of the important questions related to SCNT in goat that needed to be answered:

- Does number of donor karyoplast passages influence the viability percentage of cells?
- Does percentage viability of fresh donor karyoplast is the same with cryopreserved one?
- Do different IVM durations affect the maturation rate of cattle oocytes?
- Do different IVM durations influence the cleavage rate of cattle parthenotes?
- Which donor karyoplast is more compatible with cattle recipient cytoplast? Is it goat foetal fibroblast cells (FFC), goat ear fibroblast cells (EFC), cattle cumulus cells (CC) or all?
- Can the goat somatic nuclei be reprogrammed in cattle recipient cytoplast via interspecies SCNT?

In our university, researchers developed various goat reproductive technologies including the cloning technology using somatic cell nuclear transfer (SCNT) in order to

increase goat production at a rapid rate. There are numerous factors affecting the success of somatic cell nuclear transfer before this technique can be integrated routinely into the sustainable farm practices. These factors include the source of donor karyoplast, source of recipient cytoplast, activation and fusion of oocytes, *in vitro* culture medium and embryo transfer technique.

Cloned goat embryos developed in our laboratory using intraspecies SCNT obtained encouraging results (Kwong, 2012). However, due to insufficient source of goat oocytes, the progress of this technique is relatively slow. As an alternative, we also made an attempt to develop interspecies SCNT using cattle oocytes as recipient cytoplast and goat ear and foetal fibroblast as donor karyoplast (Soh, 2012). Dominko *et al*. (1999) reported the first interspecies SCNT experiment with the blastocyst percentages on sheep-cattle (13.9%), pig-cattle (14.3%), monkey-cattle (16.6%) and rat-cattle (0%). Except for gaur-cattle (Lanza *et al*., 2000), European Mouflon-sheep (Loi *et al*., 2001) and sand cat-domestic cat (Gomez *et al*., 2008), there was no pregnancy reported in other species including goat after transferring the interspecies SCNT cloned embryos into recipient mothers.

Most of the world's goat population is found in the rural areas of under-developed countries. Goats are important small ruminants due to its economic and social benefits to the rural communities particularly in the production of various products such as meat, milk and fibre. For example, in Nepal, with the human population of around 10 million has a goat population of 20 million with per capita consumption of 12.21 kg (Upadhyay *et al*., 2017). In contrast, Malaysia with human population of around 32 million people has a goat population around 450, 000 with per capita consumption of 0.5 kg (Jabatan Perkhidmatan Veterinar). Due to a low self-sufficiency of goat meat in our country, we imported about 90% of goats from Australia, New Zealand, India and other countries, thus, increase our food security risk (Alexandra, 2017). Generally, goat production

worldwide is inefficient due to many factors such as political, lack of appropriate technology used, poor production and reproduction as well as poor management practices (Abdullah, personal communication, August 26, 2016). In order to overcome these challenges and increase in global goat production proper planning and strategies must be implemented accordingly based on the situations of the countries involved.

One of the prerequisite to enhance goat production is through scientific creativity and innovation. Reproductive biotechnologies such as artificial insemination (AI) and multiple ovulation and embryo transfer (MOET) are traditionally known to contribute to the improvement of livestock production such as cattle, sheep and pig. However, using reproductive biotechnologies in goat production is still lagging behind. Therefore, it is timely to apply reproductive biotechnologies including the recent advancement techniques using cloning technology such as somatic cell nuclear transfer (SCNT).

1.3 JUSTIFICATION

The advancement of SCNT in livestock production is encouraging with satisfactory cloned embryo development. It is irony that this advancement is mainly in the developed countries such as USA and Europe whereby the livestock production is already efficient. In contrast the technology is lowly developed in under-developed countries whereby, this expensive technology is needed the most to increase sustainable livestock production at a rapid rate, for example, through development of superior livestock breeds and efficient production. This situation is worsening in the case of goat production. To ensure the optimum utilisation of clone technology, all factors must be taken into consideration such as providing good quality forages to be consumed by the cloned goats which are suitable to the climate of a country regardless favourable or adverse climatic condition (Srirattana, 2013). With a proper breed, nutrition and farm management it is certain that goat reproduction could be improved because it is known that goats are prolific and fertile

domestic species with conception rates of approximately 90% (Holtz, 2005). In addition, the gestation length of goats is relatively short (5 months), easy to breed, small in size and could produce high quality of meat and milk production with low amount of fats for human consumption.

Following the birth of the first cloned sheep by Wilmut *et al*. (1997), further improvements in the success rate of SCNT in other animal species have been reported. However, as indicated in the literature, research involving goats throughout the world is still scarce and left far behind. The situation is more challenging in countries like Malaysia whereby very few female goats slaughtered, resulting in difficulty of obtaining ovaries from the abattoir to be used as a source of oocytes for recipient cytoplast in intraspecies SCNT. Therefore, as an alternative to intraspecies SCNT, our preliminary results (Abdullah *et al*., 2012; Kwong *et al*., 2012; Soh *et al*, 2012) results in cloned goat embryos using cattle-goat interspecies SCNT were encouraging. However, further refined research is needed to improve this technique. This research was further conducted in goats, inter alia, to increase the understanding in intra- and interspecies SCNT in goats as well as its application in livestock species. Besides the cloning protocol per se, *in vitro* embryo production in goats is still challenging in most research laboratories (Paramio, 2010). Hence, continuous improvement of *in vitro* embryo production technique, such as appropriate *in vitro* culture medium for goat SCNT embryos is needed in order to increase cloned goat embryo production before this technique can be integrated into goat industry.

In our laboratory, we are still having a problem in getting goat ovaries from slaughterhouse in order to carry out intraspecies SCNT experiments due to low number of animals slaughtered and time constraint. There is still another way of getting goat oocytes, which is by laparoscopic oocyte pick-up (LOPU) surgery, however; the cost is much higher than taking abattoir-derived ovaries sample (Soh, 2012). Hence, we chose cattle oocyte samples from slaughterhouse as the recipient cytoplast to overcome this

problem. In this experiment, one of our objectives is to produce interspecies cloned goat embryos by using goat foetal fibroblast cell as donor karyoplast and cattle oocyte as a recipient cytoplast.

In a nutshell, up to now, only a few successful cloning of goats using interspecies SCNT have been reported worldwide with few births of cloned goat offspring. With continuous refinements of SCNT technique in goat by improving the various critical steps in cloning technique as well as IVC and ET it is hoped that in the near future numerous cloned goat embryos will be produced and subsequently resulted in high success rates in pregnancy and birth of healthy cloned goat offspring. As a technical background to the development of interspecies SCNT in goats, our laboratory has conducted goat cloning research since 2008 and satisfactorily obtained both intra- and interspecies cloned goat embryos using ear fibroblast cells (Asdiana, 2015; Kwong, 2012; Nurin, 2016) and foetal fibroblast cells (Soh, 2012) as donor karyoplast (Soh, 2012). To further improve the previous studies, therefore, the present study was conducted to compare the efficiency rate of interspecies SCNT using two different types of donor karyoplast [ear fibroblast cells (EFC) vs. foetal fibroblast cells (FFC)].

Multiple strategies have been proposed to improve intraspecies SCNT in goats including selection of the best donor karyoplast, improvement of micromanipulation protocol and culture media, however; the starting point is to obtain high quality of recipient cytoplasts (oocytes) either through laparoscopic ovum pick up (LOPU) or ovaries derived from abattoir (Menchaca *et al*., 2016) so that multiple molecular processes that will determine the fate of embryo will progress well. After goat oocytes retrieval, *in vitro* matured oocytes will be used as cytoplast recipients for intraspecies SCNT experiment. In LOPU, superovulation protocols can influence the quality of goat oocytes and subsequently the resulting cloned ruminant embryos. The age of oocytes relative to their ovulation may also be a crucial factor for the success of intraspecies

SCNT in goats, as the ability to fuse with donor karyoplasts depends on this factor (Collas & Robl, 1990). Practically, in the case of our laboratory, as for abattoir derived oocytes, there were several factors that could restrict the success of intraspecies SCNT in goats such as limited availability of needed oocytes, ovarian duration transportation, temperature of saline solution, distance from abattoir to the laboratory and age of the slaughtered animals.

1.4 APPLICATION

SCNT has the potential to generate high genetic merits in domestic animals as well as a role for the multiplication of animals and various products, yet, issues of high cost, low pregnancy and survival rate and lack of genetic diversity need to be resolved first before it can be applied in livestock improvement (Baldassarre & Karatzas, 2004).

There are limitless application possibilities of animal SCNT in research, industry, wildlife and agriculture. The fundamental background information learned from SCNT in animals could deepen the understanding of basic biological knowledge particularly in embryo development and gene expression (Santos & Dean, 2004). For example, the immediate and urgent potential of goat SCNT in biomedical application could be achieved through production of transgenic goats to produce high amount of good quality milks for pharmaceutical proteins such as insulin and other drugs.

SCNT also could be applied in agriculture to generate copies of animals with high genetic merit as well as increasing the number of possible offspring in a given herd. Another possibility is by combining SCNT with genetic modification to produce a large number of animals that can reduce negative impacts on the environment. For example, the Enviropig^{TM}; a pig that could digest plant phytate leading to less phosphate in the wastage production from the animal and hence cause less pollution to environment

(Gjerris *et al*., 2013). Extending this finding, it is most probable that this knowledge can be transferred to goat application.

Furthermore, SCNT also can be used as one of alternatives to save endangered species from extinct. International Union for Conservation of Nature (IUCN) updates their 'Red List' of endangered species annually, which includes Javan Rhino (*Rhinoceros sondaicus*), Orangutan (*Pongo pygmaeus*), Malayan Tiger (*Panthera tigris jacksoni*), Gray wolves (*Canis lupus*) and other critically endangered animals that had been listed under IUCN Red List. Kim *et al*. (2007) had successfully cloned Gray wolves (*Canis lupus*) via interspecies SCNT by injecting wolf somatic cell into an enucleated domestic dog oocyte showing the potential of interspecies SCNT as a tool for conserving critically endangered species (Jang *et al*., 2010).

Another most important application of SCNT is in human therapeutic research, to generate a preimplatation embryo for embryonic stem cells (ESC) sources. Ying *et al*. (2003) had made an attempt to transfer human somatic cell into rabbit oocyte to generate ESC. Rabbit and cattle oocytes are known as 'universal recipient' due to their availability and the ability to produce blastocyst in interspecies SCNT (Chang *et al*., 2003). The cells and tissues produced by this technique would have nuclear DNA identical to the patient's, thus, it would likely not subject to permanent immune supression in patients body system (Lazzari *et al*., 2006; Beyhan *et al*., 2007). The production of customised embryonic cells will be invaluable in human medicine for the treatment of degenerative diseases such as Alzheimer and Parkinson's disease providing no immunosuppressive treatment is required (Niemann, 2016). In summary, the cloning technology using intra- and interspecies SCNT has a wide application in livestock production for food security and safety, in human medicine to produce proteins, tissues and organs for therapeutic cloning and wildlife conservation to prevent extinction of heritage animal species.

1.5 OBJECTIVES OF THE STUDY

The main objective of this study was to produce intra- and interspecies cloned ruminant embryos using different types of donor karyoplast. Specific objectives are shown as below:

a) To produce different types of donor karyoplast for intra- and interspecies SCNT.

b) To determine the effect of *in vitro* maturation (IVM) duration and abattoir locality on maturation rate of cattle oocytes.

c) To produce *in vitro* cattle embryo without fertilisation using parthenogetic activation (PA) technique.

d) To produce cloned cattle embryo using intraspecies SCNT technique.

e) To determine the effect of different types of donor karyoplast on the efficiency rate of interspecies goat-cattle SCNT.

CHAPTER 2

LITERATURE REVIEW

2.1 ASSISTED REPRODUCTIVE TECHNOLOGIES (ART) FOR RUMINANT IMPROVEMENT

Ruminants are often regarded as remarkable contributors in economic sector of developing countries. This triggers scientists to develop various assisted reproductive technologies (ART) to facilitate livestock production at an accelerating rate (Amiridis & Cseh, 2012). It is necessary to apply recent advance technologies in livestock animals to cater the needs of human consumption and to sustain food security. Most of these technologies also proven to be effective in increasing the number of animals with high genetic trait to produce more offspring compared to natural breeding (Baldassarre & Karatzas, 2004). Various assisted reproductive techniques have been established including multiple ovulation and embryo transfer (MOET), artificial insemination (AI), *in vitro* fertilisation (IVF), gametes and embryo cryopreservation, parthenogenetic activation (PA), intracytoplasmic sperm injection (ICSI), somatic cell nuclear transfer (SCNT), stem cell and transgenic animal production.

It has been widely acknowledged that artificial insemination (AI) is the most commonly and commercially applied in ART of livestock animal species and also has becomes part of breeding management routine especially in cattle. It is mostly recommended since the cost is relatively low compared to other advanced reproductive technologies, and it offers a relatively simple method to accelerate genetic gain by selecting good quality male traits (Paramio & Izquierdo, 2014). AI has several advantages over natural breeding by using selective outstanding and superior male traits as it can enhance the rate and efficiency of genetic selection, introduce new genetic material by importing frozen semen-based instead of live animals and hence, reduce the cost of

international and trans-continental genetic material transportation. AI also could help in reducing the risk of spreading sexually transmitted diseases (Verma *et al*., 2012). AI has been performed globally for more than 3.3 million sheep and 0.5 million goats annually (Boa & Minozzi, 2006). AI involves collection of semen from selected male animal using an artificial vagina and deposition of collected semen into female's cervical tract with the aid of speculum (Holtz, 2005).

Synchronisation of oestrus and superovulation are important key components of all ART protocols and has major impact in the overall efficacy of these techniques. Various hormonal treatments have been developed in goats' ART research to optimise the number of inseminations and fertility in does including multi-injection FSH as well as a combination of FSH and eCG which also called as a 'one-shot' regimes (Baldassarre & Karatzas, 2004). It can be achieved by inserting progesterone-impregnated intravaginal devices combined with an injection of a luteolytic dose of prostaglandin (Holtz *et al.,* 2008). However, the efficiency of superovulation in goats is inconsistent due to several factors, for instance; age, breed, nutritional status, weight, health, environmental conditions as well as the dosage and timing of hormonal treatment.

Multiple ovulation and embryo transfer (MOET) is a combination technology of superovulation, fertilisation, embryo retrieval, *in vitro* culture of embryos and embryo transfer (ET) which is applied to the female animals. The most outstanding benefit of MOET technique is it could increase the number of animal offspring by reducing the number of generation interval inter-reproduction cycles. According to Nicholas and Smith (1983), MOET technique could improve animal genetic gains up to 80%. In contrast to AI, MOET still has not applied widely for genetic improvement in livestock production due to some constraints including high cost, technical complexities and unpredictable results (e.g. nutritional status, follicular status, genetics and nature) (Cognie *et al*., 2003; Gonzalez *et al*., 2004).

Embryo transfer (ET) is a promising tool to increase the number of animal progeny from one female than the usual number in normal fertilisation. ET could improve livestock production at a rapid rate as well as providing the opportunity to utilise superior genetic traits both from male and female animals. ET often integrates with other ART techniques such as MOET, embryo cryopreservation, *in vitro* fertilisation (IVF), intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT); have been successfully applied for propagation of elite breeds of goat, sheep, cattle and pig (Pawshe *et al*., 1994; Thibier & Nibart, 1995; Hazeleger & Kemp, 2001). In this technique, superovulation hormones will be administered to donor females causing the release of multiple numbers of oocytes at a controlled time. These oocytes are then fertilised through AI or other associate ART techniques and a few days later, these fertilised embryos are flushed from the uterine or oviduct of donor animals. The retrieved embryos are then examined under microscope, graded and transferred to the selected recipient females via uterine or oviductal transfer.

The transfer of embryos to recipient females often conducted by surgical means. Pregnancy will be detected using ultrasound scanning probe. With the application of ET in livestock animal breeding programme, it can help to improve and accelerate herd development through rapid expansion of high quality traits and genetic gain (Verma *et al*., 2012). However, various factors need to be monitored such as age, health status and nutrition as it can affect the outcome of this procedure (Mani *et al*., 1994).

In vitro embryo production (IVEP) comprised of three major steps which are, *in vitro* maturation (IVM), *in vitro* fertilisation (IVF) and *in vitro* culture (IVC) which is now reasonably well-established and progressing well in the past few decades (Verma *et al*., 2012). IVF has always been the most applied technology in human clinically to treat patients with infertility problems such as endometriosis and damaged fallopian tubes (Menezo *et al.,* 2000). The application of IVEP in small ruminant production allows

production of high genetic value animals from designated parents as well as providing excellent source of embryos for other emerging ART such as cloning, embryo sexing, stem cell production and transgenesis (Galli *et al*., 2014).

In vitro fertilisation (IVF) is a procedure by which matured oocytes are fertilised with spermatozoa in a laboratory dish. This technique has been well established in small ruminants' production as well as in cattle. The standard technique for IVF includes selecting viable sperm whether from fresh ejaculate or frozen-thawed sperm. Subsequently, capacitation of sperm is carried out in 5 % CO_2 and 38.5°C of temperature inside the incubator.

Intracytoplasmic sperm injection (ICSI) is one of the recent ARTs that have been used globally to treat male infertility both in humans and animals. ICSI requires intricate and precise maneuvers of micro tools on the gametes under an inverted microscope. A single sperm will be microinjected directly across the membrane of a matured oocyte which leads to fertilisation bypassing the normal oocyte-sperm interaction and selection process. ICSI has been considered efficient enough to be applied for clinical use, specifically in male infertility treatment such as azoospermia and oligospermia. This has led to a resurgence of interest due to its potential to be used in small ruminant production (Verma *et al*., 2004). The first animal produced by ICSI was a hamster by Uehara and Yanagimachi in 1976.

The production of embryos through ICSI technique has given rise to other live progenies in various animal species such as cattle (Goto *et al*., 1996), sheep (Gomez *et al*., 1998), goats (Wang *et al*., 2003), horses (Cochran *et al*., 1998) and pigs (Martin, 2000). There are several researches of ICSI focusing on small ruminants had been conducted and published worldwide including goats (Cognie *et al*., 2003; Holtz, 2005; Paramio, 2010). The first report of ICSI success in goats was conducted by Keskintepe *et*

al. (1997) with development until blastocyst stage while the first goat kid born by this technique was by Wang *et al*. (2003) using a piezo-drill method.

2.2 PARTHENOGENETIC ACTIVATION (PA)

Parthenogenesis is an asexual reproduction which carried out by a female gamete, where the growth and development of new individual could be done bypassing normal fertilisation by a male gamete (sperm) (Nakada & Mizuno, 1998). Parthenogenesis occurs naturally in aphids, nematodes, ants, snakes and some other invertebrates but can also be done in mammalian oocytes by providing appropriate stimuli through *in vitro* (Lessly *et al*., 2016). Since the past few years, parthenogenesis has attracted wide attention because of the role of activated oocytes in the field of reproductive technologies that have been established such as ICSI and SCNT. Investigating culture conditions and potential for deriving pluripotent stem cell lines and their differentiation into various cell lines that can be utilised for various tissue engineering applications such as stem cell production. The parthenogenetically activated oocytes or parthenotes comprised of maternal genome and could develop into haploid, diploid or polyploidy embryos.

PA is the production of viable embryo from female gamete with the absence of sperm and will probably develop into an adult (Beatty, 1957). This can lead to a rapid multiplication of superior females by producing identical female offsprings. The mechanism of PA in oocytes is basically to mimic the activation process in normal fertilisation which carried out by sperm causing an influx of intracellular calcium concentration (Ca^{2+}) which leads to meiosis resumption and extrusion of the second polar body to form a pronuclei (Khadijah, 2012). Consequently, this event will inactivate MPK and MAPK which allows MII arrested oocyte to complete meiosis process (Meo *et al*., 2007). In normal fertilisation, oocytes will be arrested at MII stage until they are fertilised by sperm which consists of phospholipase-C that will activate signaling pathways for

activation to take place in oocyte which leads to Ca^{2+} inside the oocyte. These series of events will release the MII oocyte from its arrest state causing the exocytosis of cortical granules, resumption of meiosis and extrusion of second polar body to occur (Versieren *et al*., 2010).

Numerous methods have been developed in PA protocol with a variety of mechanical, chemical and electrical stimuli such as using calcium ionophore and cycloheximide (Nussbaum & Prather, 1995), ionomycin (Loi *et al*., 1998), $CaCl_2$ (Machaty *et al*., 1995), electrical pulse (Kono *et al*., 1989), calcium ionophore (Kim *et al*., 1996), strontium (Meo *et al*., 2004), protein phosphorylation inhibitor (DMAP) (Liu *et al*., 1998) and Ca-EDTA (Yuh & Ryoo, 2008). Recently, combination of two activation stimuli is often applied to enhance development of activated oocytes. Activation of oocyte instigated by ionophores induces intracellular Ca^{2+} in matured oocytes causing the destruction of cyclin, reduction of MPF activity and thus leads to resumption of meiosis (Jellerette *et al*., 2006). 6-DMAP has been reported to speed up the formation of pronuclei in MII oocytes of cattle and mice (Leal & Liu, 1998).

Basically in all methods of PA, additional stimuli are required to increase intracellular Ca^{2+} concentration mimicking the action that carried out by penetrating spermatozoa during a normal fertilisation process. Various activation stimuli and protocols have resulted in the production of viable embryos for parthenogenetic as well as cloning in numerous of animal species. However, both stimuli and protocol must be optimised for application in each species and it is mandatory to understand the mechanism and effects by various activation methods. Since the birth of first viable parthenogenetic mammal, mice in 2004 (Kaguya) in Japan, significant advances have been progressed well in the field of parthenogenetic research in order to understand the molecular processes involved during genomic imprinting process which is the main barrier to

parthenogenetic development in mammals, in which the individual contains lack of paternal genetic material (Moore & Ball, 2004; Kharche & Birade, 2013).

Parthenotes are usually could not develop into full term due to the absence of paternal gene, however, they can offer a mean to study stem cell production, contribution of maternal genetic imprinting and to evaluate maternal derived factor with the absence of paternal factors at the early stage of PA embryos. In SCNT research, oocyte activation is necessary for resumption of meiosis after nuclear transfer had been conducted (Kono *et al*., 2004; Latham, 2005). Therefore, comparing cloned embryos with parthenotes could be helpful to understand the mechanism of cytoplasmic factors involved in epigenetic reprogramming within the recipient cytoplast.

2.2.1 Factors affecting parthenogenetic activation (PA)

The response and performance of oocytes to PA technique can be affected by various factors such as types of PA treatment, age of oocytes, donor strain, *in vitro* culture media, temperature, superovulation efficiency, pH, osmolarity as well as volume of the culture medium and embryo density (Khadijah, 2012).

According to Mizutani *et al*. (2004), different genetic background could influence the response of oocyte towards PA even though the activation treatment applied is similar (Li *et al*., 2005). For instance, oocytes of Sprague-Dawley rats were more susceptible activated in 1.25 mM of strontium (6 hours) and produced more cleaved PA embryos compared to Wistar strain (Kato *et al*., 2001).

Different types of activation treatment also could affect the efficiency of artificial activation in oocytes. Pathak *et al*. (2013) reported that goat oocytes exposed in ethanol (5 min) with combination of DMAP + CHX (74.0%) showed the highest cleavage rate compared to only one chemical activation per group as DMAP (42.8%) and CHX (58.2%). This significant result showed that by combining two different activation

treatments, it can enhance the signaling pathways of parthenotes development due to synergistic effect produced by both chemical activator agents.

Ongeri *et al*. (2001) mentioned that types of animal species, culture media and activation treatments applied for PA can affect the cleavage rate of parthenotes. As for *in vitro* culture, the formulation and composition of the medium is important due to different cell types possessing different type of receptors involved in cell growth, survival, differentiation and to excrete different chemicals to the environment. Extreme exposure of culture medium to different factors such as pH levels and temperature will disrupt the embryonic developments of parthenotes (Hannoun *et al*., 2010). There were several different types of basal culture medium in which depends on the animal species. For instances, Potassium Simplex Optimisation Medium (KSOM) (Erbach *et al*., 1994), Sythetic Oviductal Fluid (SOF) (Tervit *et al*., 1972; Krisher *et al*., 1999) and CR1aa and CR2 (Rosenkrans & First, 1994).

2.3 SOMATIC CELL NUCLEAR TRANSFER (SCNT)

2.3.1 Background

It has been two decades since the birth of first cloned sheep, 'Dolly' through somatic cell nuclear transfer (SCNT). The birth of Dolly in 1997 marked an important turning point in animal reproductive cloning prospects by proving that animal could be cloned by using SCNT technique and opened up various new opportunities for further research especially in biomedical research and agricultural science (Jang *et al*., 2010). Many livestock species have been cloned successfully using intraspecies SCNT technique including sheep (Schnieke *et al*., 1997; Wilmut *et al*., 1997), cattle (Cibelli *et al*., 1998; Kato *et al*., 1998; Vignon *et al*., 1998; Wells *et al*., 1998; Shiga *et al*., 1999; Zakhartchenko *et al*., 1999; Kubota *et al*., 2000), goat (Baguisi *et al*., 1999) and pig (Betthauser *et al*., 2000; Onishi *et al*., 2000; Polajeva *et al*., 2000). With further intensive and refined research, it is hoped

that SCNT could be an integral component to be applied commercially in livestock farm practices in the foreseeable future.

SCNT research has evolved so much over the years that make it possible to generate desirable and high value traits of livestock species, increase productivity of livestock industry by improving the genetic material and to conserve endangered animal species. The concept of nuclear transfer had been first developed by a German scientist; Hans Spemann in 1938 in salamander species (Vatja & Gjerris, 2006). Meanwhile, the first successful attempt of nuclear transfer in mammals was reported in murine by McGrath and Solter (1983) through the exchange of pronuclei between two types of zygotes. Majority of nuclear transfer studies during earlier phase used embryonic or foetal germ line-derived cells as donor nuclei but then, after the first report on birth of Dolly the sheep from a cultured mammary gland cell in 1997, many other species had been successfully cloned by using different types of adult somatic cells as a donor karyoplast.

SCNT procedure is a complex process because it involves numerous of critical steps and most of them still need to be further optimised. In pursuance of SCNT to be successful, every single step must be meticulously conducted as it might affect the developmental ability of reconstructed embryos. Firstly, oocytes will be collected and matured *in vitro* beforehand. After *in vitro* maturation (IVM), maternal nucleus will be removed from the oocyte (recipient cytoplast) and a donor cell (karyoplast) will be injected into recipient cytoplast. Reconstructed oocytes are then undergoing a series of artificial activation for further development (Kishigami *et al*., 2006). Subsequently, cloned embryos will be cultured for a few days before transferred into recipient does via oviduct or uterine tract for the production of healthy cloned offspring. SCNT technique of cloned-goat embryos will facilitate the production of cloned goats at a rapid rate (Abdullah *et al*., 2011). Due to difficulty in obtaining goat oocytes from the abattoir,

therefore, by using cattle oocyte as recipient cytoplast in interspecies goat SCNT would provide a promising alternative tool to produce many cloned goats within a short period.

Song *et al*. (2008) was the first to produce interspecies cloned goat blastocyst (7.9%) using cattle oocyte as recipient cytoplast. Ma *et al*. obtained blastocyst (7.4%) using goat-sheep. Our research group (Kwong *et al*., 2012; Soh *et al*., 2012), using goat-cattle interspecies SCNT of cloned blastocyst (15.09% and 12.07%), respectively. However, after embryo transfer of the interspecies cloned embryos no pregnancies were detected in their experiments. Therefore, it is timely to optimise the various factors affecting the success of interspecies SCNT in goat before it can be applied in the Malaysian goat industry. These factors include different type of donor karyoplast, passage number, duration of ovarian transportation, *in vitro* maturation of oocytes, culture medium and injection and fusion technique.

Cloning in animals is quite challenging due to many complications such as nuclear reprogramming, cell cycle synchronisations, egg activation, mitochondrial and cytoplasmic incompatibilities. The removal of maternal nucleus in oocyte could abrupt assembly of the first mitotic spindle and subsequently arrests further development of cloned embryo (Chung *et al*., 2009; Egli *et al*., 2011).

2.3.2 Intraspecies SCNT

In SCNT, a donor karyoplast is transfected into a recipient cytoplast of a same species/family/order/class. The resulting reconstructed embryo is then transferred into the uterus or oviduct tract of a suitable recipient female (e.g. doe) for further development to progress bypassing normal fertilisation by sperm and the need to wait for suitable breeding cycles (Loi *et al*., 2011). SCNT has been studied extensively and contribute exclusively in livestock animal production, biomedical research (e.g. stem cell research, drug and medicine production and transgenic animals), infertility treatment and

conservation of wild animals (Ogura *et al*., 2013). There has always been a definite interest from scientists all around the world towards the probability of producing clone livestock animals. SCNT could be one of the convenient techniques in goats and sheep farming as it can allow genotypes with the highest genetic merit to be inaugurated into commercial farms bypassing the traditional breeding procedure of upgrading (Nicholas & Smith, 1983).

To date, SCNT has been established in five species including mice, cattle, pigs, goats and sheep, though there are actually quite a number of SCNT research that has been done or still ongoing on other species such as rabbits, horses, cats, dogs and primates (Westhusin *et al*., 2001). As for mice, the earliest successful SCNT was done by Wakayama *et al*. (1998) through piezo-micromanipulation technique by using cumulus cells as donor karyoplast, resulting in the first born of cloned mice namely, *Cumulina* in 1997. After the first successful attempt of mice SCNT by Wakayama *et al*. (1998), many other researchers tried to improve the efficiency of that technique by using other different donor karyoplasts and addition of certain organic compound such as trichostatin A (TSA) as histone deacetylase inhibitor (HDACi) to produce cloned mice offspring without any deformity (Kishigami *et al*., 2006).

The first attempt of cattle SCNT was done by Cibelli *et al*. (1998) producing cattle chimeric offspring by using transgenic embryonic stem cells which derived from 60 old-day foetus. Foetal fibroblast cells then transfected with a certain gene and fused with enucleated oocytes to produce blastocyst, eventually, transgenic ESC will be formed. Out of 99 chimaeric embryos, 22 (22%) reached blastocyst stage, 10 (0.45%) were transferred into 5 recipient females cattle, giving birth to 6 (60%) calves afterwards. Following the first successful attempt by Cibelli, Kato *et al*. (2000) had evaluated various types of donor karyoplasts obtained from foetal, new-born and adult cattle resulting in no significant

difference of the blastocyst percentages which showed foetal (40%), newborn (37%) and adult (42%) (Tsunoda & Kato, 2002).

Up to now, the progress of SCNT on goats is still lag behind compared to other mammalian species albeit of vigorous efforts and success of SCNT on other animal species. First nuclear transfer on goat had been conducted by Yong and Yuqiang (1998) using blastomeres as donor karyoplast which was consequently producing cloned goat kids. However, this technique was restricted by its inability of introducing foreign gene into blastomeres (Gavin *et al*., 2014). Goat SCNT was first carried out by Baguisi *et al*. (1999) using foetal fibroblast cells as donor karyoplast along with three different phases of oocytes as recipient cytoplasts, which are metaphase II, late metaphase II and telophase II producing 3 healthy live cloned goat offspring. A year later, Chen *et al*. (2000) had also obtained live cloned goat offspring using goat oocytes derived from slaughterhouse, meanwhile, Keefer *et al*. and Reggio *et al*. (2001) using LOPU-derived oocytes.

2.3.3 Interspecies SCNT

Interspecies SCNT employs the transfer of a donor karyoplast from a species (e.g. goat) into recipient cytoplast of different mammalian species (e.g. cattle). Hence, Interspecies SCNT offers an opportunity to avoid the loss of genetic diversity thus, providing the prospect to save endangered species from extinction. One of the challenges that restrict the conservation effort through in Interspecies SCNT is due to limited availability of source of oocytes from a targeted cloned species (e.g. wild animals) (Canovas & Cibelli, 2014). Hence, Interspecies SCNT could be as an alternative to propagate wild animals' population by cloning them using other closely related species' oocytes as a recipient cytoplast (Khan *et al*., 2014). This technique is more than convenient due to scarcity of selected oocyte species. Two main prerequisites of Interspecies SCNT are that early developmental processes and events must be evolutionary conserved among mammals

and factors that regulate all these molecular events in mammalian oocytes are able to interact with other species nuclei (Beyhan *et al*., 2007).

The success of interspecies SCNT is solely depends on genetic distance between donor karyoplast and recipient cytoplast. The recipient cytoplast plays a vital role in interspecies SCNT since the dominant distribution of mitochondrial DNA (mtDNA) and enzymes which responsible for epigenetic reprogramming and modification are exclusively from recipient oocytes (Steinborn *et al*., 1998). Thus, incompatibility between mtDNA and specific enzymes in different species may abrupt epigenetic reprogramming progress in oocytes and the embryonic development.

To date, there have been an enormous number of interspecies SCNT researches involving closely related species had been conducted such as gaur-cattle (Mastromonaco *et al*., 2007), wolf-dog (Kim *et al*., 2007), wild cat-domestic cat (Gomez *et al*., 2004), cattle-sheep (Hua *et al*., 2008), domestic sheep-wild sheep (White *et al*., 1999) and goat-cattle (Soh *et al*., 2012). Live interspecies cloned offspring have been produced mostly from closely related species such as cattle-gaur (Lanza *et al*., 2000; Mastromonaco *et al*., 2007), cattle-yak (Li *et al*., 2006a, 2006b), domestic cat-wild cat (Gomez *et al*., 2004) and domestic sheep-wild sheep (Loi *et al*., 2001).

2.4 TIMELINE OF SIGNIFICANT FINDINGS OF INTRA- AND INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER IN CATTLE AND GOAT

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

Year	Author	Intra-/interspecies SCNT	Species	Significant finding
1998	Cibelli *et al.*	Intraspecies SCNT	Cattle	First report on cattle SCNT in which six cloned calves were produced using day 60 foetus as donor karyoplast.
1998	Kato *et al.*	Intraspecies SCNT	Cattle	Eight calves (four died) had been produced through cattle SCNT using cumulus cells and oviduct cells as donor karyoplast.
1999	Baguisi *et al.*	Intraspecies SCNT	Goat	Three identical female offspring had been produced using foetal somatic cells as donor karyoplast.
2000	Wang *et al.*	Intraspecies SCNT	Goat	Cloned goat (*Capra hircus*) had been produced using foetal fibroblast cell as donor karyoplast.
2001	Keefer *et al.*	Intraspecies SCNT	Goat	*In vitro* transfected and nontransfected goat foetal fibroblasts as donor karyoplast produce cloned goat offspring after nuclear transfer.
2001	Zou *et al.*	Intraspecies SCNT	Cattle	Survival rate of cloned goat embryos by WCICI technique was higher than SUZI technique.

Table 2.1: Continued.

2001	Keefer *et al.*	Intraspecies SCNT	Goat	No significant differences between granulose cells and foetal fibroblast cells as karyoplasts in the rates of pregnancy and nuclear transfer efficiency.
2003	Ohkoshi *et al.*	Intraspecies SCNT	Goat	Cloned goat offspring had been produced through nuclear transfer using anterior pituitary cells as donor karyoplast derived from in an *in vitro* culture.
2004	Kubota *et al*.	Intraspecies SCNT	Cattle	Two generations of healthy cattle were serially cloned with normal-length telomeres as to age-matched controls when they were measured at birth.
2004	Zhang *et al.*	Intraspecies SCNT	Goat	Developmental competence of reconstructed goat embryos can be improved using donor karyoplast at G0/G1 phase.
2005	Melican *et al.*	Intraspecies SCNT	Goat	More live cloned goat had been produced using donor karyoplast harvested through partial trypsinisation as well as donor karyoplast cultured in low serum versus cycling cells grown to confluence to synchronise G0/G1 stage cells.
2006	Lan *et al.*	Intraspecies SCNT	Goat	The fusibility and *in vitro* developmental potential of embryos cloned from passages 3 to 5 of foetal fibroblast were higher.

Table 2.1: Continued.

2007	Chen *et al.*	Intraspecies SCNT	Goat	First success in cloning Asian Yellow goat through whole cell intracytoplasmic injection (WCICI) combined with telophase II enucleation method.
2008	Daniel *et al.*	Intraspecies SCNT	Goat	The difference between cumulus and fibroblast membrane surface may cause higher fusion rate derived from cumulus cells to produce cloned goat embryos.
2009	Tao *et al.*	Intraspecies SCNT	Goat	Production of cloned Boer goat (*Capra hircus*) by nuclear transfer using cultured and cryopreserved ear fibroblast cells injected into abattoir derived oocytes.
2010	Akshey *et al.*	Intraspecies SCNT	Goat	Production of goat blastocyst through handmade cloning using foetal fibroblast as donor karyoplast in flat surface culture system.
2010	Dalman *et al.*	Intraspecies SCNT	Goat	Synchronisation of cells at full confluency which arrested at G0/G1 phase induced less apoptosis as to serum starvation group.
2011	Abdullah *et al.*	Intraspecies SCNT **Interspecies** SCNT	Goat	The efficiency of interspecies SCNT was similar to that of intraspecies SCNT as both to produce cloned goat embryos.
2011	Akshey *et al.*	Intraspecies SCNT	Goat	Effect of roscovitine; a cyclin dependent kinase inhibitor on donor cells and methods of activation.

Table 2.1: Continued.

2011	Tang *et al.*	Intraspecies SCNT	Goat	Cloned goat embryos cultured in mSOF medium supplemented with 10% FBS could produce healthy cloned goat offspring.
2012	Kwong *et al*.	Intraspecies SCNT **Interspecies** SCNT	Goat Cattle-Goat	Increasing glucose in KSOMaa basal medium on culture improves *in vitro* development of cloned caprine produced in both intraspecies and interspecies somatic cell nuclear transfer.
2012	Soh *et al*.	Interspecies SCNT	Cattle-Goat	Using goat FFC as donor karyoplast in goat-cattle interspecies SCNT.
2013	Zhang *et al.*	Intraspecies SCNT	Cattle-Goat	Addition of 25uM α-Lipoic acid into oocyte IVM medium improved maturation rate and antioxidant ability of oocytes and subsequently increase the percentage of cloned blastocyst.
2014	Saini *et al*.	Intraspecies SCNT	Cattle	Trichostatin A alters the expression of cell cycle controlling genes and microRNAs in donor cells and subsequently improves the yield and quality of cloned cattle embryos.

2.5 FACTORS AFFECTING SOMATIC CELL NUCLEAR TRANSFER (SCNT) EFFICIENCY

SCNT requires *in vitro* manual manipulation which involves exposure of oocytes towards external environment along with cells fluctuations in temperature, atmospheric conditions, different media, CO_2 and humidity. There are several factors that can affect the outcome of the application of SCNT in animal species including cell cycle synchronisation, egg activation, nuclear reprogramming, genomic imprinting, mitochondrial, cytoplasmic incompatibilities and technical problems that can affect the overall efficiency of the SCNT procedure involving stages of donor cycle, donor cell line passages, selection of recipient cytoplast, selection of donor cells and technical skills in SCNT technique (Soh, 2012).

2.5.1 Recipient cytoplasts source and quality

Competent and matured oocytes are a prerequisite for successful SCNT in all species (Jang *et al*., 2008). Maintenance of oocytes quality is a crucial step to ensure that the starting material for SCNT is of the top-notch. During SCNT, recipient cytoplast will determine the destiny of the embryo through epigenetic modification and responsible for multiple molecular process including reprogramming the differentiated cell to a totipotent state and controlling embryonic genome activation (Mtango & Kono, 2004). The ideal recipient cytoplast should be in unlimited availability and be well adapted to an *in vitro* maturation system. Another thing that need to be considered is whether the recipient cytoplast obtained by *in vivo* (flushing) or *in vitro* (LOPU-derived or slaughterhouse-derived oocytes). For livestock species, most of the time oocytes can be collected from slaughterhouse-derived ovaries. It is possible to produce cloned goat offspring using slaughterhouse-derived oocytes. Since then, the feasibility of using slaughterhouse-

derived oocytes as recipient cytoplasts in SCNT has been continuously inscribed by several researchers in various animal species (Gavin *et al*., 2014).

An oocyte (a recipient cytoplast) is a 'holy grail' that consists of diverse types of enzymes including the histone deacetylase (HDAC1) and the histone demethylases (JMJD2C and JMJD3) for histone remodeling (Wang *et al*., 2011; Ross & Canovas, 2016). Disorganisation of these nucleolar proteins and lower expression levels of these proteins during epigenetic reprogramming can cause aberrant in embryo development progress (Song *et al*., 2014). When a donor karyoplast injected into enucleated MII phase oocyte, premature condensation of the donor chromosomes occurs and this marked the starting point of nuclear reprogramming event to take place.

Matured oocytes are a prerequisite of SCNT either they are collected by *in vivo* or *in vitro* technique. As for *in vitro* origin sources, oocytes need to undergo *in vitro* maturation (IVM) prior to injection of donor karyoplast during SCNT. Several attempts by researchers have been made to optimise IVM of oocytes specifically in goat and cattle species. In cattle, most IVM durations from previous researches were around 22 to 24 hours.

2.5.2 Type of donor karyoplasts

Donor karyoplast will be injected into recipient cytoplast after the removal of maternal nucleus. It has been concluded that the type of donor cells could affect the efficiency of SCNT. Ever since the first report of Dolly the sheep successfully cloned using adult somatic cells, it is now widely applied in a various types of animal species to produce cloned offspring derived from different types of somatic cells originated either from adult or foetal (Prelle *et al*., 1999). Up to now, many different species of cloned animals have been produced after nuclear transfer using various types of donor karyoplast of somatic cells from tissues such as cumulus, ear, mammary gland, skin, muscle, sertoli cells, tail,

oviduct, bone marrow mesenchymal stem cell and killer T cells (Kato and Tsunoda; 2011). The most common source of donor karyoplast is dermal fibroblast as it is easy to obtain and can be cultured using standard tissue culture technique (Westhusin *et al*., 2007).

The efficiency and developmental potential of reconstructed oocytes using somatic cells from various types of tissues of adult and foetal have been examined. However, it is still remaining unclear, which donor karyoplast origins are most suitable for animal cloning (Kato *et al*., 2000). One of the profound explanations for the low success of cloning is that the reprogramming factor level of oocyte is not properly adapted providing the fact that a haploid from male gamete in normal fertilisation is constituted by donor karyoplast nuclei (Mizutani *et al*., 2014).

Researchers have evaluated different types of donor karyoplasts including gender to determine the efficiency rates of SCNT. The selection of donor karyoplast used in SCNT could impact the developmental rate of subsequent cloned embryo production (Powell *et al*., 2004). Foetal fibroblast cells (FFC) have been widely used in SCNT to produce cloned offspring because they have capacity to grow and proliferate more rapidly (Jang *et al*., 2010). FFC has been the most efficient donor karyoplast for producing cloned offspring in various types of animal species (Cibelli *et al*., 1998; Onishi *et al*., 2000; Galli *et al*., 2003; Woods *et al*., 2003). Saikhun *et al*. (2002) mentioned that FFC can produce more blastocyst compared to cumulus cells and oviductal cells (adult cells) in buffalo-bovine interspecies SCNT. This finding was in parallel with Forsberg *et al*. (2002), in which foetal cells gave higher blastocyst and calving rates compared to donor karyoplasts derived from cumulus cells. Wakayama *et al*. (2001) also found that the efficiency of SCNT was higher with FFC as donor karyoplasts (2.2%) compared to adult cells (1.7%). This indicated that FFC can be easily reprogrammed in the recipient cytoplast compared to donor karyoplasts derived from adult cells (Soh *et al*., 2012).

2.5.3 Cell cycle of donor karyoplasts

The stage of donor karyoplast cycle synchronization between donor karyoplast and recipient cytoplast has a major effect on the reprogramming process and subsequent cloned embryo production (Cibelli *et al*., 1998). The first successful effect of donor cell cycle synchronisation at G0 stage had been first described in the production of Dolly the sheep experiment by Wilmut *et al*. (1997) by using serum starvation method. In serum starvation, majority of cells are arrested in either G0 or G1-phase and has been applied in almost all successful reports of animals SCNT.

The cell cycle of donor karyoplast can influences the extent of embryo growth after SCNT procedure. After injection of donor karyoplast into the MII stage of recipient cytoplast, the nuclear envelope, which separates nucleus from cytoplasm will disintegrate causing chromosomes to condense until the activation of oocyte take place (Meena & Das, 2006). Studies have shown that this event can cause chromosomal defects in donor karyoplast because that is when a cell starting to pass genetic information to its offspring. Different approaches have been applied to enhance SCNT efficiency such as serum deprivation, chemical inhibitors and contact inhibition. These methods have been studied extensively in various animal species including goats (Baguisi *et al*., 1999), sheep (Wilmut *et al*., 1997) and cattle (Wells *et al*., 1999).

Gibbon *et al*. (2002) reported that donor karyoplast treated with roscovitine can enhance epigenetic reprogramming and pregnancy rates in cattle. Akshey *et al*. (2011) described that goat FFC treated with roscovitine yields a higher percentage of blastocyst (19.31%) compared to serum starvation method which is only 6.82%. Roscovitine is a cyclin-dependent kinase 2 (Cdk 2), an inhibitor that can suppress the effect of cyclin E, cyclin A and maturation promoting factor (MPF) in which subsequently arrest cells at G1 stage. Cells arrested at this stage are more susceptible to reprogramming by recipient cytoplast (Gibbons *et al*., 2002). Treatment of donor karyoplasts with trichostatin A

(TSA) also can help to increase blastocyst production by modifying histone acetylation patterns, thus, improve gene expression and nuclear reprogramming in mice, buffalo and goat cloned embryos, respectively (Kishigami *et al*., 2006; Chan *et al*., 2013).

2.5.4 Passages number of donor karyoplasts

Passage number is the amount of time cells have been detached from culture dish and subcultured. When working with cell lines, it is important to know how many passages that the cell lines have been subcultured due to the fact that it can influence SCNT efficiency rates (Juan *et al*., 2014). Cell lines at low passage numbers experience low risks of alterations in morphology, growth rates, protein expression and transfection efficiency compared to cells at high passage numbers (Li *et al*., 2014). Such abnormalities on donor karyoplasts will disrupt the capability of cells to reprogramme after fused with recipient cytoplast (Condic, 2008). Chacon *et al*. (2011) reported that continuous passaging of cells can affects the level of H3K9ac in cattle fibroblast; and subsequently reduce the cleavage rates of cloned cattle embryos.

High passage number also could result in chromosomal instability which correlated with telomere shortening (Westhusin *et al*., 2000). Telomere shortening is one of the major factors that cause senescence in somatic cells after culturing (Harley *et al*., 1990). Hande *et al*. (1999) reported that utilising high passage number of donor karyoplast could cause chromosomal aberrations as each passage will shorten the telomeres to the point where the length of telomere is insufficient to ensure the chromosomal stability. Thus, it would results in low efficiency of animal SCNT.

2.5.5 Donor karyoplast injection method

The most common injection methods that have been widely used by researchers worldwide in SCNT are sub-zonal injection (SUZI) and whole cell intracytoplasmic injection (WCICI). Soh *et al*. (2012) reported that SUZI method produced significantly

higher amount of 2-, 8-cell, morulae and blastocyst compared to WCICI technique. This finding also corresponded with Kawano *et al*. (2004) in pig SCNT, with the blastocyst rate for SUZI technique being higher than WCICI (18 vs. 13%), respectively. It is believed that SUZI method is less invasive compared to WCICI technique since it induces low damage on the isolated donor nuclei (Lee *et al*., 2003; Chen *et al*., 2007). Meanwhile, in WCICI technique, the plasma membrane of donor karyoplast need to be ruptured first until it form elongated structure before injection by pipetting repeatedly in and out in which can consequently injure the nucleus of donor karyoplast (Soh *et al*., 2012).

2.5.6 Method of activation

Another contributing factor that can affect SCNT efficiency is the method of activation. After the injection of donor karyoplasts, reconstructed oocytes must undergo artificial activation to progress further development. In normal fertilisation, the influx of Ca^{2+} could change the membrane potential of the oocyte in which subsequently blocking polyspermy to occur right after insemination process (Santella *et al.,* 2004).

There are several ways that can be applied to activate oocyte chemically whether by single or combined treatment. Chemicals including ionomycin (Iono), calcium ionophore (A23187), ethanol (EtOH) and 6-Dimethylaminopurine (6-DMAP) can facilitate Ca^{2+} influx into the recipient cytoplast (Nakada *et al*., 2001). Researchers have done quite a number of attempts to optimise activation protocol of SCNT in various animal species (Krivokharchenko *et al*., 2003).

2.5.7 *In vitro* culture (IVC)

Culture medium played a major role in *in vitro* production of cloned embryos due to its sensitivity as it could affect the gene expression, morphology of the embryo and subsequent embryonic growth as well as pre- or post-natally (Summers & Biggers, 2008;

Fleming *et al*., 2004). Therefore, it is necessary to understand the physiology and metabolism aspect of the embryo so that the researchers could evaluate the development and efficiency of different condition of culture systems (Gardner, 2008).

There are various types of culture medium that have been developed depending on the animal species (Feugang *et al*., 2009). The earliest medium developed was based from the composition of sheep oviductal fluid namely; Synthetic Oviductal Fluid (SOF) (Tervit *et al*., 1972). A few years later, another customary culture media known as Tissue Culture Medium-199 (TCM-199) was formulated (Eyestone *et al*., 1987).

The success of culturing cloned embryos depends on essential nutritional requirements by altering the culture conditions empirically (Barnett & Bavister, 1996). However, it is still uncertain which medium is the most effective to support the preimplantation development specifically for cloned animal embryos (Kang *et al*., 2002). There are several factors that could affect the efficiency of IVC which afterwards will influence the success rate of oocyte and embryo developments. Significant progress has been made during recent years in order to improve SCNT embryo development. Various attempts have been made on IVC culture medium such as supplementation of MEM non-essential amino acids, L-glutamine, sodium pyruvate and foetal bovine serum (FBS) in order to improve the blastocyst rate (Mitalipov *et al*., 1999; Lane *et al*., 2003).

In order to improve *in vitro* culture systems, researchers had conducted various studies in attempt to overcome the issue related to addition of serum in a culture medium either by delay in addition, removal and replacement or using different concentration levels (Bavister, 1995; Thompson *et al*., 1998; Jacobsen *et al*., 2000; Duque *et al*., 2003; Lonergen *et al*., 2003; Wirtu *et al*., 2004). Up to now, culture media have been supplemented with a various type of antioxidants, growth factors and also macromolecules. However, addition of certain compounds could cause abnormalities of cloned embryo and foetus. For example, addition of serum could cause neonatal loss

which is associated with Large Offspring Syndrome (LOS). Addition of certain hormones also could cause other anomalies on embryo and foetus such as chromosomal aberrations, pregnancy complications which would later causing a high rate of a foetus loss (Thompson *et al*., 1995, Young *et al*., 1998, Farin *et al*., 2001, Lonergen *et al*., 2004).

Growth factor components including foetal bovine serum (FBS) and bovine serum albumin (BSA) have been extensively used in IVC of cloned embryo. Previous studies have reported that serum including FBS could increase the blastocyst production and subsequent hatching blastocyst (Wang *et al*., 1997; McElroy *et al*., 2008). However, the role of serum on cloned embryo development is still remaining polemical. Some reports showed that early exposure of FBS to embryos would cause a detrimental effect on the quality of blastocyst produced (Thompson *et al*., 1995; Rizos *et al*., 2002). On the other hand, study on the effect of BSA had been conducted on in vitro mouse embryo development indicated that BSA reduce the preimplantation development rate (Fernandez *et al*., 2004).

2.6 EPIGENETIC REPROGRAMMING

In order reconstructed oocytes could develop into a healthy embryo, epigenenetic from cell-type specific must be erased through a mechanism called 'reprogramming' so that the cells could return into a 'blank slate' of genetic state and gaining totipotency (Surani, 2001). In SCNT, reprogramming happened within a short period of time, which is specifically between the injection of donor karyoplast and the initiation of embryonic transcription process. However, the timing of the process is different depending on animal species. For instance, the timing of embryonic transcription in human, cattle and sheep occur at 8-16 cell stage while in mice it occurs at 2-cell stage (Telford *et al*., 1990; Kues *et al*., 2008). Thus, it will alter the timing of reprogramming process to occur. Oestrup *et al*. (2009) reported that nuclear reprogramming in cattle occurs as early during the 3 hours

after SCNT procedure, in which the chromatin of transferred karyoplast decondenses towards the periphery of cell gradually and form the nuclear envelope.

Eventhough SCNT of mammals from various types of donor karyoplast has been successfully conducted in goat and cattle, the efficiency rate in producing live cloned offspring is still very low (<5%) due to incomplete epigenetic reprogramming. Incomplete epigenetic reprogramming could leads to irregularities in gene expression and subsequently could cause a developmental failure of cloned animals (Wang *et al*., 2004). SCNT of mammals using different somatic donor karyoplast needs the erasure of epigenetic imprints that mark the DNA, through rendering the somatic nucleus totipotent (Mann & Bartolomei, 2002).

2.6.1 DNA Methylation

DNA methylation plays a fundamental role in any of cloned animal embryos since it is one of the earliest steps in nuclear reprogramming. The function of DNA methylation is to provide the heritable DNA genetic information to the DNA which is not encoded in the nucleotide sequence. DNA methylation takes place at position 5 of the pyrimidine ring of cytosines and usually restricted to CpG dinucleotides in somatic cells (Hill *et al*., 1999). It usually occurs within 1-2 cell stage after injection of donor karyoplast takes place.

DNA methylation involves in a various range of cellular functions and pathologies such as genomic imprinting, cell differentiation, tissue-specific gene expression, X-chromosome inactivation, regulation of chromatin structure, carcinogenesis as well as aging (Jaenisch, 1997; Jeltsch, 2002; Robertson & Wolffe, 2000; Eden *et al*., 2003; Hashimshony *et al*., 2003; Gaudet *et al*., 2003;).

There are three types of catalytically active DNA methyltransferases which involved in the maintenance and establishment of DNA methylation process including

Dnmt1, Dnmt3a and Dnmt3b (Robertson & Wolffe, 2000; Bestor, 2000). Dnmt1 is a maintenance methyltransferase enzyme which is responsible for adding methyl groups during DNA replication process. The Dnmt3 family is responsible for adding methyl groups that occurs subsequently after implantation (Wang *et al*., 2004). Any dysregulation of these enzymes will abrupt the DNA methylation patterns which could consequently cause embryo lethality or abnormality in subsequent offspring phenotype.

Several researchers have reported on abnormalities in DNA methylation. The reason is mainly due to the epigenetic difference between the somatic donor karyoplast and the recipient cytoplast, subsequently causing the nucleus of donor karyoplast responds discordantly to the recipient cytoplasts which will later affects the consequent events during embryogenesis process. For instance, in the pronuclei of the maternal and paternal genome, the demethylation process is highly more coordinated than in the somatic cell genome after SCNT process (Wang *et al*., 2004).

2.6.2 Mitochondrial DNA (mtDNA)

Mitochondria is a semiautonomous organelles which play a vital role in most of the biological processes such as production of ATP through oxidative phosphorylation, apoptosis, regulation of intracellular calcium levels and cellular aging. Mitochondria in oocytes are spherical in shape and have fewer and less-prominent cristae which are different from other mitochondria in all eukaryotic organisms (Beyhan *et al*., 2007). During SCNT process, a relatively small amount of the donor karyoplast mitochondria are deposited into the reconstructing embryo which could cause mtDNA heteroplasmy. mtDNA encodes several genes and consists of a single major noncoding control region (CR) which is important for initiation of transcription and replication process.

Studies on sheep, mouse and cattle SCNT embryos indicated a high level of diversity in term of mitochondrial distribution with certain animals displaying a various

level of heteroplasmy (Han *et al*., 2003; Hiendleder *et al*., 2003; Takeda *et al*., 2003; Inoue *et al*., 2004) and others displaying a complete homoplasmy (Evans *et al*., 1999; Meirelles *et al*., 2001).

Despite a healthy live offspring have been produced by both intra- and interspecies SCNT technique over the years, probable negative effects arisen from heteroplasmy introduced by these animals could cause deficiency in cloned embryos. This is due to the incompatibility attributed by mitochondrial from maternal and donor karyoplast, which could negatively affect the function of mitochondrial and leads to suboptimal respiration for instance. Therefore, it is important to take into consideration of genetic distance between the nuclear of donor karyoplast and mitochondrial before conducting animal SCNT so that the embryo could develop to term (Brenner *et al*., 2005).

CHAPTER 3

METHODOLOGY

INTRODUCTION

The aim of this study is to produce interspecies cloned goat-cattle embryos using WCICI technique. Cattle oocytes (recipient cytoplast) were retrieved from slaughterhouse-derived ovaries and goat foetal fibroblast cells (FFC) were used as donor karyoplast to produce iSCNT cloned embryos. Cattle ovaries were collected from slaughterhouse located in Shah Alam and Banting. Cattle oocytes retrieved from these ovaries underwent *in vitro* maturation (IVM) prior to cloning and parthenogenetic experiments.

All experiments were conducted in Embryo Micromanipulation Laboratory (EMIL) and Animal Biotechnology-Embryo Laboratory (ABEL), Institute of Biological Sciences, Faculty of Science, University of Malaya. This laboratory research was conducted from October 2013 until November 2015.

3.2 EXPERIMENTAL ANIMALS

In this study, mice, goat and cattle species were used. Guidance for all aspects for the care and the use of experimental animals were in accordance with University of Malaya regulation.

3.2.1 Mice

Prior to this study, 100 female mice of BALB/c (albino) strain were used as model animal for learning curvature purpose. The mice were kept in the animal cages at room temperature and bred in the Animal House of Livetock Science Centre (LSC) in University of Malaya. Each cage was coated with dry sawdust obtained from local sawmill in Selangor. The sawdust was changed every 2 weeks to ensure the cleanliness of animal cages. Dirty cages were washed and dried out before reused. Mice were fed *ad libitum* with commercial pellet and clean drinking water.

Female mice were isolated from male at least 4 weeks before experiment prior to superovulation regime to ensure that there will be no pregnancy occurs. Female mice were injected with pregnant mare serum gonadotrophin (PMSG; 10 IU) and human chorionic gonadotrophin (hCG; 10 IU) for superovulation purpose. After 12 to 16 hours of hCG injection, oocytes were retrieved from female mice and SCNT were performed using cumulus cells as donor karyoplast.

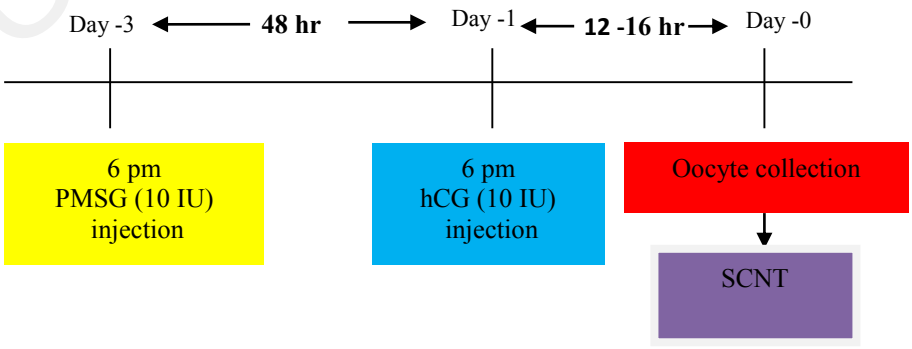


Figure 3.1: Timeline of female mice superovulation.

3.2.2 Goats

In this study, donor karyoplasts were obtained from goat foetal fibroblast tissue. Goat foetuses aged 31 and 70 old days were obtained from pregnant does (4 years old) which had been mated with Jermasia buck (more than 4 years old) in Rumpun Asia Sdn. Bhd. (RASB) Farm in Taman Kekal Pengeluaran Makanan (TPKM) Hulu Tamu, Selangor. Foetal tissue samples were obtained from does by surgery. Foetal tissues were then cultured, subpassaged, cryopreserved and stored in liquid nitrogen tank (-196°C).

3.2.3 Cattle

Cattle ovaries sample were collected from Abattoir Complex, Department of Veterinary Services of Shah Alam and Banting in Selangor depend on the availability of the female cattle. Ovaries sample were transported back to the laboratory from slaughterhouse by using thermal flask which contained normal saline solution supplemented with 0.9% NaCl (Sigma-Aldrich Co., USA) with penicillin and streptomycin to prevent bacterial contamination (Sigma-Aldrich Co., USA) at 35-37°C. The record of cattle ovary samples such as origin, history, breed, age, health status and oestrus cycle were unknown.

3.3 MATERIALS

Materials including laboratory disposables, equipments, chemicals, media and reagents used in this study are described in brief as the following.

3.3.1 Laboratory disposables

A list of laboratory disposables is described in Appendix Table 1.3 with manufacturer's name.

3.3.2 Equipment

A list of equipments used in this study is shown in Appendix table 1.1 with model number, manufacturer's and supplier's names. The most used equipments are including inverted microscope, micromanipulator attached with camer, micropuller, microforge, stage warmer, stereomicroscope, pH meter, surgical set, laminar air flow work station, liquid nitrogen tank, freezer, CO_2 incubator, centrifuge machine, water bath, autoclave and oven.

3.3.3 Chemicals, media and reagents

All of the chemicals used in this study were purchased from Sigma-Aldrich Co. (USA) unless otherwise mentioned. List of chemicals, media and reagents is presented in Appendix Table 1.2 with catalogue number, manufacturer's and supplier's name. This section will be describing several parts of experimental procedures including standard operation of tissue and embryo *in vitro* culture laboratory, preparation of media, preparation of micro tools and procedure of the experiments.

3.4 GENERAL METHODOLOGY

3.4.1 Standard operation procedure (SOP) of embryo and tissue culture laboratory

To ensure optimum embryo and tissue culture condition, all experimental activities and equipment enshrined to stringent cleanliness and sterile condition. Cleanliness and sterile environment during experimentation is needed to prevent contamination while working with cells, oocytes and embryos. Below are the conventional procedures implemented in our laboratory in favor of achieving good embryo and tissue culture environment.

3.4.1.1 The personnel

The personnel needed to wash hand with the disinfectant thoroughly first before conducting the experiment to reduce the adherence of bacteria and other microorganism which could cause contamination to the cell and embryo culture. Apart from that, researcher should be wearing a clean laboratory coat, face mask and gloves (if necessary) to protect themselves from any accidental spillage. The personnel also needed to avoid using any perfume, makeup, nail varnish or lotion which could be toxic to the embryos and subsequently compromise the results of the experiment.

3.4.1.2 The surface of work station

Inside of laminar air flow work station was sterilised by wiping with 70% ethanol and let it evaporate for about 30 minutes before commencing any experimental work. The UV radiation was applied for about 15 minutes if required. After the experiment ended, the inside surfaces of the laminar flow work station including all equipment were wiped back with 70% ethanol. Other than that, water bath also was cleaned frequently. After cleaning, the water bath was filled up with autoclaved RO water and changed every week to maintain the cleanliness.

3.4.1.3 The glassware and apparatus

All glasswares and non-disposable items were washed rigorously with tap water to remove any form of residues. The glasswares and non-disposable items were soaked overnight in a diluted detergent (7X®-PF) first before washing to remove traces of chemicals. After soaking, all labwares were cleaned using sponge with diluted detergent (7X®-PF) and rinsed thoroughly using tap water. All washed labwares were dried in the oven at 60°C. Dried labwares such as beakers, volumetric flasks and Scott bottles were

covered with aluminium foil while non-disposable items were packed in autoclavable bags with a piece of autoclave tape labelled with a researcher's name and date.

3.4.1.4 CO_2 incubator

Culturing of cell lines, oocytes and embryos were carried out in the CO_2 incubator (5%) in maximum humidified air to sustain appropriate physiological pH (pH range from 7.3 to 7.4) with similar temperature of a goat and cattle (38.5°C). Incubator was cleaned monthly in order to ensure sterility thus, providing ideal environment for the cell lines, oocytes and embryos to develop to term. Cleaning of incubator were done by wiping the inner walls, doors and racks with 70% ethanol and sterile reverse osmosis (RO) water using sterile tissues. The racks and trays inside the incubator were washed vigorously with a diluted detergent (7X®-PF) followed by rinsing with sterile RO water. After washing, all racks and trays were wiped with sterile dried tissues and sterilised using UV light for about 1 hr before putting back inside the incubator. The bottom tray was filled up with sterile RO water to provide a humidified environment inside the incubator. Opening and closing of the CO_2 incubator must be kept to minimum to prevent any contaminants from entering the incubator.

3.4.2 Preparation of media and reagents

Preparation of all media and reagents were done in the laminar air flow work station for sterility purpose. Most of the media and reagents used in this study were prepared from the water treated with reverse osmosis and electrodeionisation through Milli-Q®- RO Pulse pure water system. Apparatus such as conical flask, measuring cylinder, spatula and magnetic stirrer were autoclaved before preparing any stock or media solution. Chemicals in powdered form were weighted using digital analytical balance, whereas, chemicals in liquid form were measured using measuring cylinder, disposable pipette and

micropipette, precisely. The pH and osmolarity of the media were measured using pH meter at pH 7.2 to 7.4 and osmometer at 280 to 300 mOsm, respectively. A magnetic stirrer was used to dissolve the chemical compounds during media preparation. All prepared media and reagents were filtered using sterile disposable filter (0.22µm pore size, Sartorius) attached to syringe filter (depend on the media volume) to ensure sterility. The bottles or tubes which contained media were labelled with researcher's name and date, while the caps were sealed with parafilm before stored in refrigerator at 4°C or freezer at -20°C, respectively.

3.4.2.1 Preparation of ovary sample collection medium

Ovary collection medium contained normal saline solution, 0.9% (w/v) sodium chloride was dissolved in Milli-Q water supplemented with penicillin-G and streptomycin. This medium was used for collecting and washing cattle ovary samples from slaughterhouse. Normal saline solution possessed isotonic property which is suitable to prevent dehydration of live tissue. This medium was prepared in a 1 L Scott bottle by dissolving sodium chloride (9 g) in 1 L Milli-Q water. After preparation, the medium was autoclaved at 121°C, 20 minutes and be kept for 3 months in the refrigerator (4°C) prior to use. Before collecting the ovary samples, this medium was warmed up in the water bath at 37°C and poured inside the thermal flask (400 ml). This medium can be kept up to 3 months (Table 3.1).

Table 3.1: Composition of ovary collection medium

Chemical compound	**Catalogue number**	**Concentration**	**Quantity/litre**
NaCl	S5886	0.9 (w/v)	9.0 g
Penicillin-G	P7794	0.06 mg/ml	0.06 g

Table 3.1: Continued.

Streptomycin	S1277	0.05 mg/ml	0.05 g
Milli-Q water	-	-	1 litre

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

The IVM medium consisted of TCM-199 as a base medium supplemented with follicle stimulating hormone (FSH), oestradiol-17β, sodium pyruvate, foetal bovine serum (FBS), cystein and gentamicin. *In vitro* maturation (IVM) medium was prepared freshly and equilibrated at least 4 hours in the CO2 incubator (5%) prior to oocyte recovery.

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

Stock solution of IVM comprised of TCM-pyruvate, follicle stimulating hormone (FSH), oestradiol-17β and gentamicin sulphate salt. All of these stock solutions need to be prepared in advance before preparing IVM working medium. Each stock solution was then aliquoted into 1.5 ml microcentrifuge tube, sealed with parafilm, wrapped with aluminium foiled and labelled with name of the stock solution and date. All chemical compounds, concentration, method of preparation, method of storage as well as shelf life were depicted as in Table 3.2 below.

Table 3.2: Composition of IVM stock medium

Stock solution (catalogue number)	**Concentration (mg/ml)**	**Method of preparation/storage**	**Shelf life**
TCM-pyruvate	2.2	2.2 mg of sodium pyruvate dissolved with 1 ml of TCM-199 in microcentrifuge tube and sterile-filtered afterward.	3 days
Follicle stimulating hormone, FSH (Folltropin-V®)	5	5 mg Folltropin-V® was weighed and dissolved in 1 ml milli-Q water inside microcentrifuge tube, sealed with parafilm, wrapped in aluminium foil, labelled and stored in the refrigerator at 4°C.	6 months
Oestradiol-17β (E8875)	1	1 mg Oestradiol-17β dissolved in 1 ml ethanol (95%) inside microcentrifuge tube, sealed with parafilm, wrapped in aluminium foil, labelled and stored in the refrigerator at 4°C.	6 months

Table 3.2: Continued.

Gentamicin (G3632)	50	1 g gentamicin sulphate salt dissolved in 1 ml ethanol (95%) inside microcentrifuge tube, sealed with parafilm, wrapped in aluminium foil, labelled and stored in the refrigerator at 4°C.	6 months

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

For IVM medium preparation, TCM-199 (8.9 ml) was measured using a sterile disposable pipette (10 ml) and dispensed into a sterile tube (15 ml). Cystein (0.9 mg) was weighed by using a digital analytical balance and dissolved in the TCM-199 solution. After that, sodium pyruvate (2.2 mg) was weighed and dissolved in 1 ml of TCM-199 solution in a sterile 1 ml microcentrifuge tube. TCM-pyruvate was measured using a sterile disposable micropipette tips (100 µl), and subsequently mixed with TCM-199 solution which contained cysteamine. Other stock solutions including FSH (10 µl), gentamicin (5 µl) and FBS (1000 µl) were added to the TCM-199 solution by using sterile micropipette tips. IVM medium was mixed well and filter-sterilised by using syringe filter (0.22 µm pore size, Sartorius). After filtration of the medium, Oestradiol-17β (9.5 µl) was added. Lastly, IVM medium was sealed with parafilm, wrapped in aluminium foil and stored in the refrigerator (4°C) with a shelf life of 2 weeks (Table 3.3).

3.4.2.3 Preparation of TL-Hepes medium

TL-Hepes was used for washing, rinsing and slicing of cattle ovaries obtained from slaughterhouse as well as during searching of cattle cumulus-oocyte-complexes (COC) before transferred into the IVM medium. Apart from that, TL-Hepes also involved in embryo micromanipulation works such as during enucleation and injection in SCNT and embryo washing. This medium contained Hepes which can helps to maintain the appropriate pH, hence making it convenient to be as a base medium for micromanipulation works.

3.4.2.3 (a) Preparation of TL-Hepes stock medium

TL-Hepes stock solution was prepared by weighing all chemicals compound as depicted in Table 3.3 using digital analytical balance and dispensed into sterile conical flask contained 500 ml Milli-Q water. Milli-Q water was measured using 100 ml measuring cylinder until reaching final voulume of 500 ml. The chemicals were stirred gently by using a magnetic stirrer. Sodium lactate was added slowly into the mixture of medium. TL-Hepes was then filter-sterilised by using syringe filter (0.22 μm pore size) and stored in the refrigerator (4°C) with a shelf life of 3 months.

Table 3.3: Composition of TL-Hepes stock medium

Chemical compound	**Catalogue number**	**Concentration (mg/ml)**	**Quantity/500 ml**
NaCl	S5886	114.0	3.330 g
KCl	P5405	3.2	0.120 g
$NaHCO_3$	S5761	2.0	0.084 g
$NaH_2PO_4H_2O$	S9638	0.4	0.028 g
Na Lactate (60%)	L7900	10.0	0.93 ml

Table 3.3: Continued.

$CaCl_2.2H_2O$	C3881	2.0	0.150 g
$MgCl_2.6H_2O$	M2393	0.5	0.050 g
Hepes $C_8H_{17}N_2O_4SNa$	H3784	10.0	0.600 g
Hepes $C_8H_{18}N_2O_4S$	H3375	10.0	0.600 g
Penicillin-G	PENNA	100 IU/ml	0.0325 g
Milli-Q water	-	-	500 ml

3.4.2.3 (b) Preparation of TL-Hepes working medium

TL-Hepes working solution was prepared 100 ml each time using a sterile disposable pipette (25 ml, Falcon). TL-hepes stock solution was measured until it reaching 100 ml and dispensed into a sterile conical tube (200 ml). Gentamicin stock solution (25 µl) was measured using sterile micropipette tips and dispensed into the medium. After finished preparing TL-Hepes working solution was filter-sterilised by using syringe filter (0.22 µm pore size) before used. The working solution was stored in the refrigerator (4°C) with a shelf life of 2 weeks (Table 3.4).

Table 3.4: Composition of TL-Hepes working medium

Chemical compound	**Catalogue number**	**Quantity/100 ml**
TL-Hepes stock solution	-	99.75 ml
Gentamicin stock solution	-	25 µl
Sodium pyruvate	P4562	1.1 mg
BSA-FV	A7030	50 mg

3.4.2.4 Preparation of hyaluronidase medium

Hyaluronidase is an enzyme solution to speed up the process of removing cumulus cell (CC) after IVM of oocytes. Hyaluronidase solution was prepared by weighing hyaluronidase powder using analytical balance and dissolved in TL-Hepes working solution (1 mL) in a sterile microcentrifuge tube (1.5 ml). The prepared solution was mixed thoroughly by using vortex machine and filter-sterilised by using syringe filter (0.22 μm pore size). The solution was then aliquot into 100 μl microcentrifuge tubes, sealed with parafilm, labelled on the cover, wrapped in aluminium foil and stored in a the freezer (-20°C) with a shelf life of 6 months. Before commencing the experiment, 1 microcentrifuge tube of hyaluronidase solution was warmed up on a stage warmer to denude the COC (Table 3.5).

Table 3.5: Composition of hyaluronidase medium

Chemical compound	**Catalogue number**	**Concentration (mg/ml)**	**Quantity/10 ml**
TL-Hepes working solution	-	-	10 ml
Hyaluronidase	H4272	0.001	0.1 g

3.4.2.5 Preparation of oocyte holding medium

Oocyte holding medium comprised of TCM-199 added with 10% FBS. This medium was used to incubate oocytes in CO_2 incubator prior to micromanipulation work. TCM-199 medium was measured by using a sterile disposable pipette (5 ml) and dispensed into a sterile tube (15 ml). After that, FBS was added by using sterile micropipette tip (1000 μl) and the medium was mixed thoroughly. The prepared medium was then filter-sterilised by using syringe filter (0.22 μm pore size) and aliquot in microcentrifuge tubes (1000 μl).

After that, microcentrifuge tubes containing hyaluronidase solution were sealed with parafilm, labelled on the cover, wrapped in aluminium foil and stored in the refrigerator (4°C) with a shelf life of 3 months (Table 3.6).

Table 3.6: Composition of oocyte holding medium

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

3.4.2.6 Preparation of cytochalasin B medium

Cytochalasin B was used to inhibit polymerisation and the interaction of actin filaments of the recipient cytoplast (oocyte) and facilitate during enucleation process. Enucleation of oocyte was done by removing the nucleus with a diminutive amount of cytoplasm (10%).

3.4.2.6 (a) Preparation of cytochalasin B stock medium

Cytochalasin B was prepared by dissolving cytochalasin B powder (1 mg) in DMSO (1 ml). The solution was then aliquot (10 µl) into a microcentrifuge tube (100 µl), sealed with parafilm, labelled on the cover, wrapped in aluminium foil and stored in the freezer (-20°C) with a shelf life of 6 months (Table 3.7).

Table 3.7: Composition of cytochalasin B stock medium

Chemical compound	Catalogue number	Concentration	Quantity/ml
DMSO	D5879	-	1.0 ml
Cytochalasin B	C6762	1 mg/ml	0.001 g

3.4.2.6 (b) Preparation of cytochalasin B working medium

Before commencing the experiment, cytochalasin B that had been aliquoted (10 µl) was mixed with TL-Hepes working solution (990 µl) and the final volume was 1 ml (Table 3.8).

Table 3.8: Composition of cytochalasin B working medium

Composition	Concentration	Quantity/ml
Cytochalasin B stock solution	-	1.0 ml
TL-Hepes working solution	1 mg/ml	0.001 g

3.4.2.7 Preparation of PVP medium

In this experiment, PVP medium was used during donor karyoplast injection. PVP medium (0.1 g) was dissolved with TL-Hepes medium (1 ml) in a sterile microcentrifuge tube. The prepared medium was placed in refrigerator overnight (4°C), so that the PVP powder will dissolve completely. Then, the PVP medium was filter-sterilised through syringe filter (0.22 µm pore size) into another sterile microcentrifuge tube (1.5 ml), sealed with parafilm, labelled on the cover, wrapped with aluminium foil and kept in the refrigerator (4°C) with a shelf life of 6 months. 10 µl of PVP solution was used for making microdroplets (2-3 µl for one microdroplet) on the micromanipulation dish (Table 3.9).

Table 3.9: Composition of PVP working medium

Composition	Catalogue number	Concentration	Quantity/ml
PVP	PVP360	-	0.1 g
TL-Hepes working medium	-	-	1 ml

3.4.2.8 Preparation of activation medium

In this study, artificial activation was done by combining two different types of chemical activation medium. The chemicals used for activation were calcium ionophore (CaI) and 6-dimethylpurine (6-DMAP). Apart from SCNT, PA experiment also was done on cattle by using the same medium above.

3.4.2.8 (a) Preparation of calcium ionophore (CaI) medium

CaI is the most commonly method used for artificial oocyte activation. CaI was used to activate the oocytes during PA and SCNT experiments. CaI is essential to increase in intracellular Ca^{2+} concentration, hence mimicking the physiological cell signaling and Ca^{2+} oscillations during normal fertilisation process. This Ca^{2+} rise will affect the pattern of embryo development and implantation (Rout *et al*., 1997; Ozil *et al*., 2006).

3.4.2.8 (b) Preparation of calcium ionophore (CaI) stock medium

CaI stock solution was prepared by dissolving 0.001 g CaI ionophore in DMSO (3.82 ml). The solution was then aliquoted into a sterile microcentrifuge tubes (10 µl), sealed with parafilm, labelled on the cover, wrapped with aluminium foil and stored in the freezer (-20°C). This medium can be kept up to 6 months.

Table 3.10: Composition of calcium ionophore (CaI) stock medium

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

3.4.2.8 (c) Preparation of calcium ionophore (CaI) working medium

One microcentrifuge tube of CaI working medium (10 µl) was diluted with KSOM A (990 µl) so that the final volume can be 1 ml. On the day of the experiment, CaI working medium was warmed up in a water bath and made into microdroplets on a petri dish.

Table 3.11: Composition of calcium ionophore (CaI) working medium

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

3.4.2.8 (d) Preparation of 6-dimethylaminopurine (6-DMAP) medium

6-DMAP involves in protein dephosphorylation by triggering condensation of premature chromatin and premature mitosis in cells arrested in S-phase for the control of mitosis (Schlegel *et al*., 1990). This medium was used to activate the oocytes after 4 minutes of CaI incubation following SCNT procedure.

3.4.2.8 (e) Preparation of 6-DMAP stock medium

6-DMAP powder (0.1 g) was dissolved in Milli Q water (3.08 ml) as depicted in Table 3.10. After that, the prepared medium was aliquot in a sterile microcentrifuge tubes (10

µl), sealed with parafilm, labelled on the cover, wrapped with aluminium foil and stored inside the freezer (-20°C) with a shelf life of 6 months.

Table 3.12: Composition of 6-DMAP stock medium

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

3.4.2.8 (f) Preparation of 6-DMAP working medium

Before commencing the experiment, 6-DMAP stock solution (10 µl) was diluted with KSOM A working solution (990 µl) and the final concentration was 2 mM (Table 3.13).

Table 3.13: Composition of 6-DMAP working medium

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

3.4.2.9 Preparation of Simplex Optimisation Medium (KSOM) medium

KSOM medium was used as a basal medium for *in vitro* culture and activation purpose. It was developed by Lawitts and Biggers (1991) and had been formulated and modified to KSOM K by the Lawitts and Biggers (1993). In this study, 2 types of KSOM medium were prepared (adapted from Soh *et al*., 2012 and Kwong *et al*., 2012 methods) to culture the embryos, which, namely:

KSOM A – standard KSOM medium

KSOM B – standard KSOM medium supplemented with glucose (0.04%, 2.2mM).

3.4.2.9 (a) Preparation of Simplex Optimisation Medium (KSOM) stock medium

All of the chemicals compound were weighed using digital analytical balance and added into a measuring cylinder (100 ml). Milli-Q water was then measured using a sterile disposable pipette (25 ml) until the final volume reached 100 ml. Then, the chemicals were dissolved slowly by using a sterile magnetic stirrer and during that; sodium lactate (60%) was carefully added to the solution by using sterile micropipette tip (1000 µl). The prepared medium was filter-sterilised through syringe filter (0.22 µm pore size) and kept in the refrigerator (4°C) with a shelf life of 1 month (Table 3.14).

Table 3.14: Composition of KSOM stock medium

Composition	Catalogue number	Concentration	Quantity/100 ml
NaCl	S5886	95 mM	0.5553 g
KCl	P5405	2.5 mM	0.0186 g
KH_2PO_4	P5655	0.35 mM	0.0048 g
$MgSO_4$	M7506	0.2 mM	0.0022 g
D-glucose	G6152	0.2 mM	0.0036 g
$NaHCO_3$	S5761	25 mM	0.2101 g
$CaCl_2$	C5670	1.71 mM	0.0190 g
L-glutamine	G3126	1 mM	0.0146 g
EDTA	E9884	0.01 mM	0.0004 g
Na Lactate (60% syrup)	L7900	10.0 mM	01860 ml
Milli-Q water	-	-	99.814 ml

3.4.2.9 (b) Preparation of Simplex Optimisation Medium (KSOM) working medium

There were 2 types of KSOM working medium prepared for culturing the reconstructed oocytes after nuclear transfer; KSOM A and KSOM B. KSOM A was used to culture the embryo at early stage while KSOM B was used to culture the embryo at later stage. KSOM stock solution (9.85 ml) was measured and dispensed into a sterile centrifuge tube (15 ml). BME (100 µl) and MEM (50 µl) were measured and dispensed into the solution. BSA (0.04 g) was weighed and dissolved in the mixture. After that, the prepared KSOM A working solution was filter-sterilised with syringe-filter (0.22 µm pore size) before stored in a refrigerator (4°C) and can be kept up to 2 weeks (Table 3.13). Meanwhile, KSOM B medium (5 ml) was prepared by measuring the KSOM stock medium (4.925 ml) into a sterile centrifuge tube (15 ml). BME (50 µl) and MEM (25 µl) was added slowly into the medium. Subsequently, D-glucose (0.04%, 0.02 g, 2.2 mM) and BSA (0.02 g) were weighed and dissolved in the mixture of the medium. Lastly, the prepared KSOM B solution was filter-sterilised with syringe-filter (0.22 µm pore size) before used and stored in a refrigerator (4°C) with a shelf life of 2 weeks (Table 3.15 and Table 3.16)

Table 3.15: Composition of KSOM A working medium

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

Table 3.16: Composition of KSOM B working medium

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

3.4.3 Preparation of Donor Karyoplast Culture Medium

The media used for culturing somatic cell line is DMEM and FBS (10%). After establishing the somatic cell line, cryopreservation was done so that the cells can be stored in a liquid nitrogen tank (-196°C) and thawed prior to use. The media that were included in tissue or somatic cell culture medium were namely, tissue culture medium, PBS (-) solution, Trypsin-EDTA medium and freezing medium.

3.4.3.1 Preparation of tissue culture medium

In this study, DMEM medium (Gibco, USA) was used as a base medium for culturing somatic cell lines. This medium contains high amount of glucose, L-glutamine and phenol red. However, there were no proteins, growth factors and lipids, hence; DMEM requires addditional supplementation such as 10% foetal bovine serum (FBS). FBS contains various types of amino acids, vitamins, inorganic materials, lipids and nucleic acid derivatives to support cell growth. DMEM also contains sodium bicarbonate (3.7 g/L) which acts as a pH buffer to maintain its physiological pH in a 5-10% CO_2.

3.4.3.1 (a) Preparation of tissue culture medium for explants (DMEM X 3 P-S)

Tissue culture medium (100 ml) was prepared each time before collecting foetal fibroblast tissue sample. DMEM was measured by using sterile disposable pipette (10 ml) and dispensed into a sterile Scott bottle (100 ml). After that, P-S stock solution (300 µl) was added into DMEM medium using a sterile micropipette tip. Then, FBS (10 ml) was added into DMEM X 3 P-S medium and gently shaked until it mixed well. The prepared tissue culture medium was filter-sterilised with syringe-filter (0.22 µm pore size) before stored in a refrigerator (4°C) and can be kept up to 3 months (Table 3.17).

Table 3.17: Composition of DMEM X 3 P-S medium

Chemical compound	Catalogue number	Quantity/100 ml
DMEM	11965	90 ml
FBS	10270 (Gibco)	10 ml
P-S stock medium	-	300 µl

3.4.3.1 (b) Preparation of tissue culture medium for cell lines (DMEM X 1 P-S)

Same like 3.4.7.2 (a), DMEM was measured by using sterile disposable pipette (10 ml) and dispensed into a sterile Scott bottle (100 ml). After that, P-S stock solution (100 µl) was added into DMEM medium by using a sterile micropipette tip. Subsequently, FBS was added into DMEM X 1 P-S medium using a sterile disposable pipette (10 ml) and was shake gently. The prepared tissue culture medium was filter-sterilised with syringe-filter (0.22 µm pore size) before stored in a refrigerator (4°C) and can be kept up to 3 months (Table 3.18).

Table 3.18: Composition of DMEM X 1 P-S medium

Chemical compound	Catalogue number	Quantity/100 ml
DMEM	11150-059	90 ml
FBS	10270 (Gibco)	10 ml
P-S stock medium	-	100 µl

3.4.3.2 Preparation of PBS (-) medium

PBS medium was prepared by weighing chemical compounds that had been depicted in Table 3.17 by using digital analytical balance and dissolved in Milli-Q water (100 ml) in a sterile autoclaved Scott bottle. The chemicals were then stirred gently with a magnetic stirrer on a stirrer machine. The prepared PBS (-) medium was then filter-sterilised through syringe filter (0.22 µm pore size) before stored in a refrigerator (4°C) and can be kept up to 3 months (Table 3.19).

Table 3.19: Composition of PBS (-) medium

Chemical compound	Catalogue number	Concentration	Quantity/100 ml
NaCl	S5886	171.1 mM	9.0 g
KCl	P5405	3.35 mM	0.025 g
KH_2PO_4	P5655	10.58 mM	0.144 g
Na_2HPO_4	S5136	1.76 mM	0.025 g
Milli-Q water	-	-	100 ml

3.4.3.3 Preparation of trypsin-EDTA medium

Trypsin-EDTA was prepared to detach adherent cell from a culture dish so that the cells aggregated into single cell prior to donor karyoplast injection in SCNT experiment.

Trypsin-EDTA medium was prepared by dissolving trypsin (0.25 g) and EDTA (0.04 g) in PBS (-) medium in a sterile autoclaved Scott bottle. By using a magnetic stirrer, the chemical compounds were stirred gently on a stirrer machine. The prepared trypsin-EDTA was then filter-sterilised through syringe filter (0.22 μm pore size) before stored in a refrigerator (4°C) and can be stored up to 3 months (Table 3.20).

Table 3.20: Composition of trypsin-EDTA medium

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

3.4.3.4 Preparation of freezing medium

Freezing medium was used to kept the somatic cell at a very low temperature (-196°C) in a liquid nitrogen tank. This medium contained a cryoprotecting (DMSO) to avoid the formation of ice crystal in the cell while freezing. To prepare freezing medium, tissue culture medium (DMEM X 1 P-S + 10% FBS) was measured by using a sterile disposable pipette (10 ml) until it reached 45 ml and dispensed into a sterile autoclaved Scott bottle. After that, DMSO (5 ml) was measured and dispensed into a sterile Scott bottle containing previous medium. The prepared freezing medium was then filter-sterilised through syringe filter (0.22 μm pore size) before stored in a refrigerator (4°C) and can be kept up to 3 months (Table 3.21).

Table 3.21: Composition of freezing medium

Chemical compound	Catalogue number	Quantity/50 ml
DMSO	D5879	5 ml
DMEM X 1 P-S + FBS (10%)	-	45 ml

3.4.3.5 Preparation of P-S stock medium

P-S stock medium was prepared in advance before tissue culture medium and freezing medium preparation. This medium was used as a preventive measure to avoid bacterial contamination on cells. Penicillin was extracted from *Penicillium,* a fungi species in which can stimulates the enzymes causing the alteration of bacterial cell wall. Meanwhile, streptomycin was extracted from *Streptomyces griseus*, a bacteria species in which can inhibit protein synthesis and death in susceptible bacteria by binding onto the 30s subunit of bacterial ribosome. P-S stock medium was prepared by dissolving penicillin-G (0.3 g) and streptomycin (0.5 g) in PBS (-) medium in a sterile centrifuge tube (10 ml). Centrifuge tube containing P-S stock medium was mixed thoroughly by using vortex machine. The P-S stock medium was then filter-sterilised through syringe filter (0.22 μm pore size) before stored in a refrigerator (4°C) and can be kept up to 3 months (Table 3.22).

Table 3.22: Composition of P-S stock medium

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

3.4.3.6 Preparation of mouthpiece-controlled pipette and microneedles

In this study, a specific device namely mouth-piece controlled pipette was used to pick up or transfer the oocyte and embryo. It is made of glass pasteur pipette, rubber tube, syringe-filter and an aspirator mouthpiece. For SCNT experiment, there were different types of microneedles including holding pipette, biopsy needle and injection pipette. Mouthpiece and all microneedles were all prepared 'in-house' in the laboratory.

3.4.3.7 Cleaning and sterilisation of pasteur pipette and microneedles

The glass Pasteur pipettes were rinsed and soaked overnight with RO water. After that, the Pasteur pipettes were rinsed thoroughly with Milli-Q water before dried in the oven (56°C). Then, the dried Pasteur pipettes were packed with autoclavable bag and autoclaved (121°C, 20 minutes) for sterilising purpose. The autoclaved Pasteur pipettes were subsequently dried in the oven before using. Borosilicate capillaries were used for making microneedles prior to SCNT experiment. Borosilicate capillaries were soaked in hydrochloric acid solution (10%) overnight before autoclaved and subsequently dried properly in the oven (56°C) before using.

3.4.3.8 Preparation of mouthpiece-controlled pipette

The preparation of glass Pasteur pipette could be done by creating narrow opening by twisting the middle portion on a burner flame until the glass pipette became soft. Then, the glass pipette was withdrawn immediately from the flame and pulled in opposite directions quickly so that the inner diameter will become smaller (around 200 to 350 μm). The pulled portion of the glass pipette was cut by using a diamond stone in accordant to the type or size of oocytes used in the experiments. The tip of the glass pipette was then fire-polished immediately with a flame to smooth the end of the tip to lessen the injury of

the oocytes and embryos. For cattle species, the usual inner diameter of glass pipette used for oocytes and embryos handling are around 330 to 350 μm.

3.4.3.9 Preparation of microneedles

Microneedles including holding pipette, biopsy needle and injection pipette were prepared in-house in the laboratory for SCNT purpose. All different types of microneedles were made from thin-walled borosilicate glass capillaries with inner and outer diameter of 0.69 and 0.97 mm and 10 cm length, respectively. Two instruments were needed to fabricate all these microneedles namely, horizontal micropipette puller (P-97, Sutter Instrument, USA) and microforge (Technical Products Internationals, USA). A horizontal micropuller was already programmed with a particular quantity of heat, pulling velocity and strength (heat= 665 units, pull= 150 units, velocity= 100, time= 150 units and pressure= 500 units). This tool is used to pull the microneedle so that the tip of microneedle has a uniform and long tapering end with a length of around 10 mm. The shape of microneedle tip was further fabricated by using microforge. The function of this tool is to fabricate microneedles into holding pipette, biopsy needle and injection pipette by cutting, shape, polish and bend the microneedles. Prior to fabricating microneedles, it was essential to create a small bead first around the top of heater filament so that the filament did not make any direct contact with the microneedle. To prepare the glass bead, the filament was heated with heat level of 4 and subsequently, a microneedle was lowered onto the hot filament until it melted and formed approximately 20 to 30 μm diameter. Then, the heat was switched off immediately. The microneedles could be fabricated after the glass bead was formed on the filament.

Figure 3.2: Micropuller to pull the microneedle tips.

3.4.3.9 (a) Preparation of holding pipette

The function of holding pipette is to hold the oocyte SCNT procedure. To prepare holding pipette, the already pulled capillary was aligned parallel to filament at the same focus point. Subsequently, the pulled capillary was positioned on top of the glass bead. The filament was then heated up to 50% of its maximum capacity. After the capillary melted to the glass bead, switched off the power of heat control immediately. As soon as the capillary glass cooled down, the capillary was attached to the glass bead and eventually broken evenly after the filament relaxed from its expansion. After heating up the glass bead at 60% of its maximum capacity, the capillary was then positioned closer to the glass bead again so that the glass melted and the inside diameter will be decreased in size. After the polishing process, holding pipette was bent at 30° angle by heating the filament up to 50% of the maximum capacity horizontally.

3.4.3.9 (b) Preparation of biopsy needle

The function of biopsy needle is to remove the DNA from matured oocyte via squeezing technique. During enucleation process, the polar body along with 10% of the cytoplasm surrounding it was squeezed out after the zona pellucida layer was broken down by laser-

assisted technique. The pulled capillary was scribed using a diamond stone and fire-polished with a flame immediately to form a smooth edge. Then, the biopsy needle was positioned horizontally on the heated filament (heat level= 50% of the maximum capacity) and bent at 30° angle.

3.4.3.9 (c) Preparation of injection pipette

The function of injection pipette is to introduce a single donor karyoplast right into the oocyte's cytoplasm. Injection pipette was made by snapping the tip of a pulled capillary on a heated glass bead (heat level= 20% of the maximum capacity). The inner and outer diameters of injection pipette were around 9 μm and 10 μm, respectively. Injection pipette was then bent at 20° on a heated glass bead (heat level= 20% of the maximum capacity).

3.5 Preparation of donor karyoplast cell

For donor karyoplast preparation, the methods used were adapted from Soh *et al*. (2012), Kwong *et al*. (2012) and Asdiana *et al*. (2014).

3.5.1 Goat foetal fibroblast cell (FFC)

In this study, goat foetal fibroblast cell (FFC) aged 70 and 48 days from Rumpun Asia Sdn Bhd (RASB) Farm, Selangor were used as donor karyoplast for SCNT experiments. Goat foetuses were produced by natural mating with insertion of CIDR in does. Foetuses were obtained surgically through caesarean procedure and put in a culture dish (90 mm) containing PBS (-) medium. Then, by using a pair of surgical forceps, the head and internal organs of the foetuses were mechanically removed and the remaining tissues and bones were dissociated. The foetal fibroblast tissues were then minced by using a pair of small surgical scissor and washed thoroughly in PBS (-) (2 times) and DMEM X 3PS medium (3 times). After that, the explants were cultured in a culture dish (60 mm,

Nunclon, USA) containing tissue culture medium (DMEM X 3PS + 10% FBS) and incubated in a humidified atmosphere of CO_2 (5%) in air at 38.5°C (around 7 days). Tissue culture medium was replenished once every three days. After the cells of the explants reached 80% confluency, they were detached from the surface of culture dish using trypsin-EDTA (0.25%) and then recultured back up to passage 2. The FFC were then cryopreserved with dimethyl sulfoxide (10% DMSO) already mixed with tissue culture medium and subsequently stored in liquid nitrogen.

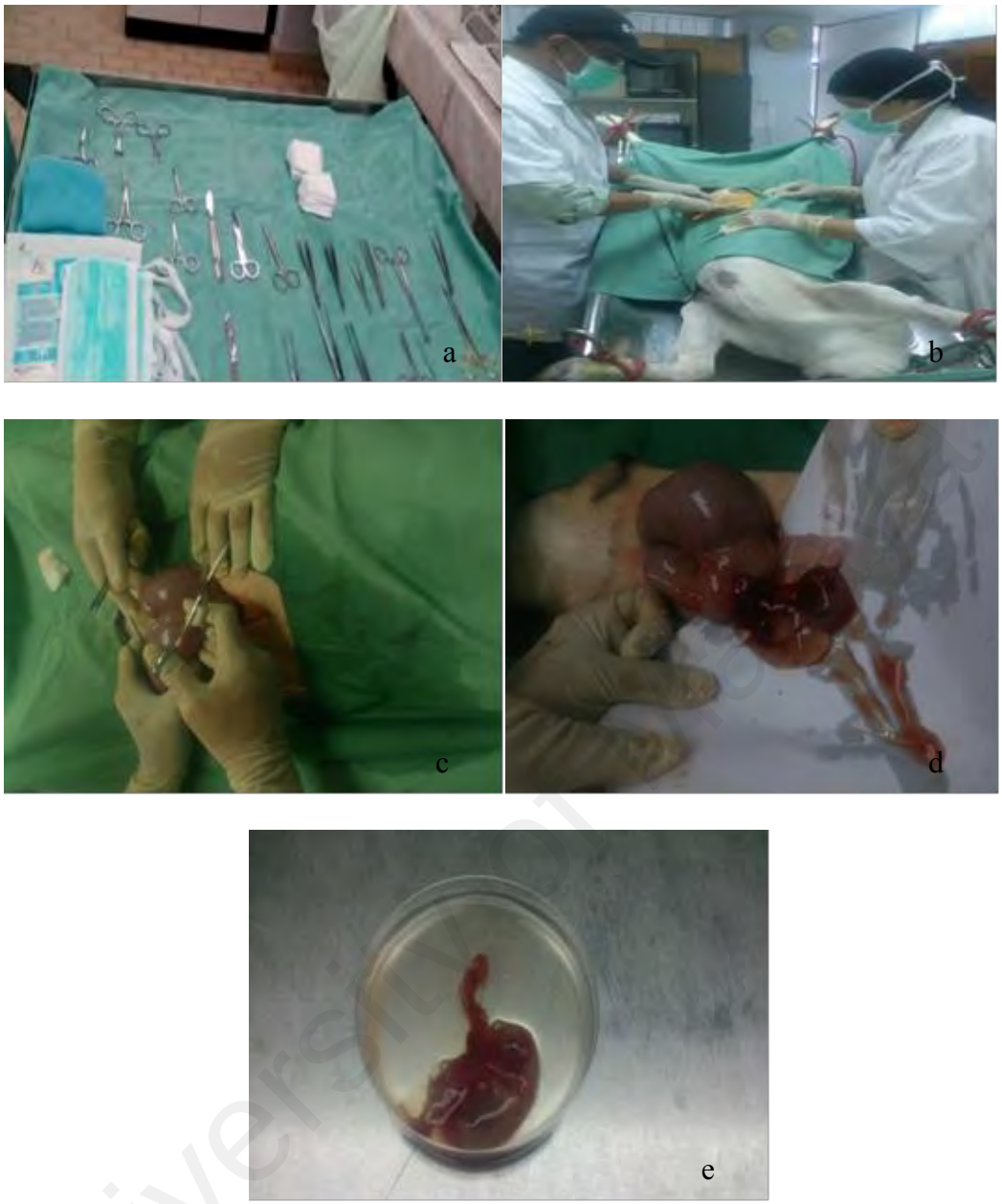


Figure 3.3: Goat foetus was collected through cesarean surgery. (a) Preparation of surgery tools and equipment before commencing goat cesarean surgery, (b) Incision was made on lower abdominal part of pregnant doe, (c) A small part of doe's uterus was cut using surgical scissor, (d) Goat foetus age 31 old days was successfully taken out from doe's womb, (e) Goat foetus was transferred into petri dish (diameter: 60 mm) which contained PBS (-) medium for cleansing purpose.

3.5.2 Goat ear fibroblast cell (EFC)

A total of 10 ear goat tissue samples from both male and female of Jermasia and Boer breeds obtained from both Livestock Science Centre (LSC), University of Malaya and Rumpun Asia Sdn Bhd farm (RASB), Selangor, were biopsied and subsequently cultured and sub-passaged until up to Passage 3 (P3) using standard tissue culture medium. Passage number was normally defined as the amount of time cells have been detached from culture dish and sub-cultured (Kwong, 2012).

Ear skin tissues were biopsied from adult goats. Prior to collecting ear skin tissue, a mixture of anaesthetic (ilium and xylezene) was administered at jugular vein of the goat. After that, part of ear goat skin selected for donor karyoplast was held and sprayed with 70% ethanol for sterilisation purpose. Ear skin was then shaved with razor blade and cut using a sterile medium size surgical scissors. Ear tissue that had been cut was then immediately washed with normal saline before placing it in the test tube containing PBS (-) medium. Then, test tubes were transported back to the laboratory by placing it in a polystyrene box with ice pack for further processing. Part of the biopsied ear skin was wiped with iodine to stop the bleeding and prevent infection.

Next, for disinfection purpose, part of the biopsied ear skin was sprayed with woundsarex. The biopsied ear tissues were washed thoroughly with PBS (-) medium and cut into small pieces using small surgical scissor in culture dish (60 mm) containing PBS (-) medium. The dissected ear fibroblast tissues were then rinsed with two droplets of PBS (-) medium and three droplets of tissue culture medium (DMEM x 3 PS + 10% FBS). Subsequently, cleaned dissected ear fibroblast tissues were placed at the centre of tissue culture dish (60 mm) and covered by sterile glass slip. After that, tissue culture medium was slowly dispensed into the culture dish containing minced goat ear fibroblast tissues until the glass cover slip was fully immersed. Goat ear fibroblast tissues (explants culture)

were then cultured until 80% confluency in tissue culture medium and incubated in 38.5°C in a humidified atmosphere of 5% CO_2 in air.

After 6 to 8 days, EFC from explants culture were harvested using Trypsin-EDTA medium and then reseeded into Passage 1 (P1) culture. Passage is the process of harvesting cells starting from explants culture to Passage 1, 2 and 3. Medium from explants culture was removed by using sterile Pasteur pipette. Adhering cell monolayer on culture dish was rinsed using PBS (-) medium to wash out any residual culture medium that could inhibit the action of trypsin enzyme. Then, EFC from explants were harvested using trypsin-EDTA medium by pipetting in and out for a few times using sterile Pasteur pipette. After cells were detached, 4 ml of tissue culture medium (DMEM x 1 PS + 10% FBS) along with 2 ml of cells suspension were added on a new petri dish (30 mm, Nunclon, USA) for subsequent passage. P1 culture was then incubated in 38.5°C in a humidified atmosphere of 5% CO_2 in air. The EFC were then cryopreserved with dimethyl sulfoxide (10% DMSO) which already mixed with tissue culture medium and subsequently stored in liquid nitrogen.

3.5.3 Cattle cumulus cells (CC)

Cumulus cells were also used as donor karyoplast for intraspecies SCNT technique. Cumulus cells were collected after denuding of cumulus oocyte complexes (COC) after *in vitro* maturation (IVM). Harvested cumulus cells were then washed in TL-Hepes medium and subsequently transferred into a microcentrifuge tube (1.5 ml) containing TL-Hepes medium (1 ml). Microcentrifuge containing TL-Hepes medium and COC was centrifuged in a centrifuge machine (3000 rpm) for 5 minutes. After centrifugation process, supernatant was removed and TL-Hepes medium (0.3 ml) was supplemented into the remaining pellet. The medium was pipetted several times with a micropipette (pipette tip size=200 ul) so that the pellet broke into single cells to ease the injection process

during SCNT later. Cumulus cells were then transferred into a holding medium before used as donor karyoplast.

3.6 Preparation of cattle recipient cytoplast

Cattle ovaries obtained from slaughterhouse at Banting and Shah Alam were used as recipient cytoplast in this experiment. The ovaries were cut while cattle being slaughtered using a pair of medium size of surgical scissors and put in the thermo flask containing normal saline supplemented with penicillin-G (60 μg/ml) and streptomycin (50 μg/ml) at 35-37°C. After reached the laboratory, ovaries were washed thoroughly with warm saline to cleanse off the remaining blood. After washing, all ovaries were placed inside a sterile beaker (100 ml) containing TL-Hepes which had already pre-warmed at 38.5°C on a stage warmer. A checkerboard cut was made on a petri dish (35 mm) to assist the process of oocyte searching. For slicing process, ovary was held with a sterile autoclaved haemostat and checkerboard slicing was made using a razor blade on the whole surface of the ovary inside the culture dish containing TL-Hepes. The sliced ovaries were then rinsed by using syringe (10 ml) containing TL-Hepes medium so that the remaining COC that still stucked inside the ovary will be flushed out. A beaker containing TL-Hepes with COC was left for 5 to 10 minutes so that the debris will settle down. Any redundant TL-hepes was sucked out by using a sterile glass pipette. Then, a petri dish (35 mm) containing TL-Hepes along with COC and debris was scanned using a stereomicroscope.

Figure 3.4: Cattle ovaries collected from abattoir.

3.6.1 *In vitro* maturation (IVM) of oocyte

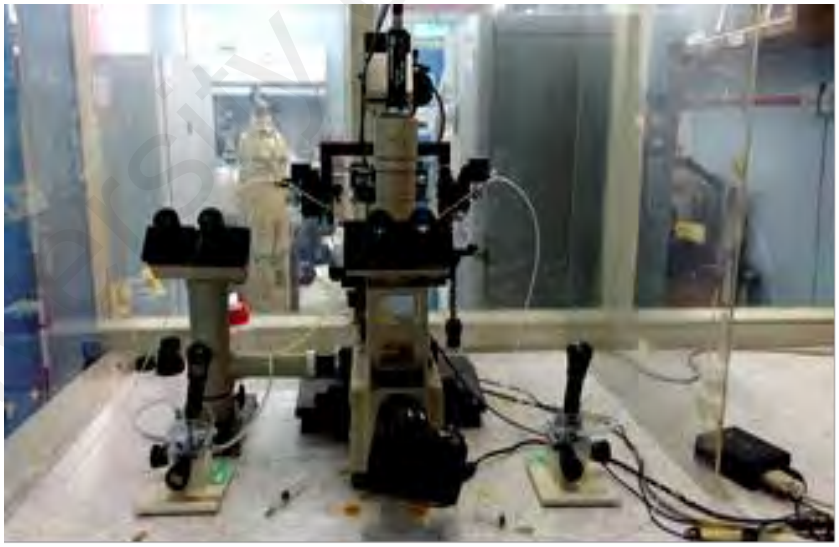
Prior to *in vitro* maturation of cattle COCs, IVM medium droplets was prepared on a small petri dish (35 mm), overlaid with silicon oil and equilibrated in the incubator (5% CO_2 in air in humidified atmosphere at 38.5°C) at least 4 hr before the experiment started. After washing the COCs with TL-Hepes, 15 to 20 COCs were cultured in each microdroplet that contained 80 µl of IVM medium. The COCs were selected based on morphology of granulose cells (more than 3 layers) for IVM. Selected COCs were incubated at 38.5°C and 5% of CO_2 in a humidified air of a CO_2 incubator.

3.6.2 Protocol of somatic cell nuclear transfer (intra- and interspecies SCNT)

In this present study, SCNT was conducted using a laser technique which involved enucleation using laser-assisted technique, whole cell intracytoplasmic injection (WCICI) and activation.

3.6.3 Preparation of SCNT micromanipulation dish

On the day of experiment, a micromanipulation dish was prepared on the lid of a polystyrene culture dish (35 mm). One TL-Hepes microdroplet (10 µl) was placed on the top centre of the lid of culture dish to align the microneedles. On the right side, three microdroplets containing TL-Hepes with cytochalasin B were placed near to the centre of the micromanipulation dish for enucleation purpose while another 3 microdroplets (10 µl) with just TL-Hepes medium were placed on the left side for injection purpose. Subsequently, four microdroplets (3 µl) of TL-Hepes with PVP for donor karyoplasts were placed on the left side of injection microdroplets. After finished preparing the microdroplets, the whole manipulation dish was overlaid with silicon oil to prevent evaporation. Micromanipulation dish was incubated at 38.5°C and 5% of CO_2 in a humidified air of a CO_2 incubator for 15 minutes before conducting the experiment.



. **Figure 3.5:** Micromanipulator for SCNT procedure.

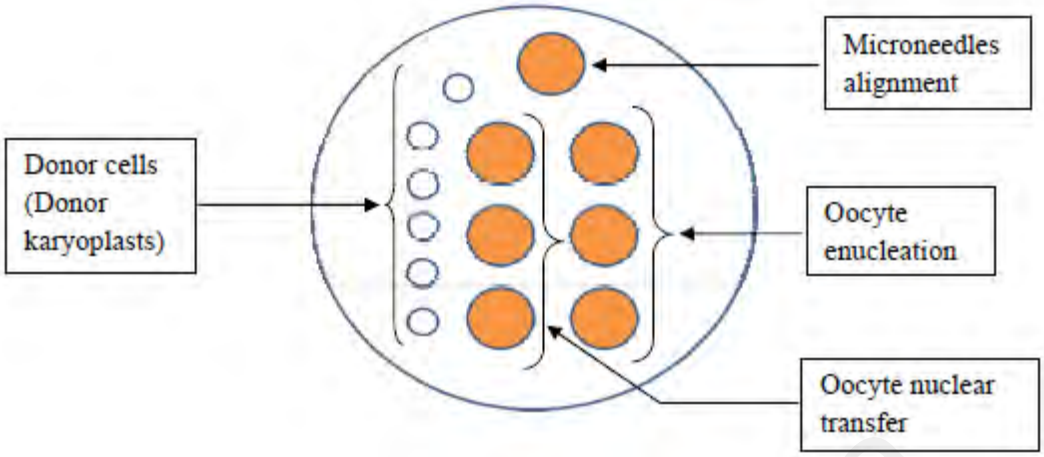


Figure 3.6: SCNT microdroplets arrangement on the micromanipulation dish.

3.6.4 Alignment of microneedles

Microneedles need to be aligned properly on the micromanipulation dish to ensure a smooth performance of both intra- and interspecies SCNT. All knobs on micromanipulator including the syringes (3 ml) were regulated to the centre of the scale. Micromanipulation dish was kept in the incubator for 15 minutes for equilibration purpose before starting the alignment of microneedles. The TL-Hepes microdroplet specifically for microneedles alignment was focused under microscope (4x objectives) until it became clear. Both holding pipette and biopsy needle were inserted into each needle holder and tightened well. Both microneedles were then placed above the TL-Hepes microdroplet. The tip of microneedles were brought down until it touched the oil and stay stilled for few minutes so that the tip of microneedles were filled in with silicon oil through capillary action. TL-Hepes microdroplet was sharply focused; the needle was moved inside the droplet close to the edge and aligned properly. Then, both enucleation needle and biopsy needle were checked under high magnification to ensure the precise alignment.

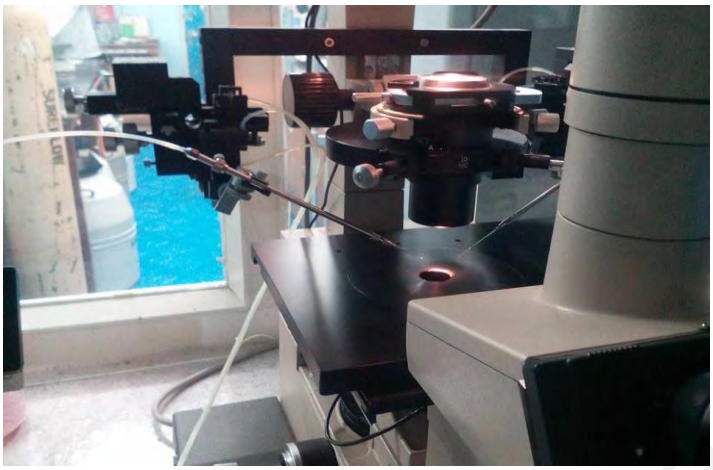


Figure 3.7: Preparing microneedles for alignment.

3.6.5 Preparation of oocytes as recipient cytoplast for enucleation

After *in vitro* maturation (IVM) of cattle oocytes, cumulus cell surrounding the oocytes were removed by pipetting repeatedly in TL-Hepes medium mixed with hyaluronidase (0.1%) in less than 5 minutes. After denuding, oocytes were washed thoroughly in TL-Hepes medium to cleanse off the hyaluronidase for maturation assessment under stereomicroscope. Oocytes with first polar body (PB-1) were identified as matured and competent were subsequently treated with TL-Hepes medium supplemented with cytochalasin B (5µg/ml) before enucleation of oocytes take place.

3.6.6 Preparation of donor karyoplasts

Cultured donor cells that reached 80% of confluency were used as donor karyoplast in this experiment. Trypsin-EDTA was used to detach the donor cells from a culture dish. Harvested cells were then washed in PBS (-) twice and incubate with trypsin-EDTA medium for 4 minutes. After incubation, a mixture of donor cells with TL-Hepes medium were centrifuged (3000rpm) for 5 minutes to obtain donor cells in a pellet form. The unused supernatant was pipetted out using sterile disposable micropipette tip (200 µl).

The pellet form of donor cells was mixed with TL-Hepes medium (50 µl) for injection of donor karyoplast preparation.

3.6.7 Enucleation of recipient cytoplast

Prior to enucleation process, selected matured oocytes were placed in the microdroplets containing a mixture of base medium (TL-Hepes) supplemented with cytochalasin B (5 µg/ml) for 10 minutes. After treated with cytochalasin B, all oocytes were transferred to enucleation dish. In this study, enucleation by laser shoot technique has been applied to all matured oocytes. The extrusion of first polar body was identified by rotating the oocyte using the tip of holding needle and biopsy needle and positioned at 12 o'clock. A slit was made on zona pellucida adjacent to the first polar body by using a laser beam. Subsequently, the first polar body along with 10% of the surrounding cytoplasm was squeezed out by using a biopsy needle at the right side which was parallel to the holding pipette on the left side (Figure 3.7). The enucleated oocytes were then washed with TL-Hepes medium (3 times) and in holding medium (3 times).

3.6.8 Injection of recipient cytoplast (intracytoplasmic injection technique)

Donor karyoplasts were harvested using the method as described in section 3.5. For injection of donor karyoplast, a single cell was aspirated gently into the injection pipette (ID: 9 µm; OD: 10 µm) and positioned near to the tip. Enucleated oocyte was rotated using holding pipette and injection needle to 3 o'clock position. The injection needle was slowly forwarded through zona pellucida until the end point of the cytoplasm at 9 o'clock position. To confirm the breakage of plasma membrane, a small amount of cytoplasm was delicately aspirated into the injection pipette. Donor karyoplast was then gently deposited into the cytoplasm. Injection pipette was delicately withdrawn and the recipient cytoplast was released from the holding pipette. After injection, reconstructed oocytes

were washed in TL-Hepes medium (3 times) and holding medium (3 times). Reconstructed oocytes were then incubated in prepared holding medium microdroplet prior to activation.

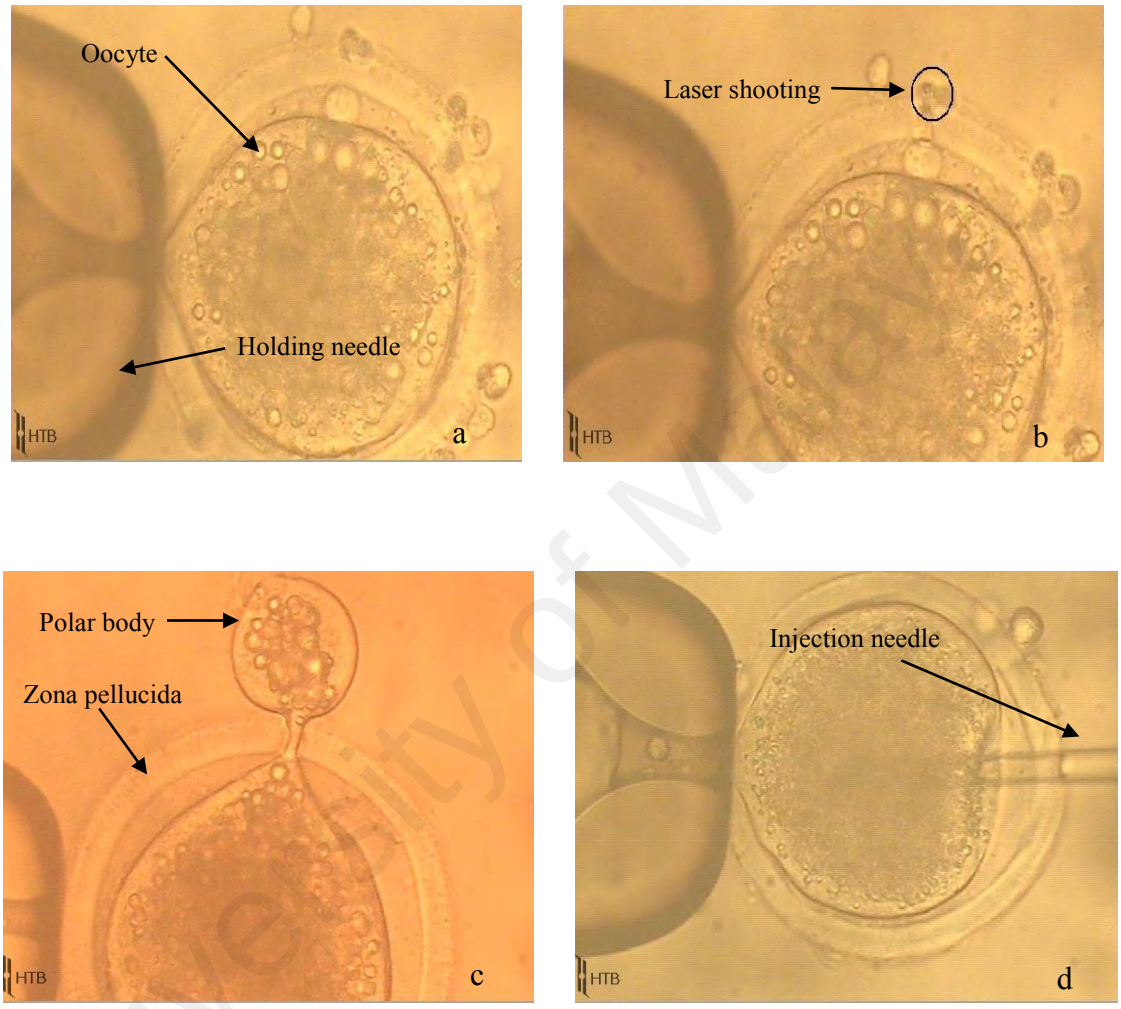


Figure 3.8: SCNT procedure. (a) Matured cattle oocyte with polar body was positioned at 12 o-clock, (b) matured cattle oocyte was enucleated using laser-shooting technique, (c) enucleated cattle oocytes with 10% of cytoplasm after squeezed using biopsy needle, (d) donor karyoplast was injected using WCICI technique.

3.6.9 Activation of the injected recipient cytoplast (oocyte)

All reconstructed oocytes were then submitted to activation with calcium ionophore (5 minutes) and 6-DMAP (4 hours). The activation dish was already prepared at least 4 hours prior to activation process and put it in incubator (5% CO_2) at 38.5°C in humidified atmosphere. Microdroplets containing activation medium (80-100 µl) were prepared on a polystyrene culture dish (Nunclon, 35 mm) and overlaid with silicon oil. All of reconstructed oocytes were first activated for 5 minutes in calcium ionophore inside the incubator (5% CO_2) at 38.5°C. Then, the reconstructed oocytes were washed 3 with 6-DMAP microdroplets (3 times) to rinse off the calcium ionophore medium and subsequently cultured in 6-DMAP microdroplets for 4 hours inside the incubator (5% CO_2) at 38.5°C.

3.7 *In vitro* culture (IVC) of cloned ruminant embryos

Microdroplets of IVC medium overlaid with silicon oil were prepared 4 hours prior to culture of cloned ruminant embryos on a polystyrene culture dish (35 mm). Then, reconstructed activated oocytes were placed in each microdroplet (10 to 15 reconstructed oocytes). After activation, all reconstructed oocytes were rinsed off with IVC medium (3 times) before transferred into the final IVC microdroplets. IVC dish containing reconstructed oocytes were then placed inside the incubator (5% CO_2) at 38.5°C under humidified atmosphere. For first 3 days, all cloned embryos were cultured in KSOM A. After 3 days, IVC medium was replenished with KSOM B medium. Cloned embryos were monitored daily under inverted microscope and the numbers of embryos were recorded.

3.8 EXPERIMENTAL DESIGN

In this present study, the researcher attempted to produce *in vitro* goat and cattle embryos through intraspecies SCNT for the cattle cloning, interspecies SCNT for goat cloning and parthenogenetic activation (PA) as a pilot study to produce *in vitro* embryos in cattle. This research consisted of four experiments as described below.

3.8.1 Effect of different IVM durations on percentage of matured cattle oocytes and subsequent *in vitro* cattle embryo production as a pilot preliminary study as a model experiment (Experiment 1)

Oocytes were obtained from cattle derived from the abattoirs at the Department of Veterinary Services (DVS) in Shah Alam and Banting, Selangor. Oocytes were collected and matured *in vitro* to metaphase II (MII) stage using standard IVM medium for cattle COCs and cultured in CO_2 (5%) incubator at 38.5°C for 23, 24, 25 and 27 hours. After IVM, matured cattle oocytes were activated using calcium ionophore (CaI) medium for 5 minutes followed by 6-Dimethylaminopurine (6-DMAP) medium for 4 hours in CO_2 (5%) incubator at 38.5°C. Number and percentages of matured cattle oocytes and embryo cleavage for PA were recorded and evaluated using SSPS software.

3.8.2 Effect of different types of donor karyoplast on viability of karyoplast (Experiment 2)

The aim of this experiment was to produce various types of donor karyoplast and to evaluate the viability of different types of donor karyoplast. Donor karyoplast were obtained from cattle and goats, which is cumulus cell (CC) from cattle, foetal fibroblast cell (FFC) and ear fibroblast cell (EFC) from goat were cultured serially in tissue culture medium (DMEM X 3PS + 10% FBS) in CO_2 (5%) incubator at 38.5°C to obtain 80% confluency. The CC was used as donor karyoplast for intraspecies SCNT; meanwhile, EFC and FFC were used as donor karyoplast for interspecies SCNT. Percent viabilities

were counted for both EFC and FFC by manual counting using a haemocytometer under inverted microscope except for CC which were used without passaging.

3.8.3 Production of cloned cattle embryos using cumulus cells (CC) as donor karyoplasts in intraspecies SCNT (Experiment 3)

The objective of this experiment was to produce cloned cattle embryos using CC as donor karyoplast using intraspecies SCNT technique. Cattle ovaries were collected from Banting abattoir, Selangor to obtain oocytes to be used as recipient cytoplasts. After *in vitro* maturation of oocytes, the CC was injected directly into the recipient cytoplast after which, the reconstructed oocytes were activated using chemical treatments (calcium ionophore and 6-DMAP). After activation, the reconstructed oocytes were cultured *in vitro* CO_2 (5%) incubator at 38.5°C for embryonic development. The cleaved cloned embryos were observed under inverted microscope and recorded daily.

3.8.4 Production of cloned goat embryos using ear fibroblast cells (EFC) and foetal fibroblast cells (FFC) as donor karyoplast in goat-cattle interspecies SCNT (Experiment 4)

The objective of this experiment was to produce cloned cattle embryos using EFC and FFC as donor karyoplasts using intraspecies SCNT technique. Cattle ovaries were collected from Shah Alam and Banting abattoirs, Selangor to obtain oocytes to be used as recipient cytoplasts. After *in vitro* maturation of oocytes, the EFC and FFC were injected directly into the recipient cytoplast after which, the reconstructed oocytes were activated using chemical treatments (calcium ionophore and 6-DMAP). After activation, the reconstructed oocytes were cultured *in vitro* CO_2 (5%) incubator at 38.5°C for embryonic development. The cleaved cloned embryos were observed under inverted microscope and recorded daily.

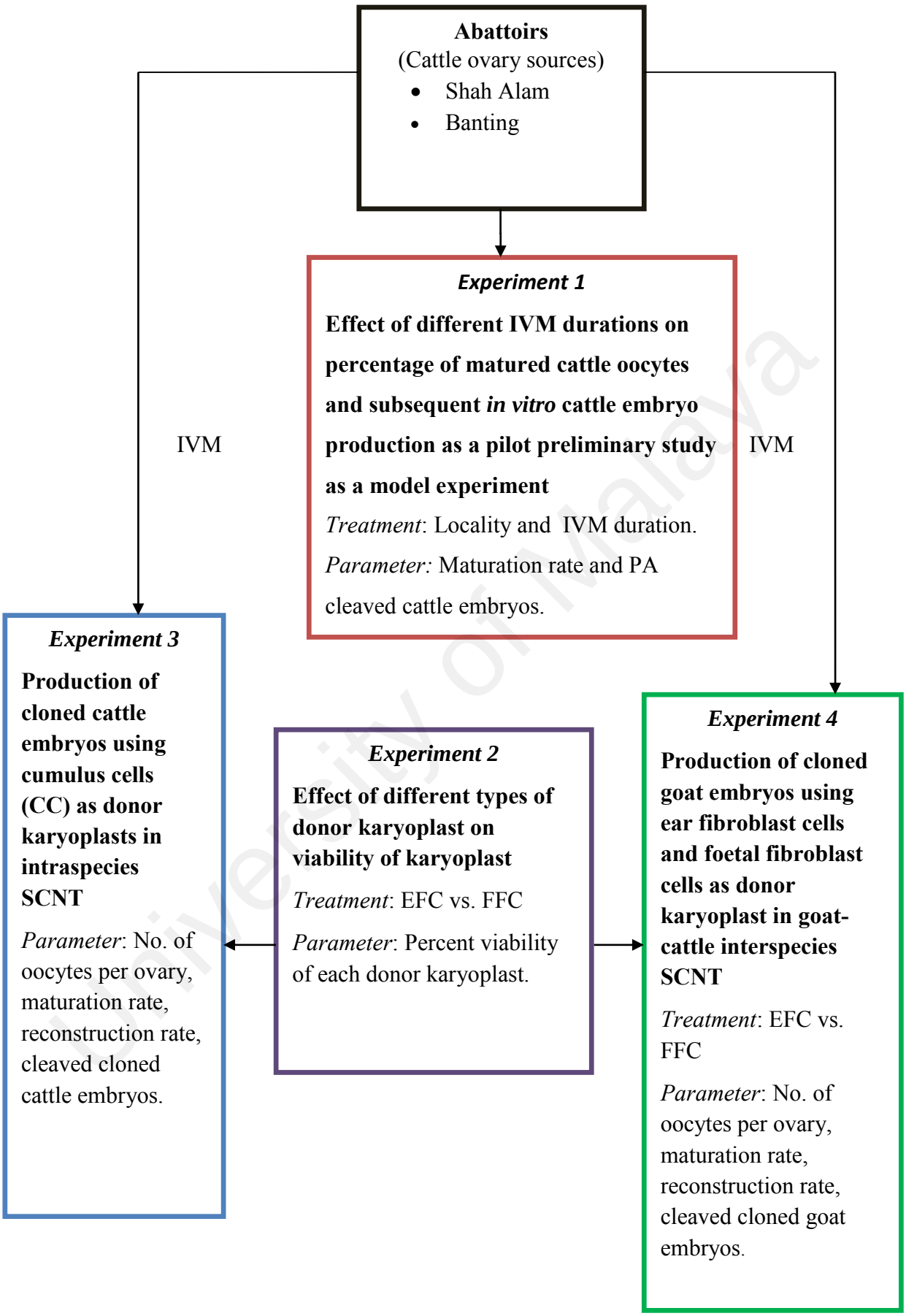


Figure 3.9: Flowchart of experimental design.

3.9 STATISTICAL ANALYSIS

All data were analysed using SPSS version 20. The differences among mean percentages for all different stages of cloned embryo development rate were analysed using one-way Analysis of Variance (ANOVA) followed by Duncan Multiple Range Test (DMRT). Differences were considered statistically significant if P values <0.05.

CHAPTER 4

RESULTS

4.1 EFFECTS OF DIFFERENT IVM DURATIONS ON PERCENTAGE OF MATURED CATTLE OOCYTES AND SUBSEQUENT *IN VITRO* CATTLE EMBRYO PRODUCTION AS A PILOT PRELIMINARY STUDY AS A MODEL EXPERIMENT (EXPERIMENT 1)

This experiment was conducted to produce and evaluate the effect of different *in vitro* maturation (IVM) durations on the maturation rate as well as the cleavage rate of cattle oocytes derived from parthenogenetic activation (PA) experiment. Cattle oocytes obtained from the abattoirs were matured *in vitro* to MII stage using a standard IVM medium for cattle COC and cultured in CO_2 (5%) incubator (38.5°C) for different IVM durations. Number of cattle ovary samples, number of oocytes obtained along with maturation rate was also recorded from both abattoirs: Shah Alam and Banting, Selangor (Table 4.1). The results of IVM rates were analysed using ANOVA as depicted in Table 4.2, which showed no significant difference between Banting and Shah Alam abattoirs.

A total of 301 cattle cumulus-oocyte-complexes (COC) from 21 ovaries were obtained from abattoirs in Banting and Shah Alam, Selangor. The average number of oocytes per ovary is equal to 14.3. The first experiment was carried out to compare the effect of IVM duration on the oocytes maturation rate and subsequently, to evaluate cattle parthenotes development between two IVM durations. The reason why PA technique was chosen because it was one of our routine controls in our laboratory to obtain *in vitro* embryos by-passing conventional fertilisation without using sperm. An average of 71.10% (214/301) of cattle oocytes were matured, which were identified by the extrusion of first polar body as observed under the inverted microscope.

The above cattle oocytes were divided into two IVM durations group which was Group 1 (22-24 hr) and Group 2 (25-27 hr). The percentages of matured oocytes for each IVM group were shown in the Table 4.2. From the results, the percentage of matured

cattle oocytes in Group 1 (22-24 hr) which was 76.29% was significantly higher than Group 2 (25-27 hr) which was 65.63%.

For subsequent experiment after IVM, cattle oocytes were artificially activated using chemicals; calcium ionophore (CaI) for 5 minutes followed by 4 hours of 6-Dimethylaminopurine (6-DMAP). The number and percentage of successfully cleaved cattle parthenotes between two IVM durations (Group 1 and Group 2) were represented in the Table 4.3. Group 1 (22-24 hr) showed significantly higher ($P<0.05$) cleavage rate of cattle parthenotes at all embryo stages compared with Group 2 (25-27 hr). Typical matured cattle oocyte with cumulus cells after IVM is shown in Figure 4.1.

The cleavage rates of cattle parthenotes obtained between Group 1 and Group 2 were 69.18% vs. 55.27% (2-cell); 53.09% vs. 38.32% (4-cell); 41.40% vs. 26.92% (8-cell) and 21.26% vs. 9.31% (morula), respectively, except for 2- and 4-cell stages in Group 1, there were significant differences for all other stages of embryo development in both Groups of IVM duration. From the results, it was noted that cattle oocytes which underwent 22-24 hours IVM duration (21.26%) were more competent to develop until morula stage than 25-27 (9.31%) hours IVM duration in PA embryos. Figure 4.2 showed typical cattle parthenotes from 2-cell until 8-cell stage, while typical cattle parthenotes of morula and compacted morula are shown in Figure 4.3.

Table 4.1: Effect of different abattoir locality on the maturation rate of cattle oocytes

Abattoir	Total number of ovaries (n)	Total number of oocytes obtained (n)	Percentage of matured oocytes (n)
Banting	12	141	71.33±2.7[a] (97)
Shah Alam	9	160	69.45±4.6[a] (113)

Means with same superscript [a] in a column showed no significant difference ($P>0.05$).
n = number of ovaries/oocytes.

Table 4.2: Percentage of matured cattle oocytes for different *in vitro* maturation (IVM) durations

Group	IVM duration (hr)	Total number of oocytes obtained (n)	Percentage of matured oocytes (n)
1	22-24	161	76.29 ± 2.1^{b} (123)
2	25-27	140	65.63 ± 2.9^{a} (91)

Means with different superscripts [a, b] in a column showed a significant difference ($P<0.05$).
n = number of ovaries/oocytes.

Table 4.3: Percentage of cleavage for cattle parthenote embryos derived from different *in vitro* maturation (IVM) durations

Group	IVM duration (hr)	Percentage of successfully cleaved cattle parthenotes based on different stages of embryo			
		2-cell (n)	4-cell (n)	8-cell (n)	Morula (n)
1	22-24	69.18 ± 3.8^{bz} (83)	53.09 ± 3.6^{byz} (64)	41.44 ± 3.6^{by} (50)	21.26 ± 1.7^{bx} (26)
2	25-27	55.27 ± 3.0^{az} (50)	38.32 ± 2.3^{ay} (35)	26.92 ± 2.2^{ax} (24)	9.31 ± 2.4^{aw} (9)

Means with different superscripts [a,b] in a column showed a significant difference ($P<0.05$).
Means with different superscripts [w,x, y, z] in a row showed a significant difference ($P<0.05$).

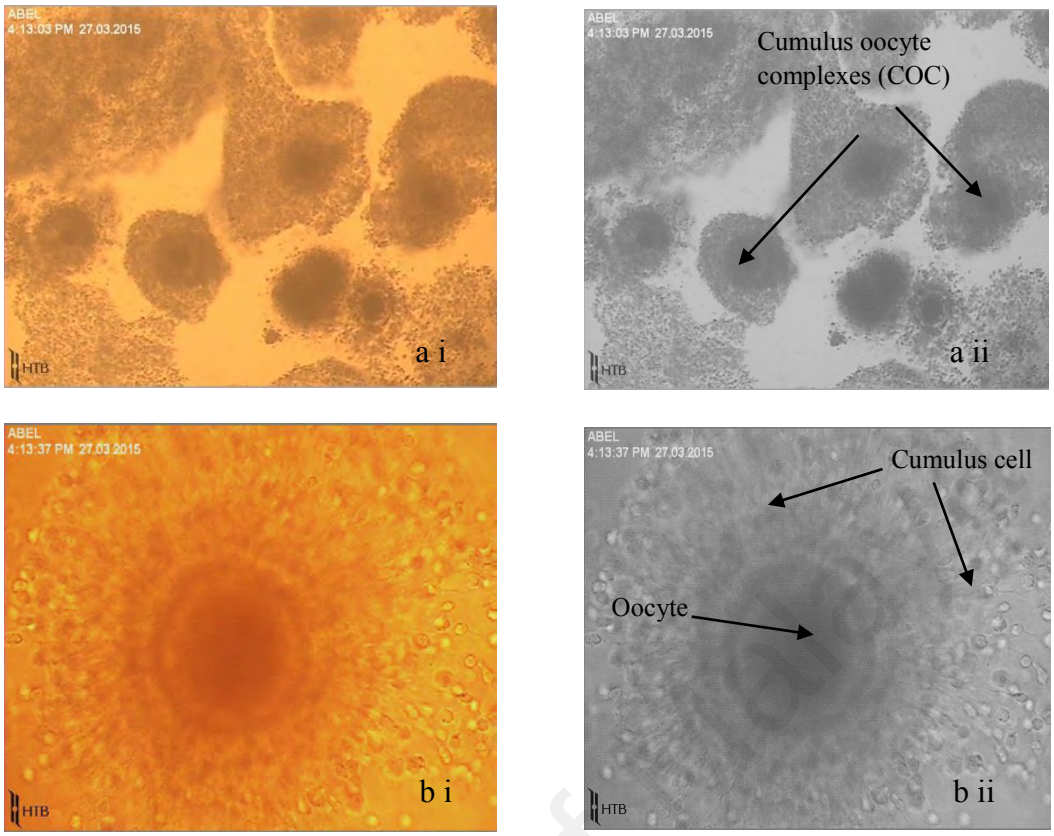


Figure 4.1: Typical matured cattle oocyte with cumulus cells after IVM (i-original photomicrograph, ii-labeled photomicrograph). (a) A group of COC after maturation, (b) a single COC after IVM with sunburst formation. Note: original magnification of photomicrographs (a) x20, (b) x100.

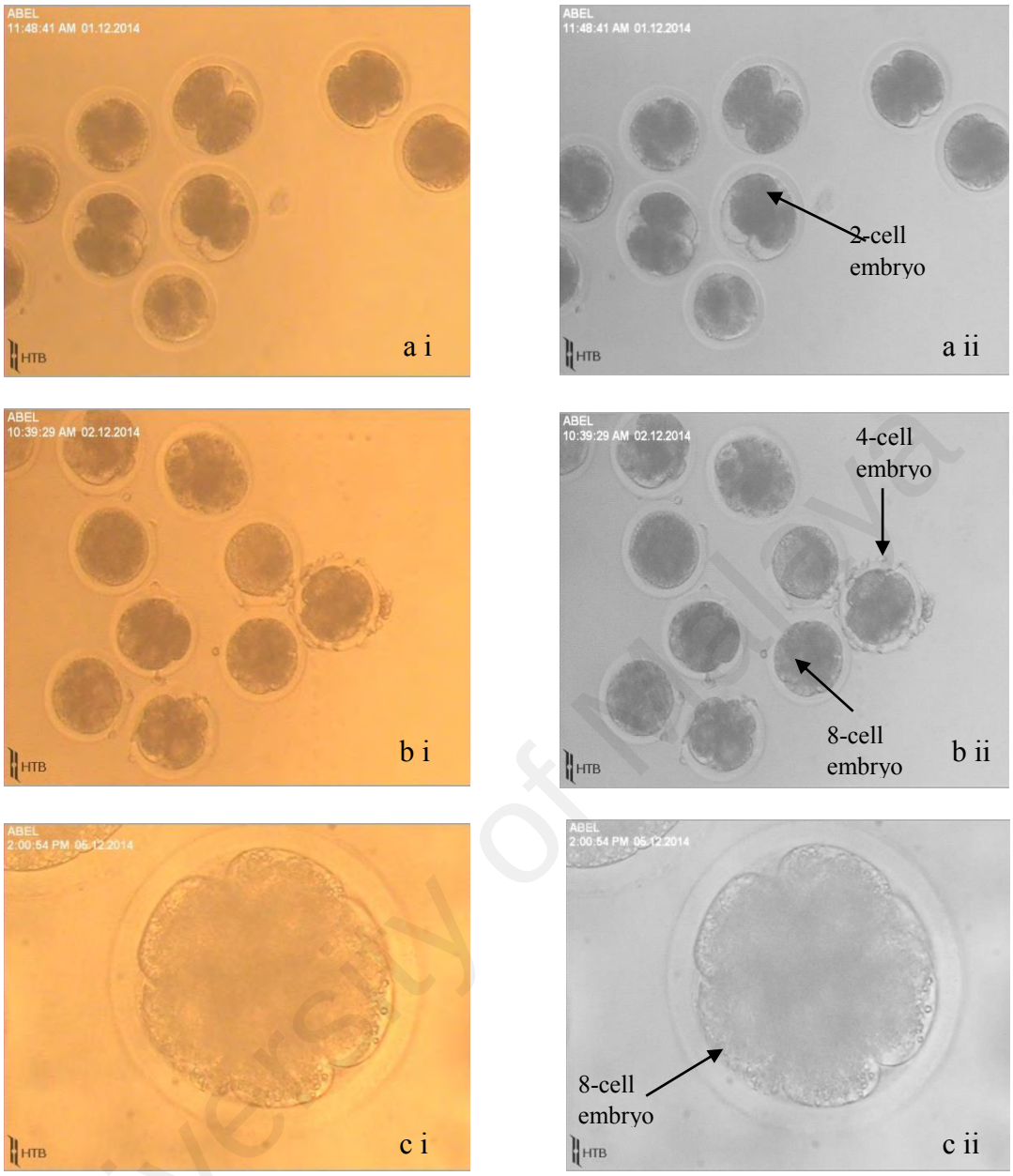


Figure 4.2: Typical cattle parthenotes (i-original photomicrograph, ii-labeled photomicrograph). (a) 2-cell stage, (b) 4-8-cell stage, (c) 8-cell stage. Note: original magnification of photomicrographs (a, b) x20, (c) x100.

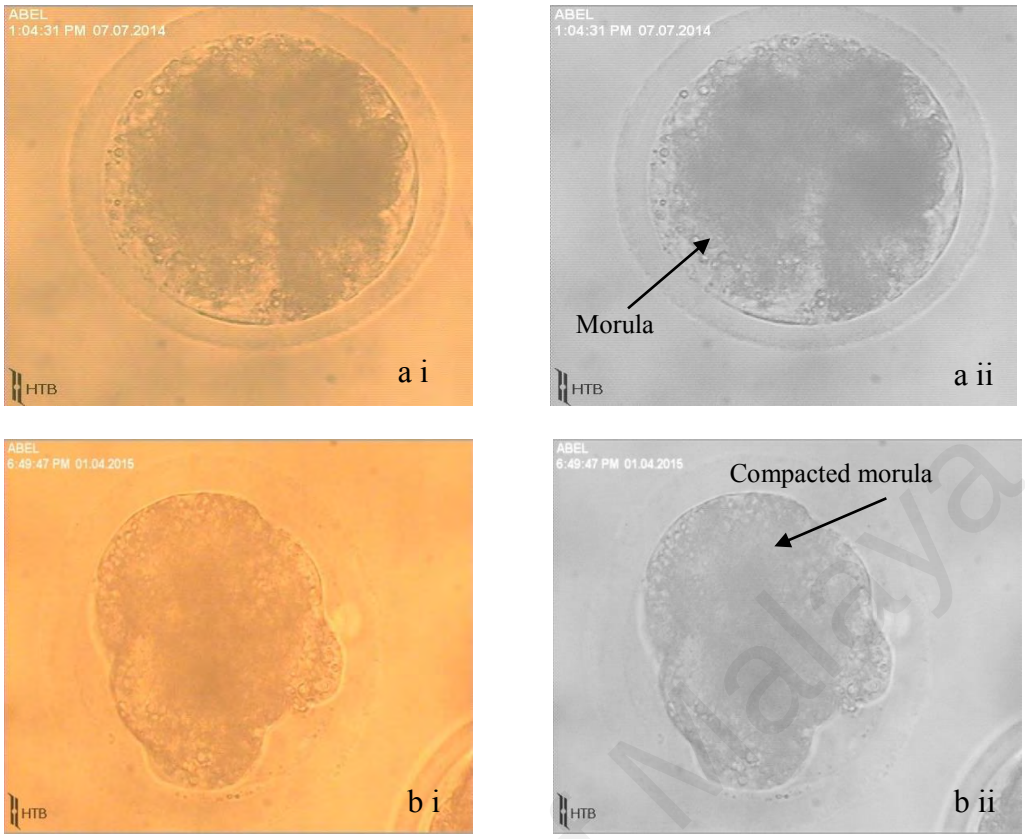


Figure 4.3: Typical cattle parthenotes (i-original photomicrograph, ii-labeled photomicrograph). (a) morula, (b) compacted morula. Note: original magnification of photomicrographs (a, b) x100.

4.2 EFFECT OF DIFFERENT TYPES OF DONOR KARYOPLAST ON VIABILITY OF DONOR KARYOPLAST INCLUDING EAR FIBROBLAST CELL (EFC) AND FOETAL FIBROBLAST CELL (FFC) (EXPERIMENT 2)

The aim of this experiment was to produce different types of donor karyoplast to be used for subsequent SCNT experiment. The results obtained are presented in Table 4.4. The effect of different types of donor karyoplast on the viability of cells was analysed using t-test. Table 4.4 shows the comparison between 3 different goat breeds which are Jermasia (EFC), Boer (EFC) and Jermasia-cross (FFC). For foetal fibroblast cell (FFC), a total of two foetuses aged 48 and 70 days were obtained by caesarean surgery. Fibroblast tissues obtained from both foetuses were cultured and subsequently sub-passage until Passage 2 (P2).

From the present results (Table 4.4), there were no significant differences in percentages of cells viability between 2 breeds of EFC (Jermasia vs. Boer) in all passages, except for P1 that Boer was significantly higher in percent viability than Jermasia (86.72% vs. 80.44%), respectively. However, there were significant differences in percentages of cell viability between fresh and frozen-thawed samples in all passages for EFC and FFC. From the Table 4.5, there was no significant difference in the effect of gender of EFC. The FFC showed significantly higher in term of cells viability as oppose to EFC. Typical 80% confluency of FFC and EFC are shown in Figure 4.4 and 4.5.

Table 4.4: Percentage of cells viability (in fresh and frozen-thawed cells) for different types of donor karyoplast

Breed	Type of donor karyoplast	Percentage of cells viability (%)					
		P1		P2		P3	
		Fresh (n)	Frozen-thawed (n)	Fresh (n)	Frozen-thawed (n)	Fresh (n)	Frozen-thawed (n)
Jermasia	EFC	80.44 ± 0.8^{az} (5)	77.73 ± 0.9^{ay} (5)	81.40 ± 0.5^{az} (5)	76.50 ± 1.6^{ay} (5)	80.46 ± 0.8^{az} (5)	72.76 ± 1.5^{ax} (5)
Boer	EFC	86.72 ± 1.0^{bz} (5)	80.75 ± 0.5^{ax} (5)	84.23 ± 1.0^{ayz} (5)	79.11 ± 0.6^{ax} (5)	81.73 ± 0.5^{ay} (5)	74.06 ± 1.1^{aw} (5)
Jermasia - cross	FFC	93.49 ± 1.4^{cy} (2)	89.03 ± 0.5^{bx} (2)	90.60 ± 0.8^{bx} (2)	88.11 ± 0.3^{bx} (2)	-	-

Means with different superscripts $^{a, b, c}$ in a column within a group showed a significant difference ($P<0.05$).
Means with different superscripts $^{w,x, y, z}$ in a row showed a significant difference ($P<0.05$).
P1, P2 and P3 = Passage 1, Passage 2 and Passage 3.
n = number of samples.

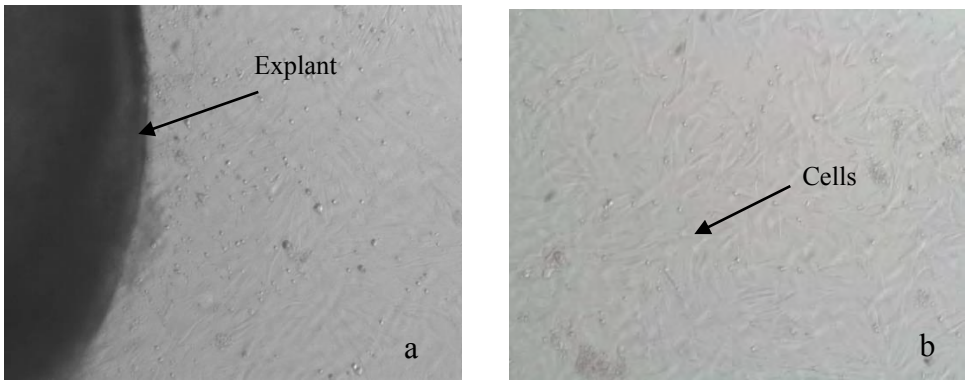


Figure 4.4: Typical 80% confluency of foetal fibroblast cells (FFC) (a) explant, (b) after passaging. Note: original magnification of photomicrographs (a, b) x40.

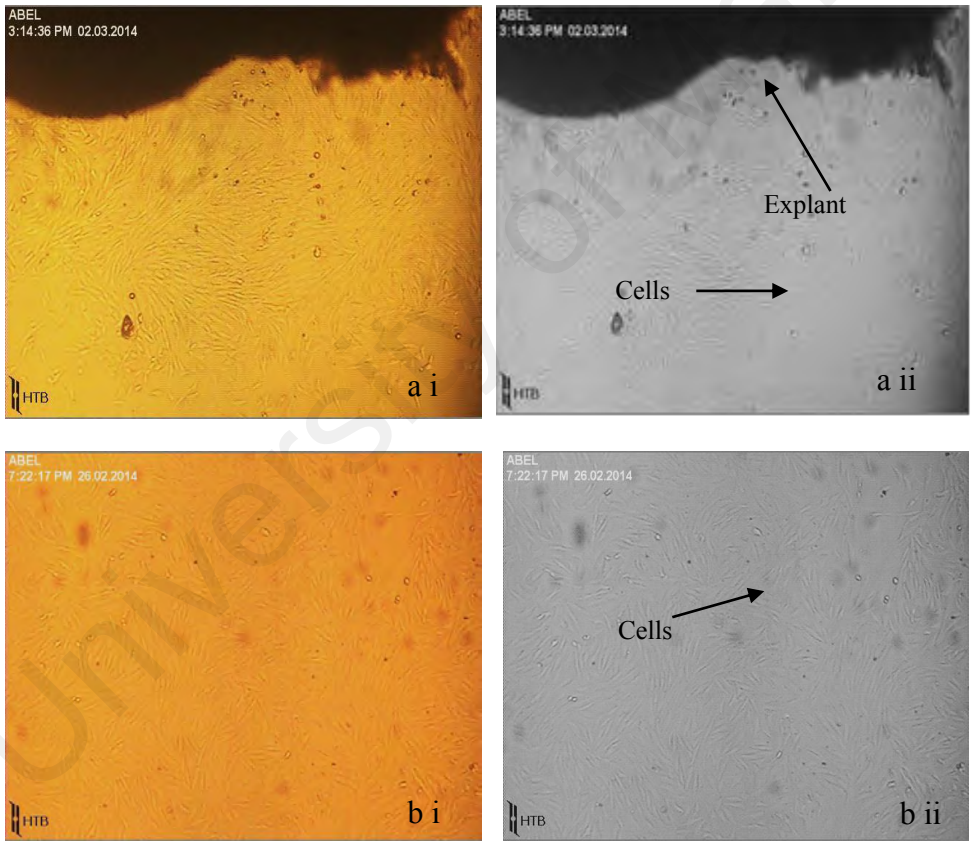


Figure 4.5: Typical 80% confluency of ear fibroblast cells (EFC) (i-original photomicrograph, ii-labeled photomicrograph). (a) explant, (b) after passaging. Note: original magnification of photomicrographs (a, b) x20.

4.3 PRODUCTION OF CLONED CATTLE EMBRYOS USING CUMULUS CELLS (CC) AS DONOR KARYOPLASTS VIA INTRASPECIES SCNT (EXPERIMENT 3)

The aim of this experiment was to produce cloned cattle embryos via intraspecies SCNT using CC as donor karyoplast. A total of 155 oocytes were matured (64.23%) and reconstructed (21.90%) out of 241 total cattle oocytes used for cattle intraspecies SCNT experiment. Cloned cattle intraspecies SCNT embryos were successfully develop to morula stage.

Table 4.7 shows the values of cleavage rates in cattle intraspecies SCNT which were 53.57% (2-cell), 33.17% (4-cell), 22.15% (8-cell) and 11.90% (morula). There were significant decreases in the cleavage rate at different stages of embryo development (2-cell to morula). The results from this intraspecies SCNT experiment was used as a basis for comparison between intra- and subsequent interspecies SCNT experiments. Figure 4.6 shows the typical cloned cattle intraspecies SCNT embryos derived from this experiment.

Table 4.5: Percentage of cells viability (in fresh and frozen-thawed cells) for different gender in goat EFC

Gender	Type of donor karyoplast	Percentage of cells viability (%)					
		P1		P2		P3	
		Fresh (n)	Frozen-thawed (n)	Fresh (n)	Frozen-thawed (n)	Fresh (n)	Frozen-thawed (n)
Female	EFC	84.29 ± 1.5^{az} (7)	79.77 ± 0.7^{ay} (7)	83.34 ± 0.9^{az} (7)	78.17 ± 1.1^{ay} (7)	81.67 ± 0.6^{ayz} (7)	73.86 ± 1.3^{ax} (7)
Male	EFC	82.03 ± 2.0^{az} (3)	78.00 ± 1.8^{ay} (3)	81.60 ± 0.5^{az} (3)	76.90 ± 2.0^{ay} (3)	79.73 ± 0.6^{ayz} (3)	72.35 ± 0.6^{ax} (3)

Means with same superscript [a] in a column within a group showed no significant difference (P>0.05).
Means with different superscripts [x, y, z] in a row showed a significant difference (P<0.05).
P1, P2 and P3 = Passage 1, Passage 2 and Passage 3.
n = number of samples.

Table 4.6: Percentage of matured and reconstructed cattle oocytes in cattle intraspecies SCNT

Donor karyoplast	Total no. of ovary	Total no. of oocytes	No. of oocytes per ovary	Percent of matured oocytes (n)	Percent of reconstructed oocytes (n)
CC	17	241	14.18	64.32 (155)	21.90 (33)

n = number of samples.

Table 4.7: Cleavage rate of cattle intraspecies SCNT

Donor karyoplast	% 2-cell (n)	% 4-cell (n)	% 8-cell (n)	% morula (n)
CC	53.57 ± 3.6^{z} (33)	33.17 ± 4.2^{y} (21)	22.15 ± 2.4^{x} (14)	11.90 ± 3.2^{w} (8)

Means with different superscripts $^{w, x, y, z}$ in a row showed a significant difference ($P<0.05$).
n = number of samples.

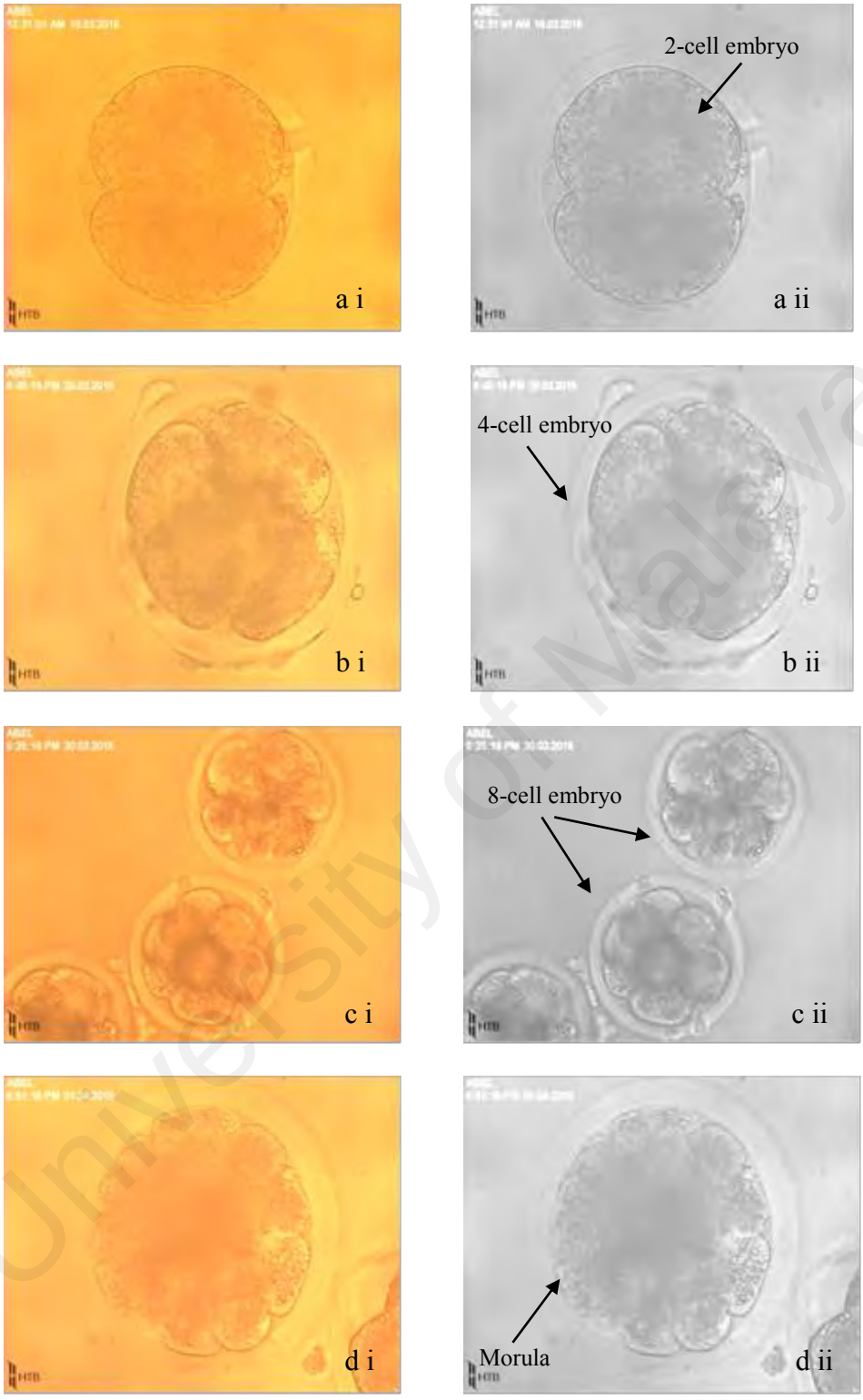


Figure 4.6: Typical cattle intraspecies SCNT embryos (i-original photomicrograph, ii-labeled photomicrograph). (a) 2-cell, (b) 4-cell, (c) 8-cell and (d) morula. Note: original magnification of photomicrographs (a, b, d) x100, (c) x40.

4.4 PRODUCTION OF CLONED CATTLE EMBRYOS USING EAR FIBROBLAST CELLS (EFC) AND FOETAL FIBROBLAST CELLS (FFC) AS DONOR KARYOPLASTS VIA INTERSPECIES GOAT-CATTLE SCNT (EXPERIMENT 4)

The aim of this experiment was to evaluate the efficiency rate of interspecies SCNT producing cloned goat embryos using 2 different sources of donor karyoplast, which were goat EFC and FFC. For EFC, out of 63.64% matured oocytes, only 26.34% were successfully reconstructed. For FFC, out of 61.76% matured oocytes, 46.23% oocytes were successfully reconstructed, which was significantly higher ($P<0.05$) than EFC.

In this experiment, the recipient cytoplasts were from cattle, meanwhile, the donor karyoplasts were derived from goats to produce cloned goat embryos. The results (Table 4.9) showed that foetal fibroblast cell (FFC) in interspecies SCNT, respectively, gave significantly higher ($P<0.05$) 2-cell (64.40% vs. 38.43%), 4-cell (54.24% vs. 24.60%), 8-cell (36.82% vs. 14.54%) and morula (22.10% vs. 7.90%) cloned goat embryos than EFC, respectively. Typical cattle interspecies SCNT embryos from 2-cell until morula stage are shown in Figure 4.7.

Table 4.8: Percentage of matured and reconstructed cattle oocytes for goat-cattle interspecies SCNT derived from different types of donor karyoplast

Donor karyoplast	Total no. of ovaries (n)	Total no. of oocytes (n)	% matured oocytes (n)	% reconstructed oocytes (n)
EFC	29	352	63.64[a] (224)	26.34[a] (59)
FFC	27	476	61.76[a] (294)	46.23[b] (136)

Means with different superscripts [a,b] in a column showed a significant difference ($P<0.05$).
n = number of ovaries/oocytes.

Table 4.9: Percentage of cleavage for interspecies goat-cattle SCNT embryos derived from different types of donor karyoplast

Donor karyoplast	% 2-cell (n)	% 4-cell (n)	% 8-cell (n)	% morula (n)
EFC	38.43±5.3[az] (59)	24.59±4.1[ay] (29)	14.54±4.1[ax] (24)	7.90±3.7[aw] (14)
FFC	64.41±6.8[bz] (136)	54.24±6.4[by] (112)	36.82±5.3[bx] (75)	22.09±4.5[bw] (45)

Means with different superscripts [a,b] in a column showed a significant difference ($P<0.05$).
Means with different superscripts [x, y, z] in a row showed a significant difference ($P<0.05$).
n = number of embryos.

Table 4.10: Percentage of cleavage for intra- and interspecies goat-cattle SCNT embryos derived from different types of donor karyoplast

Donor karyoplast	SCNT technique	% 2-cell (n)	% 4-cell (n)	% 8-cell (n)	% morula (n)
CC	Intraspecies (Cattle)	53.57 ± 3.6^{abz} (33)	33.17 ± 4.2^{ay} (21)	22.15 ± 2.4^{ax} (14)	11.90 ± 3.2^{abw} (8)
EFC	Interspecies (Goat-cattle)	38.43 ± 5.3^{az} (59)	24.59 ± 4.1^{ay} (29)	14.54 ± 4.1^{ax} (24)	7.90 ± 3.7^{aw} (14)
FFC	Interspecies (Goat-cattle)	64.41 ± 6.8^{bz} (136)	54.24 ± 6.4^{byz} (112)	36.82 ± 5.3^{abx} (75)	22.09 ± 4.5^{bw} (45)

Means with different superscripts ^{a,b} in a column showed a significant difference ($P<0.05$).
Means with different superscripts ^{w,x,y,z} in a row showed a significant difference ($P<0.05$).
n = number of embryos.

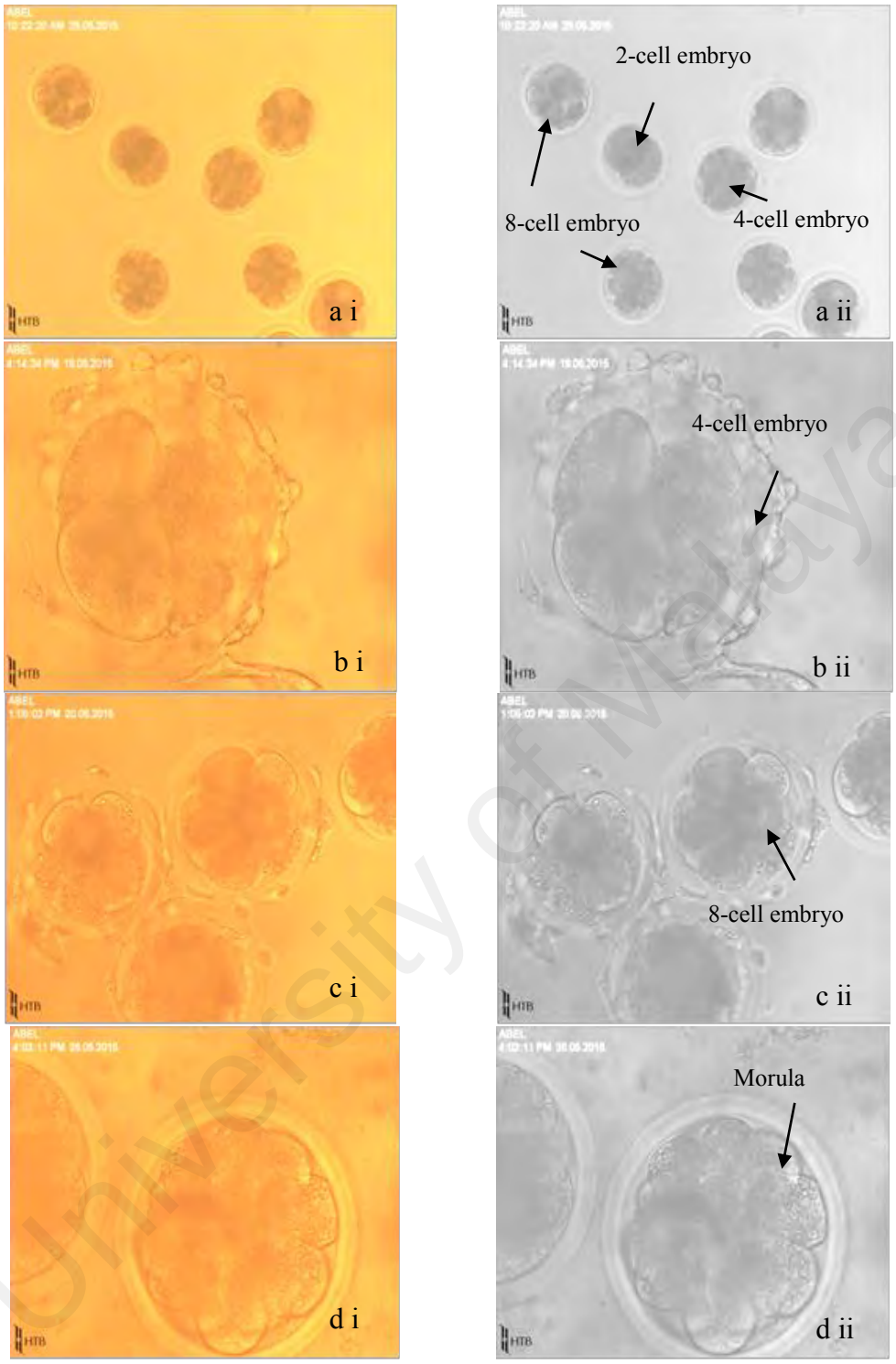


Figure 4.7: Typical cattle interspecies SCNT (i-original photomicrograph, ii-labeled photomicrograph). (a) 2-8-cell, (b) 4-cell, (c) 8-cell and (d) morula. Note: original magnification of photomicrographs (a), x20 (b) x100, (c) x40 d) x100.

CHAPTER 5

DISCUSSION

5.1 EFFECTS OF DIFFERENT *IN VITRO* MATURATION DURATIONS ON PERCENTAGE OF MATURED CATTLE OOCYTES AND SUBSEQUENT *IN VITRO* CATTLE EMBRYO PRODUCTION AS A PILOT PRELIMINARY STUDY AS A MODEL EXPERIMENT (EXPERIMENT 1)

In this experiment, cattle oocytes were matured *in vitro* using standard IVM medium. The effect of IVM duration on maturation rate of cattle oocytes was evaluated by comparing two IVM durations which were 22 to 24 hours and Group 25 to 27 hours. For the duration of 22 to 24 hours, it showed that the maturation rate was significantly than that of 25 to 27 hours. After maturation, matured cattle oocytes derived from abattoirs were subsequently activated artificially using a combination of chemical treatment calcium ionophore (5 μM CaI) for 5 minutes and followed by 6-dimethylaminopurine (2 mM 6-DMAP) for 4 hours. The cleavage rate of cattle parthenotes in two IVM durations was evaluated after parthenogenetic activation (PA) treatment. From the results, it was noted that 22 to 24 hours IVM duration showed significantly higher cleavage rate of cattle parthenotes at all stages of embryo than 25 to 27 hours IVM duration. The maturation rate of oocyte could influence the subsequent *in vitro* embryo development (Dominko & First, 1992; Van der Westerlaken *et al*., 1994). Improper maturation duration could impair the development of embryos due to abnormal chromatin and apoptosis (Dominko & First, 1992; Marston & Chang, 1964).

In the present study, the percentage of successfully matured cattle oocytes was 76.29%, which was almost similar obtained by Park *et al*. (2005), 74.50% on cattle oocytes after 24 hours of IVM treatment. These findings were in agreement with Lonergan *et al*. (1994) and Ward *et al*. (2002) which also reported that 24 hours of IVM treatment was the optimum duration for cattle oocytes to undergo maturation. The

duration of cattle oocytes to extrude their first polar body during IVM had implications on subsequent embryo development including PA. It had been reported that those oocytes that matured early were more likely to develop until blastocyst stage compared to the delayed ones (Van der Westerlaken, 1994; Dominko & First, 1997). Barcelo-Fimbres *et al*. (2015) also stated that 24 hours of IVM duration in cattle oocytes yielded higher number of blastocyst and increased in pregnancy rate.

The duration of IVM was different among various animal species. According to Kruip *et al*. (1983), it required around 19 to 25 hours for cattle *in vivo* oocytes to fully mature after the surging of luteinising hormone (LH) during oestrus cycle. Meanwhile for IVM, it took between 12 to 24 hours for cattle oocytes to extrude their first polar body (Dominko & First, 1997; Van der Westerlaken *et al*., 1994). Whereas in goat species, Kong (2010) obtained better maturation rate of goat oocytes matured at 22 to 25 hours (71.23%) when compared with goat oocytes matured at 27 to 32 hours (38.70%), respectively.

Nurin (2016) reported that no significant difference was found between maturation duration of 22-24 hours and 25-27 hours in cattle oocytes. This could be due to different sample size or other factors that could affect IVM rate of cattle oocytes. Meanwhile, Khadijah (2012) obtained higher rate of maturation in goat oocytes at 18 to 21 hours compared with 22 to 25 hours, while, Chan (2008) reported that IVM duration range from 21 to 24 hours gave higher maturation rate in goat oocytes. On the other hand, Maximo *et al*. (2017) suggested that 18 hours of IVM is the optimum duration for sheep oocytes to complete maturation. Delaying the maturation duration after 24 hours would cause oocytes degeneration such as granulation and oocyte shrinkage as well as disorganisation of mitochondrial distribution.

They also reported that excessive IVM duration (>24 hours) could result in oocyte degenerations due to disruption of oocyte ultrastructures, which would consequently

cause errors in chromosome pairing (Yu *et al*., 2011). Son *et al*. (2005) suggested that the capability of collected oocytes to develop full term is correlated with their IVM duration. Oocytes that protruded the first polar body or matured earlier in IVM medium gave better embryonic developmental competence. This finding was in parallel with the present study, in which cattle parthenotes that matured at 22 to 24 hours in IVM medium gave higher cleavage rate after PA treatment compared with 25 to 27 hours.

In this study, PA treatment was used to indicate which IVM duration resulted in better cleavage rate on cattle parthenotes. Activation process is one of the important mechanisms to determine the success of subsequent experiments such as SCNT in which it could affect the subsequent development of reconstructed oocytes or cloned embryos. Prolonged exposure of oocytes to IVM medium could result in oocyte aging in which it could increase susceptibility to activating stimuli (Hölker *et al*., 2005), decrease in MPF activity (Wilmut *et al*., 1997; Guérin *et al*., 2001; Whitaker & Knight, 2004), the onset of anaphase II (Wilmut *et al*., 1997), as well as partial exocytosis of cortical granules.

Oocyte quality could be determined by various factors including the degree of cumulus expansion, number of cumulus layers and texture of ooplasm. These criteria happened to have a significant effect on the subsequent IVM treatment, fertilisation as well as embryo development (Tanghe *et al*., 2002). Hence, in this study, cattle oocytes that surrounded by more than 3 layers of cumulus cells were selected for IVM treatment. Cumulus cells are important for cytoplasmic maturation of oocytes prior to activation process and have a major effect on subsequent embryo development after treated with activation chemicals (Khadijah, 2012). Cumulus cells are essential for transportation of nutrients and sending signals inside and outside of the oocytes which would regulate its growth and maturation (Moor & Seamark, 1986). Cumulus cells also important for subsequent embryo developments as it could synthesise intracytoplasmic glutathione

which accumulate essential compounds during the later stage of maturation (Maedomari *et al*., 2007).

It is a well-known fact that oocytes are arrested at metaphase II (MII) after maturation until they were activated whether by penetration of sperm in normal fertilisation or artificially activated using stimuli such as chemicals in an appropriate time (Yanagimachi, 1978; Whittingham & Siracusa, 1978). Otherwise, the oocytes would undergo a process of aging which would subsequently causing the decline of MPF activity (Wu *et al*., 1997), increased in cytoplasmic and DNA fragmentation due to apoptosis (Gordo *et al*., 2002) as well as impaired embryo development following fertilisation or activation (Tarin *et al*., 1998). These would lead to the incapability of oocytes to develop until blastocyst stage (Long *et al*., 1994). Oocytes arrested at MII stage had a short span of fertilisable life; hence, this would decrease the potential of oocytes to be activated by chemical stimuli if it delayed.

Other than IVM duration, the maturation rate of cattle oocytes could be influenced by some other factors such as follicle size, diameter of oocyte (Hyttel *et al*., 1997), transportation duration, storage temperature of ovary samples from abattoir to the laboratory (Yang *et al*., 1990), age of female cattle (Khatir *et al*., 1998) and developmental stage of the oocyte (Hagemann *et al*., 1999). The preparation and the composition of IVM media (Lonergen *et al*., 1997) also could affect the maturation rate of cattle oocytes such as with addition of hormones and serum could yield higher maturation rate (Avery *et al*., 1998; Zuelke & Brackett, 1990). Even though there were a number of reports on embryo development after IVM treatment, there was still lack of studies involving the effect of different IVM durations on subsequent embryo development particularly after PA experiment.

5.2 EFFECT OF DIFFERENT TYPES OF DONOR KARYOPLAST ON VIABILITY OF DONOR KARYOPLAST INCLUDING EAR FIBROBLAST CELL (EFC) AND FOETAL FIBROBLAST CELL (FFC) (EXPERIMENT 2)

The objective of this experiment was to produce different types of donor karyoplast to be used for subsequent SCNT experiment. The effect of different types of donor karyoplast including ear fibroblast cell (EFC) and foetal fibroblast cell (FFC) from Jermasia, Boer and Jermasia-cross goat breeds on percentage of cells viability were evaluated. A total of 12 goat tissue samples including 2 samples from foetus were cultured and subpassaged up to Passage 3 (P 3). From the present results, there were no significant differences in percentages of cells viability between 2 breeds of EFC (Jermasia vs. Boer) in all passages; except for P1 that Boer was significantly higher in percent cells viability than Jermasia. The FFC (Cross-jermasia) showed the highest percentage of cells ability among those 3 goat breeds. There were also significant differences in percentage of cells between fresh and frozen-thawed in all passages regardless of donor karyoplast types. The effect of gender on donor karyoplast is another important factor that needed to be studied. However, in this study there was no significant difference on gender of EFC in viability of cells. Kato *et al*. (2013) reported that there was no significant effect of donor karyoplast gender in cattle SCNT. In contrary, Wakayama & Yanagimachi (1999) mentioned that different gender could influence the efficiency rate of SCNT in mice.

The reason why the FFC and EFC were cultured up to P2 and P3, respectively, was based on previous studies that recommending early passages such as P2 and P3 fibroblasts as donor karyoplasts for SCNT to successfully produce cloned animals (Wells *et al*., 1999; Onishi *et al*., 2000; Chesne *et al*., 2002). Roh *et al*. (2000) also reported that donor karyoplast at low passage numbers able to produce more blastocyst after SCNT compared with donor karyoplast at high passage numbers. Moreover, cell lines at low passage numbers experienced low risks of alterations in morphology, growth rates,

protein expression and transfection efficiency compared to cells at high passage numbers (Li *et al*., 2014). Such abnormalities on high passage numbers of donor karyoplasts would disrupt the capability of cells to reprogramme after fused with the recipient cytoplast (Condic, 2008). Chacoon *et al*. (2011) reported that continuous passaging of cells can affects the level of H3K9ac in cattle fibroblast; thus, reducing the cleavage rates of cloned cattle embryos after SCNT. Several studies have reported that high number of donor karyoplast passages could decrease the fusion rates between donor karyoplast and recipient cytoplast and consequently reduce the blastocyst production (Wilmut *et al*., 1997; Kato *et al*., 1998; Hill *et al*., 2000; Roh *et al*., 2000; Bhuiyan *et al*., 2004). On the contrary, Kubota *et al*., (2000) reported that high passage numbers of donor karyoplasts resulted in more blastocyst production in cattle compared with low passage number of donor karyoplast. Davood *et al*. (2016) reported that biological characteristics as well as the ability of cells to growth could cease in *in vitro* cells after culturing due to various factors. Hence, effective measure was needed to ensure the diploid stability of the cells. For instance, a lower number of donor karyoplast passages were recommended to avoid changes in the genetic characteristics of the donor nucleus.

The most important criterion while preserving genetic materials of donor karyoplast from animals was genetic stability after culturing. The cells should continuously retain the same diploid character as *in vivo* cells (Davood *et al*., 2016). Prolonged culture of donor karyoplast could increase their levels of aneuploidy which would disrupt the chromosomal stability and subsequently defected in faithful transmission of chromosomes (Ono, 1971; Lengauer *et al*., 1998, Freshney, 2000). Chromosomal instability would alter the karyotype as they proliferated. Thus, it would reduce the ability of cells to growth further as observed from primary fibroblasts of Down Syndrome patients in which the cells proliferated more slowly compared with euploid control cells *in vitro* (Segal & McCoy, 1974).

In this study, the FFC showed significantly higher in term of cells viability as oppose to EFC. The FFC was claimed to have more proliferative ability and less genetic damage compared with other types of donor karyoplast (Hill *et al*., 2000). The FFC as donor karyoplast deemed to be highly potential and robust in terms of long duration cell culture, abundance of cells for genetic engineering and could result in high efficiency of blastocyst/offspring production after commencing SCNT (Marijo *et al*., 2014). From the present study, it was observed that FFC took around 3-4 days only to reach 80% confluency compared with EFC which was around 7-8 days. The FFC from early foetus could proliferate rapidly which correlated with its function in foetus for wound healing (Gurtner *et al*., 2017).

There was a significant different in percentage of cells viability between fresh and frozen-thawed cells from all different goat breeds used in this study. This result was in agreement with Singh and Sharma (2010), which showed higher percentage of cells viability (98.4%) in fresh cells compared with frozen-thawed cells (96.23%). Quick freezing was used as a cryopreservation method for donor karyoplast or cells in this study using DMSO as a cryoprotective agent. The addition of DMSO into tissue culture medium (TCM) would expose the cells into a hyperosmotic condition causing the water to diffuse out from the cells and resulting in shrinkage of the cells without damaging the cellular integrity and viability. At the same time, DMSO would enter the cells for cellular protection from cold-shock until it reaches equilibration. The cells would regain back to their original size and volume after thawing which would cause the fluid from the medium reinfuses into the cells. Simultaneously, DMSO would be diffused out into the medium. The cryoinjury of cells could be avoided if freezing technique was conducted properly to assure and maintain the viability of cells (Aflah, 2014).

Cell cryopreservation technology could be applied in developing the fibroblast cell banks specifically for wildlife conservation as a nucleus source for biological

research to preserve valuable genetic materials from endangered species (Li *et al*., 2009). As such, different types of fibroblasts have been biopsied and cultured from various animal species for various applications, including donor karyoplasts for SCNT, feeder layer for embryonic stem cells, tissue engineering as well as research in wound healing properties. Moreover, donor karyoplasts including EFC and FFC have been established from several animal species to develop fibroblast cell bank (Davood *et al*., 2016). Establishment of fibroblast cell banks also had been recorded from some ruminant breeds including Luxi cattle (Liu *et al*., 2008), Simmental cattle (Li *et al*., 2009), Taihang black goat (Guan *et al*., 2005), Cashmere goat (Islam & Zhou, 2007) and Mongolian sheep (Liu *et al*., 2011).

In a nutshell, there were no significant differences between gender and passages (except for P1 in Boer and Jermasia) studied in this experiment, which indicated that the donor karyoplast could be successfully produced from EFC and FFC regardless of the factors involved. The FFC showed the highest percentage of cell viability, showing that it is the most optimum to be used as donor karyoplast for subsequent SCNT experiment. This experiment was deemed to be useful for future studies particularly in biology of cell, stem cell, tissue engineering, genomics, genetics as well as providing donor karyoplast materials for future SCNT experiments.

5.3 PRODUCTION OF CLONED CATTLE EMBRYOS USING CUMULUS CELLS (CC) AS DONOR KARYOPLASTS VIA INTRASPECIES SCNT (EXPERIMENT 3)

A total of 155 oocytes were matured (64.23%) out of 241 total cattle oocytes used for cattle intraspecies SCNT experiment. Cloned cattle intraspecies embryos were successfully develop to morula stage. However, the rate was lower compared with previous researchers in our laboratory which could be due to different experimental set up as well as early learning curve period in SCNT protocol for the researcher in this study.

Abdullah *et al*. (2011) reported that cattle intraspecies SCNT with cleavage rate of 77.70% and 46.10% respectively for 2-cell (77.70%) morula (46.10%) which were higher than the present study. From the present study, it showed that the values of cleavage rates in cattle intraspecies SCNT. The present results of cleavage (2-cell-morula: 11.90%) for intraspecies SCNT cattle cloning were generally considered lower when compared with previous findings in ABEL laboratory. The exact reason is not known, however, it could be due to different laboratory set-up, different time, different samples, different procedures as well as personnel skill in cloning technique.

Intraspecies SCNT is a cloning technique that transfect a donor karyoplast into a recipient cytoplast of a same species. In this study, cumulus cells (CC) were used as a donor karyoplasts to produce cattle intraspecies SCNT embryos. CC are clusters of differentiated ovarian granulosa cells that surround the oocytes. These cells undergo terminal differentiation after the surging of LH during ovulation phase (Ono *et al*., 2001). CC functions in providing the physical support and microenvironment which are essential for oocyte to growth and develop further. CC has been widely used as donor karyoplast in cloning of mice (Wakayama *et al*., 1998; Campbell *et al*., 2007), cattle (Kato *et al*., 1998), pig (Cheong *et al*., 2000) and in goat (Zou *et al*., 2001). Previous research in mice showed that cloned embryos using donor karyoplast derived from follicular epithelial cells yielded higher number of blastocysts compared with CC (Kato *et al*., 1999). The findings from the present experiment generally agreed with previous researchers, indicating that CC maybe less suitable to be used as karyoplast under the current laboratory set-up. The results from this intraspecies SCNT experiment was used as a basis for comparison between intra- and interspecies SCNT experiments.

History of the animals also could be one of the important factors that determine the success of intraspecies SCNT. The first step to produce successfully cloned animal is to obtain developmentally competent oocytes. The oocytes must have the ability to

resume and achieve meiosis (matured), ready to be fertilised, develop into viable embryo and subsequently give birth to healthy offspring after gestation. In this study, oocytes were retrieved from abattoir-derived cattle ovaries. Abattoir ovaries were cheap and a large number of oocytes could be easily obtained, however, it would compromise the quality of oocytes since the history of the animals, such as age and origin was unknown.

Asdiana (2014) reported that low cleavage rate using CC as donor karyoplast could be due to source of CC and number of passage. In this study, CC was freshly harvested after IVM to be used as donor karyoplast for SCNT experiment. According to Lin *et al*. (2010), using fresh CC as donor karyoplast showed lower cloned embryo developmental rate when compared with Passage 4 of CC. This could be due to different in cell cycle stages in both donor karyoplast. Cell cycle consists of growth arrest phase (G_0), DNA prophase synthesis (G_1), DNA synthesis (S), preparation of mitosis (G_2) and Mitosis phase (M) (Hayes *et al*., 2005). Donor karyoplast at G_0 or G_1 phase was considered to be most suitable for subsequent SCNT experiment. There was a high circumstance that freshly harvested CC was already at S phase (DNA synthesis). Meanwhile, passage culture of CC usually arrested at G_0 or G_1 phase. Hence, the coordination between cell cycle stage and donor karyoplast is important to boost cloning efficiency rate (Wells *et al*., 2003).

5.4 PRODUCTION OF CLONED CATTLE EMBRYOS USING EAR FIBROBLAST CELLS (EFC) AND FOETAL FIBROBLAST CELLS (FFC) AS DONOR KARYOPLASTS VIA INTERSPECIES GOAT-CATTLE SCNT (EXPERIMENT 4)

The present results indicated that foetal fibroblast cell (FFC) in interspecies SCNT, respectively, gave significantly higher cleavage rate in all embryo stages which are, 2-cell, 4-cell, 8-cell and morula of cloned goat-cattle interspecies embryos than EFC, respectively. This experiment indicated that both donor karyoplasts are suitable to be used as donor karyoplast; however, FFC gave higher efficiency rate compared with EFC in

producing cloned goat embryos via interspecies SCNT technique. It is believed that different types of donor karyoplasts may have profound effects on the ability of SCNT embryos to develop to term. Fibroblast cells have been regarded as the most commonly used as donor karyoplast in animal SCNT (Hosseini *et al*., 2008).

High cloned embryo developmental rate obtained from FFC was shown in this study. Marijo *et al*., (2014) reported that FFC have constitutionally long G_1 phase, thus, they are already at quiescent point of the cell cycle at any time, in which making them as the most efficient donor karyoplast compared to EFC and CC. Furthermore, FFC were believed to have high proliferation rate and less genetic damage than any other adult somatic cells. Jang *et al*. (2004) also reported that FFC gave the lowest apoptotic rate (0.060) when compared with skin fibroblast cell (0.089) in cattle SCNT.

The origin of donor karyoplast could affect the ability of the cloned SCNT embryos to develop beyond blastocyst stage (Cho *et al*., 2002; B. Oback & D. Wells, 2002; Galli *et al*., 2003; Batchelder *et al*., 2005). Santos *et al*. (2003) reported that types of donor karyoplast used for SCNT could influence the DNA methylation process as well as gene expression patterns during early development of the cloned embryos.

Hence, choosing an ideal donor karyoplast which is developmentally competent and also able to reprogramme in recipient cytoplast is one of the most critical steps before conducting SCNT experiment (Cho *et al*., 2002; Santos *et al*., 2003).

A number of studies suggested that FFC could be fully reprogrammed following SCNT experiment. The current result was in agreement with several reports in animal SCNT which suggested that FFC are predominant to adult somatic cells as donor karyoplast (Kato *et al*., 2000; Wakayama & Yanagimachi; 2001; Forsberg *et al*., 2002) in term of embryo developmental rate. Shah *et al*. (2009) reported that higher embryo developmental rate was obtained using FFC as donor karyoplast in buffalo SCNT compared with adult fibroblast cells, as well as in cattle species (Saikhun *et al*., 2002) and

pig (Lee *et al*., 2007). This is the second study in our laboratory using FFC as donor karyoplast to produce cloned goat-cattle interspecies embryos after Soh (2012). However, the present result was still considered low compared with Soh (2012); obtained 24.45% of morula and 12.08% of blastocyst. The reason for the lower cleavage rate in the present study was not known, however, it could be due to lower samples size, lower quality of recipient cytoplast source, different techniques including using micromanipulation facilities and technical skill of the personnel.

Even though cloned goat embryos were successfully produced using EFC in this study, the developmental rate was still considered low compared with previous studies done by researchers in ABEL. Kwong (2012) obtained 32.75% morula and 8.54% of blastocyst resulted from cloned goat-cattle interspecies experiments. Asdiana (2014) obtained 23.24% of cloned goat morula using EFC as donor karyoplast, while Nurin (2016) obtained 19.05% morula of interspecies cloned goat-cattle embryos, using same type of donor karyoplast. This could be due to *in vitro* culture conditions which is a critical factor for cloned embryo development. For example, medium and culture duration, contamination, degeneration of oocytes, handling of oocytes, oocytes source, technical, personnel as well as procedure applied.

Since CC and EFC were relatively easy to obtain and resulted in minor injury of the chosen animals, they were often used as donor karyoplast whether in intra- and interspecies SCNT study (Yang *et al*., 2010). From the present results, CC gave higher cleavage rate than EFC. This finding was in agreement with Hosseini *et al*. (2008) in which CC gave higher cleavage rate in sheep SCNT compared with EFC. This could be due to different SCNT technique used, which was CC for intraspecies for and EFC for interspecies.

It is worth to note that, the efficiency rate of SCNT is still greatly affected by the capability of recipient cytoplast to operate towards a foreign genome (Keith, 2004). This

is why closely related species or genera between recipient cytoplast and donor karyoplast results in more successful interspecies SCNT, which is of practical important in conservation of wildlife species. For example, an attempt to conserve endangered species of sheep, *Ovis orientalis musimon* was successfully done by transferring donor karyoplast into recipient cytoplast of domestic sheep (Loi *et al*., 2001). Another example, viable offspring of Zebu cattle also have been produced by transfecting a nuclei from *Bos indicus* somatic cells into *Bos taurus* recipient cytoplast (Meirelles *et al*., 2001). In contrary, extreme combinations between donor karyoplast and recipient cytoplast resulted in early arrested of cloned embryo development. For instance, the fusion between fibroblast cell of mouse and cattle oocytes failed to develop past 8-cell due to inappropriate gene expression (Arat *et al.,* 2001).

Therefore, it is important to study the genetic distance between donor karyoplast and recipient cytoplast to avoid incompatible genomic regulation and metabolic mechanism as suggested by Soh (2012). Hence, cattle oocytes were used in this study as recipient cytoplast along with goat donor karyoplast, with approach of interspecies SCNT technique as adapted from previous researchers (Kwong, 2012; Soh, 2012; Asdiana, 2014; Nurin, 2016) from ABEL laboratory which deemed to be feasible in order to produce cloned goat embryos. Furthermore, cattle oocytes were proven to be a universal recipient cytoplast as it could support the differentiation of nuclei introduced by donor karyoplast from different species.

5.5 GENERAL DISCUSSION

SCNT is a very complicated process which requires reprogramming of the donor karyoplast through recipient cytoplast from a differentiated state to a totipotent condition. Typically, the *in vitro* embryo production (IVEP) of cloned mammals by SCNT involves numerous complexes of steps, including oocyte/egg retrieval, culture of donor karyoplast,

in vitro maturation of oocyte (IVM), activation of reconstructed oocyte and embryo transfer (ET). The misstep at one of any SCNT protocols would affect the efficiency of the whole subsequent cloning procedure.

Although this study successfully produced cloned intra- and interspecies ruminant embryos, there were quite a number of drawbacks and weaknesses while carried out the experiments due to various factors such as resources, physical, personal and time constraints. There were several circumstances resulting in low developmental rates of cloned interspecies goat-cattle SCNT in this study. Such circumstances were in conjunction with source of oocyte/egg (recipient cytoplast), type of donor karyoplast, IVM duration, and activation of oocyte followed by developmental of reconstructed embryos. Thus, before integrating SCNT technique into a ruminant management for future industries and wildlife conservation, it is important to conduct more refined studies, while providing possible solution to improve the efficiency rate of this technique.

5.5.1 Constraints of the study

Belows are the constraints faced by the author while conducting this study.

i) Laboratory facilities and skill acquisition

Good laboratory facilities, practices (SOP) and quality control (QC) are compulsory in order to obtain successful SCNT experiment results. It is worth to mention that the current laboratory design, facilities and equipment were not in par with the standard laboratory to conduct SCNT research comprehensively due to many factors and limitations. The design of ART laboratory should be a risk-free environment by providing aseptic facilities to reduce the exposure of contamination or infection from any source. Cleaning and decontamination of *in vitro* culture facilities and work station such as CO_2 incubator and laminar flow should be done regularly which was not fully applied in ABEl laboratory

due to many constraints in infrastructure and financial. Comprehensive learning skills particularly in preparing the microtools, media, handling with oocytes and embryos, culturing, cryopreservation of donor karyoplast and micromanipulation technique for SCNT was earned earlier before conducting experiments. Speed and aseptic technique are very crucial aspects while dealing with oocytes or embryos. Excessive exposures towards outside environment would consequently cause the death of the oocyte or embryo due to elevated pH level and fluctuation in temperature. Therefore, the ruminant experiments were conducted after the author optimising her skills in SCNT using mice model due to the latter of easy to maintain and handling, efficient production and reproduction as well as lower cost.

ii) Adequate source of recipient cytoplast

Sufficient and constant supply of oocytes is one of the factors that could contribute success of SCNT embryo production. Difficulty in getting an adequate number of good quality oocyte was one of the main problems encountered by the author in order to carry out proper SCNT experiment in this study. Due to low number of goat population as well as number of goat slaughtering in local abattoirs (Ministry of Agriculture and Agro-based Industry Malaysia, 2014), it is difficult to get goat oocytes in order to conduct intraspecies SCNT to produce cloned goat embryo. This condition worsened by the lack of history record of cattle oocytes used in this study such as age and origin as well as unsterile environment of abattoir that could compromise the quality of oocytes obtained.

iii) Ovary transportation duration

Cattle ovaries were collected from local abattoir which located more than 70 km away from the laboratory. Due to the long distance, it would somehow cause the decrease of normal saline solution temperature during transportation of the ovaries to the laboratory.

Therefore, it compromised the quality of oocytes collected from those ovaries which consequently resulted in low cloned embryo developmental rates. The standard temperature of normal saline should be not less than 35°C. The decrease of sub-physiological temperatures of oocytes could elicit aberrancies at all stages of meiosis (Moor & Crosby, 1985; Heyman *et al*., 1986; Pickering *et al*., 1990; Aman & Parks, 1994).

iv) Early cloned and parthenote embryos developmental arrest

One of the main obstacles faced by the author was the *in vitro* embryo development failed to develop past morula stage. This could be due to various reasons such as IVC media, change in IVC environment, delay while handling the oocyte or embryo during experimentation, in which could lead to degeneration of both, activation treatment and others. Hence, it is important to troubleshoot every factor that could affect further embryo development.

5.5.2 Improvement for the current study

Despite of several imperfections in this study, the overall findings from the present study could be a basis for more refined research to optimise the protocol and techniques of *in vitro* cloned ruminant embryo production in future. The suggested aspects for future studies including:

a) Optimisation of donor karyoplasts including passage numbers, cell cycle synchronisation, trichostatin treatment (to improve blastocyst rate) and different types of somatic cell type.
b) Using different SCNT techniques such as aspiration, fusion and activation by electrical pulse to increase blastocyst rate.

c) Using LOPU as another oocyte source alternative to increase high quality recipient cytoplast in goat for intraspecies SCNT.

CHAPTER 6

CONCLUSION

This study was carried out to evaluate the effects of different donor karyoplast types derived from cattle and goat on the efficiency of developmental competence of cloned embryos after intra- and interspecies SCNT. The effect of IVM maturation duration on parthenogenetic activation (PA) and IVM rate were carried out as well in order to select which IVM duration produce optimise result for subsequent SCNT experiments. From this research, the conclusions could be made as follows:

a) Different types of donor karyoplast were successfully produced and cryopreserved to be used as donor karyoplast for subsequent SCNT experiment.
b) A range of 22-24 hours was the optimum IVM duration for subsequent PA and SCNT experiments.
c) Cattle parthenotes were successfully produced using parthenogenetic activation (PA) technique up to morula stage.
d) Cloned cattle and goat embryos could be produced using different types of donor karyoplast; CC (intraspecies SCNT), EFC (interspecies SCNT) and FFC (interspecies SCNT).
e) FFC gave significantly higher cleavage rate of goat-cattle interspecies cloned embryo production compared with EFC.

In a nutshell, with optimisation of karyoplast, cytoplast and IVC system, *in vitro* ruminant embryos, specifically for cattle and goat cloned embryos could be produced efficiently using advanced reproductive techniques such as pathenogenetic activation, intra- and interspecies SCNT techniques. These cloned embryos could be biosources for

the applications in livestock production for food security and safety, wildlife conservation for sustainable fauna and flora as well as denegerative diseases in human medicine.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publication

1. Siti Haslinda M. S., Asdiana A., Wan Khadijah, W. E., & Abdullah R. B. (2018). Successful cleavage of cloned goat embryos using ear fibroblast cell and fetal fibroblast cell as donor karyoplast in interspecies SCNT. *Indian Journal of Animal Sciences*. (ISI, accepted).