PRODUCTION OF CLONED INTRA- AND INTERSPECIES CAPRINE EMBRYOS THROUGH SOMATIC CELL NUCLEAR TRANSFER TECHNIQUE WITH SPECIAL REFERENCE TO DONOR KARYOPLAST TYPE AND RECIPIENT CYTOPLAST SOURCE

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2014

PRODUCTION OF CLONED INTRA- AND INTERSPECIES CAPRINE EMBRYOS THROUGH SOMATIC CELL NUCLEAR TRANSFER TECHNIQUE WITH SPECIAL REFERENCE TO DONOR KARYOPLAST TYPE AND RECIPIENT CYTOPLAST SOURCE

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DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCE
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KUALA LUMPUR

2014

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ORIGINAL LITERARY WORK DECLARATION

Name of Candidate : Asdiana Amri

Registration/Matric No : SGR110060

Name of Degree : Master of Science

Title of Dissertation : Production of Cloned Intra- and Interspecies Caprine Embryos Through Somatic Cell Nuclear Transfer Technique with Special Reference to Donor Karyoplast Type and Recipient Cytoplast Source

Field of Study : Reproductive Biotechnology

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ABSTRACT

In Malaysia, low goat population and high demand for such products from goat meat, milk and manure contribute to increase goat production to ensure food security and to improve the strategic and socio-economic status of the citizen, especially the rural population and also possibly for the commercialisation of goats. Therefore, using advanced assisted reproductive techniques (ART) through intra- and interspecies somatic cell nuclear transfer (SCNT) in order to produce cloned caprine embryos and subsequently offspring for propagation of genetically improved goat population at a rapid rate. The main objective of this research was to produce *in vitro* cloned caprine embryos through SCNT with special reference to recipient cytoplast source and donor karyoplast type. Briefly, caprine oocytes were obtained from two different oocyte sources (laparoscopic oocyte pick-up and abattoir), meanwhile bovine oocytes were only obtained from abattoir-derived oocytes. The collected oocytes were cultured in *in vitro* maturation medium (IVM) in CO_2 incubator (5%) at 38.5^{o}C for 21 to 24 hours (LOPU-derived oocyte) and 24 to 27 hours (abattoir-derived oocyte) for maturation. After maturation, cumulus oocyte complexes (COC) were removed by denuding in hyaluronidase (0.2%). The matured oocyte was confirmed by the extrusion of the first polar body (PB1) and enucleated by using laser technique followed by injection of the donor karyoplast (fresh cumulus cell or ear fibroblast cell) into the successful enucleated oocyte using whole-cell intracytoplasmic injection (WCICI) technique. The successful reconstructed oocytes were activated by using combination of calcium ionophore (CaI) for 5 minutes followed by 6-dimethylaminopurine (6-DMAP) for 4 hours which then were cultured in KSOM medium in CO_2 incubator (5%) at 38.5^{o}C for further development. Observation of the cleavage of reconstructed embryo was carried out daily.

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In Experiment 1, evaluation of the effect of oocyte sources on quantity and quality of oocytes obtained, maturation rate as well as subsequent *in vitro* culture performance of SCNT embryos were performed. There were no significant differences in the percentages of oocyte quality in LOPU versus abattoir: Grade B (36.24±2.6 vs. 33.23±2.7, respectively) and C (24.56±3.3 vs. 28.11±2.9, respectively); however, significant differences were shown in Grade A (32.46±4.4 vs. 19.80±4.4, respectively) and Grade D (6.74±1.7 vs. 18.86±2.0, respectively). Oocytes retrieved from LOPU showed the highest maturation rate was obatained from Grade A (77.62±6.7%) followed by Grade B (76.63±4.3%), Grade C (39.64±7.8%) and Grade D (21.97±8.5%), whereby Grades A and B were significantly higher than Grades C and D ($P<0.05$). Meanwhile in oocytes retrieved from abattoir showed no significant differences in maturation rates among the groups: Grades A (46.94±7.7%), C (63.80±7.8%) and D (58.24±7.4%), however, Grade C oocytes were significantly higher than those of Grade B (39.54±5.6%). When comparing the 2 oocyte-sources according to respective grade of oocytes, LOPU-derived oocytes was significantly higher ($P<0.05$) than abattoir-derived oocytes on the maturation rate in good quality of oocytes of Grades A and B. In *in vitro* developmental of embryo after SCNT, there were significant differences in cleavage rate of cloned embryos in LOPU versus abattoir: 2-cell (86.84±7.0 vs. 41.04±11.3%, respectively), 4-cell (84.03±8.5 vs. 35.06±10.8%, respectively), 8-cell (71.12±6.8 vs. 24.30±9.9%, respectively) and morula (47.59±7.2 vs. 16.48±7.2%, respectively).

In Experiment 2, the effect of time interval (36 to 40 hours; 66 to 70 hours; 71 to 75 hours) from PMSG/ hCG injection to LOPU on caprine stimulation response, oocyte recovery and maturation rate were investigated. These 3 different time intervals had significant differences in number of stimulated follicles and number of oocytes recovered.

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However, there was no significant difference in number of ovulated follicles (presence of corpus luteum). The recovered oocytes were randomly cultured in IVM medium for the maturation process. During IVM, time interval of 66 to 70 hours gave the highest survival rate (95.07±2.3%) followed by time interval of 71 to 75 hours (92.29±2.3%) and 36 to 40 hours (78.73±4.1%), which correspondingly to the maturation rate (75.64±3.4, 65.30±3.6 and 53.92±2.1%, respectively).

In Experiment 3, the effect of different types of donor karyoplast on the cleavage rate of cloned caprine embryos after SCNT was conducted. There was no significant difference in the enucleation rate and injection rate of two different types of fresh cumulus cell versus ear fibroblast cell (97.61±2.4 vs. 100.00% and 95.15±3.4 vs. 94.32±4.0%, respectively). The cleavage rate of cloned caprine embryo using ear fibroblast cell as donor karyoplast were significantly higher than the cumulus cell (82.76±5.1 vs. 57.17±5.6%, 75.97±7.5 vs. 46.38±7.1%, 64.49±9.8 vs. 27.25±8.7% and 50.82±10.4 vs 15.59±7.0%, respectively).

In Experiment 4, the efficacy of two different SCNT approaches, namely intraspecies SCNT and interspecies SCNT on the cleavage rate of cloned caprine embryos, and attempts of transferring of cloned embryo into surrogate mother were conducted. There were no significant differences between intra- and interspecies in respective maturation rate (66.08±3.8 and 59.68±4.9%, respectively), enucleation rate (98.90±1.1 and 96.95±2.3%, respectively) and injection rate (94.70±2.6 and 100%, respectively). The cleavage rates of intraspecies cloned embryos were significantly higher ($P<0.05$) than those of interspecies in all cases of preimplantation developmental stage: 2 cell (75.67±3.7 vs. 55.06±4.1%), 4 cell (64.86±5.4 vs. 48.58±4.5%), 8 cell (53.14±5.7 vs. 34.21±5.3%)

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and morula (38.55±5.1 vs. 23.24±3.9%). A total of 29 cloned embryos from the both SCNT approaches were transferred into the uterine horn of 8 recipient does. Unfortunately, no pregnancy was detected.

In conclusion, due to low population of goat in Malaysia, the difficulties in supply of caprine oocytes, the production of *in vitro* caprine embryos through assisted reproduction technique such as IVF, ICSI and SCNT was limited. Therefore, to choose recipient cytoplast source and donor karyoplast type was very important to maximise the efficacy in production of cloned caprine embryos and subsequent live offspring. Beside, the interspecies SCNT approach by using different species of recipient cytoplast (bovine oocyte) will be an alternative for the production of cloned caprine embryos in future which could be transferred into surrogate mother to develop full-term.

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ABSTRAK

Di Malaysia, populasi kambing yang rendah dan permintaan tinggi untuk produk dari kambing seperti daging, susu dan baja menyumbang kepada peningkatan pengeluaran kambing untuk memastikan keselamatan makanan dan dan juga strategik untuk meningkatkan status sosio-ekonomi terutamanya penduduk luar bandar dan juga untuk memungkinkan pengkomersialan kambing. Oleh itu, dengan menggunakan bantuan teknik pembiakan (ART) melalui sel somatik pemindahan nuklear intra- dan interspesies (SCNT) untuk menghasilkan klon embrio dan seterusnya anak klon hidup untuk menggandakan populasi kambing pada kadar yang pesat. Tujuan utama kajian ini adalah untuk menghasilkan klon embrio kaprin secara *in vitro* melalui SCNT dengan rujukan khas kepada sumber penerima sitoplast dan jenis penderma karyoplas. Secara ringkas, oosit kaprin diperolehi daripada dua sumber yang berbeza (pengambilan oosit secara laparoskopi (LOPU) dan rumah penyembelihan), sementara itu oosit lembu hanya diperolehi daripada rumah penyembelihan. Oosit yang diperolehi akan di kultur di dalam medium pematangan *in vitro* (IVM) dalam inkubator CO_2 (5%) pada suhu 38.5^{o}C selama 21 hingga 24 jam (oosit LOPU yang diperolehi) dan 24 hingga 27 jam (oosit rumah penyembelihan yang diperolehi) untuk kematangan. Selepas kematangan, kompleks oosit kumulus (COC) diditanggalkan di dalam medium hyaluronidase (0.2 %). Oosit matang disahkan dengan pengeluaran badan kutub pertama (PB1) dan dipilih untuk dienukleasi dengan mengeluarkan spindelnya menggunakan teknik laser diikuti dengan suntikan sel penderma (sel kumulus segar atau sel fibroblas telinga) ke dalam oosit telah dienukleasi dengan jayanya menggunakan teknik suntikan seluruh sel ke dalam sitoplasma (WCICI). Oosit yang telah berjaya direkonstruksi di aktifkan dengan menggunakan gabungan c*alcium ionophore* (CaI) selama 5 minit diikuti dengan *6-dimethylaminopurine* (6-DMAP) selama

4 jam yang kemudiannya dikultur ke dalam medium KSOM dalam inkubator CO2 (5%) pada cuhu 38.5oC untuk perkembangan lanjut. Pemerhatian ke atas belahan rekonstruksi embrio telah dilakukan setiap hari.

Dalam Eksperimen 1 , penilaian kesan sumber oosit kepada kuantiti dan kualiti oosit, kadar kematangan dan juga seterusnya kultur secara *in vitro* pada embrio SCNT telah dilakukan. Tiada perbezaan yang signifikan dalam peratusan kualiti oosit dalam LOPU lawan rumah penyembelihan: Gred B (36.24±2.6 vs. 33.23±2.7%, masing-masing) dan C (24.56±3.3 vs. 28,11±2.9% masing-masing), di mana perbezaan yang signifikan ditunjukkan dalam Gred A (32.46±4.4 vs. 19.80±4.4, masing-masing) dan Gred D (6.74±1.7 vs 18.86±2.0, masing-masing). Oosit diperolehi daripada LOPU menunjukkan kadar kematangan yang tertinggi diperolehi daripada Gred A (77,62±6.7%) diikuti dengan Gred B (6.63±4.3%), Gred C (39,64±7.8%) dan Gred D (21.97±8.5%), di mana Gred A dan B adalah lebih tinggi daripada Gred C dan D ($P< 0.05$). Sementara itu, oosit diperolehi daripada rumah penyembelihan tidak menunjukkan perbezaan yang ketara dalam kadar kematangan antara kumpulan-kumpulan: Gred A (46.94±7.7%) , C (63,80±7.8%) dan D (58.24±7.4%), bagaimanapun, Gred C oosit adalah lebih tinggi dengan bererti berbanding dengan Gred B (39.54±5.6%). Apabila membandingkan 2 sumber oosit mengikut gred masing-masing oosit, oosit diperolehi daripada LOPU adalah lebih tinggi ($P<0.05$) daripada oosit yang diperolehi daripada rumah penyembelihan pada kadar kematangan bagi oocsit berkualiti tinggi daripada Gred A dan B. Dalam perkembangan *in vitro* embrio selepas SCNT, terdapat perbezaan yang bererti dalam kadar belahan klon embrio daripada sumber LOPU berbanding rumah penyembelihan: 2-sel (86.84±7.0 vs, 41,04± 1.3 %, masing-masing), 4-sel (84,03±8.5 vs. 35.06±10.8%, masing-masing), 8-sel (71,12±6.8% vs 24.30±9.9%, masing-masing) dan morula (47.59±7.2 vs. 16.48±7.2%, masing-masing) .

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Dalam Eksperimen 2, kesan selang masa (36 hingga 40 jam; 66 hingga 70 jam; 71-75 jam) dari suntikan PMSG/hCG sehingga LOPU ke atas tindak balas stimulus, perolehan oosit dan kadar kematangan oosit telah disiasat. Bagi ketiga-tiga selang masa menghasilkan perbezaan yang ketara ditunjukan pada bilangan stimulasi folikel dan bilangan oosit yang diperolehi. Walau bagaimanapun, tiada perbezaan yang ketara dalam bilangan folikel yang dirangsang (kehadiran korpus luteum). Oosit yang perolehi secara rawak dikultur di dalam medium IVM untuk proses kematangan. Semasa IVM, selang masa 66 hingga 70 jam memberi kadar hidup tertinggi (95.07±2.3%) diikuti dengan selang masa 71 hingga 75 jam (92.29±2.3%) dan 36 hingga 40 jam (78,73 ± 4.1), sepadan dengan kadar kematangan oosit (75,64±3.4, 65,30±3.6 dan 53.92±2.1, masing-masing).

Dalam Eksperimen 3, kesan jenis karyoplas penderma pada kadar belahan klon embrio berhubung dgn selepas SCNT telah dilakukan. Tiada perbezaan yang ketara dalam kadar enukleasi suntikan daripada dua jenis sel kumulus segar berbanding telinga sel fibroblast (97.61±2.4 vs. 100.00% dan 95.15±3.4 vs. 94.32±4.0%, masing-masing). Kadar belahan klon embrio menggunakan jenis sel fibroblas telinga sebagai penderma karyoplas adalah lebih tinggi berbanding sel kumulus (82.76±5.1 vs. 57.17±5.6%, 75.97±7.5 vs 46.38±7.1%, 64.49±9.8 vs. 27.25±8.7% dan 50.82±10.4 vs. 15.59±7.0%, masing-masing).

Dalam Eksperimen 4, keberkesanan dua pendekatan SCNT yang berbeza, iaitu intraspesis SCNT dan interspesies SCNT kadar belahan embrio berhubung dgn kambing jantan diklon dan percubaan memindahkan embrio klon ke ibu tumpang telah dijalankan. Tidak ada perbezaan yang ketara antara intra-dan interspesies dalam kadar kematangan, masing-masing (66.08±3.8 dan 59.68±4.9, masing-masing), kadar enukleasi (98,90±1.1 dan 96.95±2.3%, masing-masing) dan kadar suntikan (94.70±2.6 dan 100%, masing-

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masing). Kadar belahan klon intraspesies embrio menunjukkan keertian yang tinggi (P<0.05) berbanding dengan klon interspesies dalam semua kes peringkat perkembangan prapenempelan: 2-sel (75.67±3.7 vs. 55.06±4.1%), 4-sel (64.86±5.4 vs. 48.58±4.5%), 8-sel (53.14±5.7 vs. 34.21±5.3%) dan morula (38.55±5.1 vs. 23.24±3.9%). Sebanyak 29 embrio klon daripada 2 pendekatan SCNT telah dipindahkan ke dalam tanduk rahim 8 penerima betina. Malangnya, tidak ada kebuntingan dikesan.

Kesimpulannya, disebabkan oleh populasi kambing yang rendah di Malaysia, kesukaran bekalan oosit kambing untuk penghasilan embrio kambing secara *in vitro* melalui ART seperti IVF, ICSI dan SCNT adalah terhad. Oleh itu, pemilihan sumber penerima sitoplas dan penderma jenis karyoplas adalah sangat penting untuk memaksimumkan keberkesanan dalam penghasilan klon embrio kambing dan berikutnya kelahiran anak klon hidup. Selain itu, pendekatan interspesies SCNT dengan menggunakan spesies yang berbeza bagi penerima sitoplas (oosit lembu) akan menjadi satu alternatif untuk penghasilan klon embrio kambing yang menghasilkan klon anak selapas dipindahkan ke ibu tumpang pada masa akan datang.

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ACKNOWLEGDEMENTS

First and foremost, my grateful to Almighty God who gave me strengths and health to finish my Master's study successfully. I would like to express my deepest gratitude to my supervisors Professor Dr. Ramli Abdullah and Professor Dr. Wan Khadijah Embong for their excellent guidance, caring, patience, and providing me with an excellent environment for doing my Masters's degree during this 2 and a half years. I appreciate them to let me experience the research on embryo cloning field and practical issues beyond the textbooks, patiently corrected my writing and findings grants for financial purpose to support my research. I am thankful for their help in laparoscopic oocyte pick-up (LOPU) surgery for caprine oocyte source prior to my cloning experiments as well as embryo transfer surgery.

I am thankful to my parents, Mr. Amri Kalie and Mrs. Darmi Sudirman and my siblings for their love and support throughout my life. I also wish to thank my fiance, Mr. Nizan Marjuki for his fully support and understanding during my study. Without their support and encouragement it would have been impossible for me to finish my research work.

I really appreciate the effort of my senior Dr. Kwong Phek Jin, Ms. Soh Hui Hui, Mrs. Goh Siew Ying, Mrs. Azieatul Ashikin Abdul Aziz, Mrs. Nor Farizah Abdul Hamid and Mrs. Siti Khadijah Idris to teach me in gaining personnel skill and knowledge in this embryo research. I am grateful for the cooperation of all ABEL members, Mr. Parani Baya, Dr. Mohammad Mijanur Rahman, Mr. Md. Rokibur Rahman, Ms. Nurin Farhana Norzaiwin and Ms. Siti Haslinda Mohd Sharif who were willingly helping me throughout

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my studies and also for being great lab mates, exchange of ideas and for being a good friends.

I also would like to thank to Mr. Mohd Nor Azman Mat Nong, Mr. Razali Jonit and other workers at the Institute of Biological Sciences (ISB) Mini Farm for their help in managing and taking care of goata before and after surgeries. Lastly, thank you to University of Malaya which provided me a research grant IPPP (PG149-2012B) for funded this project.

Sincerely,

Asdiana Amri

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LIST OF PUBLICATIONS AND PRESENTATIONS

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Asdiana, A., R. B. Abdullah and W.E Wan Khadijah. 2013. Developmental Potential of Mouse Single Blastomere Derived from Isolated 2-, 4-, and 8-Cell Embryos into Blastocyst and Inner Cell Mass (ICM) Outgrowths. *In Vitro* Cellular and Development-Animal. *(Under review).*

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University of Malaya

TABLE OF CONTENTS

ABSTRACT	i
ABSTRAK	v
ACKNOWLEDGMENTS	ix
LIST OF PUBLICATIONS AND PRESENTATIONS	xi
TABLE OF CONTENTS	xii
LIST OF TABLES	xx
LIST OF FIGURES	xxiii
LIST OF ABBREVIATIONS AND SYMBOLS	xxv
LIST OF APPENDICES	xxvi

CHAPTERS

Chapter 1

1.0 INTRODUCTION	1
1.1 BACKGROUND	1
1.2 STATEMENT OF PROBLEMS	5
1.3 JUSTIFICATION	7
1.4 APPLICATION	9
1.5 OBJECTIVES	11

Chapter 2

2.0 REVIEW OF LITERATURE	12
2.1 BACKGROUND	12

University of Malaya

2.2 TYPES OF CLONING 15
2.2.1 Reproductive Cloning 15
2.2.1.1 Intraspecies SCNT 15
2.2.1.2 Interspecies SCNT 18
2.3 SUPERSTIMULATION 21
2.4 SOURCE OF OOCYTES 22
2.4.1 Laparoscopic Oocyte Pick-up (LOPU) 22
2.4.2 Abattoir 23
2.5 FACTORS AFFECTING SOMATIC CELL NUCLEAR TRANSFER 24
2.5.1 Technical Skill on Micromanipulation of Oocyte 24
2.5.2 *In Vitro* Maturation 24
2.5.3 Enucleation 26
2.5.4 Donor Karyoplast 27
2.5.5 Injection of Donor Karyoplast 28
2.5.6 Activation 29
2.5.7 *In vitro* Embryo Culture 32
2.5.8 Nuclear Reprogramming 34
2.5.9 DNA methylation 35
2.5.10 Epigenetic Modification 36
2.5.10.1 Genomic imprinting 37
2.5.10.2 X-chromosome inactivation 39

University of Malaya

Chapter 3

3.0 MATERIALS AND METHODS 41

3.1 INTRODUCTION 41

3.2 MATERIALS 41

3.2.1 Experimental Animals 42

3.2.2 Facilities 42

3.2.3 Equipment 42

3.2.4 Lab-wares 43

3.2.5 Chemical, Reagent and Medium 43

3.3 METHODOLOGY 43

3.3.1 Cleanliness and sterilisation of research laboratory 44

3.3.2 Preparation of Medium 45

3.3.2.1 Preparation of normal saline solution 45

3.3.2.2 Preparation of ovary collecting medium 46

3.3.2.3 Preparation of phosphate buffer saline without calcium chloride (Ca^{2+}) and magnesium chloride (Mg^{2+}), PBS (-) 47

3.3.2.4 Preparation of penicillin G sodium salt and streptomycin sulphate salt stock solution [100x] 48

3.3.2.5 Preparation of oocyte retrieval medium 48

3.3.2.6 Heat-inactivation foetal bovine serum, FBS 49

3.3.2.7 Preparation of *in vitro* maturation (IVM) medium 50

3.3.2.8 Preparation of oocytes manipulation medium 52

3.3.2.8 (a) Preparation of TL-Hepes medium 52

University of Malaya

(i) Preparation of TL-Hepes stock medium 52

(ii) Preparation of TL-Hepes working medium 53

3.3.2.8 (b) Preparation of hyaluronidase solution 54

3.3.2.8 (c) Preparation of incubated-oocyte holding medium 55

3.3.2.8 (d) Preparation of cytochalasin B, CB 55

(i) Preparation of CB stock solution 55

(ii) Preparation of CB working solution 56

3.3.2.8 (e) Preparation of activation medium 57

(i) Preparation of calcium ionophore, CaI solution 57

(i.a) preparation of CaI stock solution 57

(i.b) preparation of CaI working solution 58

(ii) Preparation of 6-dimetylaminopurine, (6-DMAP) 58

(ii.a) preparation of 6-DMAP stock solution 58

(ii.b) Preparation of 6-DMAP working solution 59

3.3.2.8 (f) Preparation of potassium simplex optimisation medium with amino acid, KSOMaa 59

***(i)** Preparation of KSOMaa stock medium* 59

(ii) Preparation of KSOMaa working medium 60

3.3.2.9 Preparation of medium for establishment of ear fibroblast cell (EFC) 62

3.3.2.9 (a) Preparation of 0.25% Trypsin EDTA 62

3.3.2.9 (b) Preparation of culture medium for ear fibroblast cell (EFC) 62

3.3.2.9 (c) Preparation of freezing medium for ear fibroblast cell (EFC) 63

3.3.3 Preparation of Microtools 64

3.3.3.1 Preparation of mouth pipette assembly 64

University of Malaya

3.3.3.2 Preparation of mouthpiece-controlled pipette 65

3.3.3.3 Preparation of microneedles for SCNT manipulation 65

3.3.3.3 (a) Preparation of holding pipette 66

3.3.3.3 (b) Preparation of biopsy needle and injection needle 67

3.3.4 General Experimental Procedure 68

3.3.4.1 Preparation of caprine donor karyoplast (ear fibroblast cell) 68

3.3.4.1 (a) Isolation of primary of EFC 69

3.3.4.1 (b) Sub-passage of EFC 69

3.3.4.1 (c) Cryopreservation of EFC 70

3.3.4.1 (d) Thawing of EFC 70

3.3.4.2 Preparation of caprine donor karyoplast (fresh cumulus cell) 71

3.3.4.3 Preparation of caprine and bovine recipient cytoplast 71

3.3.4.3 (a) Superstimulation in goat 71

3.3.4.3 (b) Goat oocyte retrieval 74

(i) Laparoscopic oocyte pick-up (LOPU) 74

(ii) Ovary slicing 76

3.3.4.3 (c) Grading of goat oocytes 77

3.3.4.3 (d) *In vitro* maturation (IVM) 77

3.3.4.3 (e) Assessment of matured oocyte 78

3.3.4.4 Intra- and interspecies somatic cell nuclear transfer (SCNT) procedure 78

3.3.4.4 (a) Preparation of micromanipulation dish 78

3.3.4.4 (b) Alignment of microtools 80

3.3.4.4 (c) Enucleation (laser shoot technique) 81

University of Malaya

3.3.4.4 (d) Donor cell injection (WCICI) 82

3.3.4.4 (e) Chemical activation 83

3.3.4.4 (f) IVC 83

3.3.4.4 (g) Embryo transfer 83

3.3 EXPERIMENTAL DESIGN 84

3.4.1 Effect of Different Sources (LOPU Versus Abattoir) of the Oocytes on Oocyte Yield, Oocyte Grade and Maturation Rate (Experiment 1) 84

3.4.2 Effect of Time Interval from Hormonal Treatment to Laparoscopic Oocyte Pick-Up (LOPU) on Goat Stimulation Response, Oocyte Recovery and Maturation Rate (Experiment 2) 86

3.4.3 Production of In Vitro Cloned Embryos Through Intra- and Interspecies Somatic Cell Nuclear Transfer Using Ear Fibroblast Cell as Karyoplast (Experiment 3) 88

3.4.4 Effect of Different Donor Karyoplasts (Cumulus Cell versus Ear Fibroblast Cell) on Cleavage Rate of Cloned Caprine Embryo (Experiment 4) 90

Chapter 4 92

4.0 RESULTS 92

4.1 EFFECT OF OOCYTE SOURCES (LOPU VERSUS ABATTOIR) ON OOCYTE YIELD, OOCYTE QUALITY, MATURATION RATE AND SUBSEQUENT SCNT PERFORMANCE (EXPERIMENT 1) 92

4.1.1 Effect of Two Different Oocyte Sources (LOPU versus Abattoir) on Distribution of Oocyte Quality 92

4.1.1 Effect of Two Different Sources (LOPU versus Abattoir) on Maturation Rate According to the Quality of Oocytes 93

4.1.3 Cleavage Rate of Cloned Embryos from Two Different Oocyte Sources (LOPU versus Abattoir) 94

4.2 EFFECT OF TIME INTERVAL FROM HORMONAL TREATMENT TO LAPAROSCOPIC OOCYTE PICK-UP (LOPU) ON CAPRINE STIMULATION RESPONSE, OOCYTE RECOVERY AND MATURATION RATE (EXPERIMENT 2) 104

University of Malaya

4.2.1 Effect of Time Interval from PMSG/ hCG treatment to LOPU on Stimulated Follicle, Ovulated Follicle and Oocyte Retrieval Rate 104

4.2.2 Effect of Time Interval from PMSG/ hCG treatment to LOPU on Distribution of Oocyte Quality, Survival Rate and Maturation Rate 105

4.3 EFFECT OF DIFFERENT TYPES OF DONOR KARYOPLAST (CUMULUS CELL VS. EAR FIBROBLAST CELL) ON PRODUCTION OF CLONED EMBRYOS (EXPERIMENT 3) 112

4.3.1 Effect of Different Types of Donor Karyoplast on the Manipulation Efficiency for the Production of Intraspecies SCNT Cloned Caprine Embryos 112

4.3.2 Effect of Different Types of Donor Karyoplast on the Cleavage Rate of Cloned Embryos Following Intraspecies SCNT 113

4.4 PRODUCTION OF CLONED EMBRYO THROUGH INTRA- AND INTERSPECIES SCNT APPROACHES USING CAPRINE EAR FIBROBLAST CELL (EXPERIMENT 4) 117

4.4.1 Efficacy of Producing Cloned Caprine Embryos using Intraspecies versus Interspecies SCNT 117

Chapter 5

5.0 DISCUSSION

5.1 EFFECT OF OOCYTE SOURCES (LOPU VERSUS ABATTOIR) ON OOCYTE YIELD, OOCYTE QUALITY, MATURATION RATE AND SUBSEQUENT SCNT PERFORMANCE (EXPERIMENT 1) 128

5.1.1 Effect of Two Different Oocyte Sources (LOPU versus Abattoir) on Distribution of Oocyte Quality 128

5.1.2 Effect of Two Different Sources (LOPU versus Abattoir) on Maturation Rate According to the Quality of Oocytes 131

5.1.3 Cleavage Rate of Cloned Embryos from Two Different Oocyte Sources (LOPU versus Abattoir) 134

5.2 EFFECT OF TIME INTERVAL FROM HORMONAL TREATMENT TO LAPAROSCOPIC OOCYTE PICK-UP (LOPU) ON CAPRINE STIMULATION RESPONSE, OOCYTE RECOVERY AND MATURATION RATE (EXPERIMENT 2) 136

University of Malaya

5.3 EFFECT OF DIFFERENT TYPES OF DONOR KARYOPLAST (CUMULUS CELL VS. EAR FIBROBLAST CELL) ON PRODUCTION OF CLONED EMBRYOS (EXPERIMENT 3) 139

5.4 PRODUCTION OF CLONED EMBRYO THROUGH INTRA- AND INTERSPECIES SCNT APPROACHES USING CAPRINE EAR FIBROBLAST CELL (EXPERIMENT 4) 142

5.5 GENERAL DISCUSSION 145

5.1.1 Source of Oocyte (Recipient Cytoplast) 146

5.1.2 Donor Karyoplast Types 150

5.1.3 Summary of the Significant Findings 151

5.1.4 Constraint of Studies and Future Direction 152

Chapter 6

6.0 CONCLUSIONS 156

REFERENCES 157

APPENDICES

Appendix 1: LIST OF MATERIALS 184

Appendix 2: STATISTICAL DATA 189

Appendix 3: LIST OF PUBLICATIONS AND PRESENTATIONS

University of Malaya

LIST OF TABLES

Table		Page
2.1	Timeline of significant findings of successful cloning in various species	14
2.2	Timeline of significant findings of successful intraspecies cloning in caprine	16
2.3	Recent and relevant iSCNT with special references to species and types of dnor cell (Adapted from Sebastian and José, 2013)	19
3.1	Composition of ovary collection medium	46
3.2	Composition of phosphate buffer saline without calcium chloride (Ca2+) and magnesium chloride ($Mg^{2+)}$, PBS (-)	47
3.3	Composition of ovary collection medium	48
3.4	Composition of penicillin G sodium salt and streptomycin sulphate salt stock solution [100x]	49
3.5	Preparation of stock solution for IVM medium	50
3.6	Composition of IVM medium	51
3.7	Composition of TL-Hepes stock medium	53
3.8	Composition of TL-Hepes working medium	54
3.9	Composition of hyaluronidase solution	54
3.10	Composition of hyaluronidase solution	55
3.11	Composition of CB stock solution	56

University of Malaya

3.12 Composition of CB working solution solution 56

3.13 Composition of CaI stock solution 57

3.14 Preparation of CaI working solution 58

3.15 Composition of 6-DMAP stock solution 58

3.16 Composition of 6-DMAP working solution 59

3.17 Composition of KSOMaa stock medium 60

3.18 Composition of KSOMaa A working medium 61

3.19 Composition of KSOMaa B working medium 61

3.20 Composition of 0.25% trypsin EDTA 62

3.21 Composition of culture medium for EFC 63

3.22 Composition of freezing medium for EFC 63

3.23 Charaterisation of COC (According to Rahman *et al.,* 2009) 77

4.1 Distribution (%; mean±SEM) of oocyte quality obtained from different sources 95

4.2 Number (mean±SEM) of oocyte per ovary for different grades of oocytes obtained from different source 96

4.3 Maturation rate of oocyte according to its grade obtained from different sources 98

4.4 Number (%; mean±SEM) of matured oocyte quality obtained from different source 99

University of Malaya

4.5	Maturation rate, enucleation rate and injection rate of oocyte from different oocyte sources	101
4.6	Cleavage rate of reconstructed oocyte after SCNT from different sources	102
4.7	Stimulated follicle, ovulated follicle and oocyte recovery (mean±SEM) for different time intervals from PMSG/ hCG treatment to LOPU	106
4.8	Distribution of oocyte quantity and quality retrieved from LOPU with different time interval of PMSG/ hCG treatment to LOPU	108
4.9	Survival rate and maturation rate of goat oocyte according to the time interval from PMSG/hCG to LOPU	109
4.10	Maturation rate, enucleation rate and injection rate of oocytes using different types of donor karyoplast	114
4.11	Cleavage rate of reconstructed oocytes using different types of donor karyoplast	115
4.12	Cleavage rate of intraspecies SCNT reconstructed oocyte and caprine parthenotes embryo	120
4.13	Cleavage rate of interspecies SCNT, caprine and bovine parthenotes embryo	121
4.14	Percent of maturation, reconstruction oocyte and in vitro development of intraspecies and interspecies SCNT reconstructed oocyte using goat ear fibroblast cell	122
4.15	Percent of maturation, reconstruction oocyte and in vitro development of intra- and inter-SCNT reconstructed oocyte using goat ear fibroblast cell	123
4.16	Embryo transfer of intra- and interspecies cloned embryos	125

University of Malaya

LIST OF FIGURES

Figure		Page
3.1	Mouthpiece. It consists of mouthpiece, silicone tube, syringe filter and Pasteur pipette.	64
3.2	Pulling Pasteur pipette. The pipette was pulled over a blue flame part of the Bunsen burner.	65
3.3	Micropuller.	66
3.4	Microforge.	66
3.5	Preparation of holding needle.	67
3.6	Preparation of biopsy and injection needle.	68
3.7	Oestrus synchronisation and hormonal stimulation.	73
3.8	Preparation of surgical set on surgery table before LOPU.	74
3.9	Procedures of LOPU.	75
3.10	Ovarian slicing. The ovary was clamped using a haemostat and sliced using a razor blade.	76
3.11	Enucleation dish.	79
3.12	Injection dish.	79
3.13	Alignment of microneedles on the micromanipulator.	80
3.14	Enucleation technique.	81

University of Malaya

3.15	Injection of donor karyoplast through WCICI technique.	82
3.16	Flow chart of methodology of Experiment 1.	85
3.17	Flow chart of methodology of Experiment 2.	87
3.18	Flow chart of methodology of Experiment 3.	89
3.19	Flow chart of methodology of Experiment 4.	91
4.1	Comparison between two different sources of oocytes on distribution of oocytes quality.	97
4.2	Comparison between two different sources of oocytes on maturation rate after *in vitro* maturation.	100
4.3	Comparison between two different sources of oocytes on developmental rate after intraspecies SCNT.	103
4.4	Comparison between three different time intervals on stimulated follicles and number of oocytes retrieved.	107
4.5	Comparison between three different time intervals on distribution of oocyte quality.	110
4.6	Different grades of oocytes and matured oocytes.	111
4.7	Comparison between two different types of donor karyoplast on cleavage rate of cloned caprine embryos.	116
4.8	Comparison between two approaches of SCNT technique on cleavage of cloned caprine embryo.	124
4.9	Cleavage of caprine cloned embryos at different stage.	126
4.10	*In vitro* developmental of caprine parthenotes embryos at different stage.	127

University of Malaya

LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
^{o}C	degree Celcius
µl	microlitre
µm	micrometer
mm	millimeter
cm	centimeter
mg	milligram
l	litre
ml	millilitre
g	gramme
mOsm	millimosmole
O_2	oxygen
CO_2	carbon dioxide
pH	hydrogen potential
w/v	weight/volume
v/v	volume/volume
kV	Kilovolt
6-DMAP	6-dimethylaminopurine

University of Malaya

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
CR1	Charles Rosenkrans medium
DC	direct current
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffer saline
EFC	ear fibroblast cell
EMiL	Embryo Micromanipulation Laboratory
ET	Embryo transfer
FBS	foetal bovine serum
FCS	foetal calf serum
FSH	follicular stimulating hormone
GAHP	good animal husbandry practices

University of Malaya

GV	Germinal vesicle
GVBD	germinal vesicle breakdown
hCG	Human Chorionic Gonadotrophin
ID	internal diameter
interSCNT	interspecies somatic cell nuclear transfer
intraSCNT	intraspecies somatic cell nuclear transfer
IO	ionomycin
iSCNT	interspecies
IVC	*in vitro* culture
IVEP	*in vitro* embryo production
IVM	*in vitro* maturation
IVP	*in vitro* production
KSOM	potassium simplex optimization medium
LH	Luteinizing hormone
LOPU	laparoscopic oocyte pick-up
MAP	mitogen-activated protein
MII	metaphase II
MPF	maturation promoting factor
SOF	synthetic oviductal fluid medium
NaTuRe	Nuclear Transfer and Reprogramming Laboratory

University of Malaya

NEBD	breakdown of the nuclear envelope
OGS	oestrus goat serum
OR	Oocyte recovery
PA	parthenogenetic activation
PB1	first polar body
PBII	second polar body
PCC	premature chromatin condensation
PIP2	phosphatidylinostitol (4,5)-bisphosphate
PLCζ	phospholipase C zeta
PMSG	Pregnant mare's serum gonadotrophin
PVP	polyvinylpyrrolidone
RO	reverse osmosis
ROS	reactive oxygen species
SCNT	somatic cell nuclear transfer
SUZI	sub-zonal injection
WCICI	whole cell intracytoplasmic injection
XCI	X chromosome inactivation

University of Malaya

LIST OF APPENDIX TABLES

Appendix table		Page
1.1	List of equipment/instrument	184
1.2	List of chemical, reagents and media	186
1.3	List of lab-wares and disposable	188

University of Malaya

Chapter 1

1.0 INTRODUCTION

University of Malaya

Chapter 1

1.0 INTRODUCTION

1.1 BACKGROUND

Population of goat in Malaysia was 537,000 (FAO Statistics Division 2012) which is insufficient to support the local goat meat consumption. Therefore, 95% of goat meat was imported annually from other countries such as Australia and New Zealand. In other words, approximately 10 million of goats are required to be slaughtered per year to meet the local consumption of Malaysian populations (per capita consumption 0.5 kg per person per year). Due to low population of goats and high demand of products from goat such as meat, milk and manure, production of goats for food security and safety is imminent and strategic in order to increase the sosio-economic status particularly the rural population as well as for the advanced goat commercialisation. This is mainline with Malaysian government policy to make agriculture as the third engine of economic growth besides manufacturing and services sectors which also give impact of economic growth in Malaysia.

In Malaysia, all stake-holders including government, agency, research institution, private sector, and small-holder have to conduct SWOT analysis before implementing the goat production programme in the country. Some of the pertinent constraints to be taken into consideration are lack of appropriate technologies, improper breeding programme, poor management practices, poor nutrition, lack of marketing mechanism, non-commercialisation and small scale production system. For example, there is no goat breed that is locally adapted to

University of Malaya

Malaysian environment suitable for the sustainable goat industry. Therefore, it is imperative to establish goat breeds through research and development that are genitically superior in meat and milk traits. This can be achieved through systematic goat breeding using advanced reproductive technologies (ARTs) such as *in vitro* fertilisation (IVF), croyopreservation, intractoplasmic sperm injection (ICSI), somatic cell nuclear transfer (SCNT), artificial insemination (AI) and embryo transfer (ET).

Somatic cell nuclear transfer (SCNT) and parthenogenetic activation (PA) are parts of ART that can be used in goat breeding as an attempt to produce superior goat offspring at a rapid rate without involving sperm or paternal genome. In reproductive cloning, briefly, SCNT is a laboratory technique for creating a cloned embryo with inserting a donor nucleus (donor karyoplast) after removing the genetic material (enucleation) of an oocyte (recipient cytoplast). Partheno-activation is a form of asexual reproduction where growth and development of embryos occur without fertilisation but with help of electrical or chemical stimulus.

A mammal created by parthenogenesis would have double doses of maternally imprinted genes and lack paternally imprinted genes, leading to developmental abnormalities. It has been suggested that defects in placental folding or interdigitation are one cause of parthenote abortive development. Hagemann *et al.* (1998) reported that parthenogenetic sheep embryos are only capable to survive until Day 21. This is proving that both sets of chromosome from maternal and paternal genomes are required for successful to full-term development. Therefore, without genetic modification, the parthenogenetic embryos were unsuccessful to develop full term due to the failure to develop trophectoderm and endoderm-extraembryonic tissues.

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In order to propagate the number of goats using SCNT, there are two approaches were used in this study: intraspecies SCNT (using recipient cytoplast and donor karyoplast from same species) and interspecies SCNT (using recipient cytoplast and donor karyoplast from different species). Failure to reprogramme the donor genome is thought to be a main reason for the low efficiency of cloning (Rideout *et al.,* 2001), (Dean *et al.,* 2001) and (Mann and Bartolomei, 2002). Various strategies have been employed to improve the success rate of SCNT. Most of these focus on the donor cell, including: a) cell type or tissue of origin, b) passage number, c) cell cycle stage and d) use of chemical agents and cellular extracts to modify the donor cell's epigenetic state . The influence of various oocyte enucleation, fusion and activation methods on cloning efficiency has also been analysed to a lesser extent. Besides, inefficient of gene printing results in a limited success rate in developmental, abnormalities and genetic disease presence in cloned offspring.

In our laboratory, we were actively conducting various research activities in various goat reproductive techniques for the past 30 years, however, for SCNT research in goat were only started in year 2008. Therefore, there are still many issues to be solved to get better results in SCNT performance. For example, lack of source of goat ovaries in Malaysia which is due to low goat population and few numbers of female were slaughtered in local abattoir due to restriction of Malaysian law to prevent the slaughter of female goats. Consequently, insufficient number of oocytes was obtained to conduct various experiments in reproductive techniques.

Up to date, various species have been successfully cloned intraspecies in various laboratories over the world such as sheep (Wilmut *et al.,*1997*,* cow (Kato *et al.,*1997), pig (Polejaeva *et al.,* 2000), rabbit (Chesne *et al.,* 2002), monkey (Meng *et al.,* 1997) and goat

University of Malaya

(Baguisi *et al.,* 1999). However, for interspecies SCNT are mostly successful by cloned the closely related species (Behyan *et al.,* 2007) such as gaur-cattle (Lanza *et al.,* 2000), mouflon-sheep (Loi *et al.,* 2001) and African wild cat-domestic cat (Gomez *et al.,* 2004). Even though our research group obtained encouraging results in producing cloned embryos using both SCNT approach, there still have problems in producing live cloned goats in our laboratory.

Prior to SCNT, there are many factors could affect the developmental efficiency of cloned embryos, such as oocytes harvesting technique (Wani *et al.,* 2000), quality of oocytes, *in vitro* maturation of oocytes and type of donor karyoplast (Srirattana *et al.*, 2010). Since then, to surmount this problems, there is still a wide scope of research needs to be conducted from source of collected oocyte, oocyte retrieval from stimulated does, oocyte maturation, preparation of different type of donor karyoplast up to cloned embryos transfer into the recipient does to improve the technical and elucidate the biological factors influencing the success rate in SCNT technique.

University of Malaya

1.2 STATEMENT OF PROBLEMS

Somatic cell nuclear transfer (SCNT) technique has a complex protocol which involved a series of steps to be carried out. These include the preparation of somatic cell (donor karyoplast), retrieval of oocytes, enucleation of oocytes (recipient cytoplast), injection or fusion of somatic cell into enucleated oocyte, oocyte activation and culture method for further development of reconstructed oocytes. This complex protocol could affect the efficiency of getting cloned caprine embryo as well as live and health offspring. Many researchers conducted various experiments in SCNT as an effort to improve and solve the technical constraints relating to a various factors influencing the efficiency of cloned embryo production. Many successful finding were reported in the literature, however, caprine somatic cell nuclear transfer (SCNT) efficiency is still lower compared to other domestic animal species such as bovine, ovine and porcine.

Low developmental competence of caprine oocytes after SCNT has been reported in the literature. For example, Baldassarre *et al.,* (1995) reported that shortening of the gonadotrophin stimulation-follicular aspiration interval (24 to 48 hours) had no effect on the quantity of oocyte retrieved as well as the developmental rate after *in vitro* embryo production (IVEP) technique. In contrast, Abdullah *et al.* (2008) suggested 60 to 72 hours of prolonged interval from hormonal treatment to LOPU could improve oocyte retrieval rate and oocyte quality for IVM and embryo production. However, there is still lack of information of time interval from hormonal treatment prior to LOPU in order to recover large number of oocytes. Hence, this study was conducted to investigate the appropriate interval time to perform LOPU after hormonal treatment in obtaining large number of oocytes as well as to maximise the usage of recovered oocytes for SCNT experiment which could increase the developmental of cloned embryos.

University of Malaya

Among the somatic cell types tested, various laboratories reported that cumulus cells give the highest efficiency in SCNT with the least number of abnormalities in cloned animals (Tian *et al.* 2003). Cumulus cell is the first type of nuclear donors used for somatic cloning in mice (Wakayama *et al.,* 1998) and cattle (Wells *et al.,* 1999). In rabbits, producing live cloned offspring could be possible from freshly collected cumulus cells (Chesne *et al.,* 2002), however, Yang (2005) observed that the developmental rate of using fibroblast cells was higher than cumulus cells. In goat, Daniel *et al.* (2008) obtained better SCNT performance by using cumulus cell as donor karyoplast. Therefore, an appropriate choice of donor nuclei is the question of primary importance on the development competence of cloned embryos.

There are several issues still remain to be answered in this study. Some of these questions are listed as described below:

a) What is the effect of time interval from PMSG/hCG treatment to laparoscopic oocyte pick-up (LOPU) on the stimulation response and oocyte recovery rate?
b) Do the sources (LOPU versus abattoir) of caprine oocytes affect the oocyte quality, maturation rate and subsequent developmental rate of embryos?
c) What is the effect of different types of donor karyoplast (ear fibroblast cell) on intra- (caprine-caprine) and inter-species (bovine-caprine) developmental rate in goat?
d) Is the caprine ear fibroblast compatible with bovine cytoplasm after SCNT?
e) Is there any difference in cleavage rate of cloned caprine embryos using different donor cell (cumulus cell vs. ear fibroblast cell)?
f) Is there any difference in obtaining pregnancy from intra- and inter-species SCNT embryos?

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1.3 JUSTIFICATION

In Malaysia, goat is a "goodwill" animal, in which it is an important source of animal protein for human food in the form of milk, meat and their bi-products since they can be used by the multi-ethnicity as well as religion, culture and diversity of Malaysian population. In addition, goat manure is gaining popularity to be used as organic fertiliser which indirectly gives benefits to supplement the income of the farmers and to sustain the ecological balance in livestock industry. Malaysian government gives a high priority to expand the goat farming via transfer of appropriate technologies to the farmers such as artificial insemination and embryo transfer in order to increase rapidly the genetically superior goat population for commercialisation in the country.

Scientists at the University of Malaya have been embarking on goat reproductive biotechnology research since 1980s. Some of the techniques established for the caprine include sperm freezing, artificial insemination, oestrus synchronisation, superovulation, laparoscopic oocytes pick-up (LOPU), *in vitro* maturation, fertilisation, intracytoplasmic injection, cryopreservation of oocytes, zygotes and embryos, embryo transfer, radioimmunoassay, somatic cell nuclear transfer (SCNT) and stem cell studies.

In this study, the oocytes were retrieved from LOPU and slaughtered goat ovaries obtained from local abattoir. Due to low goat population in the country and low number of female goat slaughtered at the abattoir, LOPU was carried out repeatedly as proposed by Baldassarre *et al*. (2003) even though the cost much higher. Therefore, to increase the recovery rate and maturation rate of oocytes retrieved from LOPU, the author do the oestrus synchronisation and study the time interval to perform the LOPU after giving hormonal

University of Malaya

treatment to the goat in order to obtain a large number of oocytes for various experiments in cloning.

In our laboratory or even in Malaysia, both intra- and interspecies SCNT was successfully developed since year 2008 using different types of donor karyoplast such as cumulus cell, ear fibroblast cell (EFC) and foetal fibroblast cell (FFC). However, the developmental rate of cloned embryos is still low. Therefore, the objective of present study was to optimise the technique of SCNT consequently improved the production of cloned embryos and offspring through embryo transfer.

University of Malaya

1.4 APPLICATION

In animal breeding, somatic cell nuclear transfer (SCNT) or cloning had been emerged as a new technology to generate multiple copies of genetically elite farm animals consequently, produce transgenic animals for pharmaceutical protein production or xeno-transplantation and to preserve the endangered species. There are many significant improvements have been made in SCNT protocols in the past years which now allow to embarking on practical applications. Efficient transgenic animal production through SCNT can provide numerous opportunities for agriculture and biomedicine industry. Regulatory agencies around the world have agreed that food derived from cloned animals and their offspring is safe and there is no scientific basis (Tian *et al.,* 2003). The main areas of application of SCNT are: reproductive cloning, therapeutic cloning and basic research.

In order to increase the number of goat population in Malaysia, SCNT is an efficient technique to be applied. However, due to difficulties of getting goat oocytes from abattoir, inter-SCNT is new approaches which use bovine oocyte as recipient oocyte (cytoplast). SCNT cloning technique was very important for genetic diversity conservation and utilisation of indigenous local breeds. For example, Katjang goat which is highly resistance to the regional diseases and more tolerance with local climate could be produced using SCNT in which the population of Katjang goats could be increase at a rapid rate. Besides, sustaining the local indigenous livestock breeds, another application is in the field of agriculture whereby the number of imported goats for slaughter could be reduced significantly. Thus, these will help in the import-export balance in the country as well as food security and safety.

University of Malaya

In essence, SCNT could play a vital role in socio-economic development of Malaysia to ensure sustainability of agriculture for food security and safety including livestock production using SCNT technology, improved goat breeds of high genetic merits for meat and milk production could be produced in large numbers as well as at a rapid rate. As SCNT technologies developing steadily, it is evident that it can transform from animal farming to animal pharming in the future especially using embryonic stem cells for production of various cell lines for the production of proteins, cells, tissues and organs for the treatment of degenerative disease such as Parkinson's disease, Alzheimer's disease and heart disease.

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1.5 OBJECTIVES

The objectives of this study were:

a) To compare the effect of LOPU and abattoir sources of the oocytes on oocyte yield, oocyte grade and maturation rate
b) To investigate the effect of the time interval from hormonal treatment to laparoscopic oocyte pick-up (LOPU) on the goat stimulation response and oocyte recovery
c) To produce cloned embryos *in vitro* through intra- and interspecies somatic cell nuclear transfer using ear fibroblast cell as donor karyoplast
d) To evaluate the effect of different donor karyoplast (cumulus cell versus ear fibroblast cell) on developmental rate of cloned embryos
e) To obtain produce intra- and inter-species cloned caprine embryos after embryo transfer through oviduct and uterus embryo transfer technique

University of Malaya

Chapter 2

2.0 REVIEW OF LITERATURE

University of Malaya

Chapter 2

2.0 REVIEW OF LITERATURE

2.1 BACKGROUND

Since the birth of Dolly in 1997 through somatic cell nuclear transfer (SCNT) technique (Wilmut *et al.,*, 1997), the animal cloning procedure was soon extended successfully to mice (Wakayama *et al*., 1998) and had been accomplished in various other species including farm animals such as cattle (Kato *et al.,* 1998), pigs (Polejaeva *et al.,* 2000), sheep (Wilmut *et al.,* 1997), horses (Galli *et al.,* 2003), and goats (Baguisi *et al.* 1999). The application of SCNT technique in farm animals is gaining popularity to produce cloned offspring which have identical genetics to the cloned animals. In addition, SCNT technique has a marked impact on the pharmaceutical industry, especially in regenerative medicine to treat chronic diseases such as cardiovascular, Alzheimer and Parkinson diseases.

In Malaysia, the consumption of goat meat and its dairy products is increased annually. However, genetically improved goat breeds for industry as well as for food security and safety are currently not available in the country. Therefore, in order to alleviate this problem, it is important to incorporate modern animal technologies such as assisted reproductive technologies (ART) into good animal husbandry practices (GAHP) to produce superior goat breeds. One way to achieve this is through reproductive cloning whereby the goat population can be multiplied in a short time frame (Abdullah *et al.,* 2011).

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Female goats are prohibited from slaughtering due to low goat population in Malaysia. Therefore, it was a constraint to obtain oocytes from abattoir ovaries to be used in reproductive cloning research in this country. One alternative was to obtain caprine oocytes (as recipient cytoplast) from laparoscopic oocyte pick-up (LOPU) for intraSCNT procedure. Bovine oocytes (as recipient cytoplast) were obtained from abattoir cattle ovaries as they were more available than the goat ovaries for interSCNT procedure.

In 2008, our research team have successfully developed the SCNT technique and obtained caprine cloned embryos from intraSCNT (caprine oocyte as recipient cytoplast) and interSCNT (bovine oocyte as recipient cytoplast) (Kwong *et al.,* 2011; Soh, 2012; Goh, 2012). However, it is still difficult to produce viable cloned goat offspring in Malaysia. Therefore, in order to facilitate the increase of goat population for viable, profitable and sustainable in goat industry, SCNT technical constraints must be overcome as well as appropriate goat management ecosystem must be established.

After the first cloned in mammals in 1997 (Wilmut *et al.,* 1997), reproductive cloning has continued to advance whereby nearly 20 different mammalian species have been successfully cloned. Specific in goat, Baguisi *et al.* (1999) were the first to report on goat cloned offspring obtained from SCNT using somatic cell as donor karyoplast. Table 2.1 shows the timeline of species that have been cloned and developed to full-term.

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Table 2.1: Timeline of significant findings of successful cloning in various species

Year	Author	Species	Significant finding
1997	Wilmut *et al.*	Sheep (Dolly)	Sheep cloned by nuclear transfer from a cultured cell line
1997	Kato *et al.*	Cow	Eight calves cloned from somatic cells of a single adult
1998	Wakayama *et al.*	Mice	Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei
2000	Polejaeva et al.	Pig	Cloned pigs produced by nuclear transfer from adult somatic cells
2002	Chesne *et al.*	Rabbit	Cloned rabbits produced by nuclear transfer from adult somatic cells
2004	Gomez *et al.*	Wildcat	Birth of African wildcat cloned kittens born from domestic cats
2005	Lee *et al.*	Dog	Dog cloned from adult somatic cells
2006	Li *et al.*	Ferret	Cloned ferrets produced by somatic cell nuclear transfer
2008	Galli *et al.*	Horse	First somatic cell nuclear transfer in horses
2010	Wani *et al.*	Camel	Production of the first cloned camel by somatic cell nuclear transfer

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2.2 TYPES OF CLONING

Types of cloning can be divided into two classes, namely reproductive cloning and therapeutic cloning. Generally, the reproductive cloning was used to produce cloned embryos that were genetically identical to the original adult (Zavos, 2003), whereas therapeutic cloning using embryos was aimed to produce embryonic stem cell (ESC) population with special identical genetic characteristics. In other words, it was suggested that ECS was self-renewal whereby the ESC could divide and duplicate themselves for a prolonged period of time without differentiating and maintained its pluripotency to differentiate into various type of cells (Cibelli *et al.,* 2001).

2.2.1 Reproductive Cloning

A few decades ago, the areas of farm animal research using embryo technology, especially in somatic cell nuclear transfer (SCNT) and stem cell had increased significantly. In this species, SCNT was focused to optimise and improve the breeding programme in order to increase the animal population at a rapid rate. There are two SCNT approaches in reproductive cloning have been proposed, that is intra- and interspecies SCNT.

2.2.1.1 Intraspecies SCNT

Generally, intraspecies SCNT involved the recipient cytoplast and donor karyoplast of the same species. Kwong *et al.* (2012) reported the cleavage and blastocyst rates obtained from intraspecies SCNT were significantly higher than interspecies SCNT. In 1999, the first

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cloned kid was born using intraspecies SCNT; however, the overall efficiency is still low. In caprine studies, various laboratories worldwide carried out experiments mainly to improve the caprine intraspecies cloning efficiency as listed in Table 2.2.

Table 2.2: Timeline of significant findings of successful intraspecies cloning in caprine

Author	Year	Significant findings
Baguisi *et al.*	1999	First report on caprine SCNT, obtaining of 3 healthy identical female offspring.
Keefer *et al.*	2001	Both *in vitro* transfected and non-transfected caprine foetal fibroblast could develop to full-term development following nuclear transfer.
Reggio *et al.*	2001	First report of cloned-caprine produced from nuclear transfer using cytoplast derived from abattoir ovaries.
Zou *et al.*	2001	Survival rate of cloned-caprine embryos, obtained by injection, was higher than that derived from fusion (62.7 and 45.9%, respectively).
Keefer *et al.*	2002	No significant differences in rates of pregnancy and nuclear transfer efficiency between granulosa cells and foetal fibroblast cells.
Das *et al.*	2003	Reported that 3.0 resulted in better electrofusion, cytochalasin B blocked sycnhronised cells and fast growing skin fibroblast cells of caprine could be used for nuclear transfer.
Baldassarre *et al.*	2007	LOPU followed by IVP is an efficient method for the 'reproductive rescue' of valuable female goats that have acquired reduced fertility primarily as a result of increased age.
Zhang *et al.*	2004	Donor cell cycle at stage G0/G1 might be efficient ways to improve developmental competence of reconstituted caprine embryos

University of Malaya

		than stage G2/M.
Melican *et al.*	2005	More offspring were produced utilising karyoplast cultures in low serum versus cycling cells grown to confluence to synchronised G0/G1 stage cells. In addition, more live offspring were produced using donor cells harvested by partial compared with complete trypsinisation.
Lan *et al.*	2006	The fusibility and *in vitro* development potential of embryos reconstructed from foetal fibroblast at passage 20-25 were significantly lower than those of embryos reconstructed from foetal fibroblast at passage 3-5, and cloning efficiency of long term cultured cells was low (0.5%).
Chen *et al.*	2007	The method of telophase II enucleation combined with whole cell intracytoplasmic injection (WCICI) could properly reprogramme somatic cells and WCICI could provide efficient and less labour-intensive protocol in Asian Yellow goat cloning.
Daniel *et al.*	2008	2.0–2.5 kV/cm resulted in optimum fusion of reconstructed oocytes. In addition, fusion efficiency and embryo development observed in cumulus cell line was significantly higher than fibroblast cell line.
Wang *et al.*	2008	Successfully developed effective method to screen transgenic donor cells and improved production efficiency of transgenic embryos.
Tao *et al.*	2009	Optimisation of SCNT protocol and proper hCG treatment led to successful production of live cloned offspring.
Dalman *et al.*	2010	The use of full confluency (100%) was suitable for cell cycle synchronisation because it arrested cells at the G0/G1 phase and also induced less apoptosis in comparison with serum starvation group.
Abdullah *et al.*	2011	Cloned caprine embryos could be produced *in*

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		vitro via both intra- and interspecies SCNT approaches in which efficacy of interspecies SCNT approach was comparable to that of intraspecies SCNT approach.
Akshey *et al.*	2011	First report of effect of treatment of donor cells with rescovitine and different activation methods on hand-made cloned embryo production in caprine
Tang *et al.*	2011	mSOF medium supplemented with 10% FBS could better support the development of cloned caprine embryos, and the blastocyst cultured in this medium could produce live cloned offspring.
Kwong *et al.*	2012	Increasing glucose in KSOMaa basal medium culture Day 2 improves *in vitro* development of cloned caprine blastocyst produced via intra- and interspecies SCNT.
Kwong *et al.*	2013	First report of using bone marrow mesenchymal cell as donor karyoplast.

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2.2.1.2 Interspecies SCNT

Interspecies SCNT (iSCNT) studies had two purposes: to elucidate the roles of interaction between the recipient cytoplast and donor karyoplast during reprogramming and subsequent embryo development; and to practically produce offspring after SCNT procedure, for specific application such as prevention of endangered wildlife species extinction and rapid improvement and multiplication of rare-valuable livestock species.

Song *et al.* (2008) reported the first attempt on production of caprine cloned interspecies embryos using enucleated bovine oocytes as recipient cytoplast and caprine

foetal fibroblast cell as the donor karyoplasts which resulted 7.9% of blastocyst production. In a separate laboratory, Ma *et al.* (2008) obtained 7.4% of blastocyst after injection of goat foetal fibroblast cell into the enucleated sheep oocyte. Recently, our research group has successfully obtained 8.5% of blastocyst of the cloned interspecies caprine-bovine using enucleated bovine as the recipient cytoplast (Kwong *et al.*, 2012[8]).

To date, interspecies (iSCNT) technique have been used in more than 50 studies in various species such as cats, dogs, mice, rabbits, sheep, cattle, goats and pigs (Beyhan *et al.,* 2007; Loi *et al.,* 2011).

Table 2.3: Recent and relevant iSCNT with special references to species and types of donor cell (Adapted from Sebastian and José, 2013)

Recipients	Species of donor	Types of donor cells	References
Cow (*Bos taurus*)	Chimpanzee (*Pan troglodytes)*	Adult fibroblasts	(Wang *et al*., 2009)
	Human (*Homo sapiens*)	Cumulus cells	(Chung *et al*., 2009)
	Human (*Homo sapiens*)	Fibroblasts	(Li *et al*., 2008)
	Rhesus monkey (*Macaca mulatta*)	Ear skin fibroblasts	(Song *et al*., 2009)
	Monkey (*Macaca fascicularis*)	Skin fibroblasts	(Lorthongpanich *et al*., 2008)
	Domestic cat (*Felis catus*)	Oviductal fibroblasts	(Thongphakdee *et al*., 2008)
	Marbled cat (*Pardofelis marmorata*)	Ear skin fibroblasts	(Imsoonthornruksa *et al*., 2011)
	Goat (*Capra hircus*)	Ear skin fibroblasts	(Tao *et al*., 2008)
	Eland antelope (*Tauro tragusoryx*)	Epithelial cells	(Nel-Themaat *et al*., 2008)
	Pig	Transgenic cells	(Uhm et al., 2007)

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	(*Sus scrofa*)		
	Sheep (*Ovis aries*)	Fetal fibroblasts	(Hua *et al*., 2008)
	Sei whale (*Balaenoptera borealis*)	Fetal fibroblasts	(Bhuiyan *et al*., 2010)
	Rhesus monkey (*Macaca mulatta*)	Ear skin fibroblasts	(Kwon *et al*., 2011)
	Goat (*Capra hircus*)	Ear skin fibroblasts	(Kwong *et al*., 2012)
	Dog (*Canis domesticus*)	Fetal fibroblasts	(Hong *et al*., 2012)
	Water buffalo (*Bubalus bubalis*)	Fibroblasts	(Srirattana *et al*., 2012)
	Gaur (*Bos gaurus*)	Somatic cells	(Srirattana *et al*., 2012)
	Mouse (*Mus spretus*)	Mitochondria	(Takeda et al., 2012)
Mouse (*Mus musculus*)	Human (*Homo sapiens*)	Cumulus cells	(Chung *et al*., 2009)
Rabbit (*Oryctolagus cuniculus*)	Human (*Homo sapiens*)	Cumulus cells	(Chung *et al*., 2009)
	Red panda (*Ailurus fulgens*)	Ear fibroblasts	(Tao *et al*., 2009)
	Human (*Homo sapiens*)	Foreskin fibroblasts	(Shi *et al*., 2008)
	Cynomologus monkey (*Macaca fascicularis*)	Fibroblasts	(Yamochi *et al*., 2012)
	Asian elephant (*Elephas maximus*)	Ear skin fibroblasts	(Sathanawongs and Jarujinda, 2010)
Pig (*Sus scrofa*)	Dog (*Canis domesticus*)	Tail and dewclaw fibroblasts	(Sugimura *et al*., 2009)
	Rat (*Rattus novergicus*)	Fetal fibroblasts and cumulus cslls	(Sugawara *et al*., 2009)
	Bovine (*Bos taurus*)	Transgenic fibroblasts	(Uhm *et al*., 2007)
	Mouse (*Mus spretus*)	Zygotes	(Amarnath *et al*., 2011)
Dog (*Canis domesticus*)	Gray wolf (*Canis lupus*)	Skin fibroblasts	(Oh *et al*., 2008)
Sheep (*Ovis aries*)	Goat (*Capra hircus*)	Fetal fibroblasts	(Ma *et al*., 2008a,b)
	Human (*Homo sapiens*)	Somatic cells	(Hosseini *et al*., 2012)
	Goat	Fetal fibroblasts	(Ma *et al*., 2011)

University of Malaya

	(*Capra hircus*)		
Goat (*Capra hircus*)	Human (*Homo sapiens*)	Neural stem cells	(Sha *et al*., 2009)
Cat (*Felis catus*)	Marbled cat (*Pardofelis marmorata*)	Ear skin fibroblasts	(Imsoonthornruksa *et al*., 2012)
	Bovine (*Bos taurus*)	Fibroblasts	(Imsoonthornruksa *et al*., 2011)
	Black-footed cat (*Felis nigripes*)	Skin fibroblasts	(Gomez *et al*., 2011)

University of Malaya

2.3 SUPERSTIMULATION

In goat superstimulation programme, gonadotrophin treatment resulted in significant variation in quantity and quality of oocytes recovered from the donor does which consequently yielded low *in vitro* embryo production (IVEP) after *in vitro* maturation (IVM), IVF and *in vitro* culture (IVC). Pregnant mare's serum gonadotrophin (PMSG) and follicular stimulating hormone (FSH) are widely used for superstimulation with the ultimate goal to maximise the number of oocytes or embryos obtained. Even though most successful superstimulation protocols are using FSH, under unaccustomed to handling the animals, superstimulation can be achieved with a single dose of PMSG compared to FSH which must be injected at 12 hr intervals for 3 or 4 consecutive days (Armstrong *et al*., 1983; Pendleton *et al*., 1992).

A long half-life of PMSG is the main disadvantage for follicular growth which maintaining stimulated and high blood oestradiol concentration after ovulation that is important in inducing the synthesis and release of prostaglandin $F_{2\alpha}$, and subsequently resulting in early luteal regression (Battye *et al*., 1988). Superstimulation using PMSG in

goats normally resulted in stimulation and subsequent ovulation, at approximately 48 and 77 hr later, respectively (Cameron *et al*., 1988). Prior to LOPU, administration of PMSG at 36 and 48 hr showed no significant difference in number of follicle and recovery rate in caprine (Baldasaree *et al*., 2002). In our previous study, a prolonged time interval of LOPU at 60 and 72 hr after FSH-hCG could improve oocytes retrieval rate, oocyte quality, maturation rate and goat embryo production (Abdullah *et al*., 2008).

2.4 SOURCE OF OOCYTES

Prior to *in vitro* embryo production (IVEP), oocytes were obtained from both stimulated follicle through laparoscopic oocyte pick-up (LOPU) and non-stimulated ovaries obtained from abattoir (Brett *et al.,* 2001).

2.4.1 Laparoscopic Oocyte Pick-up (LOPU)

LOPU is an efficient technique to be used for oocyte recovery from live animal which are medium size farm animal such as goat, sheep and pig. Moreover, oocyte recovery from LOPU has the appropriate timing of oocyte maturation as LOPU were subjected to oestrus synchronisation and ovarian stimulation (Baldassaree *et al*., 2002). In addition, LOPU gave less traumatic to the live animals which could be used for the repetition of LOPU for many times during the reproductive life of the desired and valuable genetic females (Baldassaree *et al*., 2007). Usually, LOPU resulted more than 5 oocytes aspirated from stimulated follicles per donor and less ovarian damage observed after several cycles of LOPU (Alberio

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et al., 2002; Baldassarre and Karatzas, 2004). In addition, Tervit *et al.,* (1992) reported that LOPU can be repeated at short intervals as early as 4 days.

2.4.2 Abattoir

Generally, oocytes obtained from local abattoir are the easiest method and cheapest in cost. However, in Malaysia, the number of abattoir ovaries obtained is very limited due to low number of females were slaughtered and high competition in getting ovaries with other institution. The most commonly used recover methods in sheep are slicing (Wahid *et al.,* 1992), aspiration of visible follicles (Watson *et al.,* 1994) or follicular dissection (Fukui *et al.,* 1988). Wani *et al.* (2000) demonstrated that in sheep, the puncture of whole ovarian surface is a simple and efficient method of recovering a high number of morphologically normal oocytes compared to slicing and aspiration technique.

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2.5 FACTORS AFFECTING SOMATIC CELL NUCLEAR TRANSFER

There are many factors have been suggested which could affect the developmental potency after somatic cell nuclear transfer (SCNT).

2.5.1 Technical Skill on Micromanipulation of Oocyte

SCNT has a complex procedure which involves a series of steps including: enucleation, injection of donor karyoplast, activation and *in vitro* embryo culture. Every step needs technical skill, such as preparing of microtools, handling of oocytes and handling of micromanipulator. During SCNT procedure, these technical skills are very important for the overall efficiency of the cloning outcome.

2.5.2 *In Vitro* Maturation

In vitro maturation (IVM) refers to the maturation in culture of immature oocytes after retrieval that progress from prophase I stage through meiosis I to reach metaphase II (MII). This matured state of meiosis is prerequisite before oocytes could be used as recipient cytoplast for SCNT procedure. In early prenatal life of mammals, primary oocytes enter the meiosis cycle and arrested at prophase 1 (GV) stage before the time of ovulation. In *in vivo* oocyte maturation, the meiosis cycle was resuming under the influence of hormonal stimulus (Dieleman *et al.,* 1983a; 1983b; Callesen *et al.,* 1986). The oocytes retrieved from follicle undergo maturation by further culturing them in suitable conditions (Edwards, 1965; Thibault *et al.,* 1987).

University of Malaya

The maturation of oocytes is a complex process involving the rearrangement of certain organelles and a polarisation of the cell cortex. The most evident changes in oocyte structure during the final stages of meiosis are those concerned with nuclear maturation which proceeds in a series of steps highlighted by: 1) dissolution of the nuclear envelope or germinal vesicle breakdown (GVBD), 2) chromatin condensation and chromosome formation, 3) assembly of the first meiotic spindle, 4) movement of the spindle to the cell periphery, 5) formation and extrusion of the first polar body (as asymmetric cytokinetic event), and 6) formation and positioning of the second meiotic spindle (David, 1984). Besides, during IVM, the cytoplasmic maturation causes changes in distribution and organisation of organelles at MII stage that involves the synthesis of specific protein, (Schultz and Kopf, 1995), releasing of cortical granules, releasing calcium from intracellular stores (Carroll *et al.,* 1996), thus, able to relocalise mitochondria and to condense sperm head (Thibault, 1987). After IVM, the matured oocytes were usually confirmed by the extrusion of first polar body (PBI) that could undergo nuclear and ooplasmic maturation to be used for *in vitro* embryo production (IVEP). The success of IVM procedure was very important and correlated in developmental of IVEP.

Appropriate microenvironment for IVM is needed for oocytes to undergo maturation process. There are various maturation media that have been developed in different laboratory over the world. Generally, the IVM media contained base medium (TCM-199), heat inactivated serum, pyruvate, hormones and other additional supplements (Ongeri *et al.,* 2001; Izquierdo *et al.,* 2002). Supplementation of gonadotrophin (FSH and LH) and oestradiol-17β in caprine IVM media are reported to enhance maturation rate significantly (Keskintepe *et al*., 1994; Izquierdo *et al.,* 2002), subsequently increase the blastocyst rate

University of Malaya

(Pawshe and Totey, 2003). Besides, other additional supplements in IVM media such as cysteamine or cystein and vitamins could improve the embryo development (De Matos and Furnus, 2000; Bormann *et al.,* 2003).

The time required for IVM varied in different species (Zhang *et al.,* 1995; Coonrod *et al.,* 1994). In their caprine research, the authors suggested that IVM duration requirement ranged from 16 to 32 hours (Pawshe *et al.,* 1994; Crozet *et al.,* 1995; Sharma *et al.,* 1996). The difference in maturation rate for different duration of IVM could be affected by the variation in the culture condition and source of oocytes [Kwong, (2012). Samaké *et al,* (2000)] reported 100% maturation was obtained after 24 hours of oocyte IVM duration retrieved from superstimulation and oestrus synchronisation by laparotomy and ovariectomy methods. However, oocytes retrieved from LOPU method after prolonging gonadotrophin treatment for 72 hours achieved maturation rate of 82%, following 27 hours of IVM (Abdullah *et al.,* 2008). Sharma *et al.* (1996) reported that oocytes collected from non-stimulated abattoir achieved the highest maturation rate (72%) after 32 hours of IVM. Recently, our group demonstrated oocyte obtained from LOPU achieved highest maturation rate (87%) at 21 hours, on the other hand, oocyte derived from non-stimulated ovaries abattoir showed the highest maturation rate (80%) after 24 hours of IVM (Kwong, 2012).

2.5.3 Enucleation

In SCNT procedures, the matured oocytes have to be enucleated (remove the DNA) in order to prepare the recipient cytoplast. Generally, prior to enucleation, the matured oocyte with an extrusion of first polar body (PB1) were pretreated with mycotoxin or cytochalasin B to

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destabilise the cytoskeleton for DNA removal which was washed out immediately after enucleation process. Enucleation could be carried out by aspiration or squeezing technique to remove approximately one third of ooplasm underneath the PB1 (Mc Grath *et al.,* 1983; Kwong, 2012; Soh, 2012). This mechanical enucleation could change the morphology of ooplasm, resulting in an altered metabolic activity of manipulated oocyte subsequently reducing the reconstructed oocyte developmental potential after injection of donor karyoplast into the enucleated oocyte (Greising and Jonas, 1998).

Hoechst 33342 stain (Tsunoda *et al.,* 1988) and some chemical means (Russell *et al.,* 2005) eased the enucleation process. However, these techniques were potentially harmful which could affect the reprogramming and subsequent development of the reconstructed embryos (Tatham *et al.,* 1995; Silva *et al.,* 1999).

2.5.4 Donor Karyoplast

There were multiple factors in SCNT procedure that caused the low success rate to produce cloned embryos and live offspring (Tian *et al.,* 2003). One of the important factors was the type of donor karyoplast. Briefly, prior to SCNT experiments, donor karyoplast from the animals with the desired genetic traits was firstly obtained and cultured.

In SCNT procedure, donor karyoplasts were inserted into the enucleated oocytes prior to activation and cultured for further development. In caprine SCNT, there were many types of donor karyoplasts had been successfully used, including mammary epithelia (Zhang *et al.,* 2008; Yuan *et al.,* 2009), cumulus cells (Zou *et al.,* 2001; Keefer *et al.,* 2002), foetal fibroblasts (Baguisi *et al.,* 1999; Reggio *et al.,* 2001; Keefer *et al.,* 2002; Melican *et al.,*

University of Malaya

2005; Soh *et al.,* 2012) and skin (Abdullah *et al.,* 2011; Kwong *et al.,* 2012; Wan *et al.,* 2012). Recently, Kwong *et al.* (2013) reported that SCNT in caprine, using bone-marrow mesenchymal as donor karyoplast, was successful to developed cloned embryos until hatched blastocyst stage. However, until now it is still unable to suggest which type of donor karyoplasts is the most suitable for caprine nuclear transfer procedure.

2.5.5 Injection of Donor Karyoplast

Injection of a donor karyoplast into an enucleated oocyte could only be performed following the enucleation step. Generally, there were two different techniques commonly used to insert the donor karyoplast: 1) whole cell intracytoplasmic injection (WCICI), which donor cell was injected directly into the recipient cytoplast, and 2) sub-zonal injection (SUZI), which donor karyoplast was transferred into the perivitelline space followed by electrofussion with direct current (DC) pulse.

For WCICI technique, the donor karyoplast membrane was broken by repeated pipetting in and out before can be inserted into the recipient cytoplast (Dao *et al.,* 2002) to ensure the successful fusion between recipient cytoplast and donor karyoplast. Normally, the breaking of donor karyoplast membrane was carried out by piezo-driven technique which needed a machine to provide an electrical current in order to remove the donor karyoplast membrane. Thus, as an alternative using a piezo-driven machine, repeat pipetting method could be more practical and cheaper.

Lee *et al.* (2003) suggested that WCICI method was less labour intensive without involving special equipment that could result in high porcine blastocyst rate (37%). Kawano

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et al. (2004) reported that reconstruction rate of porcine oocyte using direct injection was significantly higher than cell fusion technique (89 vs. 48%, respectively). However, blastocyst rate of the reconstructed oocyte showed less efficient result than the direct transfer cell (equivalent to WCICI; 14 vs. 18%, respectively). Additionally, the two methods have no significant difference in total cell number, inner cell mass and trophectoderm cell number in blastocyst, suggesting that either technique could be used in SCNT. In caprine, Zou *et al.* (2001) reported that injection method showed high cleavage rate than fusion method by using the cumulus cell as donor karyoplast. Soh *et al.* (2012) reported high cleavage rate and blastocyst rate was obtained in SUZI method compared to WCICI technique using foetal fibroblast cell as donor karyoplast.

2.5.6 Activation

Oocyte activation was referred to the changes that induced by fertilisation which marked with extrusion of second polar body (PBII), formation of a pronucleus and exocytosis of cortical granules. Resumption MII stage of matured oocyte was the first consequences of fertilisation which initiated further embryo development (Schultz and Kopf, 1995; Ducibella and Fissore, 2008; Ito *et a*l., 2011; Ito and Kashiwazaki, 2012).

Up to date, activation in all mammalian species required an increase in the intracellular concentration of calcium (Striker, 1999). During normal fertilisation, penetration of sperm triggered the repetitive rises of intracellular Ca^{2+} signal that most commonly referred to Ca^{2+} oscillation (Miyazaki *et al.,* 1992) which caused oocyte activation. Saunders *et al.* (2002) reported a sperm component called phospholipase C zeta

University of Malaya

(PLCζ), is responsible for these oscillations. PLCs are known to hydrolyze phosphatidylinostitol (4,5)-bisphosphate (PIP_2) into diacyl glycerol (DG) and inositol 1,4,5-trisphosphate (IP_3) (Rebechi and Pentyala, 2000; Berridge, 2002) which were powerful signaling molecules which controlled intracellular Ca^{2+} release during fertilisation process (Berridge, 2002).

In SCNT, an artificial activation was needed to initiate nuclear reprogramming and further development of embryo due the lack of sperm to induce fertilisation step since the enucleated oocytes was fused with somatic cells (Campbell, 1999; Wells *et al.,*1999). This artificial activation was designed to mimic the interaction of normal sperm-oocyte or to replicate the effect of Ca^{2+} oscillation in order to stimulate the biochemical and physiological events that exited from the MII arrest and entered the embryonic cell cycle. There were two types of kinases, namely maturation promoting factor (MPF) and mitogen-activated protein (MAP), which was important for the calcium-stimulated events to complete the meiosis process, pronuclear formation and DNA synthesis (Collas *et al.,* 1993); Velhac *et al.,* 1994; Moos *et al.,* 1996).

Briefly, chemical activation protocol relied on exposing eggs to calcium ionophore, ethanol or ionomycin to maintain the increase of intracellular concentration of Ca^{2+} to initiate the step of activation by releasing the maturing oocyte from the MII arrest (Cuthbertson, 1983; Kline and Kline, 1992). During the transition from MII stage to interphase, the long-lasting Ca^{2+} oscillation was required, and this was responsible for degradation of cyclin B (MPF regulatory component) which was required to inactivate the MPF and at the same time to exit the maturing oocyte from metaphase II phase to anaphase II and telophase II (Nixon *et al.,* 2002). It should be reminded that a single exposure to Ca^{2+}

was insufficient to complete the degradation of cyclin B, and this subsequently maintained the low level of MPF activity. Therefore, concurrent activation combination with other kinase inhibitor, such as cycloheximide (CHX) and 6-dimethylaminopurine (6-DMAP) was required for successful transition from MII into interphase stage which was important for further embryo development.

Different artificial oocyte activation protocols had been developed in different mammalian species. In mice SCNT, cloned pups could be produced from various chemical activation protocols, including $SrCl_2$, ethanol and direct current (DC) pulse (Kishigami *et al.,* 2007). Kishigami and Wakayama (2007) reported that the application of $SrCl_2$ was the most commonly used chemical in oocyte activation since their finding proved that single exposure to $SrCl_2$ could initiate activation without adding any protein synthesis or kinase inhibitor.

However, single chemical oocyte activation in livestock animals did not achieved high success rates. For example, in bovine and porcine, parthenogenetic activation using single $SrCl_2$ (without other compound combination) was low (Yamazaki *et al.,* 2005; Wang *et al.,* 2008). On the other hand, combinations of ionomycin (IO) or calcium ionophore (CaI) with 6-dimethylaminopurine (6-DMAP) gave the successful optimal results in oocyte activation protocol in the same species (Loi *et al.,* 1998; Wells *et al.,* 1999; Akagi *et al.,* 2003). Another approach to activate oocyte was to apply electrical DC pulse in presence of extracellular Ca^{2+} oscillation. Ozil (1990) and Ozil *et al.* (2005) obtained high rates of blastocyst stage by using electrical DC pulse.

University of Malaya

2.5.7 *In vitro* Embryo Culture

Vatja and Gjerris (2006) suggested that production of transgenic domestic animals from somatic cell nuclear transfer (SCNT) technique promise huge implication in agriculture and biomedical industry. *In vitro* culture of the reconstructed embryos was a critical stage for further embryo development, pregnancy subsequently producing live cloned offspring (Campbell *et al.,* 2007). The efficiency to produce embryos through *in vitro* technique was low compared to *in vivo* due to the suboptimal condition of *in vitro* culture system, where the embryos cultured outside the womb of females that could affect the morphology of embryo, embryonic growth, gene expression, postnatal growth and phenotype (Kruip and denDaas, 1997; Summer and Biggers, 2003; Fleming *et al*., 2004; Thomson *et al*., 2007).

In order to improve the efficiency of embryo production through *in vitro* technique, optimization of culture media formulation must be used to mimic the *in vivo* environment inside female womb (Summers and Biggers, 2003). Over the years, specifically in caprine, many researches had been used and improved the culture medium to culture cloned caprine embryos, including: TCM 199 (Baguisi *et al*., 1999), Charles Rosenkrans medium (CR1) (Guo *et al*., 2002; Lan *et al*., 2006; Chen *et al*., 2007), synthetic oviductal fluid (SOF) (Melican *et al*., 2005) and potassium simplex optimization medium (KSOM) (Kwong *et al.,* 2012; Wan *et al*., 2012), supplemented with serum or chemical defined compound such as: essential and non essential amino acids, glucose, glutamine, gluthathione, taurine, cystemine, β-merchaptoethanol and EDTA.

Generally, serum such as foetal bovine serum (FBS), foetal calf serum (FCS) and oestrus goat serum (OGS) had been widely added into the culture medium. Serum contain

University of Malaya

growth factor which beneficial for embryo development which could increase the developmental rate until blastocyst stage (Pinyopummintr and Bavister, 1994; Van Langendonckt *et al*., 1997). Beside the addition of serum and chemically known compound, there was another stressor that could affect the *in vitro* developmental of embryo, including: inappropriate formulation, inappropriate culture system, technical issues and lack of quality control and quality assurance (Gardner, 2004). These types of stressor could trigger a response mechanism in embryo that affect the homeostatic balance subsequently lower in the embryo developmental, pregnancy and live offspring.

Therefore in SCNT, *in vitro* culture (IVC) medium formulation, culture system and technical issue should be optimsed in order to provide a similar *in vivo* environment and subsequently enhance the developmental embryos (Dominko *et al.,* 1999; Sugimura and Sato, 2011). Lorthongpanich *et al.* (2008) suggested that supplementation of appropriate nutrients in IVC medium, energy source and growth factors were the main key factors to the successes in production of cloned embryos.

In addition, the metabolism of molecular oxygen is important in developmental of embryo (Houghton *et al*., 1996; Thompson *et al*., 1996). However, a higher concentration of oxygen during IVC could cause formation of reactive oxygen species (ROS). Johnson and Nasr-Esfahani (1994) suggested that ROS have deleterious effects on cells, including: lipid peroxidation, oxidative modification of proteins and DNA damage. Therefore, to minimise the formation of ROS in embryos and maintained the pH of IVC medium, the environment for embryo culture with low concentration of oxygen and carbon dioxide (5-8%) was needed. Most researcher had been used atmosphere CO_2 (5%) in air to culture embryos in various species, such as mice, caprine, bovine, porcine, ovine including human.

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Besides the formulation of culture medium, Gardener (1994) reported that culture system such as gas phase, embryo incubation volume and group size of embryo were integrated for the development of embryo. Embryo cultured in small volume of IVC medium could enhance embryo development subsequently getting high rates of viability after embryo transfer (Wiley *et al*., 1986; Paria and Dey, 1990; Lane and Gardner, 1992). This is due to the effect of specific embryo-derived autocrine/paracrine factor(s) that stimulate development when culture a group of embryos in small volume of IVC medium. In contrast, embryos cultured in large volume could cause dilution factor that becomes ineffectual (Gardner, 1994).

2.5.8 Nuclear Reprogramming

In somatic cell nuclear transfer (SCNT), an oocyte can be reprogrammed to enter embryonic developmental state, which described the resting (inactivate) donor cell (karyoplast) memory that could be reactivated after inserted into a recipient cytoplast (Wilmut *et al.,* 1997). The success of SCNT producing cloned offspring created from insertion of differentiated donor karyoplast with desired genetic traits into a recipient cytoplast have challenged the theory of a natural process of nuclear reprogramming. Conventionally, nuclear reprogramming was described as a process of reversing a terminal differentiated unipotent somatic nucleus (donor karyoplast) to a totipotent stage (embryo); in other words, it could be differentiated into all types of cells, tissues or organs

Even though SCNT was successful in various species including: mouse (Wakayama *et al*., 1998), cattle (Cibelli *et al*., 1998; Kato *et al*., 1998; Kubota *et al*., 2000), goat (Baguisi

University of Malaya

et al., 1999), pig (Polejaeva *et al*., 2000), cat (Shin *et al*., 2002; Yin *et al.,* 2005), rabbit (Chesne *et al*., 2002), mule (Woods *et al*., 2003) and horse (Galli *et al*., 2003), however, the success to develop full-term cloned offspring is still low (3-10%). In bovine, high rate (up to 90%) of abnormal foetal death and abortion had been observed in cloned pregnancy (Alexander *et al*., 1995; Hasler *et al*., 1995; Forar *et al*., 1996). Additionally, premature birth was frequently observed in cloned pregnancy (Taneja *et al*., 2001). It was suggested that the abnormalities of cloned offspring were due to incomplete reactivation of genes in the donor cells.

2.5.9 DNA methylation

It was generally accepted that development of *in vivo* embryos was affected by acetylation and metyhlation of genomic DNA that cause changes in configuration of chromatin. DNA methylation occurred at predominantly CpG dinucleotides affecting numerous key genomic functions (Takahashi, 2004). DNA methylation was the first step in nuclear reprogramming, and it is important to note that *Pou5f1/Oct-4* played a significant role in maintaining the pluripotentcy at early embryonic stage (Niwa *et al.,* 2000; Simonsson and Gurdon, 2004). Consequently, DNA methylation was believed to be cell fate determinant and cell type specific gene expression (Shi *et al.,* 2003).

In SCNT, a massive epigenetic reprogramming was demonstrated by reducing level of methylation in cloned embryos. Thus, gene-specific DNA methylation could affect the efficiency of cloning and *in vitro* culture which showed an aberration of pattern of global DNA methylation (Dean *et al*., 2001; Kang *et al.,* 2001). Perturbation of the normal wave of

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demethylation and remethylation at early developmental of embryo could be associated with the abnormalities in cloned animals. The maintenance of elevated methylation levels during cleavage was thought to be related due to presence of the somatic form of DNMT1 [DNA (cytosine-5)-methyltransferase 1], brought by the somatic donor cell nucleus into the cloned embryo and probably interfering with the genome-wide demethylation process that takes place in conventionally produced preimplantation embryos (Reik *et al.,* 2001).

Epigenetic reprogramming must occur within a short interval between transfer of the donor karyoplast into the recipient cytoplast and the initiation of embryonic transcription, however, whereby the timing of the said interval varied from species to species. For example in mouse, embryonic transcription begins at the 2-cell stage, in the pig at the 4-cell stage, and in sheep, cattle, and humans at the 8 to 16-cell stage (Kues *et al.,* 2008; Telford *et al.,* 1990). Niemann and Wrenzycki (2000) suggested that the deviations from normal pattern of mRNA expression were observed in preimplantation embryo and were maintained during foetal development until birth. This was due to epigenetic mechanisms which the effects of this early period of culture only manifested later in development.

2.5.10 Epigenetic Modification

Epigenetics is the study of heritable changes in gene activity that are not caused by changes in the DNA sequence; it also can be used to describe the study of stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable. Holliday (1987) suggested that epigenetics could be defined as nuclear inheritance that is not based on differences in DNA sequences. During cell differentiation, gene inactivation occurred

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due to epigenetic modification which involved changes in DNA methylation, histone acetylation, chromatin configuration as well as other mechanisms. These epigenetic modifications control precise tissue-specific expression whereby different tissues expressing different proteins while having the same genetic make-up.

The production of live cloned births was relatively low (1-10%). Most of the failures were found to be during gestation period and birth. Additionally, those cloned that survived had variety of abnormalities during their birth that was believed to be due to inappropriate epigenetic reprogramming. Genomic imprinting and X-chromosome inactivation were two main types of epigenetic modification that actively occurred in somatically cloned animals because somatic cloning bypassed the natural process of parental-specific.

2.5.10.1 Genomic imprinting

"Parental conflict hypothesis" was a hypothesis which was widely used to describe the evolution of genomic imprinting (Moore and Haig, 1991). Genomic imprinting was an epigenetic phenomenon by which certain genes can be expressed in a parent of origin specific manner. Basic feature of genomic imprinting was two paternal-maternal alleles of a given gene were expressed differentially. In early embryo development, the functional differences were present between paternal and maternal genomes. In paternal genome, it exerted its effects in the extraembryonic tissues, whereas the maternal genome influenced in the embryo developmental (McGrath and Solter, 1983; Surani *et al*., 1984).

In mammals, genomic imprinting was very important in order to maintain the embryo development, foetal growth and function of placenta (Barlow, 1995). In recent

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years, it had become apparent that embryo culture could adversely affect the faithful retention of epigenetic information during early embryogenesis which led to loss of imprinting information occurred in cloned embryos (Doherty *et al.,* 2000; Mann *et al.,* 2004; Kwong *et al.,* 2006; Watkins *et al.,* 2008; Banrezes *et al.,* 2011).

For example in mouse cloned blastocysts, it showed that there were disruptions in imprinted gene expression and methylation whereby only 4% displayed a normal blastocyst mode of expression for five imprinted genes studied. Imprinting abnormalities were also reported for clones in other species: cattle (Yang *et al.,* 2005; Suzuki *et al.,* 2009; Suzuki *et al.,* 2011), pig (Wei *et al.,* 2010) and sheep (Young *et al.,* 2003). This genomic imprinting abnormalities may contribute to abnormal placenta growth and development (Guillomot *et al.,* 2010), although some expressed imprinted genes may not be problematic (Arnold *et al.,* 2006).

In SCNT, there are many aberrant phenotypes were described in cloned foetal or offspring due to abnormalities associated with either deletion or mutation in imprinted genes or aberrant expression of genes. Current data indicate that the correct expression profiles of the IGF2 gene and other members of this gene family are critical for normal embryonic and foetal development. Some SCNT protocols may be associated with faulty reprogramming of imprinted loci, specifically of the IGF gene family (Niemann, 2014).

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2.5.10.2 X-chromosome inactivation

In mammals, sex determination mechanism was based on chromosome with the primary sex determinant, whereby the presence of XX and XY chromosomes for female and male offspring, respectively (Chang *et al.,* 2006). X chromosome consisted of approximately 3 to 5% of the genome which encoded over 1000 genes with a diverse range of functions, while Y chromosome was comparatively gene-depleted, consisting of a variable amount of DNA that encoded less than 100 genes, mainly involved in sex determination and fertility. The *SRY* gene (sex determining region Y) was considered to be the initiator of a cascade of events that led to the development of a testis. Absence of SRY resulted in a different cascade of gene expression culminating in ovarian development.

The regulation of X chromosome posed an interesting challenge in cloning protocol. In mammals, males have one copy while females have two copies of the X chromosome. This creates a situation in which there is an unequal gene dosage among males and females. During evolution, this was solved by a process termed X chromosome inactivation (XCI; Heard *et al.,* 1997; Lyon,1999), that is the random transcriptional silencing of one of the two X chromosomes in somatic cells of females during early development. XCI occurrred by the process of epigenetic modification, in which the inactivated X chromosome was hyper-methylated in DNA and hypo-acetylated on histones. Therefore, proper XCI was essential for embryonic development. Inactivation of both X chromosomes in mouse embryos could led to embryonic lethality, while having more than one active X chromosome was deleterious to extraembryonic development and also caused early embryonic death in mice (Wang *et al.,* 2001).

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In mice, although the inactive X of the donor cells could be successfully reactivated by the recipient cytoplast, there is heterogeneity within SCNT blastocysts for X-inactivation, with cells showing 0, 1 or 2 inactive X-chromosomes (Nolen *et al.,* 2005). Female SCNT-derived embryos inherited 1 active and 1 inactive X chromosome from the donor cell.

University of Malaya

Chapter 3

3.0 MATERIALS AND METHODS

University of Malaya

Chapter 3

3.0 MATERIALS AND METHODS

3.1 INTRODUCTION

The research was conducted in Animal Biotechnology-Embryo Laboratory (ABEL) and Embryo Micromanipulation Laboratory (EMiL) at Institute of Biological Sciences (ISB) as well as Nuclear Transfer and Reprogramming Laboratory (NaTuRe) at Institute of Research Management and Consultancy (IPPP), University of Malaya, Kuala Lumpur, Malaysia.

The oocytes used in this project were collected from two sources, that is ovaries from abattoir in Shah Alam, Selangor and laparoscopic oocyte pick-up (LOPU) after oestrus synchronisation and superovultation. Caprine cloned embryos were developed by using somatic cell nuclear transfer. This study was conducted from November 2011 to November 2013.

3.2 MATERIALS

The materials that were used included experimental animals, physical facilities, equipment and chemicals.

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3.2.1 Experimental Animals

Goat's breeds that were used for laparoscopic oocyte pick-up (LOPU) were Boer crossbred and local mixed-breed, and the age is 2 to 5 years old. Before the start of the surgery, the goats underwent oestrus synchronisation for superovulation procedures.

3.2.2 Facilities

The research facilities that had been used at the University of Malaya included ISB Mini Farm (source of the experimental animals; goat and cow) and 3 main laboratories, which were NaTuRe (for oocyte retrieval), ABEL and EMiL (for ovaries slicing taken from abattoir, *in vitro* maturation and *in vitro* culture for caprine and bovine experiments).

3.2.3 Equipment

All the equipment and instruments that had been used in the trials are listed in Appendix Table 1. The common used equipment in this research included inverted microscope with micromanipulators, micropuller, microforge, XYClone laser shoot, CO_2 incubators, stereomicroscope, surgical set, laparoscopic system, oven, liquid nitrogen tank, laminar flow, stage warmer, water bath, ultrapure water system and pH meter.

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3.2.4 Lab-wares

Numerous lab-wares were used in this research, such as measuring cylinder, beaker, magnetic stirrer, scot bottle (100, 200, 500, 1000 and 2000 ml), tissue culture dish, centrifuge tube (15 and 50 ml), microcentrifuge tube (0.5 and 1 ml), aluminium foil, disposable glove, autoclave bag, blades, glass pipette, micropipette, 4-well culture dish and tissue paper as listed in Appendix Table 3.

3.2.5 Chemical, Reagent and Medium

In general, the chemicals, reagents and media that were used to prepare the various media for the experiments were mainly purchased from Sigma Aldrich Co., USA, unless otherwise stated, as listed in Appendix 2.

3.3 METHODOLOGY

The description of the research methodology was divided into 4 sections as listed below;

1) Cleanliness and sterilisation of research laboratory

2) Preparation of medium

3) Preparation of microtools

4) Experimental procedure

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3.3.1 Cleanliness and Sterilisation of Research Laboratory

In this research, the cleanliness and sterilisation in laboratory was very important in order to control the embryo environment as well obtaining favourable outcomes. The aseptic practices and precautions were applied in research laboratory to minimise the possibilities of getting contamination in cell culture.

Washing hand with the disinfectant before and after any experiments was a must to minimise the adherence of microorganism on the skin, which can bring diseases to human and contamination to the cell culture. Besides, wearing a clean lab-coat, face mask and glove (if necessary) must be practised in the laboratory to keep the environment of embryo clean.

Before beginning any experiment, all the surfaces of working bench, laminar flow hood and microscope stage ought to be wiped with alcohol (70%) for sterilisation purpose. If any spillage occurred, the surface should be wiped immediately with the alcohol (70%). At the end of the experiments, the entire working surface was again wiped with alcohol (70%) to maintain the sterility.

All the glassware and apparatus such as magnetic stirrer, measuring cylinder, Scott bottle, beakers and etc. was soaked in diluted detergent (7X®-PF) right away after the experiments. The lab-wares were brushed and rinsed in tab water followed by rinsing in reverse osmosis (RO) water. Subsequently, the lab-wares were dried upside down in room temperature (27°C). Glassware such as beaker, measuring cylinder, volumetric flask were covered with aluminium foil, while screw-cap tubes were put in a autoclave bag and sealed which was later ready to autoclave for sterilisation purpose. Finally, after completing the

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sterilisation process, all the lab-wares were dried in oven (60°C) before placing them in appropriate cabinets.

On the other hand, for non-autoclavable lab-wares such as mouth-pipette, oocyte collecting tube, syringe, culture dish, etc. were sterilised using the application of UV light inside the laminar flow hood for at least 2 hours.

3.3.2 Preparation of Medium

All the media that were used in this research were prepared before the start of the experiments. Preparing the media must be carried out under laminar flow hood for aseptic purpose. For medium preparation, purified milli-Q water (Milipore, Ireland) was used as base solution. The pH and osmolarity were measured by using pH meter (7.2-7.4) and osmometer (280-300 mOsm), respectively. The prepared media were filtered into Scot bottle by using disposable filter (0.22 µm). The media were labeled before store in refrigerator at 3-5°C or freezer at -20°C.

3.3.2.1 Preparation of normal saline solution

In general, normal saline is a solution of 0.9% (w/v) of sodium chloride (NaCl). Normal saline was also known as physiological or isotonic saline. Normal saline was prepared by adding 9 g NaCl into 1 litre milli-Q water. Normal saline was kept at room temperature (27°C) for 3 months.

University of Malaya

3.3.2.2 Preparation of ovary collecting medium

Oocyte collecting medium (OCM) consisted of normal saline supplemented by penicillin G sodium salt (60 µg/ml) and streptomycin salt (50 µg/ml). The purpose of the medium was for washing and collecting ovary from abattoir. Table 3.1 shows the composition of chemicals in preparing 1000 ml OCM. The OCM was kept at room temperature (27ºC) with shelf life of 1 month for future use. Prior to ovary collection, the medium was warmed in water bath at 37ºC to maintain the temperature and cell tonicity of the collected ovary from abattoir before slicing.

Table 3.1: Composition of ovary collection medium

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

(Storage temperature: 27 ºC; shelf life: 1 month)

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

The PBS (-) solution was used as a base medium of certain stock solution as well as a form of balanced salt solution. It is also used for washing and suspending cells. This solution was prepared without adding (Ca^{2+}) and (Mg^{2+}). A list of composition to prepare PBS (-) was depicted in Table 3.2. The stock solution was filtered by filter-sterilised (0.22µm pose size) before and kept in the refrigerator at 3-5 ºC with shelf life of 3 months.

Table 3.2: Composition of phosphate buffer saline without calcium chloride (Ca^{2+}) and magnesium chloride (Mg^{2+}) , PBS (-)

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

(Storage temperature: 3-5 ºC; shelf life: 3 months)

University of Malaya

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

Table 3.3 was show the composition of chemical to prepare the penicillin G sodium salt and streptomycin sulphate salt stock solution with 100x concentration. The stock solution was filtered by filter-sterilised (0.22µm pose size) before aliquot in centrifuge tube (1.5 ml) and kept in the freezer at -20 ºC with shelf life of 6 months.

Table 3.3: Composition of ovary collection medium

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

(Storage temperature: -20 ºC; shelf life: 6 months)

3.3.2.5 Preparation of oocyte retrieval medium

In this study, Dulbecco's phosphate buffer saline (DPBS) was used as flushing medium for oocytes retrieval. This medium played important role to maintain the physiological pH range (7.2-7.4), viability and cell tonicity. DPBS medium was supplemented by polyvinylpyrrolidone (PVP) as a source of surfactant, in order to prevent the oocyte from stick on the surface of culture dish during oocyte manipulation.

University of Malaya

The DPBS medium was prepared according the composition in Table 3.3 and stir at moderate speed to dissolve and mix the chemical. Finally, the medium was filtered by filter-sterilised (0.22µm pose size) before kept in refrigerator at 3-5 ^oC with shelf life of 1 month.

Table 3.4: Composition of penicillin G sodium salt and streptomycin sulphate salt stock solution [100x]

Chemical component (catalogue number)	Final concentration	Quantity
PBS tablets (Dulbecco A, BR0014G)	1x	5 tablets
PS stock [100x]	1x	0.5 ml
PVP (P-360)	0.1%	0.5 g
Mili-Q water		500 ml

(Storage temperature: 3-5 ^oC; shelf life: 1 month)

3.3.2.6 Heat-inactivation foetal bovine serum, FBS

Serum is derived from clotted blood and remains popular as media supplement for cell culture. It provides a wide array of function such as deliver nutrients, growth and attachment factors and also protects the cell from oxidative damage and apoptosis.

In this study, foetal bovine serum (FBS) was used as a supplement in some of the cell culture media. FBS was purchased from Gibco® which was stored frozen at -20^oC and not heat-inactivated. Before heat-inactivation, the frozen FBS was thawed in refrigerator (3-5^oC) to avoid or limit the amount of precipitation. The thawed FBS was heated in water bath (56^oC) for 45 minutes to destroy the heat-sensitive complement protein which can

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promote cell lysis from an immune reaction between cell and the serum proteins. The temperature of water bath was monitored accurately to avoid protein denaturation.

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

In vitro maturation medium was prepared freshly which consists of TCM-199 as base medium supplemented with TCM-pyruvate, follicle stimulating hormone (FSH), oestradiol-17β, gentamicin sulphate salt, cystein and FBS. A list of stock solution used to prepare the IVM medium is depicted in Table 3.5.

Table 3.5: Preparation of stock solution for IVM medium

Stock solution (catalogue number)	Final concentration	Method of preparation
Follicle stimulating hormone, FSH (Folltropin®-V)	5 mg/ml	5 mg of Folltropin®-V powder was dissolved in 1 ml solvent in microcentrifuge tube (1.5 ml), sealed with parafilm, wrapped with aluminium foil and stored at -20ºC with shelf life 6 months
Gentamicin sulphate salt (G3632)	50 mg/ml	1 g of gentamicin sulphate salt was dissolved in 20 ml of DPBS, sterile filtered, aliquot in microcentrifuge tube (1.5 ml), sealed with parafilm, wrapped with aluminium foil and stored at 3-5ºC with shelf life 12 months
Oestradiol-17β (E8875)	1 mg/ ml	1 mg of oestradiol-17β was dissolved in 1 ml of filtered ethanol, 95%, aliquot in microcentrifuge tube (1.5 ml), sealed with parafilm,

University of Malaya

		wrapped with aluminium foil and stored at 3-5ºC with shelf life 6 months
TCM-pyruvate	2.2 mg/ml	2.2 mg of sodium pyruvate was dissolved in 1 ml of TCM-199, sterile filtered, aliquot in microcentrifuge tube (1.5 ml), sealed with parafilm, wrapped with aluminium foil and stored at 3-5ºC with shelf life of 1 week

The composition of IVM medium is listed in Table 3.6. The medium was filtered by filter-sterilised (0.22 µm pore size) before kept in refrigerator at 3-5ºC with shelf life of 1 week. Typically, a day prior preparation is required with equilibration in 5% of CO_2 incubator for at least 4 hours before used.

Table 3.6: Composition of IVM medium

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

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solution

(Storage temperature: 3-5°C; shelf life: 1 week)

* Oestradiol-17β stock solution was added 1 µl/ml of final volume after filtered.

3.3.2.8 Preparation of oocyte manipulation medium

The embryos were established from somatic cell nuclear transfer (SCNT) and parthenogenetic activation (PA). The solutions required during the experiment were TL-Hepes, hyaluronidase solution, incubated-oocyte holding solution, cytochalasin B, activation medium and culture medium.

3.3.2.8 (a) Preparation of TL-Hepes medium

In general, TL-Hepes medium was used for oocyte collection, washing and manipulation activities outside the CO_2 incubator. It consists of hepes to maintain the pH of the medium at 7.2-7.4.

(i) Preparation of TL-Hepes stock medium

The chemical components of TL-Hepes stock medium are listed in Table 3.7. All the chemicals were weighed using a digital balance before dispensing into the conical flask containing 500 ml of Milli-Q water and dissolved slowly by stirring gently using a magnetic stirrer. The TL-Hepes stock medium was stored in a refrigerator (3-5°C) with shelf life of 3 months.

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Table 3.7: Composition of TL-Hepes stock medium

Chemical component (catalogue number)	Final concentration	Quantity/ 500 ml
NaCl (S5886)	114 mM	3.330 g
KCl (P5405)	3.2 mM	0.120 g
$NaHCHO_3$ (S5761)	2.0 mM	0.084 g
$NaH_2PO_4.H_2O$ (S9638)	0.4 mM	0.028 g
$CaCl_2.2H_2O$ (C3881)	2.0 mM	0.150 g
$MgCl_2.6H_2O$ (M2393)	0.5 mM	0.050 g
Hepes: $C_8H_{17}N_2O_4SNa$ (H3784)	10 mM	0.600 g
Hepes: $C_8H_{18}N_2O_4S$ (H6147)	10 mM	0.600 g
Penicillin G (PEN-NA)	100 IU/ml	0.0325 g
Phenol red powder (P3532)	-	0.0025 g
Na lactate, 60% syrup (L7900)	10 mM	0.93 ml
Milli-Q water	-	500 ml

(Storage temperature: 3-5°C; shelf life: 3 months)

(ii) Preparation of TL-Hepes working medium

TL-Hepes working solution (100 ml) was prepared by adding gentamicin stock solution (50 µl), sodium pyruvate (0.0044 g) and bovine serum albumin- fraction V (BSA-FV) (0.1 g) into 99.95 ml of TL-Hepes stock medium as depicted in Table 3.8.

University of Malaya

Table 3.8: Composition of TL-Hepes working medium

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

(Storage temperature: 3-5°C; shelf life: 2 weeks)

3.3.2.8 (b) Preparation of hyaluronidase solution

Hyaluronidase solution was used for denuding the cumulus cells from COCs after *in vitro* maturation. Briefly, 0.2% hyaluronidase solution was prepared by dissolving (0.1 g) hyaluronidase powder and (0.05 g) PVP-360 in (50 ml) mDPBS as presented in Table 3.9. The solution was aliquot (150 µl) and stored in freezer (-20°C) with shelf life of 6 months.

Table 3.9: Composition of hyaluronidase solution

Chemical component (catalogue number)	Final concentration	Quantity
Hyaluronidase (H4272)	0.2%	0.1 g
PVP-360 (P360)	0.1%	0.05 g
mDPBS	1x	50 ml

(Storage temperature: -20°C; shelf life: 6 months)

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3.3.2.8 (c) Preparation of incubated-oocyte holding medium

During a long procedure of SCNT, the oocytes were kept in a medium of incubated-oocyte holding which is containing of TCM-199 supplemented with FBS (10%) (Table 3.10). The medium was filtered-sterilised using syringe filter (0.22 µm pore size) and incubated (5% CO_2, 38.5°C) at least 3 hours prior to use.

Table 3.10: Composition of hyaluronidase solution

Chemical component (catalogue number)	Final concentration	Quantity
TCM-199 (11150-059)	-	4.5 ml
FBS (16000-044)	10%	0.5 ml

(Storage temperature: 3-5°C; shelf life: 1 week)

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3.3.2.8 (d) Preparation of cytochalasin B, CB

Cytochalasin B is a cell-permeable mycotoxin which inhibits the formation of contractile microfilament as well induces the nuclear extrusion. In SCNT procedure, it is used as a relaxant agent where the zona pellucida of the oocyte is not rigid and can be easily pierced during enucleation.

(i) Preparation of CB stock solution

CB stock solution was prepared by dissolving 1 mg of CB powder in 1 ml of DMSO as presented in Table 3.11. Briefly, the stock solution was aliquoat (10 µl) into

microcentrifuge tube (1.5ml), wrapped in aluminium foil and stored in freezer (-20°C) with shelf life of 6 months.

Table 3.11: Composition of CB stock solution

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

(Storage temperature: -20°C; shelf life: 6 months)

(ii) Preparation of CB working solution

CB working solution was prepared freshly on the day of the experiment. TL-Hepes (990 µl) was added into the microcentrifuge tube (1.5 ml) containing (10 µl) CB stock solution as shown in Table 3.12.

Table 3.12: Composition of CB working solution solution

Chemical component (catalogue number)	Final concentration	Quantity
Cytochalasin B (C6762)	1 mg/ml	10 µl
TL-Hepes	-	990 µl

(Storage temperature: -20°C; shelf life: 6 months)

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3.3.2.8 (e) Preparation of activation medium

In SCNT and PA procedures, calcium ionophore (CaI) and 6-dimetylaminopurine (6-DMAP) were used as an activation medium. These two activation chemicals were used in combination whereas the manipulated oocytes were placed in CaI for 5 minutes followed by 6-DMAP for 4 hours.

(i)Preparation of calcium ionophore, CaI solution

(i.a)Preparation of CaI stock solution

The composition of CaI stock solution was listed in Table 3.13. It was prepared by dissolving 0.001 g of CaI in 3.82 ml of DMSO and was aliquot (10 µl) in 1.5 ml microcentrifuge tube and stored in freezer (-20°C) with shelf life of 6 months.

Table 3.13: Composition of CaI stock solution

Chemical component (catalogue number)	Final concentration	Quantity
Calcium ionophore (C7522)	500 µM	0.001 g
DMSO (D5879)	-	3.2 ml

(Freshly prepared on the day of usage)

University of Malaya

(i.b) Preparation of CaI working solution

The CaI working solution was freshly prepared by mixing 10 µl of CaI stock solution with 990 µl of equilibrated *in vitro* culture (IVC) medium as listed in Table 3.14.

Table 3.14: Composition of CaI working solution

Chemical component (catalogue number)	**Final concentration**	**Quantity**
Calcium ionophore stock solution	5 µM	10 µl
IVC medium	-	990 µl

(Freshly prepared on the day of usage)

(ii)Preparation of 6-dimetylaminopurine, 6-DMAP

(ii.a) preparation of 6-DMAP stock solution

The composition of 6-DMAP stock solution was listed in Table 3.15. It was prepared by dissolving 0. 1 g of 6-DMAP in 3.08 ml of Milli-Q water and was aliquot (10 µl) in 1.5 ml microcentrifuge tube and stored in freezer (-20ºC) with shelf life of 6 months.

Table 3.15: Composition of 6-DMAP stock solution

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

(Storage temperature: -20ºC; shelf life: 6 months)

University of Malaya

(ii.b) Preparation of 6-DMAP working solution

The 6-DMAP working solution was freshly prepared by mixing 10 µl of 6-DMAP stock solution with 990 µl of equilibrated *in vitro* culture (IVC) medium as listed in Table 3.16.

Table 3.16: Composition of 6-DMAP working solution

Chemical component (catalogue number)	Final concentration	Quantity
6-DMAP stock solution	2 mM	10 µl
IVC medium	-	990 µl

(Freshly prepared on the day of usage)

3.3.2.8 (f) Preparation of potassium simplex optimisation medium with amino acid, KSOMaa

After manipulation through SCNT or PA, the embryos were cultured in *in vitro* culture medium known as KSOMaa for further development. Bigger *et al.* (2000) further modified the KSOM with supplementation of amino acid. In 2011, Kwong *et al.* found that additional D-glucose into the medium increase the developmental rate of bovine and caprine embryos.

(i) Preparation of KSOMaa stock medium

Typically, KSOMaa stock medium was prepared by diluted the chemicals component in 200 ml of Milli-Q water (Table 3.17) by stirring gently using magnetic stirrer. The KSOMaa stock medium was stored in refrigerator (3-5ºC) with shelf life of 2 months.

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Table 3.17: Composition of KSOMaa stock medium

Chemical component (catalogue number)	Final concentration	Quantity/ 200 ml
NaCl (S5886)	95.0 mM	1.1106 g
KCl (P5405)	2.50 mM	0.0372 g
KH2PO4 (P5655)	0.35 mM	0.0096 g
MgSO4	0.20 mM	0.0048 g
Na lactate, 60% syrup (L7900)	10.0 mM	0.372 ml
Sodium pyruvate (P4562)	0.20 mM	0.0044 g
D-Glucose (G6152)	0.20 mM	0.0072 g
NaHCO3 (S5761)	25.0 mM	0.4202 g
CaCl2 (C5670)	1.71 mM	0.0384 g
L-Glutamine (G3126)	1.0 mM	0.0292 g
EDTA (E9884)	0.01 mM	0.0008 g
Milli-Q water	-	199.63 ml

(Storage temperature: 3-5ºC; shelf life: 2 months)

(ii) Preparation of KSOMaa working medium

The KSOMaa working medium was prepared in 2 types:

a) KSOMaa A- standard KSOMaa (Table 3.18)

b) KSOMaa B- KSOMaa supplemented with D-glucose (2.78 mM) (Table 3.19)

The medium was filtered by filter-sterilised (0.22µm pore size) before kept in refrigerator at 3-5ºC with shelf life of 1 month. A day prior to manipulation was needed for

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the preparation of this medium as well as it required equilibration in CO_2 (5%) incubator for at least 4 hours before experimentation.

Table 3.18: Composition of KSOMaa A working medium

Chemical component (catalogue number)	**Final concentration**	**Quantity**
KSOMaa stock solution	1x	20 ml
BSA-V (A6003)	0.4%	0.08 g
MEM non-essential amino acids solution [100x] (M7145)	-	100 µl
BME amino acid solution [50x] (B6766)	-	200 µl

(Storage temperature: 3-5°C; shelf life: 1 week)

Table 3.19: Composition of KSOMaa B working medium

Chemical component (catalogue number)	Final concentration	Quantity
KSOMaa A working medium	1x	10 ml
D-glucose (G6152)	2.78 mM	0.0046 g

(Storage temperature: 3-5°C; shelf life: 1 week)

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3.3.2.9 Preparation of medium for establishment of ear fibroblast cell (EFC)

3.3.2.9 (a) Preparation of 0.25% Trypsin EDTA

Trypsin EDTA is widely used for detachment of adherent cells. In preparation of EFC, this cell culture was attached to each other which is mediated by cell surface glycoprotein and Ca^{2+}. In order to detach the EFC from the petri dish during sub-passage of EFC, trypsin (0.25%) was supplemented with ethylenediaminetetraacetic acid (EDTA).

The trypsin EDTA Trypsin (0.25%) was prepared using PBS (-) as the base medium, and the composition is listed in Table 3.20. The solution was mixed well using a magnetic stirrer and filter-sterilised using a syringe filter (0.22 µm pore size) before kept in a refrigerator (3-5ºC).

Table 3.20: Composition of 0.25% trypsin EDTA

Chemical component (catalogue number)	Final concentration	Quantity/ 200ml
Trypsin (T4799)	0.25%	0.5 g
EDTA (E9884)	1.37 mM	0.08 g
PBS (-)	-	200 ml
PS stock [100x]	1x	200 µl

(Storage temperature: 3-5ºC; shelf life: 3 months)

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3.3.2.9 (b) Preparation of culture medium for ear fibroblast cell (EFC)

Ear fibroblast cell (EFC) was used as donor karyoplast in producing cloned embryo from somatic cell nuclear transfer (SCNT). During preparation of EFC, 2 different concentrations of penicillin-streptomycin were used which were primary culture (3X) and

sub-passage (1X). The EFC culture medium was prepared using a composition as listed in Table 3.21.

Table 3.21: Composition of culture medium for EFC

Chemical component (catalogue number)	Final concentration	Quantity
Dulbecco's modified eagle medium, DMEM (D5796)	-	90 ml
FBS (16000-044)		10 ml
Penicillin-streptomycin		300 µl (3X) or 100 µl (1X)

(Storage temperature: 3-5ºC; shelf life: 1 month)

3.3.2.9 (c) Preparation of freezing medium for ear fibroblast cell (EFC)

The EFC was frozen in liquid nitrogen (-196ºC) to be kept before used and thawed when needed in SCNT experiments. The freezing medium was prepared by adding of foetal bovine serum (FBS; 20%) and dimethyl sulfoxide (DMSO;cryoprotectant; 20%) into DMEM. The composition of freezing medium is listed in Table 3.22.

Table 3.22: Composition of freezing medium for EFC

Chemical component (catalogue number)	Final concentration	Quantity/100 ml
DMEM (D5796)	60%	60 ml
FBS (16000-044)	20%	20 ml
DMSO (5879)	20%	20 ml

Storage temperature: 3-5ºC; shelf life: 1 week)

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3.3.3 Preparation of Microtools

3.3.3.1 Preparation of mouth pipette assembly

Mouth pipette was used during the manipulation of oocytes and embryos. The mouth pipette was hand-made using 2 pipette tips (1000 µl) as an aspirator mouthpiece and as Pasteur pipette holder, silicone tube (5 mm diameter), a syringe filter (0.22 µm pore size) and a narrow opening pulled Pasteur pipette (Figure 3.1).

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

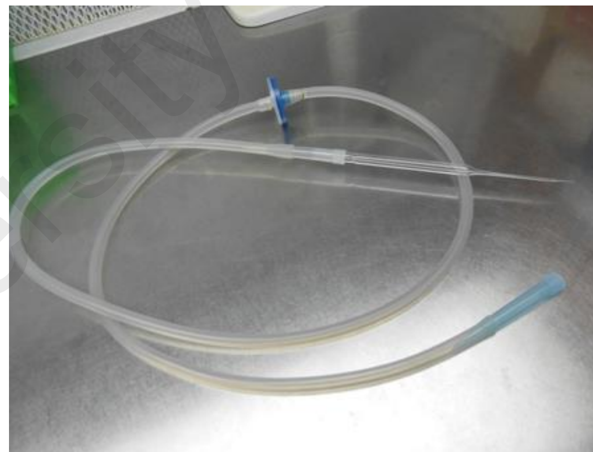

Figure 3.1: Mouthpiece. It consists of mouthpiece, silicone tube, syringe filter and Pasteur pipette.

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3.3.3.2 Preparation of mouthpiece-controlled pipette

The mouthpiece-control is an important part of the mouthpiece-pipette assembly. It was prepared by pulling an existing glass Pasteur pipette in a flame into the appropriate internal diameter (200-300 µm) so that it could fit the oocytes and embryos for experimental manipulation. The internal diameter of the capillary tube was very important to transfer the oocytes and embryos safely without injury. If the Pasture pipette was used in different media, it needed to be washed with the medium that to be transferred into before picking up any oocytes or embryos.

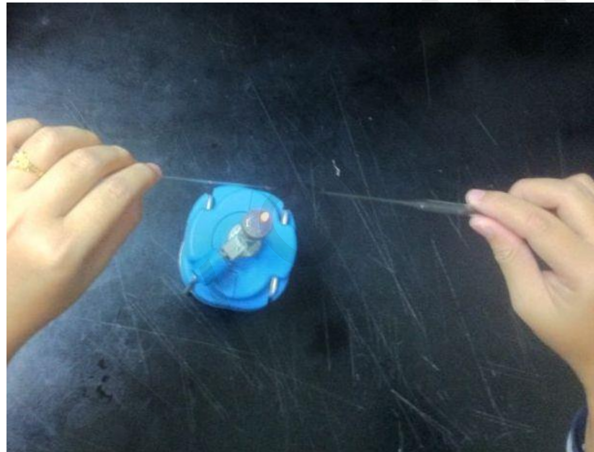

Figure 3.2: Pulling Pasteur pipette. The pipette was pulled over a blue flame part of the Bunsen burner.

3.3.3.3 Preparation of microneedles for SCNT manipulation

Three microneedles were used during SCNT procedure, namely holding pipette, biopsy needle and injection needle. The main equipment in preparing the microneedles for the micoromanipulator were micropuller (to pull the borosilicate glass capillary) (Figure 3.3) and microforge (to cut and bend the microneedle) (Figure 3.4).

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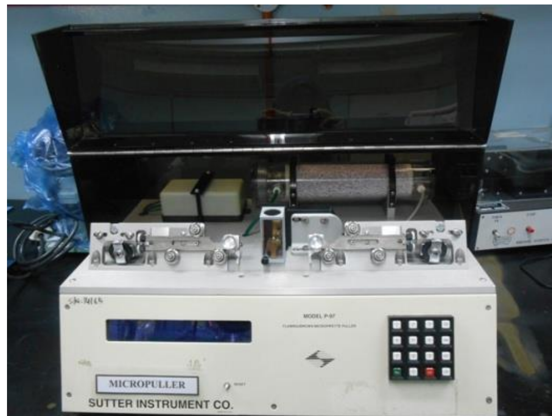

Figure 3.3: Micropuller,

Figure 3.4: Microforge.

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3.3.3.3 (a) Preparation of holding pipette

Holding pipette was used to hold the matured oocyte during SCNT procedure (i.e. during enucleation of DNA and injection of donor karyoplast). The micropuller was set with the parameter (heat= 665 units, pull=150 units, velocity=100, time=150 units, pressure=500 units) before pulling the borosilicate glass capillary. Briefly, the pulled capillary was

aligned and focused where the breaking point of the capillary was placed on the glass bead of the filament. The filament was heated to about 30-50% as the capillary was melted to the glass bead form and the heat was immediately turned off. As the capillary cooled, the capillary was broken evenly with blunt cut. Again, 30-50% heat was directed towards the tip of the capillary so that the inside diameter of holding pipette shrunk. The capillary was then bent at 30° to allow a horizontal displacement on the microscope stage. The preparation of holding pipette is shown in Figure 3.5.

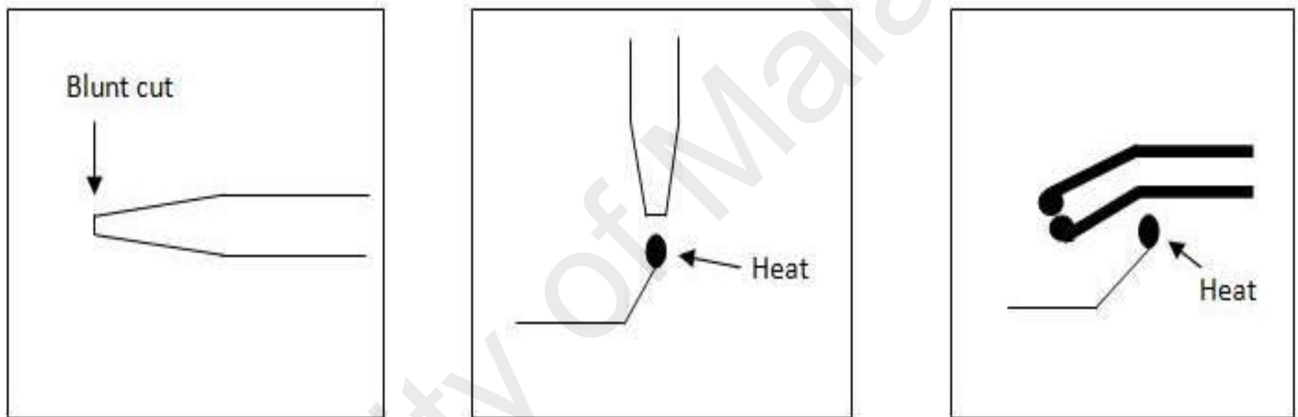

Figure 3.5: Preparation of holding needle. a) Blunt cut of holding pipette using glass bead, b) heat was directed towards the tip of pipette to obtain desired internal diameter and c) bending of holding pipette using heat.

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3.3.3.3 (b) Preparation of biopsy needle and injection needle

Biopsy needle and injection needle were used for enucleation of DNA and injection of donor cell during SCNT procedure, respectively. They were different in the diameter of the capillary tip but both had the blunt cut. The diameter for biopsy needle was bigger (30 µm) than the diameter for injection needle (10 µm).

The pulled capillary was aligned and focused accordingly where the breaking point of the capillary was placed on the glass bead of the filament. As the capillary cooled, the capillary was broken evenly with blunt cut. Lastly, the capillary with blunt cut was then bent at 30° to allow a horizontal displacement on the microscope stage (Figure 3.6).

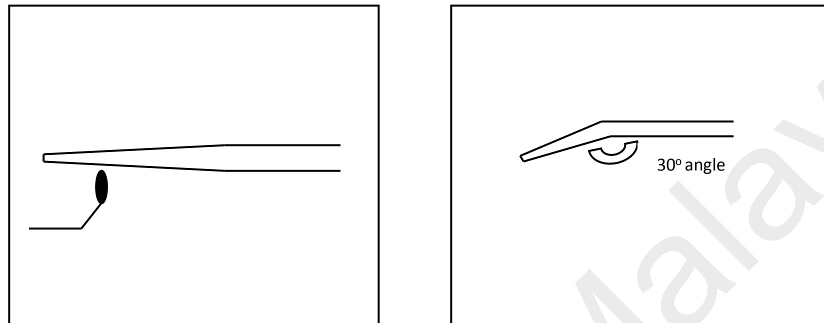

Figure 3.6: Preparation of biopsy and injection needle. a) Blunt cut using heat and b) bending of biopsy needle and injection needle using heat.

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3.3.4 General Experimental Procedure

3.3.4.1 Preparation of caprine donor karyoplast (ear fibroblast cell)

In production of cloned embryos through SCNT technique, besides cumulus cell, the other type of donor karyoplast used was caprine ear fibroblast cell (EFC). The preparation of EFC involved the following steps: 1) isolation of primary EFC, 2) sub-passage of EFC, 3) cryopreservation of EFC and 4) thawing of EFC.

3.3.4.1 (a) Isolation of primary of EFC

The goat was anaesthetised via intramuscular injection (i.m.) (1 ml) of mixed Xylazine hydrochloride and Ketamin hydrochloride (1:50) before started the ear biopsy. The area of ear tissue (circa 20 mm) was shaved to remove the hair which was then swabbed with EtOH (70%) before cut using a pair of surgical scissors. The biopsied ear was washed with mDPBS (3 times) before kept in mDPBS at 4°C and transported to the laboratory.

The biopsied ear was washed again with mDPBS solution (3 times) before cleaning the remaining hair on the surface of ear tissue. Then, the ear tissue was sprayed with EtOH (70%) and washed again in a series of primary culture medium supplemented with DMEM medium (90%), FBS (10%) and penicillin-streptomycin (3x). The dermal layer was dissected from cartilage using a razor blade. Subsequently, the dermal layer was cut into small pieces using a sterile surgical scissors and was then arrange at the centre of Petri culture dish containing primary culture medium with white surface facing down. Finally, a glass cover slip was put on top of the dermal tissue to avoid from floating. The explants ear tissue culture was incubated in CO_2 (5%) incubator at 38.5^{o}C until the outgrowth was observed (usually at Day 5 onwards).

3.3.4.1 (b) Sub-passage of EFC

After the EFC reached 80-90% confluent, the cells were ready to be sub-passaged. All the culture media were removed and washed with PBS (-) for 3 times to remove the remaining media. The MEF cells could be harvested by trypsinisation step by adding trypsin-EDTA medium into the culture dish and incubated for 2-3 minutes (to detach the cells from the culture dish). The trypsin-EDTA medium containing the detached EFC was transferred

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into conical tube (15 ml). The culture medium was added with a ratio of 1.5-fold of the typsin-EDTA and centrifuged at 3000 rpm for 5 minutes. The supernatant was removed and culture medium (1 ml) added into the remaining pellet followed by a gentle sucked in-out before seeded and cultured in culture dish (60 mm) in CO_2 (5%) incubator at 38.5°C.

3.3.4.1 (c) Cryopreservation of EFC

Freezing medium which contained cryoprotective agent (DMSO) (0.5 ml) was dispensed into each cryovial followed by added up the culture medium containing EFC cells (0.5 ml) so that the final volume was 1 ml. The cryovial was placed overnight in a freezer (-80°C) before transferred into a liquid nitrogen tank.

3.3.4.1 (d) Thawing of EFC

The cryovial was removed from the liquid nitrogen tank and directly warmed in a water bath (37°C) for 3 to 5 minutes. All the medium was aspirated out and transferred into a conical tube (15 ml) and culture medium [DMEM supplemented with FBS (10%) and PS (1X)] was added with ratio of 1.5-fold of the medium and centrifuged at 3000 rpm for 5 minutes. The supernatant was removed and culture medium (1 ml) was added into the remaining pellet. The medium was pipetted several times (8-10) to break the pellet into single cells. Next, EFC cells were then seeded and cultured in a culture dish in CO_2 (5%) incubator at 38.5°C.

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3.3.4.2 Preparation of caprine donor karyoplast (fresh cumulus cell)

Fresh cumulus cells (another type of donor karyoplast) were used as donor karyoplasts. The cumulus cells were harvested during denuding of oocytes after maturation. The cumulus cells were collected and washed in TL-hepes medium, which were then transferred into a microcentrifuge tube (1.5 ml) containing TL hepes (1 ml) and centrifuged at 3000 rpm for 5 minutes. The supernatant was removed and TL-hepes medium (0.3 ml) was added into the remaining pellet. The medium was pipetted several times (5-8) to break the pellet into single cells and were transferred into holding medium before used as donor karyoplasts.

3.3.4.3 Preparation of caprine and bovine recipient cytoplast

3.3.4.3 (a) Superstimulation in goat

In order to retrieve the oocytes through laparoscopic oocyte pick-up (LOPU) technique, the goat was prepared by superstimulation protocol (oestrus synchronisation and hormonal stimulation) as shown in Figure 3.7. The oestrous cycle was synchronised by the insertion of a controlled internal drug release device (CIDR®, 0.3 g progesterone,Company) into the vagina of the donor doe for 14 days (Day 0 at 0900 hr). The CIDR was inserted deeply in the doe's vagina with the help of CIDR applicator coated with the lubricant gel.

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At approximately 36 hours prior to CIDR removal (Day 13 at 0900 hr) Cloprostenol, Estrumate® (125 µg) was administered intramuscularly (i.m.) which is important for regression of corpus luteum. Upon CIDR removal (Day 13 at 0900 hr), the does were superstimulated by administration of PMSG (Folligon®) (1200 IU) and hCG (Ovidrel®) (250 IU) {Day 13 at 1800 hr).

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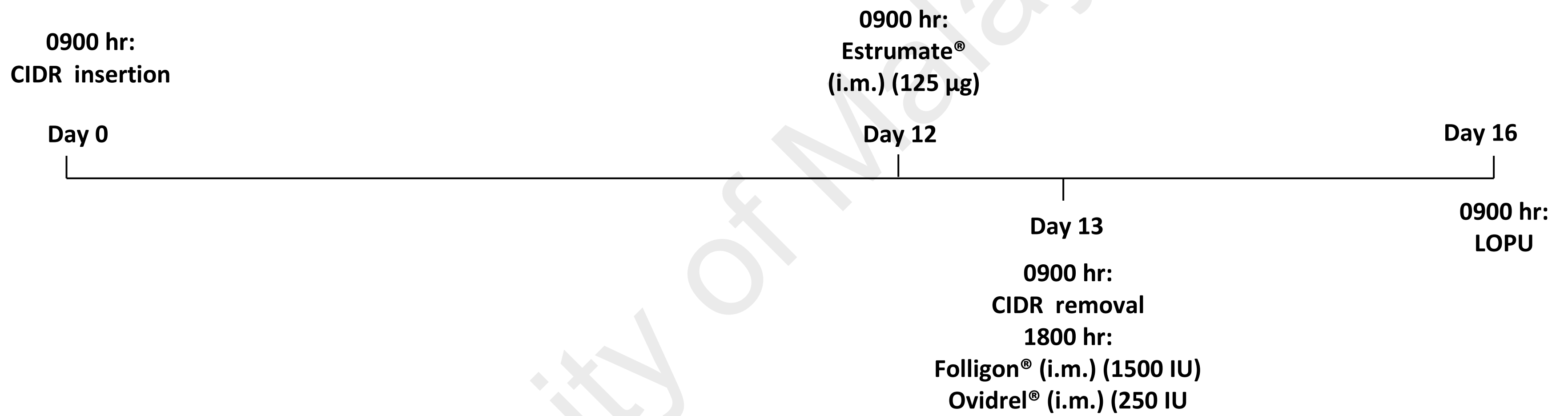

Figure 3.7: Oestrus synchronisation and hormonal stimulation.

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3.3.4.3 (b) Goat oocyte retrieval

(i) Laparoscopic oocyte pick-up (LOPU)

The treated does were isolated in off-feed pen which feed and water was removed 24 hr prior to LOPU. The does were given anesthesia (1 ml/30 kg body weight) by intramuscular (i.m.) administration of mixed xylazine hydrochloride and ketamine hydrochloride (1:50)

Surgical sites of the abdominal and inguinal areas were cleaned with Hibiscrub before shaved and was scrubbed with surgical iodine. Before oocyte collection, the aspiration and flushing system machine (V-MAR 5100 and V-MAR 4000, Cook) were prepared with 50 ml syringe of flushing medium connected to the aspiration needle and collecting tube. The trocar which connected to the plastic tubing that was connected to a CO_2 tank was inserted through a small incision (3-5 cm) near the umbilicus for CO_2 gas insertion. After gas insertion, the laparoscope which connected with the light source and camera system was inserted through the trocar sheath to facilitate the visualisation inside the abdomen. The edge of the ovary was held using a pediatric grasper and the ovarian follicles were punctured, flushed and aspirated by the OPU needle. The collecting tubes were sent to the embryo room for oocyte collection.

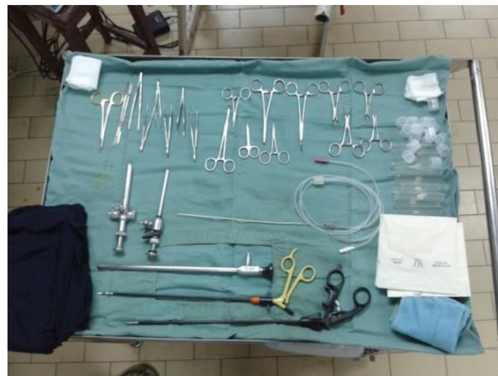

Figure 3.8: Preparation of surgical set on surgery table before LOPU.

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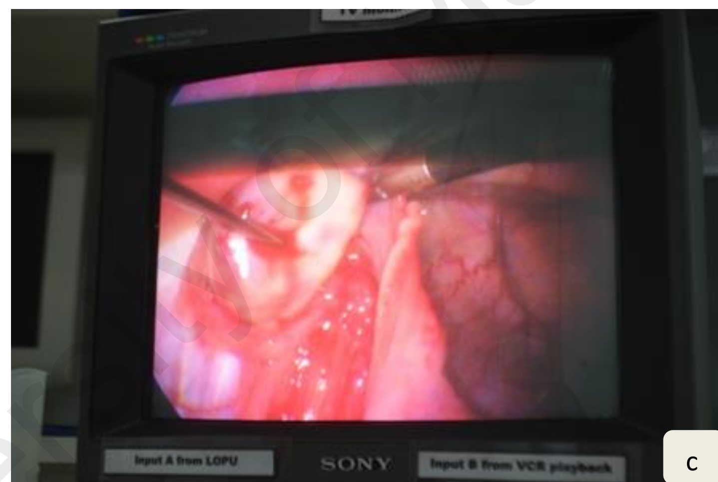

Figure 3.9: Procedures of LOPU. a) Assessment of ovary before LOPU, b) LOPU was carried out by the surgeons and c) the visible follicle was aspirated by using a OPU needle.

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(ii)   Ovary slicing

Another source of oocytes was obtained from local abattoir. The ovaries were collected within 3 hours after the females were slaughtered. The collected ovaries were washed and kept in a flask containing normal saline supplemented with penicillin-G (60 μg/100 ml) and streptomycin (50 μg/100 ml) at 35ºC before transported to the laboratory. The ovaries were washed in normal saline at least 3 times to remove the blood prior to slicing of ovaries.

The ovary was wiped with sterile tissue and held with a sterile haemostat on a culture dish (60 mm diameter) containing pre-warmed (37ºC) oocyte collection medium (TL-Hepes) (4 ml) and sliced using a sterile stainless razor blade held with another heamostat. The ovaries were sliced in checkerboard incisions to the entire surface of the ovaries. The sliced ovaries were then rinsed in a beaker (50 ml) containing TL-Hepes medium (30 ml).

Subsequently, the sliced material was transferred into embryo room for COC pick-up under a stereomicroscope attached with a stage warmer (38.5ºC). The COC were collected using mouthpiece-controlled pipette and were transferred into a culture dish (35 mm) containing pre-warm (38.5ºC) TL-Hepes (2 ml).

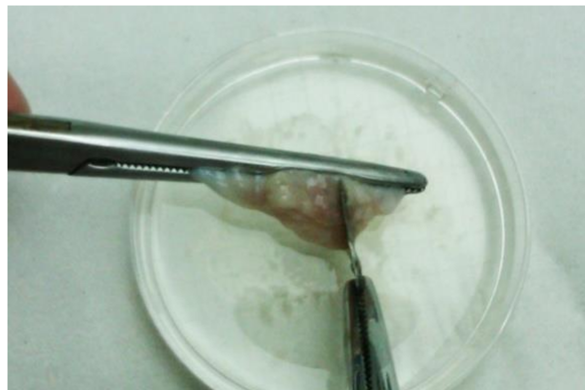

Figure 3.10: Ovarian slicing. The ovary was clamped using a haemostat and sliced using a razor blade.

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3.3.4.3 (c) Grading of goat oocytes

The collected oocytes from LOPU and ovarian slicing were washed in TL-Hepes medium (3 times) before graded into 4 different grades according to cumulus cell layer surrounded the oocyte. Table 3.23 presents the charaterisation of COC of different grades.

Table 3.23: Charaterisation of COC (According to Rahman *et al.,* 2009)

Grade	Characterisation of COC
Grade A	COC more than 5 layers of cumulus cells
Grade B	COC with 3-5 layers of cumulus cells
Grade C	COC with 1-2 of cumulus cells
Grade D	Incomplete cumulus cells and cumulus-free oocyte (naked oocyte)

3.3.4.3 (d) *In vitro* maturation (IVM)

After oocyte grading, the oocytes were washed in IVM medium at least 3 times before transferred into droplet (60 µl) of IVM medium which prepare and incubate in CO_2 (5%) at 38.5^{o}C one day prior to IVM procedure. Each grade of oocytes (10-15) was accumulated in one droplet.

The duration of IVM was different, depending on the source of the oocytes source:

a) LOPU: 21-24 hours of IVM

b) Abattoir (ovarian slicing): 24 to 27 hours of IVM

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3.3.4.3 (e) Assessment of matured oocyte

After IVM, the oocytes were transferred into a droplet (100 µl) of hyaluronidase (0.2%) for denuding process to remove the cumulus cell surrounded the oocytes. Denuding was performed less than 5 minutes by repeated pipetting (40-50 times) using micropipette (200 µl) adjusted to 90 µl. The naked oocytes were rotated by using a mouthpiece-controlled pipette and assessed under stereomicroscope by protrusion of first polar body (PB1). Only matured oocytes were kept and transferred into incubated holding medium [TCM-199 supplemented with FBS (10%)] before used for oocyte manipulation in various experiments.

3.3.4.4 Intra- and interspecies somatic cell nuclear transfer (SCNT) procedure

Intra- and interspecies SCNT procedures are as follows: 1) preparation of micromanipulation dish, 2) alignment of microtools, 3) enucleation, 4) injection, 5) chemical activation and 6) *in vitro* culture (IVC)

3.3.4.4 (a) Preparation of micromanipulation dish

Generally, the manipulation medium was prepared on the day of SCNT experiment. The manipulation media that were used included: TL-Hepes (alignment of microtools) , TL-Hepes supplemented with stock CB (10%) (enucleation medium) and TL-Hepes supplemented with PVP-360 (1 g/ml) (injection medium).

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The manipulation medium was prepared on the lid of a polystyrene culture dish (35 mm) with dispensing droplets (15 µl) and covered with the mineral oil (Figures 3.11and 3.12).

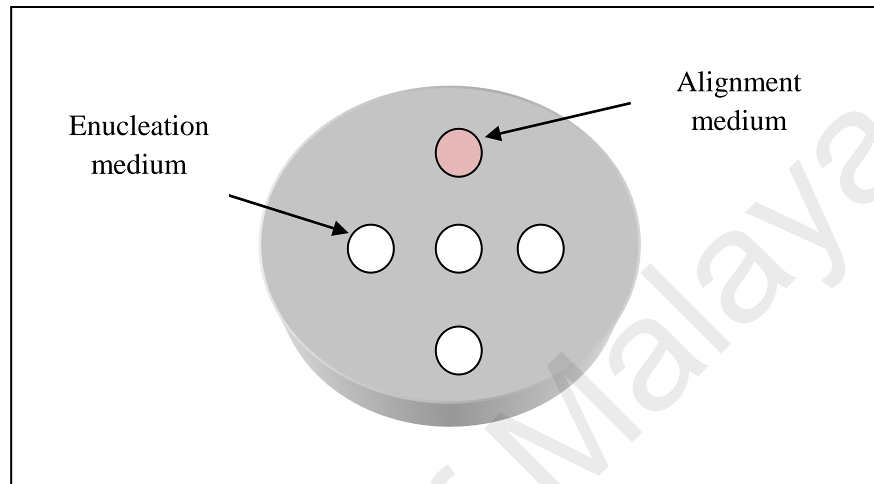

Figure 3.11: Enucleation dish.

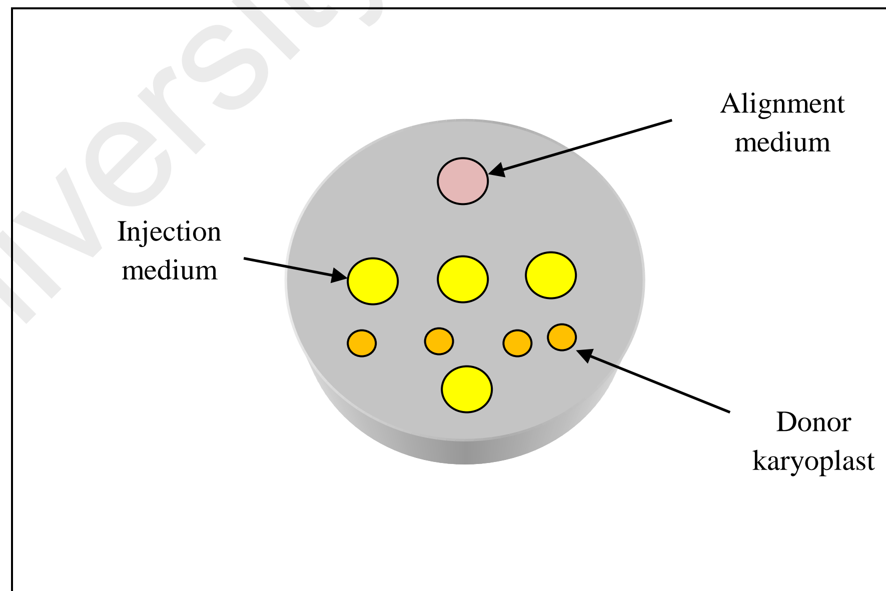

Figure 3.12: Injection dish.

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3.3.4.4 (b) Alignment of microtools

Prior to micromanipulation procedure, the crucial step was to set up the microtools (holding needle and enucleation needle or injection needle) to the electrode holder of micromanipulator. Technical skill was needed to set up these microtools to ensure the microtools were in right position and SCNT procedure went smoothly.

Firstly, alignment medium on prepared manipulation dish was put on the stage and focused to get a sharp image. The holding needle and enucleation needle were inserted into the electrode holder at both sides and adjusted to the centre on top of the focused alignment medium. The position of both microtools was lowered down until the tip touch the droplets. Subsequently, the image of both microtools was focused and aligned so that the working tips were parallel with the manipulation dish (Figure 3.13).

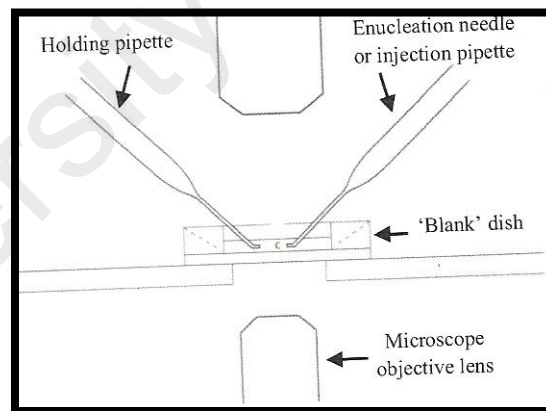

Figure 3.13: Alignment of microneedles on the micromanipulator. Adapted from Kwong (2013).

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3.3.4.4 (c) Enucleation (laser shoot technique)

Enucleation was referred to removing the nucleus (DNA) of donor oocytes for preparing the cytoplast before injection of donor karyoplast. Laser shoot technique has been used for enucleation. The oocyte was held by the holding pipette whereby the extrusion of first polar body (PBI) at 12 o'clock position was carried out. A hole was made on zona pellucida near the PBI by using a laser beam. The oocyte was pushed using a biopsy needle at another side that was parallel to the holding pipette in order to enucleate the DNA and PBI (Figure 3.14).

Figure 3.14: Enucleation technique. a) PB1 with cytoplasm (10%) was removed through a hole of zona pellucida made by laser shoot and b) the successful of enucleated oocytes.

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3.3.4.4 (d) Donor cell injection (WCICI)

Prior to donor cell injection, donor karyoplast was prepared and suspended into DMEM culture medium and kept in CO_2 (5%) incubator at 38.5°C.

Once the preparation of donor karyoplast was completed, the biopsied needle was changed with injection needle and aligned as describe in Section 3.3.4.4 (b). The hole that was made during enucleation was put at 3 o'clock position and held firmly by a holding pipette. The karyoplast was pipetted into the injection needle. Subsequently, the karyoplast was pipetted in and out for few times (2-3) to break the plasma membrane before injected into the recipient cytoplast through whole cell intracytoplasmic injection (WCICI) technique (Figure 3.15).

Figure 3.15: Injection of donor karyoplast through WCICI technique.

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3.3.4.4 (e) Chemical activation

After donor cell injection, the reconstructed oocytes were chemically activated with calcium ionophore (CaI) for 5 minutes followed by 6-dimetylaminopurine (6DMAP) for 4 hours.

3.3.4.4 (f) IVC

The reconstructed oocytes were washed and cultured in equilibrated IVC medium in CO_2 (5%) incubator at 38.5^{o}C. Development of reconstructed embryos was assessed daily until Day 7.

3.3.4.4 (g) Embryo transfer

Embryo transfer (ET) of cloned embryos (morula stage) was carried out at Day 5 of SCNT. The cloned embryos were transferred into the oestrus synchronised recipient does via oviduct transferred method. The recipient does were synchronised and superovulated as described in Section 3.3.4.3 (a).

Prior to ET, corpus luteum (CL) was examined using laparoscopic procedure by making a small incision (circa 3-5 mm) at the lower abdomen near the udder of the does to allow the insertion of trocar which connected to the plastic tubing that was connected to a CO_2 tank for gas insertion. After gas insertion, the laparoscope which connected with the light source and camera system was inserted through the trocar sheath to facilitate the visualisation inside the abdomen. Another small incision (circa 3-5 mm) was made at the

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left side and parallel with the first incision. The uterus was held using a pediatric grasper and pull out to the outside of the body for embryo transfer. In embryo room, the cloned embryos were loaded into an ET tube with the IVC medium and ready to transfer into the uterus of the recipient does.

3.4 EXPERIMENTAL DESIGN

3.4.1 Effect of Different Sources (LOPU Versus Abattoir) of the Oocytes on Oocyte Yield, Oocyte Grade and Maturation Rate (Experiment 1)

The objective of this experiment was to evaluate the effect of different sources of oocyte on the quantity and quality of oocyte, maturation rate and cloned-caprine embryo developmental rate after SCNT. The oocytes were obtained from two different sources: a) abattoir and b) laparoscopic oocyte pick-up (LOPU). After oocyte recovery, the cumulus-oocyte complexes (COCs) were graded into 4 different grades according to the cumulus cell layer as described in Section 3.3.4.3 followed by culturing in IVM medium in CO_2 (5%) incubator at 38.5ºC as described in Section 3.3.4.4.

After maturation, the COCs were denuded in hyaluronidase (0.2%) by pipetting in and out for 3 minutes and then transferred into TL-Hepes medium for determination of matured oocyte (extrusion of first polar body) under a stereomicroscope. The matured oocytes were subjected to SCNT which were finally cultured in IVC medium for further development. The flow of this experiment was shown in Figure 3.16. The data were collected and analysed by using ANOVA to determine the differences between the treatments.

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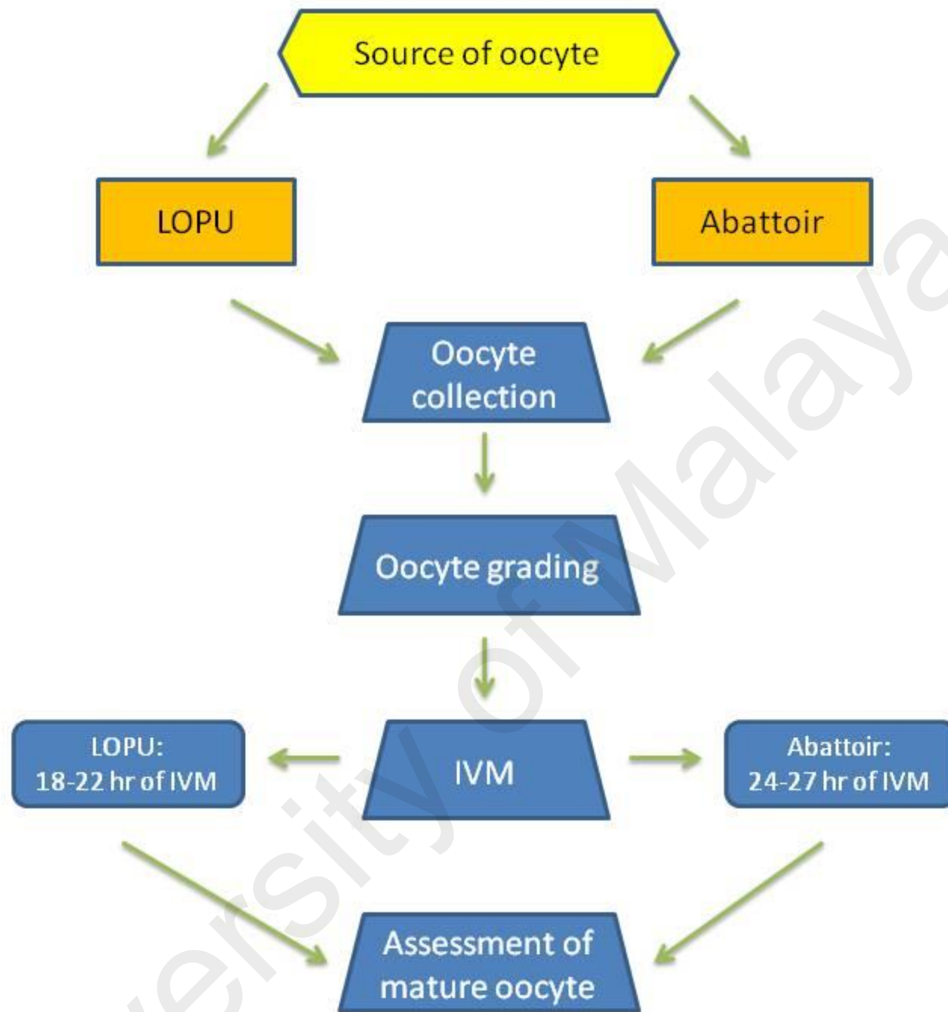

Figure 3.16: Flow chart of methodology of Experiment 1.

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3.4.2 Effect of Time Interval from Hormonal Treatment to Laparoscopic Oocyte Pick-Up (LOPU) on Goat Stimulation Response, Oocyte Recovery and Maturation Rate (Experiment 2)

This experiment was designed to investigate the effect of three different time intervals from PMSG-hCG treatment to LOPU on goat stimulation response, oocyte recovery and maturation rate. A total of 48 goats were used for oestrus synchronisation with controlled internal drug release device (CIDR) for 14 days followed by administration of 1200 IU of PMSG and 250 IU of hCG (i.m.). LOPU was performed at 3 different time intervals: 1) 36 to 40 hours, 2) 66 to 70 hours and 3) 71-75 hours after PMSG/hCG treatment. The numbers of stimulated follicles, ovulated follicles and oocyte recovery were recorded. The retrieved oocytes were washed and graded (Grades A, B, C and D) before cultured into *in vitro* maturation (IVM) medium for 21 to 24 hours. The matured oocytes were evaluated by extrusion of a first polar body (PBI). The flow of this experiment is shown in Figure 3.17. The data were collected and analysed by using ANOVA to determine the differences between the time intervals.

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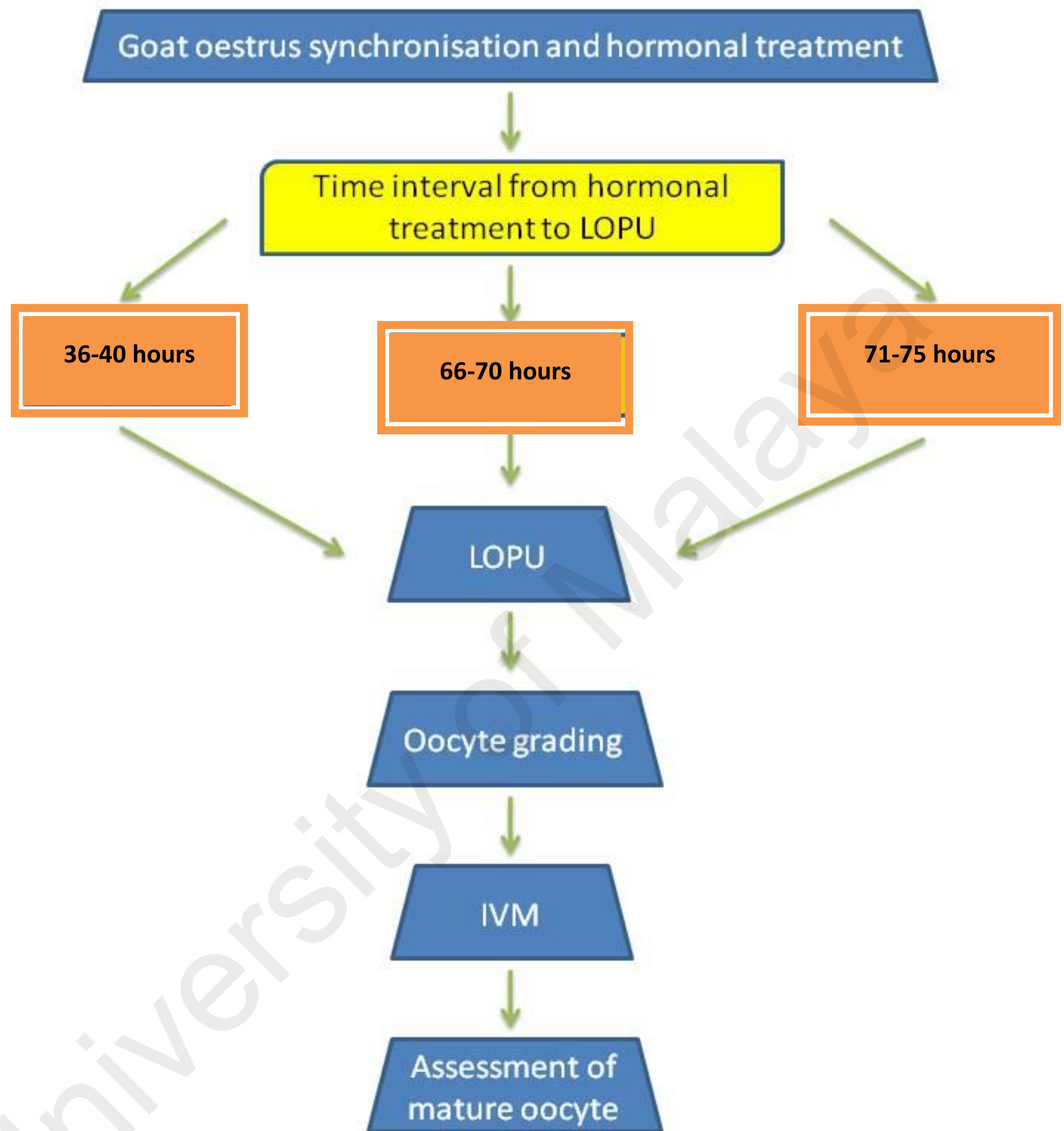

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Figure 3.17: Flow chart of methodology of Experiment 2.

3.4.3 Production of *In Vitro* Cloned Embryos Through Intra- and Interspecies Somatic Cell Nuclear Transfer Using Ear Fibroblast Cell as Karyoplast (Experiment 3)

Cloned caprine embryos were produced from two different types of SCNT: a) intraSCNT and b) interSCNT. In the former, donor karyoplast and both recipient cytoplast were obtained from same species (caprine), meanwhile in later were obtained from different species (caprine and bovine, respectively).

Ear fibroblast cells (EFC) were cultured and prepared prior SCNT as described in Section 3.3.4.1. In interSCNT experiment, the oocytes were obtained from bovine ovaries collected from abattoir while for intraSCNT, the oocytes were obtained from LOPU and abattoir. The collected oocytes were cultured in IVM medium for oocyte maturation before used as the recipient cytoplast [intraSCNT (caprine) or interSCNT (bovine)]. After maturation, the matured oocytes were selected by confirmation of extrusion of a first polar body (PBI). The experiment was continued with enucleation, injection of a donor karyoplast (EFC) and chemical activation as described in Section [3.3.4.4 (c), 3.3.4.4 (d) and 3.3.4.4 (e), respectively] before culture in IVC medium for further development. The flow of this experiment is shown in Figure 3.18.

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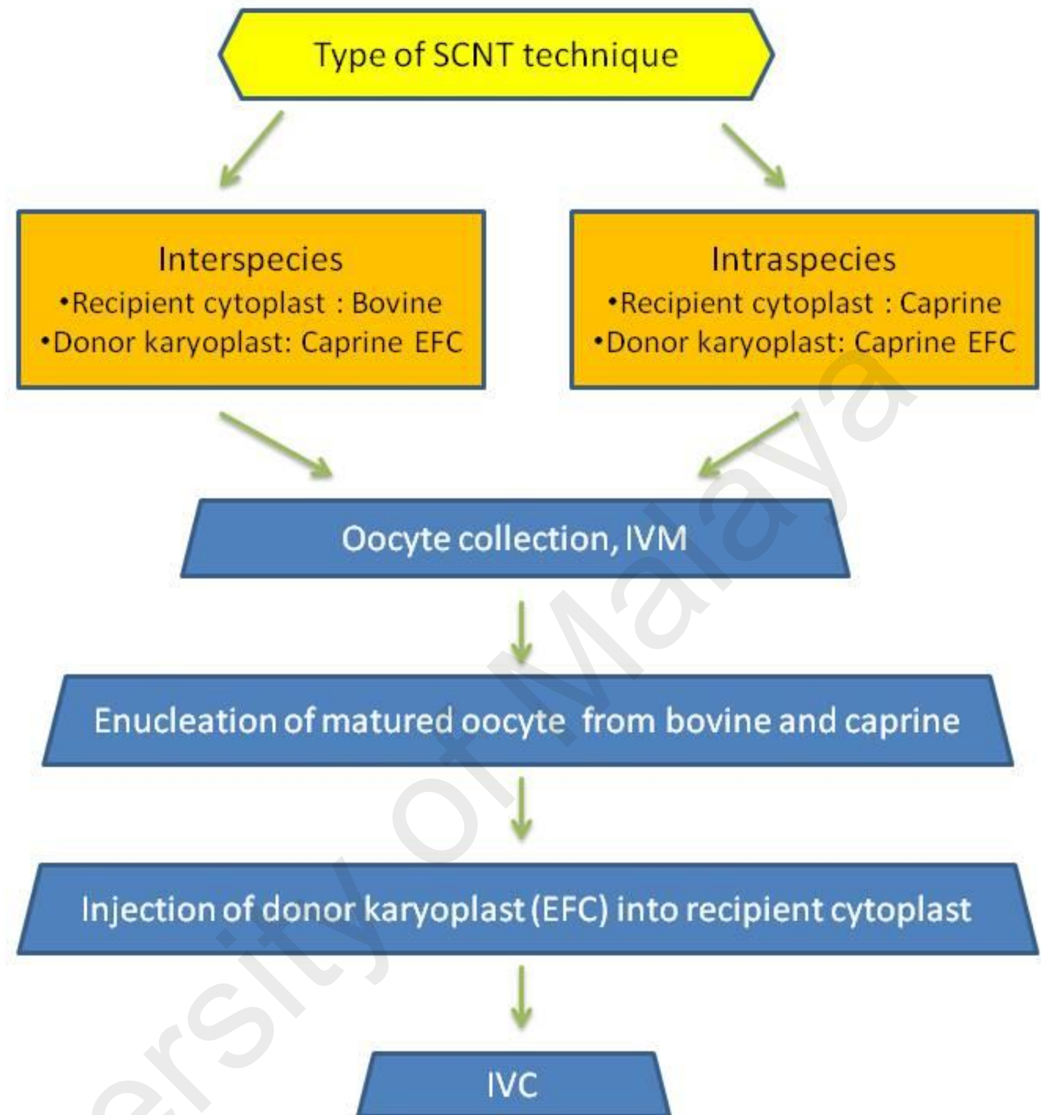

Figure 3.18: Flow chart of methodology of Experiment 3.

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3.4.4 Effect of Different Donor Karyoplasts (Cumulus Cell versus Ear Fibroblast Cell) on Cleavage Rate of Cloned Caprine Embryo (Experiment 4)

Two different types of donor karyoplasts were used in the production of intraSCNT caprine embryos. The cleavage rate of intraSCNT caprine using two different types of donor karyoplasts , namely a) cumulus cell and b) ear fibroblast cell were investigated. The collected oocytes were cultured in IVM medium for maturation before underwent SCNT procedure. After enucleation, the recipient cytoplasts were subjected into two groups for injection of different types of donor karyoplasts. The successful reconstructed embryos were activated before cultured in IVC medium for further development. The successful enucleated, injected and developmental competence embryos were recorded. A flow of the methodology that was involved in this experiment is presented in the flow chart as illustrated in Figure 3.19.

The cloned embryos were transferred into recipient does (surrogate mother) which underwent oestrus synchronisation and superovulation as describe in Section 3.3.4.3 (a). On Day 5 after CIDR removal and hormonal treatment, the recipient does were transferred into surgery room for embryo transfer procedure. Prior to embryo transfer (ET), the recipient doe ovaries were examined for the presence of a corpus luteum (CL) by using laparoscopic procedure. Only does with the presence of CL (at least 1 CL) could be used as recipients for ET. On the other hand, the cloned embryos (morula stage) were prepared and loaded into ET tube before transferred into the uterus. Pregnancy of the recipient does can be diagnosed as early as 36 days after ET by real-time ultrasound scanning (SSC500V; Aloka, Japan).The data were collected and analysed by using ANOVA to determine the differences between two different types of donor karyoplasts.

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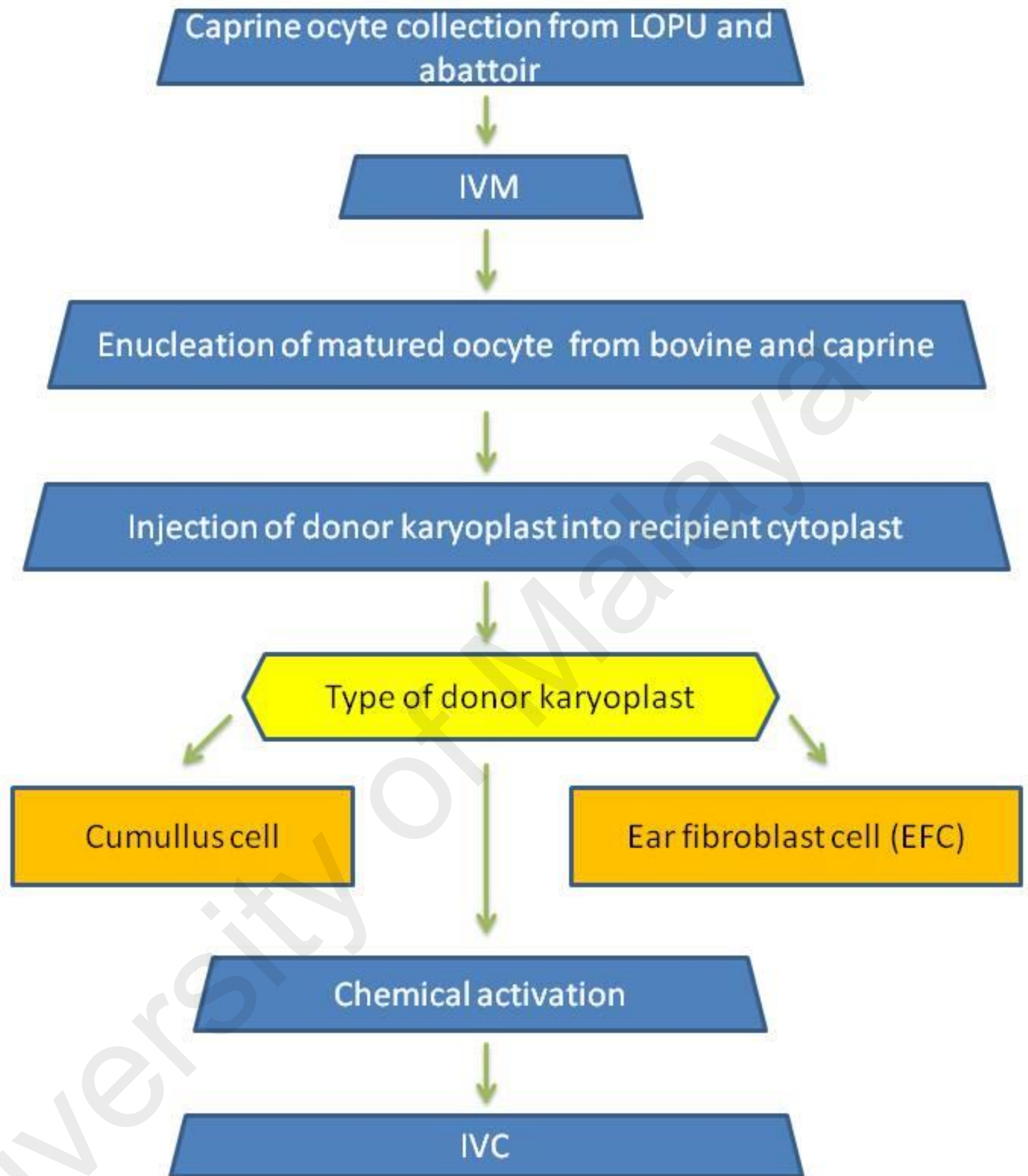

Figure 3.19: Flow chart methodology of experiment 4.

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Chapter 4

4.0 RESULTS

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Chapter 4

4.0 RESULTS

4.1 EFFECT OF OOCYTE SOURCES (LOPU VERSUS ABATTOIR) ON OOCYTE YIELD, OOCYTE QUALITY, MATURATION RATE AND SUBSEQUENT SCNT PERFORMANCE (EXPERIMENT 1)

This experiment was carried out to evaluate the effect of source of oocytes on quantity and quality of oocytes obtained, maturation rate as well as subsequent *in vitro* culture performance of SCNT embryos. The oocytes were derived from two different sources: abattoir (non-stimulated goat) and laparoscopic oocytes pick-up (LOPU; stimulated goat). The number of oocytes, percentage of oocyte quality (Grades A, B, C and D); maturation rate; reconstruction rate and cleavage rate after SCNT are presented in Tables 4.1, 4.2, 4.3 and 4.4, respectively.

4.1.1 Effect of Two Different Oocyte Sources (LOPU versus Abattoir) on Distribution of Oocyte Quality

A total of 499 oocytes were retrieved from two different oocyte sources: LOPU (236; 22 ovaries) and abattoir (263; 18 ovaries) were grouped into 4 different grades (Grades A, B, C and D). Table 4.1 shows there were no significant differences in the percentages of oocyte quality in LOPU vs. abattoir: Grade B (36.24±2.6 vs. 33.23±2.7%, respectively) and C (24.56±3.3 vs. 28.11±2.9%, respectively); however, significant differences were shown in Grade A (32.46±4.4 vs. 19.80±4.4%, respectively) and Grade D (6.74±1.7 vs. 18.86±2.0%, respectively).

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In term of number of oocytes retrieved per ovary, Grade B (3.91±0.4) followed by Grade A (3.55±0.7) was significantly higher than Grade C (2.50±0.46) and Grade D (0.77±0.2) in LOPU-derived oocytes. In abattoir, Grades B (4.94±0.5) and C (4.28±0.6) oocytes showed significantly higher than Grades A (2.78±0.4) and D (2.61±0.3) (Table 4.2).

Figure 4.1 shows the comparison between two different sources on the distribution of oocytes quality. It was clearly shown that there was significant difference ($P<0.05$) in distribution of oocytes quality in Grade D between the two different sources.

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4.1.1 Effect of Two Different Sources (LOPU versus Abattoir) on Maturation Rate According to the Quality of Oocytes

The retrieved oocytes that were grouped in different grades were cultured in IVM medium for maturation process are described in Section 3.3.4.3. The matured oocytes were confirmed by the extrusion of first polar body (PBI). Oocytes retrieved from LOPU showed the highest maturation rate was obatained from Grade A (77.62±6.7%) followed by Grade B (76.63±4.3%), Grade C (39.64±7.8%) and Grade D (21.97±8.5%), whereby Grades A and B were significantly higher than Grades C and D ($P<0.05$).

However, oocytes retrieved from abattoir showed no significant differences in maturation rates among the groups: Grades A (46.94±7.7%), C (63.80±7.8%) and D (58.24±7.4%), however, Grade C oocytes were significantly higher than those of Grade B (39.54±5.6%). When comparing the 2 oocyte sources according to respective grade of oocytes, LOPU-derived oocytes was significantly higher ($P<0.05$) than abattoir-derived oocytes on the maturation rate in good quality of oocytes of Grades A and B. The data are presented in Table 4.3 and 4.4.

4.1.3 Cleavage Rate of Cloned Embryos from Two Different Oocyte Sources (LOPU versus Abattoir)

A total of 108 oocytes from LOPU and 202 oocytes from abattoir were subjected to intraspecies SCNT. The total maturation rate of oocytes, successful rate of enucleated oocytes and successful rate of injected oocytes were analysed and presented in Table 4.5. LOPU-derived oocyte was significantly higher ($P<0.05$) than abattoir-derived oocyte (76.87±9.1 vs. 67.89±3.8%), but no significant difference ($P>0.05$) was detected in successful rate of enucleated oocytes and successful rate of injected oocytes.

Table 4.6 shows the cleavage rate of cloned embryos at 2-, 4-, 8 cell and morula for both sources of oocytes. There were significant differences in cleavage rate of cloned embryos in LOPU versus abattoir: 2-cell (86.84±7.0 vs. 41.04±11.3%, respectively), 4-cell (84.03±8.5 vs. 35.06±10.8%, respectively), 8-cell (71.12±6.8 vs. 24.30±9.9%, respectively) and morula (47.59±7.2 vs. 16.48±7.2%, respectively).

Graphical presentation of the results for the effect of oocytes sources on cleavage rate of cloned embryo is depicted in Figure 4.3.

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Table 4.1: Distribution (%; mean±SEM) of oocyte quality obtained from different sources

Source of oocyte	No. of ovaries	No. of oocytes retrieved	Percent distribution of oocytes quality (n)			
			Grade A	Grade B	Grade C	Grade D
LOPU	22	236	32.46 ± 4.4^{byz} (78)	36.24 ± 2.6^{az} (86)	24.56 ± 3.3^{ay} (55)	6.74 ± 1.7^{ax} (17)
Abattoir	18	263	19.80 ± 4.4^{ay} (50)	33.23 ± 2.7^{az} (89)	28.11 ± 2.9^{az} (77)	18.86 ± 2.0^{by} (47)

*Calculation was based on oocytes retrieved from each ovary
^{ab} Means with different superscripts in a column within a group were significantly different (P<0.05)
^{xyz} Means with different superscripts in a row within a group were significantly different (P<0.05)

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Table 4.2: Number (mean±SEM) of oocyte per ovary for different grades of oocytes obtained from different source

Source of Oocyte	No of ovaries	No. of oocytes retrieved/ ovary	Number of oocytes per ovary (n= total number)			
			Grade A	Grade B	Grade C	Grade D
LOPU	22	10.73 ± 0.9^{a}	3.55 ± 0.7^{ayz} (78)	3.91 ± 0.4^{az} (86)	2.50 ± 0.46^{ay} (55)	0.77 ± 0.2^{ax} (17)
Abattoir	18	14.61 ± 1.2^{b}	2.78 ± 0.4^{ay} (50)	4.94 ± 0.5^{az} (89)	4.28 ± 0.6^{bz} (77)	2.61 ± 0.3^{by} (47)

*Calculation was based on oocytes retrieved from each ovary
^{ab} Means with different superscripts in a column within a group were significantly different (P<0.05)
^{xyz} Means with different superscripts in a row within a group were significantly different (P<0.05)

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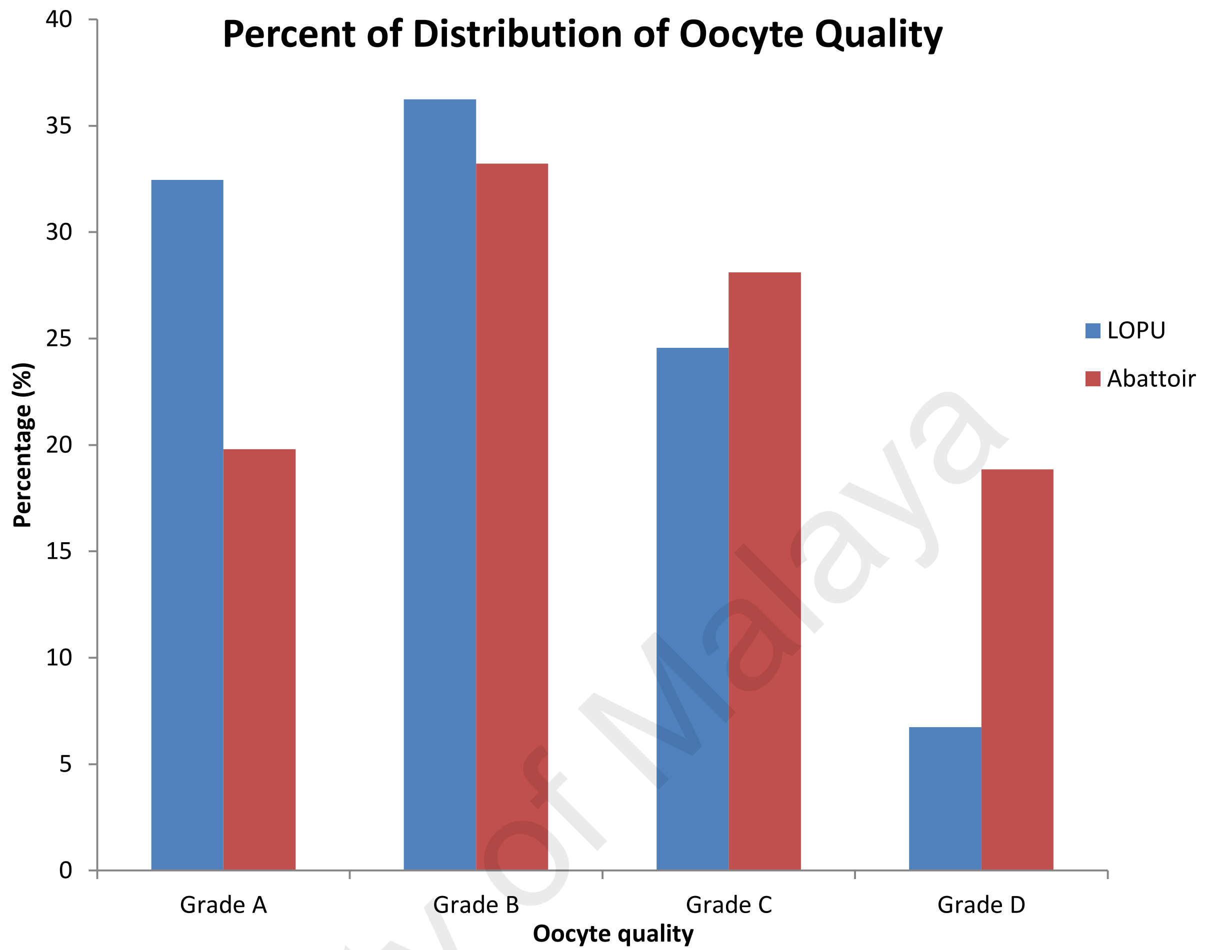

Figure 4.1: Comparison between two different sources of oocytes on distribution of oocyte quality.

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Table 4.3: Maturation rate of oocyte according to its grade obtained from different sources

Source of Oocyte	No. of oocytes retrieved/ ovary	Overall maturation rate (%)	Matured oocyte (%)			
			A	B	C	D
LOPU	10.73 ± 0.9^{a}	68.56 ± 3.8^{b} (160/236)	77.62 ± 6.7^{bz} (61/78)	76.63 ± 4.3^{bz} (63/86)	39.64 ± 7.8^{ay} (30/55)	21.97 ± 8.5^{ay} (6/17)
Abattoir	14.61 ± 1.2^{b}	53.26 ± 4.2^{a} (136/263)	46.94 ± 7.7^{ayz} (24/50)	39.54 ± 5.6^{ay} (40/89)	63.80 ± 7.8^{bz} (45/77)	58.24 ± 7.4^{byz} (27/47)

*Calculation was based on oocytes retrieved from each ovary.
() Number in bracket means the number of matured oocyte over total of oocytes used
^{ab} Means with different superscripts in a column within a group were significantly different ($P<0.05$)
^{yz} Means with different superscripts in a row within a group were significantly different ($P<0.05$)

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Table 4.4: Number (mean±SEM) of matured oocyte quality obtained from different source

Source of Oocyte	No. of oocytes retrieved/ ovary	No. of matured oocytes/ ovary	Number of oocytes per ovary (n=total number)			
			Grade A	Grade B	Grade C	Grade D
LOPU	10.73±0.9[a]	7.27±0.7[a]	2.77±0.5[bz] (78)	2.86±0.3[az] (86)	1.36±0.3[ay] (55)	0.27±0.1[ax] (17)
Abattoir	14.61±1.2[b]	7.56±0.7[a]	1.33±0.2[ax] (50)	2.22±0.4[ayz] (89)	2.50±0.3[bz] (77)	1.50±0.2[bxy] (47)

*Calculation was based on oocytes retrieved from each replication.
[ab] Means with different superscripts in a column within a group were significantly different ($P<0.05$)
[xyz] Means with different superscripts in a row within a group were significantly different ($P<0.05$)

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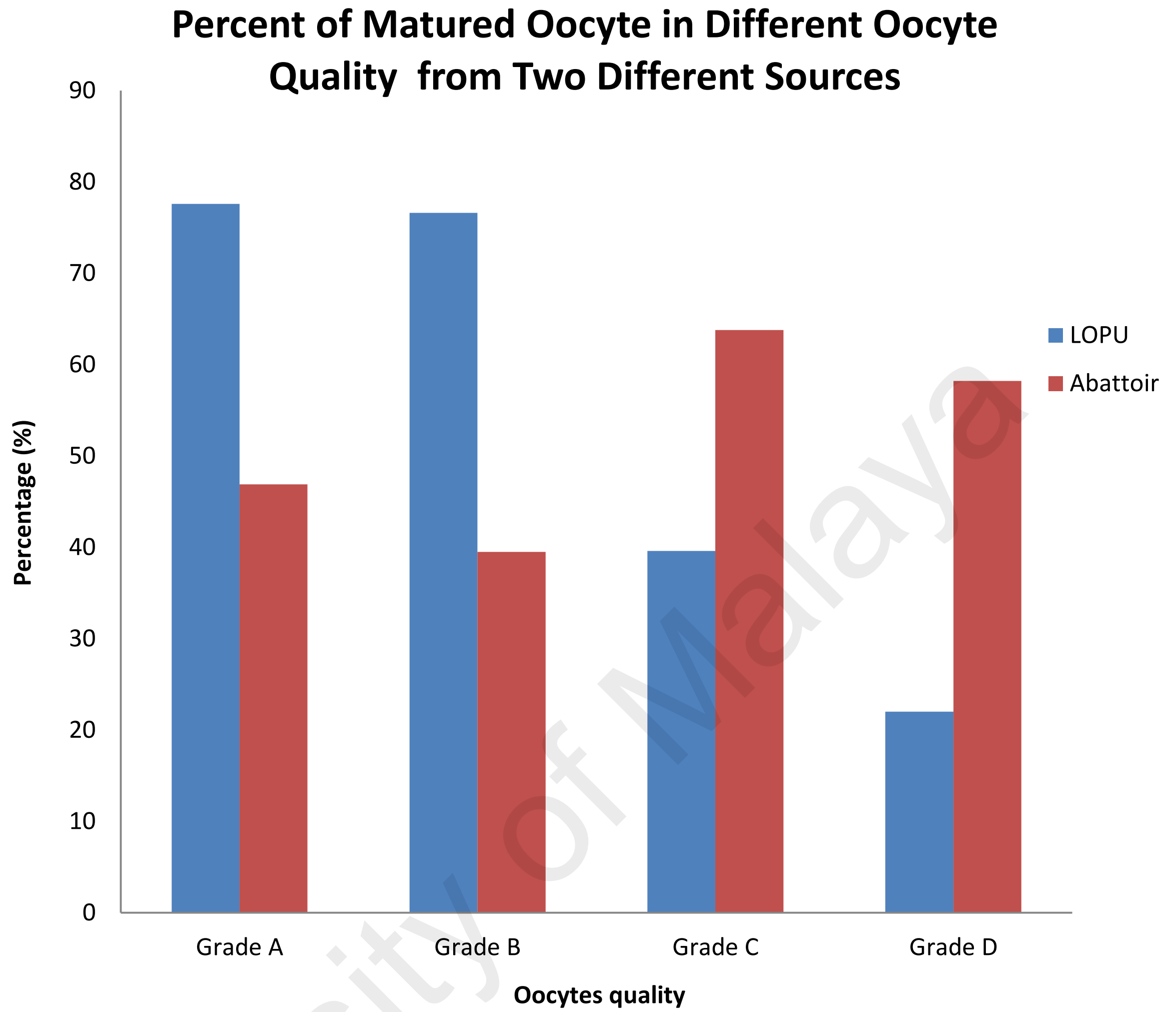

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Figure 4.2: Comparison between two different sources of oocytes on maturation rate after *in vitro* maturation.

Table 4.5: Maturation rate, enucleation rate and injection rate of oocyte from different oocyte sources

Source of oocytes	Total no. of oocytes	Percent of matured oocytes (MII)	Percent of enucleated oocytes	Percent of injected oocytes
LOPU	108	76.18±3.4[a] (82/108)	92.06±5.3[a] (75/82)	94.32±4.0[a] (70/75)
Abattoir	202	67.89±3.8[a] (136/202)	93.81±2.9[a] (127/136)	99.23±3.5[a] (125/127)

*Calculation was based on oocytes retrieved from each replication.
() Number in bracket means the number of matured oocytes or enucleated oocytes or injected oocytes over total of oocytes used
[ab] Means with different superscripts in a column within a group were significantly different ($P<0.05$)

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Table 4.6: Cleavage rate of reconstructed oocyte after SCNT from different sources

Source of oocytes	No. of reconstructed oocytes	Percent of cleaved intra-SCNT cloned embryos (n=total number)			
		2-cell	4-cell	8-cell	Morula
LOPU	70	86.84 ± 7.0^{bz} (59)	84.03 ± 8.5^{bz} (57)	71.12 ± 6.8^{bz} (49)	47.59 ± 7.2^{by} (32)
Abattoir	125	41.04 ± 11.3^{az} (58)	35.06 ± 10.8^{az} (50)	24.30 ± 9.9^{az} (35)	16.48 ± 7.2^{az} (23)

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

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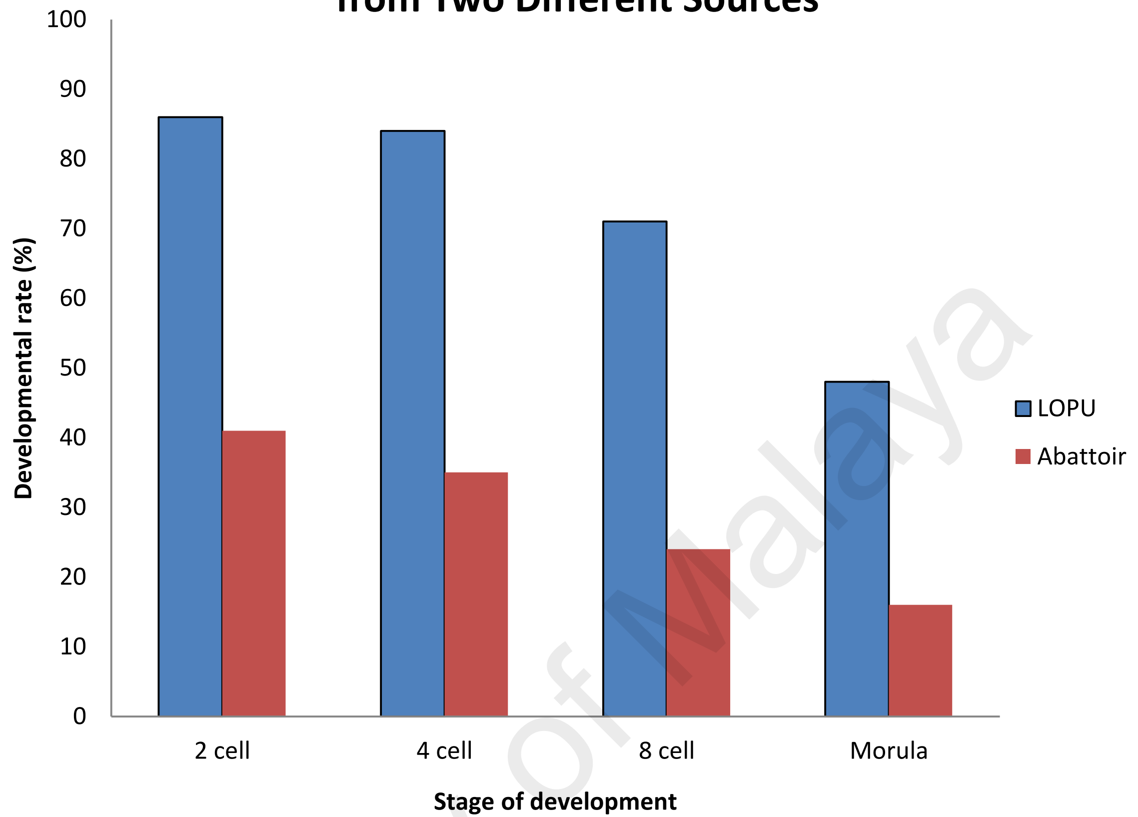

Figure 4.3: Comparison between two different sources of oocytes on developmental rate after intraspecies SCNT.

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4.2 EFFECT OF TIME INTERVAL FROM HORMONAL TREATMENT TO LAPAROSCOPIC OOCYTE PICK-UP (LOPU) ON CAPRINE STIMULATION RESPONSE, OOCYTE RECOVERY AND MATURATION RATE (EXPERIMENT 2)

This experiment was conducted to evaluate the effect of time interval from PMSG/ hCG injection to laparoscopic pick-up oocyte (LOPU) on caprine stimulation response, oocyte recovery and maturation rate. The rates of stimulated follicles, ovulated follicles and oocytes retrievals were analysed and depicted in Table 4.7. The results for the distribution of oocytes quality after LOPU in 3 different intervals after PMSG/ hCG injection are shown in Table 4.8. Maturation rate of oocytes according to time interval group is shown in Table 4.9.

4.2.1 Effect of Time Interval from PMSG/ hCG treatment to LOPU on Stimulated Follicle, Ovulated Follicle and Oocyte Retrieval Rate

A total of 39 goats were used in this experiment to determine the effect of 3 different time intervals (36 to 40; 66 to 70; 71 to 75 hours) from hormonal treatment to LOPU on the number of stimulated follicles, number of ovulated follicles and oocyte retrieval rate. Prior to follicle aspiration during LOPU surgery, the ovaries were assessed to indicate the number of stimulated follicles and ovulated follicles (presence of corpus luteum; CL) through a camera system.

Time interval of 36 to 40 hours showed the highest number of stimulated follicles and oocyte recovery rate (16.42±2.1 and 15.25±2.4, respectively) followed by time interval of 71 to 75 hours (14.5±1.6 and 11.00±1.4, respectively) and time interval of 66 to 70 hours

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(11.00±1.4 and 9.17±0.9, respectively). These 3 different time intervals had significant differences in number of stimulated follicles and number of oocyte recovered. However, there was no significant difference in number of ovulated follicles (presence of corpus luteum). The data were analysed and presented in Table 4.7. Figure 4.4 shows the comparison between the 3 different time intervals on number of oocytes retrieved .

4.2.2 Effect of Time Interval from PMSG/ hCG treatment to LOPU on Distribution of Oocyte Quality, Survival Rate and Maturation Rate

After ovarian assesment, the stimulated follicles were aspirated using a OPU needle. A total of 532 oocytes were recovered during LOPU surgery at 3 different time intervals from hormonal treatment. During the process of searching oocytes from the collecting tube, they were assesed and grouped into 4 different grades (Grades A, B, C and D). There were no significant differences in the percentages of distribution of oocytes quality in 3 different time intervals. The data are presented in Table 4.8. Figure 4.5 shows the comparison between 3 different time intervals on the distribution of oocytes quality.

The recovered oocytes were randomly cultured in IVM medium for the maturation process. Before evaluation of MII oocytes, the survival rates of oocytes were counted. Time interval of 66 to 70 hours gave the highest survival rate (95.07±2.3%) followed by time interval of 71 to 75 hours (92.29±2.3%) and 36 to 40 hours (78.73±4.1%). Subsequently, shows the similar result for the maturation rate (75.64±3.4, 65.30±3.6 and 53.92±2.1%, respectively). The data were analysed and shown in Table 4.9.

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Table 4.7: Stimulated follicle, ovulated follicle and oocyte recovery (mean±SEM) for different time intervals from PMSG/ hCG treatment to LOPU

Time intervals (hour)	No. of goats	Stimulated follicle (SF)		Ovulated follicle (OF)		Oocyte recovery	
		Total	No. of SF/goat	Total	No. of OF/goat	Total	No. of oocyte /goat
36 - 40	12	197	16.42±2.1[b]	9	0.75±0.3[a]	183	15.25±2.4[b]
66 - 70	12	132	11.00±1.4[a]	12	1.00±0.7[a]	110	9.17±0.9[a]
71 - 75	15	203	14.5±1.6[ab]	24	1.6±0.6[a]	165	11.00±1.4[ab]

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

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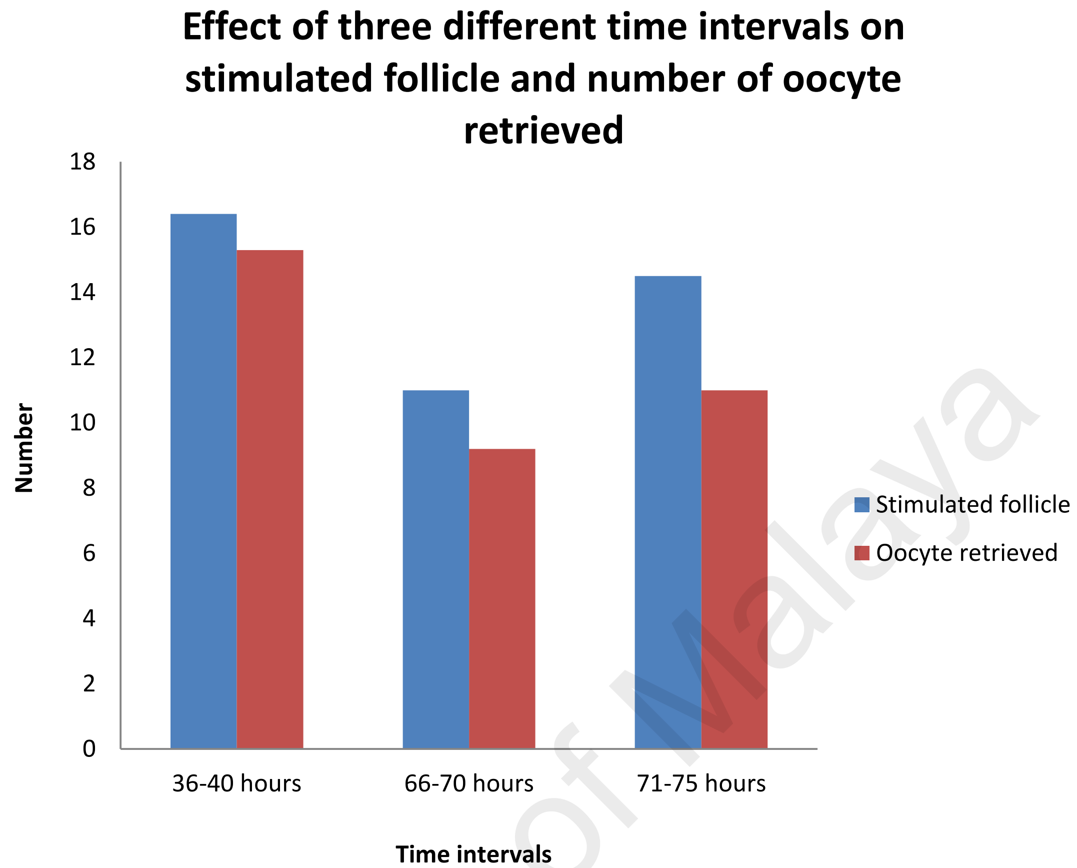

Figure 4.4: Comparison between three different time intervals on stimulated follicles and number of oocytes retrieved.

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Table 4.8: Distribution of oocyte quantity and quality retrieved from LOPU with different time interval of PMSG/ hCG treatment to LOPU

Time interval (hour)	No. of oocytes retrieved	Distribution of oocyte quality (%)			
		Grade A (n)	**Grade B (n)**	**Grade C (n)**	**Grade D (n)**
36 - 40	183	34.04 ± 3.8^{az} (62)	33.98 ± 3.8^{az} (63)	15.65 ± 2.2^{ay} (26)	16.34 ± 2.2^{ay} (32)
66 - 70	110	34.06 ± 4.4^{az} (44)	34.37 ± 3.2^{az} (44)	17.52 ± 3.3^{ay} (21)	14.06 ± 3.0^{ay} (17)
71 - 75	165	29.18 ± 3.6^{az} (56)	36.00 ± 4.7^{az} (72)	17.80 ± 2.3^{ay} (32)	17.03 ± 3.5^{ay} (29)

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

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Table 4.9: Survival rate and maturation rate of goat oocyte according to the time interval from PMSG/hCG to LOPU

Time interval (hr)	Total no. of IVM oocytes	No. of oocytes/ goat (Mean±SEM)	Survival oocytes/ goat (Mean±SEM)	Survival rate (n) (Mean±SEM)	Matured oocytes/ goat (Mean±SEM)	Maturation rate (n) (Mean±SEM)
36 - 40	183	15.25 ± 2.4^{b}	11.92 ± 1.9^{a}	78.73 ± 4.1^{a} (143/183)	8.33 ± 1.4^{a}	53.92 ± 2.1^{a} (100/183)
66 - 70	110	9.17 ± 0.9^{a}	8.58 ± 0.8^{a}	95.07 ± 2.3^{b} (103/110)	7.00 ± 0.8^{a}	75.64 ± 3.4^{c} (84/110)
71 - 75	165	11.00 ± 1.4^{ab}	10.57 ± 1.0^{a}	92.29 ± 2.3^{b} (148/110)	8.0 ± 1.2^{a}	65.30 ± 3.6^{b} (112/165)

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

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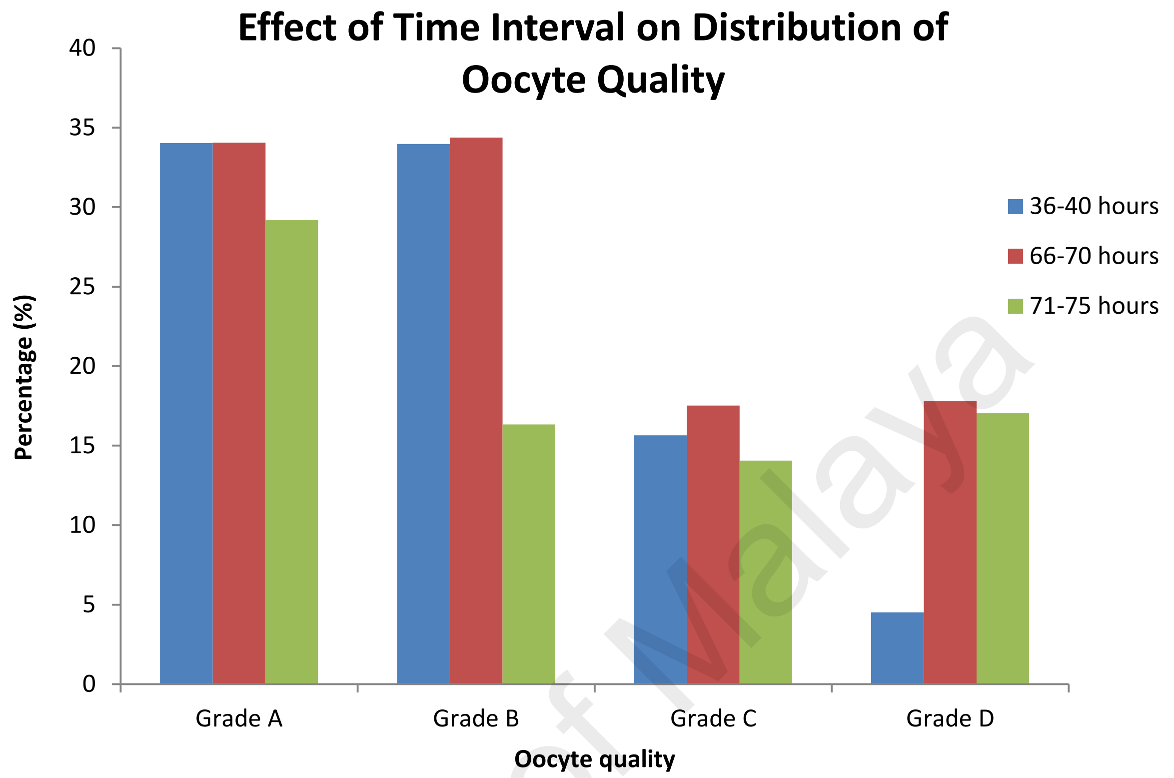

Figure 4.5: Comparison between three different time intervals on distribution of oocyte quality.

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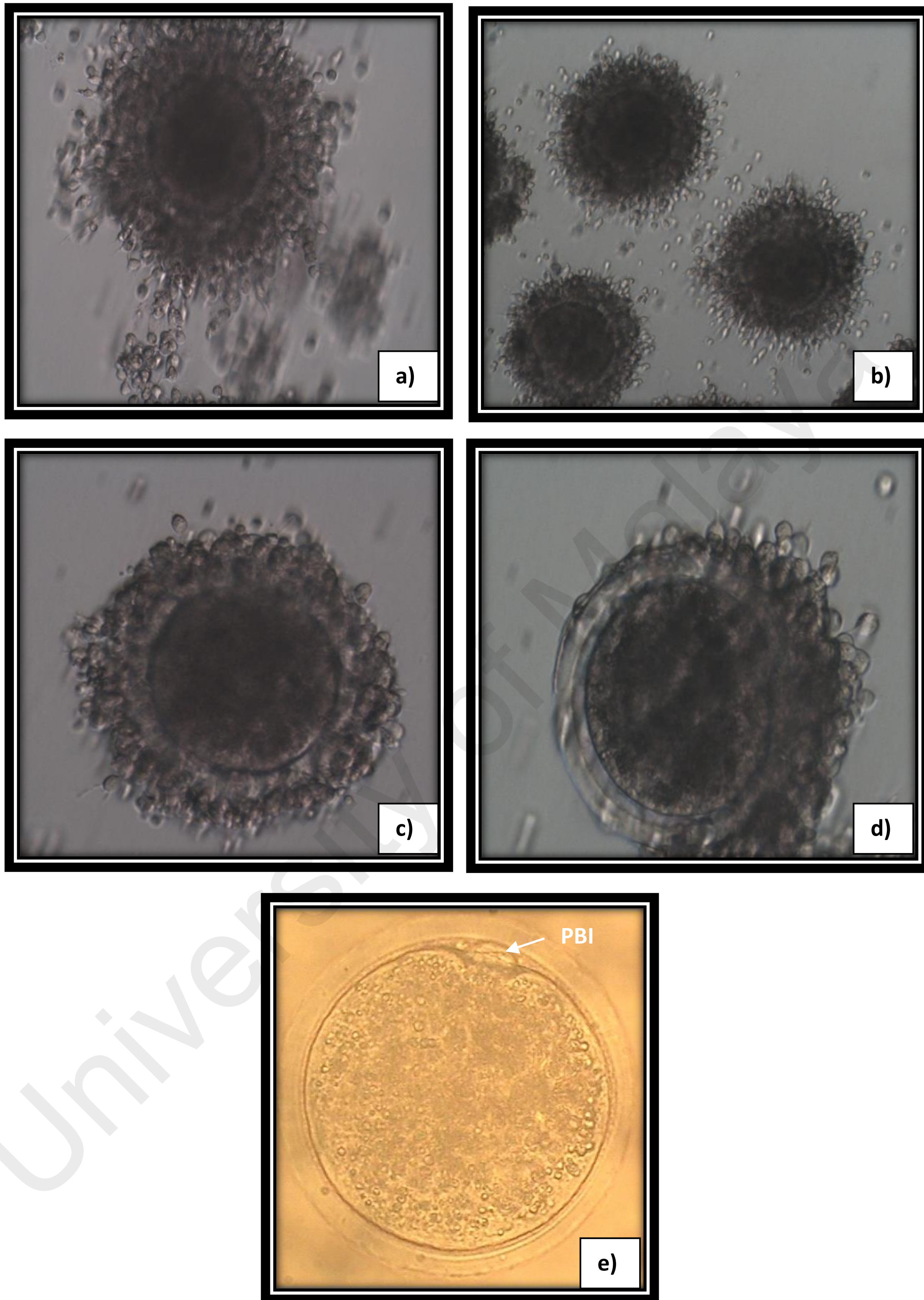

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Note: (Orginal magnification of photomicrographs (a,c,d): 100x; (b): 40x; (e): 200x)

Figure 4.6 (a-e): Different grades of oocytes and matured oocytes: (a) Grade A; (b) Grade B; (c) Grade C; (d) Grade D and (e) matured oocytes confirmed by the extrusion of first polar body (PBI).

4.3 EFFECT OF DIFFERENT TYPES OF DONOR KARYOPLAST (CUMULUS CELL VS. EAR FIBROBLAST CELL) ON PRODUCTION OF CLONED EMBRYOS (EXPERIMENT 3)

This experiment was designed to compare two different types of donor karyoplast (cumulus cell versus ear fibroblast cell) on the efficiency of successfully reconstructed oocytes as well as the production of cloned caprine embryos following intraspecies SCNT. The maturation rate, successful enucleation rate and successful injection rate were analysed and presented in Table 4.10. The cleavage rate of cloned caprine embryos obtained from two different types of donor karyoplast were analysed and summarised in Table 4.11.

4.3.1 Effect of Different Types of Donor Karyoplast on the Manipulation Efficiency for the Production of Intraspecies SCNT Cloned Caprine Embryos

A total of 203 cumulus oocyte complexes (COC) were randomly recovered from a combination of abattoir and LOPU sources, and subsequently cultured in *in vitro* maturation (IVM) medium as described in Section 3.3.4.3 (d). There were a total 133 of matured caprine oocytes which were confirmed by the extrusion of first polar body (PBI). There was no significant difference in the injection rate of two different types of fresh cumulus cell versus ear fibroblast cell (97.61±2.4 vs. 100.00% and 95.15±3.4 vs. 94.32±4.0%, respectively) (Table 4.10).

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4.3.2 Effect of Different Types of Donor Karyoplast on the Cleavage Rate of Cloned Embryos Following Intraspecies SCNT

Table 4.11 shows the cleavage rate of cloned embryos following intraspecies SCNT using two different types of donor karyoplast. The overall cleavage rates of cloned embryos from 2-, 4-, 8-cell and morula for both types of donor karyoplast were 71.46±7.4, 62.83±8.5, 47.82±10.0 and 37.63±8.8%, respectively. The cleavage rate of cloned caprine embryo using ear fibroblast cell (EFC) as donor karyoplast were significantly higher than the cumulus cell (82.76±5.1 vs. 57.17±5.6%, 75.97±7.5 vs. 46.38±7.1%, 64.49±9.8 vs. 27.25±8.7% and 50.82±10.4 vs. 15.59±7.0%, respectively).

Figure 4.7 shows the comparison of cleavage rate after intraspecies SCNT using two different types of caprine donor karyoplast. It was clearly shown that the type of EFC used as donor karyoplast in intraspecies SCNT gave higher cleavage rate compared with the type of cumulus cell.

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Table 4.10: Maturation rate, enucleation rate and injection rate of oocytes using different types of donor karyoplast

Type of donor karyoplast	Total no. of oocytes	Percentage of reconstructed oocytes (%)		
		Maturation rate (n)	Enucleation rate (n)	Injection rate (n)
Cumulus cell	99	68.33±6.9[a] (66)	97.61±2.4[a] (65)	95.15±3.4[a] (61)
Ear fibroblast	104	64.12±4.3[a] (67)	100.00[a] (67)	94.32±4.0[a] (62)
Total	203	66.08±3.8	98.90±1.1	94.70±2.6

(n) Total number of oocytes used
[a]Means with similar superscript in a column within a group were not significantly different (P>0.05)

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Table 4.11: Cleavage rate of reconstructed oocytes using different types of donor karyoplast

Type of donor karyoplast	Total no. of reconstructed oocyte	Cleavage rate (%)			
		2-cell	4-cell	8-cell	Morula
Cumulus cell	61	57.17 ± 5.6^{az} (38)	46.38 ± 7.1^{ayz} (31)	27.25 ± 8.7^{ay} (18)	15.59 ± 7.0^{ax} (14)
Ear fibroblast	62	82.76 ± 5.1^{bz} (52)	75.97 ± 7.5^{bzy} (48)	64.49 ± 9.8^{bzy} (41)	50.82 ± 10.4^{by} (31)
Total	133	71.46±7.4	62.83±8.5	47.82±10.0	37.63±8.8

(n) Total number of oocytes used
^{ab} Means with different superscripts in a column within a group were significantly different (P<0.05)
^{xyz} Means with different superscripts in a row within a group were significantly different (P<0.05)

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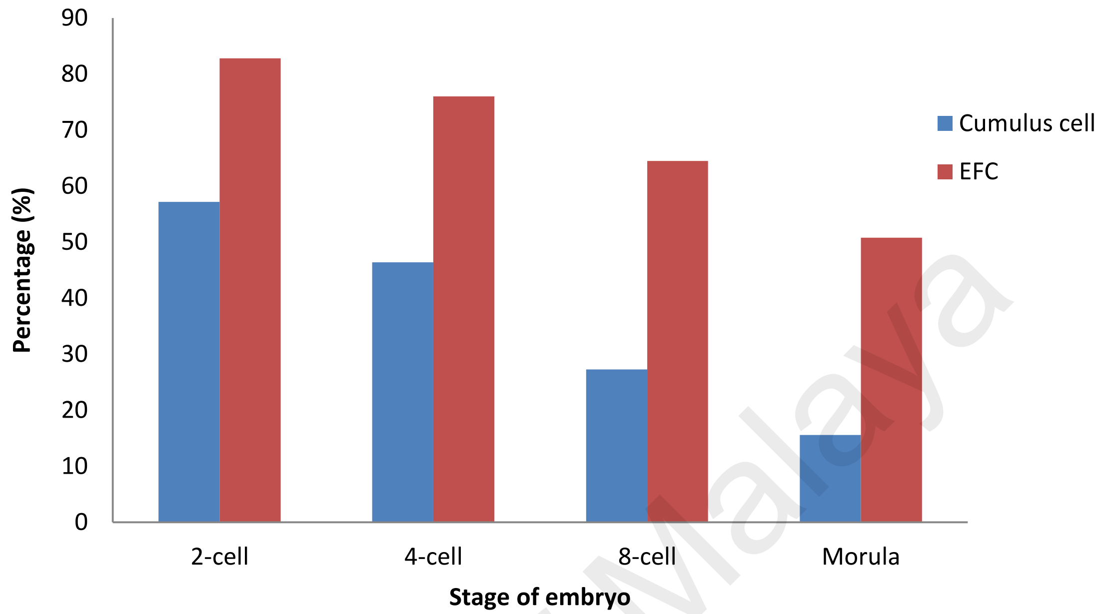

Figure 4.7: Comparison between two different types of donor karyoplast on cleavage rate of cloned caprine embryos.

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4.4 PRODUCTION OF CLONED EMBRYO THROUGH INTRA- AND INTERSPECIES SCNT APPROACHES USING CAPRINE EAR FIBROBLAST CELL (EXPERIMENT 4)

This study was designed to evaluate the efficacy of two different SCNT approaches, namely intraspecies SCNT and interspecies SCNT on the cleavage rate of cloned caprine embryos. The efficiency of cleavage rate of cloned intraspecies (caprine-caprine) embryos was compared with the caprine parthenotes that served as the control group (Table 4.12), while for cloned interspecies (caprine-bovine) embryos was compared with the caprine and bovine parthenotes (Table 4.13). The comparisons on cleavage rate between two SCNT approaches are shown in Tables 4.14 and 4.15. The attempts on cloned embryo transfer were also conducted to test the pregnancy ability of both the intraspecies and interspecies cloned caprine embryos (Table 4.16).

4.4.1 Efficacy of Producing Cloned Caprine Embryos using Intraspecies versus Interspecies SCNT

A total of 123 successfully injected caprine intraspecies cloned embryos and 110 activated caprine oocytes (parthenotes) were cultured in IVC medium and observed daily from Day 2 to Day 8 of *in vitro* development. The cleavage rates of cloned intraspecies (caprine-caprine) embryos were not significantly different from parthenote embryos (caprine). The results of intraspecies cloned caprine embryos versus caprine parthenotes at different stages of embryos were 75.67±3.7 vs. 73.18±8.5% (2-cell), 64.86±5.4 vs. 70.33±8.4% (4-

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cell), 53.14±5.7 vs. 54.64±7. 2% (8-cell), 38.55±5.1 vs. 40.10±5.6% (morula) and 0 vs. 7.36±5.0% (blastocyst). The data were analysed and presented in Table 4.12.

In a different trial, cloned caprine embryos were produced through interspecies (caprine-bovine) SCNT approach, which bovine oocytes were used as the recipient cytoplasts and the caprine ear fibroblasts cells as the donor karyoplasts. The cleavage rates of cloned interspecies embryos were evaluated and compared with the competency of caprine and bovine parthenotes (Table 4.13). Successfully injected caprine interspecies cloned embryos (109) and activated oocytes of caprine and bovine (97 and 159, respectively) were cultured in the same culture system. The cleavage rates of interspecies cloned embryos versus bovine parthenotes embryos at 2-cell to morula stage were similar ($P>0.05$), however, were significantly different ($P<0.05$) from caprine parthenotes at 4-cell to morula stage. No blastocyst in interspecies cloned embryos was obtained. In the case of bovine and caprine parthenotes, there was no difference in the percent of blastocyst (11.64±3.3 vs. 7.36±5.0%) obtained. The data were analysed and presented in Table 4.14.

The production of cloned caprine embryos through two different SCNT approaches (intra- and interspecies SCNT) was evaluated in term of the successful of manipulation procedure and cleavage rate. There were no significant differences between intra- and interspecies in respective maturation rate (66.08±3.8 and 59.68±4.9%, respectively), enucleation rate (98.90±1.1 and 96.95±2.3%, respectively) and injection rate (94.70±2.6 and 100%, respectively) (Table 4.15). The cleavage rates of intraspecies cloned embryos were significantly higher ($P<0.05$) than those of interspecies in all cases of preimplantation developmental stage: 2-cell (75.67±3.7 vs. 55.06±4.1%), 4-cell (64.86±5.4 vs. 48.58±4.5%), 8-cell (53.14±5.7 vs. 34.21±5.3%) and morula (38.55±5.1 vs. 23.24±3.9%).

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Figure 4.8 clearly shows clear graphical presentation in the significant differences ($P<0.05$) in development of cloned embryos between the 2 SCNT approaches.

A total of 29 cloned embryos derived (8-cell and morula stage) from the 2 SCNT approaches were transferred into the uterine horn of 8 recipient does (Table 4.16). Unfortunately, after using ultrasound scanner on Day 36 of gestation, no pregnancy was detected.

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Table 4.12: Cleavage rate of intraspecies SCNT reconstructed oocyte and caprine parthenote embryo

Type of SCNT	Percent cleaved cloned goat embryos				
	2-cell	4-cell	8-cell	Morula	Blastocyst
Intraspecies (caprine-caprine)	75.67 ± 3.7^{az} (94/123)	64.86 ± 5.4^{ayz} (80/123)	53.14 ± 5.7^{ay} (66/123)	38.55 ± 5.1^{ax} (47/123)	0^{a}
PA (caprine)	73.18 ± 8.5^{az} (68/97)	70.33 ± 8.4^{az} (65/97)	54.64 ± 7.2^{ayz} (51/97)	40.10 ± 5.6^{ay} (37/97)	7.36 ± 5.0^{ax} (7/97)

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

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Table 4.13: Cleavage rate of interspecies SCNT, caprine and bovine parthenotes embryo

Type of SCNT	Cleavage rate (%)				
	2-cell	4-cell	8-cell	Morula	Blastocyst
Interspecies (caprine-bovine)	55.06 ± 4.1^{az} (61/109)	48.58 ± 4.5^{az} (53/109)	34.21 ± 5.3^{ax} (38/109)	23.24 ± 3.9^{ax} (26/109)	0^{ax}
PA (caprine)	73.18 ± 8.5^{az} (68/97)	70.33 ± 8.4^{bz} (65/97)	54.64 ± 7.2^{byz} (51/97)	40.10 ± 5.6^{byz} (37/97)	7.36 ± 5.0^{abx} (7/97)
PA (bovine)	64.27 ± 5.18^{az} (99/159)	56.93 ± 5.6^{abyz} (86/159)	46.71 ± 4.3^{aby} (71/159)	34.59 ± 5.3^{abxy} (54/159)	11.64 ± 3.3^{bw} (19/159)

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

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Table 4.14: Percent of maturation, reconstruction oocyte and in vitro development of intraspecies and interspecies SCNT reconstructed oocyte using goat ear fibroblast cell

Type of SCNT	Total no. of oocyte	Maturation rate (n)	Enucleation rate (n)	Injection rate (n)
Intraspecies (caprine-caprine)	203	66.08±3.8[a] (133)	98.90±1.1[a] (132)	94.70±2.6[a] (123)
Interspecies (bovine-caprine)	198	59.68±4.9[a] (112)	96.95±2.3[a] (109)	100[a] (109)

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

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Table 4.15: Percent of maturation, reconstruction oocyte and in vitro development of intra- and inter-SCNT reconstructed oocyte using goat ear fibroblast cell

Type of SCNT	Total no. of reconstructed oocyte	Percent cleaved cloned goat embryos			
		2-cell	4-cell	8-cell	Morula
Intraspecies (caprine-caprine)	123	75.67 ± 3.7^{bz} (94)	64.86 ± 5.4^{byz} (80)	53.14 ± 5.7^{by} (66)	38.55 ± 5.1^{bx} (47)
Interspecies (bovine-caprine)	109	55.06 ± 4.1^{az} (61)	48.58 ± 4.5^{az} (53)	34.21 ± 5.3^{ay} (38)	23.24 ± 3.9^{ay} (26)

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

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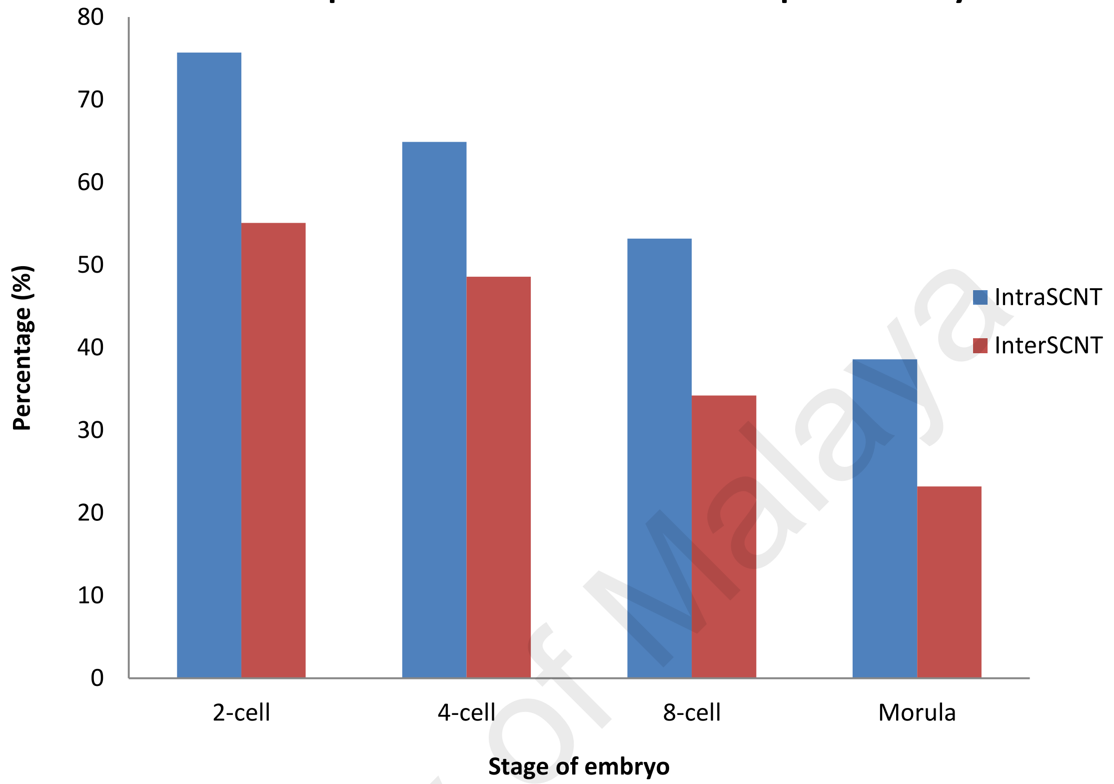

Figure 4.8: Comparison between two approaches of SCNT technique on cleavage of cloned caprine embryo.

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Table 4.16: Embryo transfer of intra- and interspecies cloned embryos

Recipient ID synchronised	Type of caprine embryo transferred	No of embryo transferred (embryo stages)		Pregnancy detection
		Left	Right	
G109001	Intraspecies SCNT	0	3 (Morula) 1 (8-cell)	0
1012	Intraspecies SCNT	1 (8-cell)	3 (Morula)	0
0058	Intraspecies SCNT	1 (Morula)	2 (Morula) 1 (4-cell)	0
12040	Intraspecies SCNT	1 (Morula)	1 (Morula) 1 (8-cell)	0
11028	Interspecies SCNT	1 (Morula) 1 (8-cell)	1 (Morula) 1 (8-cell)	0
11025	Interspecies SCNT	1 (Morula) 1 (8-cell)	1 (Morula)	0
0067	Interspecies SCNT	1 (Morula)	1 (Morula)	0
G108012	Interspecies SCNT	3 (Morula)	2 (Morula)	0

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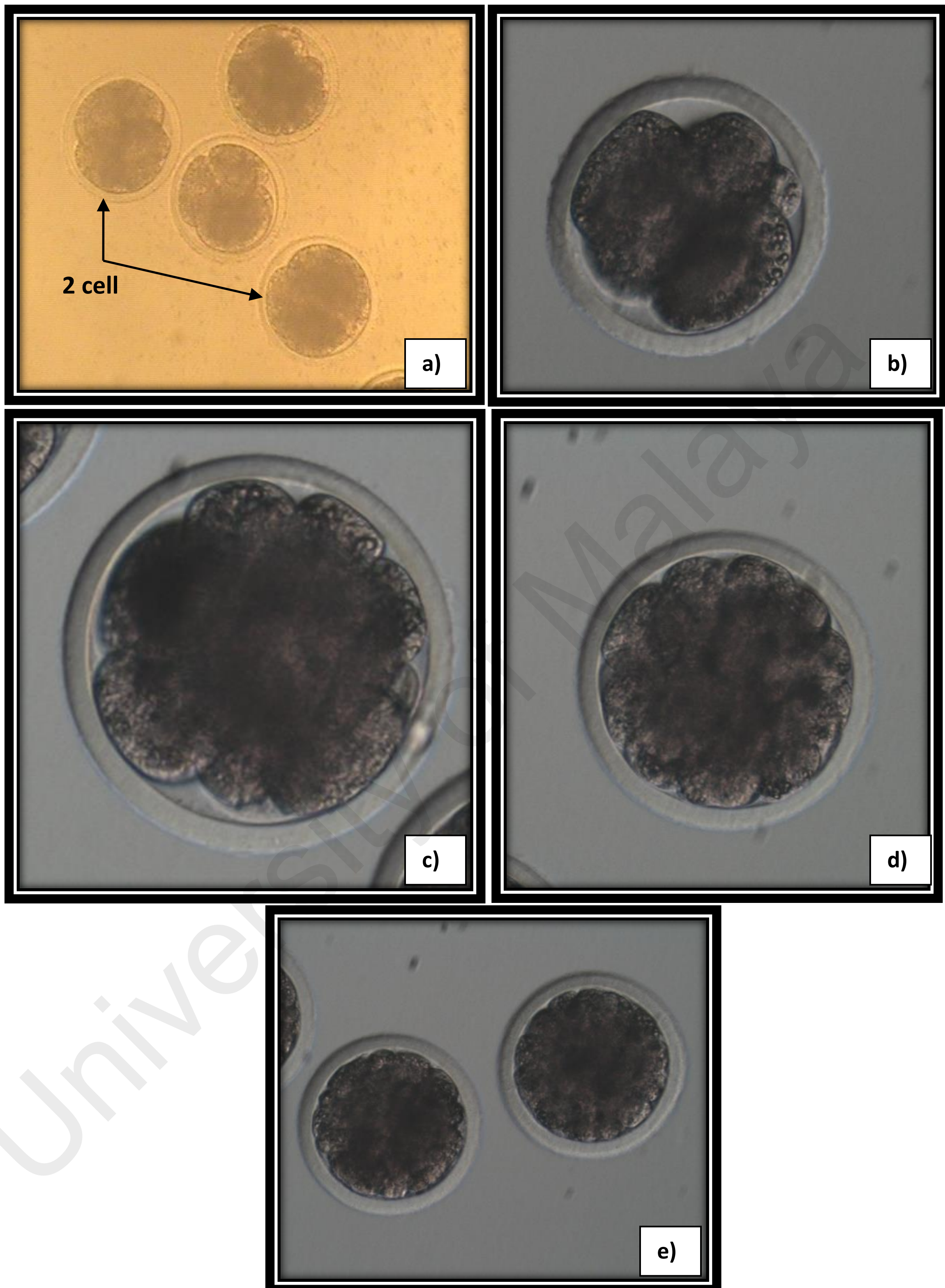

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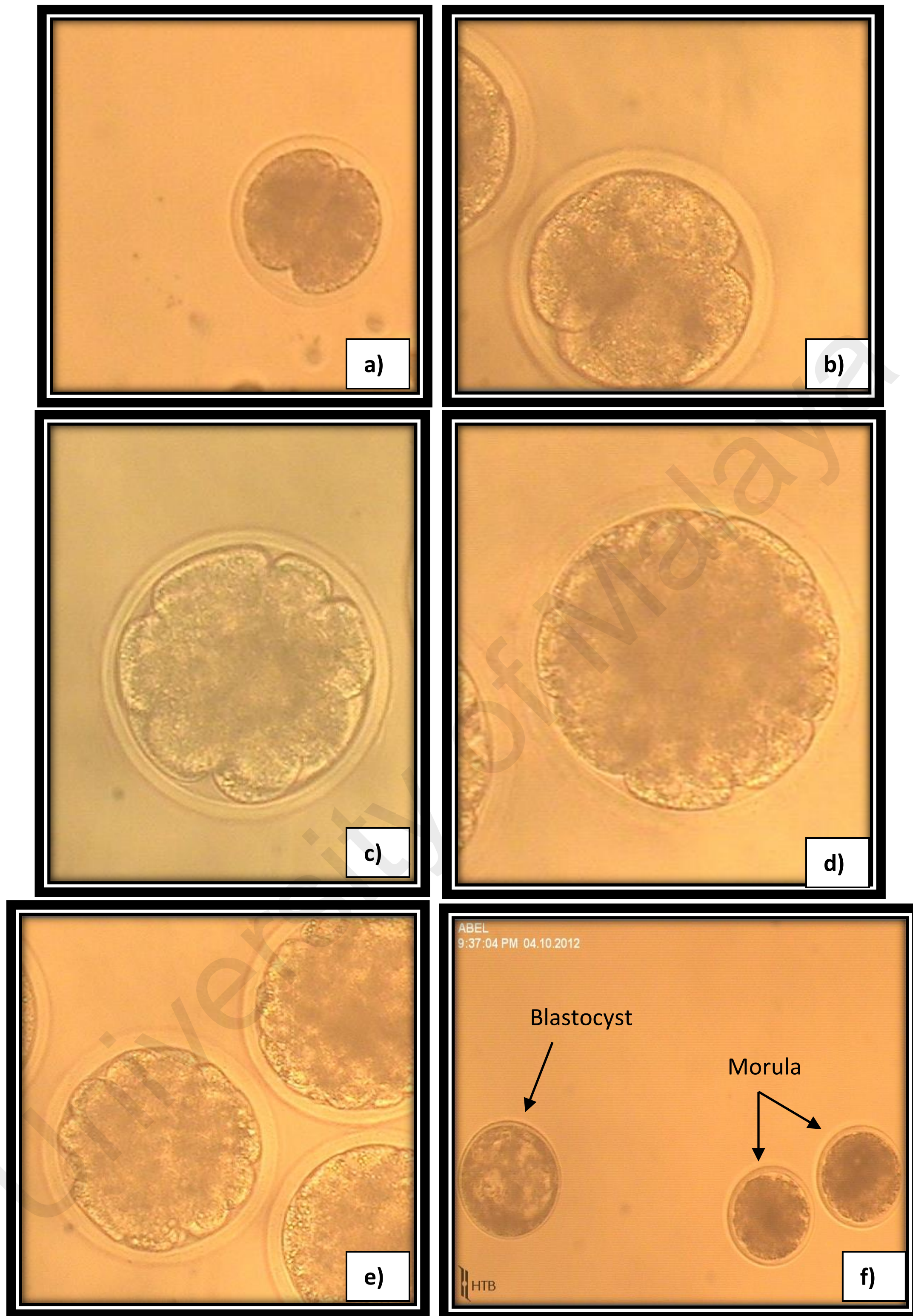
Note: (Orginal magnification of photomicrographs (a): 40x; (b,c,d): 200x; (e): 100x)

Figure 4.9: Cleavage of caprine cloned embryos at different stage: (a) 2 cell; (b) 4 cell; (c) 8 cell; (d) 16 cell; and (e) morula.



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Note: (Orginal magnification of photomicrographs (a,f): 40x; (b,c,d,e): 200x)

Figure 4.10: *In vitro* developmental of caprine parthenotes embryos at different stage: (a) 2 cell; (b) 4 cell; (c) 8 cell; (d) 16 cell; (e) morula and (f) blastocyst.

Chapter 5

5.0 DISCUSSION

University of Malaya

Chapter 5

5.0 DISCUSSION

5.1 EFFECT OF OOCYTE SOURCES (LOPU VERSUS ABATTOIR) ON OOCYTE YIELD, OOCYTE QUALITY, MATURATION RATE AND SUBSEQUENT SCNT PERFORMANCE (EXPERIMENT 1)

This experiment was designed to evaluate the effect of oocyte sources (laparoscopic oocyte pick-up; LOPU versus abattoir ovaries) on the oocyte yield, oocyte quality, maturation rate and subsequent SCNT performance.

5.1.1 Effect of Two Different Oocyte Sources (LOPU versus Abattoir) on Distribution of Oocyte Quality

In the present experiment, oocytes were obtained from two different sources, namely; LOPU and ovaries derived abattoir. Oocyte recovery (OR) from LOPU was conducted after ovarian stimulation using PMSG/hCG as described in Section 3.3.4.3 (a). The number of oocyte retrieved (10.73±0.9 per doe) from LOPU was similar to the results obtained by Baldassarre *et al.,* (2002) who obtained 10.0±5.7 and 10.1±5.6 at 36 and 48 hours post-PMSG stimulation. In other finding using pFSH for hormonal treatment, Kwong (2012) reported the number of oocyte retrieved (11.0±86 per doe) after 71 hours of post-ovarian stimulation. Besides, Abdullah *et al.* (2008) reported number of oocyte retrieved per doe were 11.4±1.7 and 16.1±3.2 at 60 and 72 hours post-ovarian stimulation, respectively. Baldassarre and Karatzas (2004) and Gibbons *et al.* (2007) reported that the minimum

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number of oocyte retrieved per doe is usually >5 oocytes due to individual variation in the response to gonadotropin treatment which are in general agreement with this present finding.

The number of oocytes retrieved from abattoir-derived ovaries source was higher compared to the number of oocytes retrieved from LOPU. This outcome could be due to the different techniques used during oocyte retrieval from ovarian follicles. As for LOPU, only visible follicles (size of follicle: 2 to 3 mm) were aspirated by using OPU needle, meanwhile in oocyte source abattoir-derived ovaries, all the surfaces of ovary were sliced without considering the follicular size. Even though ovarian slicing was a better technique to collect a large number of oocytes from ovaries derived abattoir for IVM-IVF programme (Martino *et al.,* 1994), this technique fails to select oocytes of a particular follicular diameter which reflects the stage of meiosis of the oocyte that may require a different IVM durations to prepare for various reproductive techniques such as IVF, ICSI and SCNT. Besides, in ovarian slicing technique, searching of oocytes may take a longer time because of difficulties due to the presence of undesired ovarian tissues in oocyte collection medium. Hence this could compromise the quality of oocyte thus reducing the maturation and subsequent *in vitro* developmental competence of embryos.

Although low number of oocyte retrieved from LOPU than slicing abattoir-derived ovaries, the oocyte recovery procedure could be performed repeatedly (circa 5 times) on the same doe. This could be achieved by utilising appropriate aspiration technique and grasping instrument whereby the doe's reproductive tract for example the Fallopian tube and uterus was less traumatic and minimal adhesion. It has been shown that when using LOPU, the retrieved oocytes from does of known age and health status could easily be determined which was believed to be important factors affecting the oocyte quantity and

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quality (Amstrong, 1983; Baldassarre *et al.,* 2002). Conversely, in slicing abattoir-derived ovaries source, in some cases (e.g. in Malaysia), the unknown records of origin, breed, age, health condition and phase of oestrous cycle might affect the distribution of oocyte quantity and quality and its subsequent *in vitro* developmental competence.

In this experiment, the morphology of different grades of oocytes (Grades A, B, C and D) were categorised according Rahman *et al.* (2009). The result showed that oocyte derived from LOPU obtained slightly better compared to those derived from abattoir ovaries on distribution of oocyte quantity and quality. It showed that in LOPU, the majority of oocyte quality was in the categories of Grade A and B (69%) which was higher than reported by Abdullah *et al.* (2008) (55%), but lower than reported by Kwong (2012) (79%) and (>80%) (Koeman *et al*., 2003). The preservation of cumulus cell surrounded the oocyte was very important in order to grade the collected oocytes. Therefore, to avoid the detachment of cumulus cell, Baldassarre *et al.* (1994; 2003) suggested the appropriate needle size and the vacuum pressure must be selected properly during LOPU.

Furthermore, oocyte maturation rate showed significantly different between LOPU source and abattoir. This could be due to the wide range of sizes of follicles (2-5 mm) sliced from abattoir ovaries including those of smaller follicles (<2 mm). Oocytes retrieved from the small follicles <2 mm in diameter were less meiotic competence, thus, lower maturation rate in abattoir-derived oocyte was obtained (Motlik et al., 1984). Besides, due to unknown phases of oestrous cycle (oestrus, metoestrus, dieoestrus and prooestrus) resulted from natural hormonal secretion during the oestrous cycle, the cumulus oocyte complexes (COCs) were derived from different follicular and meiotic stages comprising of both viable and atretic follicles (Rodriguez-Gonzalez *et al.,* 2002). Amstrong (2001) reported that oocytes derived from prepubertal oocyte donors have lower quality compared

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to adult donors. Therefore, understanding the history and exact physiological state of oocyte donor derived from abattoir is important to avoid negative outcomes in quantity and quality of oocytes obtained, and the subsequent developmental competence of embryos *in vitro* and *in vivo.*

5.1.2 Effect of Two Different Sources (LOPU versus Abattoir) on Maturation Rate According to the Quality of Oocytes

This present experiment was undertaken to evaluate meiotic competency according to the quality of oocytes (Grades A, B, C and D). The oocytes of different grades derived from different sources: LOPU versus abattoir were cultured same formulation *in vitro* maturation (IVM) medium using IVM duration: 21 to 24 hours and 24 to 27 hours, respectively. The result showed that maturation rate of oocytes derived from LOPU was significantly higher than abattoir-derived oocytes (68.5% vs. 53%). This result, even though slightly lower, was in agreement to the findings reported by Kwong (2012) who obtained high maturation rate in LOPU-derived oocyte (79.6%) compared to abattoir-derived oocytes (69.7%).

The time required for IVM varies among different species. Earlier studies reported that IVM of goat oocytes should be optimised at least 27 hours (Rho *et al.,* 2001) or even 32 hours (Sharma *et al.,* 1996) for abattoir source. Longer time was needed for the abattoir-derived oocytes compared to the LOPU-derived oocytes due to lack of hormonal stimulation in the former thus resulted in collection of oocytes at earlier stages of meiosis (e.g. GV stage). A recent study reported shorter IVM duration for LOPU (18 to 21 hours)

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than slaughterhouse oocytes (24 to 27 hours) was probably due to the latter oocyte collection from stimulated goats and already primed for maturation (Tan *et al.,* 2012).

Kwong (2012) reported another factor that could contribute to the variation might be due to the chemical components supplemented into the IVM medium. Basically, tissue culture medium-199 (TCM-199) is used as a basic medium for IVM of caprine oocytes but other supplement such as hormones, vitamins along with different protein supplements is added to get well-defined medium. Protein supplements are important for optimum maturation and subsequent fertilisation. A lot of experiments have been conducted on different protein supplements, for example, foetal bovine serum (FBS) (Tajik and Esfandabadi, 2003), foetal calf serum (FCS) (Rho *et al.* 2001), steer serum (SS) (Jiménez-Macedo *et al.* 2007), oestrus goat serum (OGS) (Kharche *et al.* 2006), oestrus sheep serum (OSS) (Kharche *et al.* 2009), bovine serum albumin (BSA) (Rajikin *et al.* 1994), peritoneal fluids from rabbit or goat (Malik *et al.* 1999), follicular cells (Jiménez-Macedo *et al.* 2005) and follicular fluids (Cognié *et al.* 2004). Guignot *et al.* (2006) reported that oocytes requirements during maturation may differ according to their origin; abattoir or LOPU-derived oocytes and, thus, the same maturation media could have different efficiencies on both sources. In most of the studies, the basic medium is supplemented with hormones and different concentrations of serum (Cognié *et al.,* 2004; Paramio, 2010). However, all complex supplements such as foetal calf serum, oestrus goat/ sheep serum or follicular fluid lead to a lack of reproducibility and risk of pathogen contamination, thus, there is a trend to use defined or at least semi-defined maturation media, but this information for caprine oocyte is still incipient (Joanna *et al.,* 2014).

Even though there was a significant difference in overall maturation rate between LOPU-derived oocyte and abattoir-derived oocyte sources, when the comparison was

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made in their respective grades (Grades A, B, C and D), only grade A and B from LOPU were significantly higher than abattoir-derived oocytes. This could be due to the abattoir-derived ovaries were sliced all over the surface of ovary including the small size (<2 mm) of follicle resulting in heterogeneous oocyte sizes. Motlik *et al.* (1984) reported that the oocytes retrieved from the small follicles less than 2 mm diameter were less meiotic competence, thus, lower maturation rate in abattoir-derived oocyte might be attributed to the heterogeneity in oocyte size.

In LOPU-derived oocyte, the maturation rate of Grades A and B were significantly higher than Grades C and D. Leibfiied-Rutledge *et al.* (1989) reported that the developmental competence of bovine oocytes was reduced when the number of attached cumulus cells was reduced. However, conflicting results were reported by Kim *et al.* (1996), who reported no difference in developmental competence, even though the cumulus cell numbers from COCs were decreased which was in agreement with the result obtained in abattoir-derived oocytes.

In general, the finding in this experiment demonstrated that LOPU-derived oocytes gave better meiotic competency compared to the abattoir-derived oocytes. Currently, oocytes used for *in vitro* production (IVP) of embryos were collected from abattoir-derived ovaries which provided a cheaper cost of large number of oocytes or by LOPU which could be used repeatedly from given females with high economic or genetic merit that were helpful for research and improvement of IVP conditions. Hence, both oocyte sources are equally important to be studied.

University of Malaya

5.1.3 Cleavage Rate of Cloned Embryos from Two Different Oocyte Sources (LOPU versus Abattoir)

The aim of this study was to evaluate the effect of 2 different oocyte sources (LOPU- versus abattoir-derived oocytes) on cleavage rate after SCNT. The result showed cleavage rate of cloned caprine embryos from LOPU-derived oocyte was significantly higher than abattoir-derived oocyte sources (87% vs. 41%). This result deviated from findings reported by Reggio *et al.* (2001), who reported that no significant difference on cleavage rate of cloned embryos derived from stimulated goats (LOPU) and non-stimulated goats (abattoir-derived ovaries) (57% vs. 56%).

Although goat oocytes could be recovered in relatively large numbers from abattoir ovaries, the oocytes frequently had reduced development potential compared to *in vivo* matured or immatured oocytes collected after gonadotrophin treatment (Cognié *et al.,* 2003). Poor development potential started with maturation that limited the suitability of these oocytes for biotechnology application, and slows the application of *in vitro* embryo production to commercial scale for the purpose of embryo transfer (Khandoker *et al.,* 2001).

There is a general agreement that the oocyte source played an important role (Yang *et al.,* 1998) in developmental competency. The *in vitro* meiotic and developmental competence of oocytes is related to follicle size, oestrous cycle stage and the level of atresia influenced by other follicles, mainly the dominant follicle (Pavlok *et al.,* 1992; Lonergan *et al.,* 1994; Machatkova *et al.,* 1996; Hagemann, 1999). Oocytes will acquire an intrinsically greater *in vitro* developmental capacity if the follicles reach 7 mm in size. On

the other hand, in ovaries with the irrespective follicle size could cause lower developmental potential of embryos.

In different experiment, Rahman *et al.* (2009) obtained fertilisation rate, cleavage, and morula development rates were significantly higher in LOPU compared to abattoir oocytes. Wang *et al.* (2003) obtained high cleavage rate (71 to 90%) using better quality LOPU-derived oocytes (>2 cumulus cell (CC) layers). Conversely, in abattoir-derived oocytes, low cleavage rate range from 51 to 75% was reported in different laboratory using good quality oocytes with at least 2 layers of CC (Keskintepe *et al.,* 1997; Jiménez-Macedo *et al.*, 2007).

In this experiment, the majority of the oocytes from LOPU (Grades A and B) acquired competence for development up to the 8-cell stage, only low percentage of embryos possessed the ability to form morula. However, the results showed morula developmental rate was significantly higher in LOPU compared to abattoir-derived oocytes. Low cleavage rate of cloned embryos from abattoir-derived oocytes compared to LOPU-derived oocyte could be related to the quality of the oocytes used, SCNT technique and IVC media (Wang *et al.,* 2003; Jiménez-Macedo *et al.,* 2005). Gardner *et al.* (2000) and Donnay *et al.* (2002) reported that glucose seemed to be toxic to early cleaving embryos, whereas morula needs some glucose in the medium for further development. This statement was confirmed by Kwong *et al.* (2012) where increased of glucose in culture medium on culture Day 2 improved *in vitro* development of cloned caprine blastocysts after somatic cell nuclear transfer (SCNT).

Therefore, it is very important to know the source and quality of oocytes in order to increase the *in vitro* production (IVP) of embryos from various ART techniques such as

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IVF, ICSI and SCNT. This information could be useful to predict the fate of embryos in *in vitro* maturation, oocytes manipulation, development of embryos and subsequent pregnancy after embryo transfer.

5.2 EFFECT OF TIME INTERVAL FROM HORMONAL TREATMENT TO LAPAROSCOPIC OOCYTE PICK-UP (LOPU) ON CAPRINE STIMULATION RESPONSE, OOCYTE RECOVERY AND MATURATION RATE (EXPERIMENT 2)

This experiment was conducted to evaluate the effect of time interval from hormonal treatment to laparoscopic oocyte pick-up (LOPU) on caprine stimulation response, oocyte recovery and maturation rate. The oocytes were successfully retrieved through LOPU technique as early as 36 hours of time interval from gonadotrophin treatment using PMSG/hCG to LOPU. During LOPU procedure, the ovulation rate increased as the time interval increased 36 to 75 hours. Ritar *et al*. (1984) reported that ovulation time could be observed at 56 hours after hormonal treatment. These different results could be due to the low dosage (200-600 IU) of PMSG used for hormonal stimulation. It shows that, low dosage of gonadotrophin might delay the ovulation time compared to high dosage (Ritar *et al.,* 1984; Baldassarre *et al.,* 2003)

High number of visible follicles per goat was obtained at 36-40 hours (16.4) time interval from PMSG/hCG treatment to LOPU compared to previous reported when at 36 hours (11.5) prior to LOPU (Baldassaree *et al*., 2002). This result could be due to high dosage of administration of PMSG (1200 IU) were used in this experiment. However, compared to our previous findings, the visible follicles were significantly higher when

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used FSH-hCG for hormonal treatment prior to LOPU (Abdullah *et al*., 2008). Time interval group of 36 to 40 hours gave the highest oocyte recovery rate followed by 71 to 75 hours and 66 to70 hours. However, the survival rate of the retrieved oocytes from group of 36 to 40 hr was significantly lower than group 71 to 75 hours and 66 to 70 hours. These results could be due to the retrieved oocytes had not undergone full meiotic competence, and cytoplasmic maturation failed to mature at early phase of follicular development. Further, *in vitro* environment had inadequacies to support oocyte maturation consequently has lower developmental compared to *in vivo* matured oocytes (Sutton *et al*., 2003). Besides, different stage of maturation of oocyte could be observed if too long interval between stimulation and aspiration which was undesirable (De, 1992).

Moreover, the present study showed that the oocytes quantity and quality was not improved at any time interval from PMSG-hCG treatment to LOPU which was in contrast to the previous findings using FSH (Abdullah *et al*., 2008). This was probably due to FSH treatment that gave better results than PMSG prior to LOPU, whereby it increased the diameter of ovarian follicles (2 to 4 mm) that subsequently resulted in higher oocyte recovery (Baldassarre *et al*., 1994). It is interesting to note that, in group of 36 to 40 hours, there was occurrence of small follicular size and premature follicular regression with yellowish in colour correlated with low oocyte survival rate after maturation. Small follicular size may lead to less capacity to acquire developmental competence during follicular development as described by Khatir *et al*. (2007). Besides, at started of follicular phase, less granulosa cells present could give inappropriate microenviroment for the developing oocytes. Granulosa cell monolayer also supported cytoplasmic maturation of growing oocytes to enhance the maturation rate, activate fertilisation and subsequently improve cleavage rate (Teotia *et al*., 2001).

University of Malaya

There are still many factors that could contribute in oocyte recovery through LOPU method such as oestrus synchronisation, oocyte recovery (OR) cycle, age, breed and live weight of goat (Anna *et al*., 2013). Besides, aspiration system was also important consideration that affected oocyte recovery. The ability of laparoscopist, aspiration circuit, suction pressure, the diameter and length of the puncture needle could directly influence success of oocyte recovery (Rodriguez *et al*., 2006).

In vitro maturation enabled the recovery oocytes to reach maturity at metaphase II (MII) stage which was very important for further development after IVP technique. Culture medium for IVM also affected the maturation rate in which high maturation rate (approximately 70%) was obtained by using TCM-199 based medium supplemented with FSH and oestradiol-17β for IVM medium equivalent to that reported by Yadav *et al*. (2010) but lower than Gall *et al*. (1996) and Samake *et al*. (2000). On the positive side, the ovulated follicle [developed corpus luteum (CL)] of the donor does indicate that the does could be used as recipients for production of offspring through embryo transfer (ET) after IVP following LOPU. The advantage of donor-cum-recipient concept is to maximise the usage of a goat whereby in a LOPU cycle the donors with CL can be used as recipients for ET programme.

In summary, considering the quality of oocytes, recovery, survival and maturation rates of oocytes, 66 to 70, and 71 to 75 hours interval time were recommended for LOPU in goat compared to 35 to 40 hours time interval using PMSG-hCG treatment. In spite of occurrence of some ovulated follicles, this option provided a high number of oocytes for various experiments as well as the same donors could be used as recipients in goat ET programme.

University of Malaya

5.3 EFFECT OF DIFFERENT TYPES OF DONOR KARYOPLAST (CUMULUS CELL VS. EAR FIBROBLAST CELL) ON PRODUCTION OF CLONED EMBRYOS (EXPERIMENT 3)

This experiment was to investigate the effect of donor karyoplast type (cumulus cell; CC versus ear fibroblast cell; EFC) on the successful enucleation rate, successful injection rate and subsequent cleavage rate of cloned embryos. The results showed that there was no significant difference in successful enucleation rate and successful injection rate for both donor karyoplast types which was similar to the findings reported by Jongki *et al.* (2004) and Hosseini *et al.* (2008). However, the cleavage rate at 2-cell to morula stage showed EFC (83 to 51%) was significantly higher than CC (57 to 16%). This result were similar to findings reported by Kato *et al.* (2000) who reported high cleavage rate was showed in EFC (83%) compared to CC (73%). In contrast, Jongki *et al.* (2004) reported that cleavage rate of cloned bovine embryos from donor karyoplast of cumulus cell (86%) gave higher percentage than ear fibroblast cell (75%) and foetal fibroblast cell (63%).

Low cleavage rate of cloned caprine embryos used cumulus cell as donor karyoplast could be due the source of cumulus cell and donor cell passage. In this experiment, fresh cumulus cells were harvested after IVM of matured oocytes to be used as donor karyoplast. Lin *et al.* (2010) reported that Passage 4 of cumulus cells showed higher developmental rate compared to fresh cumulus cells. This was mainly described as the effect of the cell cycle (Kitiyanant *et al.,* 2003). Hayes *et al.* (2005) reported that the cell cycle stages include G_0 (growth arrest phase), G_1 (DNA prophase synthesis), S (DNA synthesis phase), G_2 (preparation of mitosis) and M (mitosis phase). There was a high

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possibility of fresh cumulus cells was at S phase (DNA synthesis) while passage culture of cumulus cell was at G0/G1 phase.

Cell-division cycle of the karyoplast was very important to lead success in SCNT developmental embryos. Karyoplasts in the G0 or G1 phase were considered to be the most suitable for nuclear transfer. Campbell *et al.* (1996; 1999b) and Stice *et al.* (2000) reported that the synchronisation of cell cycle at G-phase (G_0 or G_1) donor karyoplast was required to be injected into the successful enucleated oocyte at metaphase II (MII). The MII oocyte has a high concentration of maturation promoting factor (MPF), which can cause premature chromatin condensation (PCC) and breakdown of the nuclear envelope (NEBD). Therefore, when a G-phase nucleus was transferred, developmental competency of the reconstructed embryos was normal without any appearance of DNA damage (Campbell, 1999). In contrast, when S-phase nucleus was transferred, the active DNA replication would result in pulverised chromosomes and subsequently caused extensive DNA damage resulted in abnormal or retarded developmental reconstructed embryos.

High cleavage rate from donor karyoplast of EFC was obtained in this experiment. This could be due to the EFC was used from Passages 3 to 6. Hill *et al.* (2000) reported that, even though some studies showed little effect of the cultured cells as donor karyoplast, it could enhance the development of the reconstructed oocytes after SCNT (Zakhartchenko *et al.*, 1999b; Saikhun *et al.* 2002; Kitiyanant *et al.,* 2003). However, the effects of donor cell passages on embryo development were still controversial. Most studies had used early passage fibroblasts as donor cells for SCNT to produce successful cloned animals (Cibelli *et al.,* 1998; Schinieke *et al.,* 1997; Wells *et al.,* 1999; Onishi *et al.,* 2000; Polejaeva *et al.,* 2000). In contrast, Kubota *et al.* (2000) reported that use of long-term cultured (up to Passage 30) EFC derived from an aged bull did not compromise

University of Malaya

their cloning competence in terms of full-term development, and resulted in higher developmental rates than those derived from use of early passage. Wells *et al.* (1999) reported that the number of donor cell passages did not show any differences on the development of somatic cell nuclear transfer (SCNT) embryos. It was very important to synchronise the donor cells and the recipient cells situated at their respective cell cycle stages, where failed to perform cell synchronisation could inhibit the activation and pronuclear formation of the reconstructed oocytes.

The efficiency of developmental embryos after SCNT of different donor karyoplast type could be due to genetic differences in the cell donors. It had been postulated that some cell populations may have chromatin modifications that prevented their nuclear reprogramming (Campbell and Wilmut, 1996). Furthermore, possible disruptions to the regulation of (imprinted) genes could result from the culture of the cell line and led to perturbations in embryo and foetal development (Walker *et al.,* 1996).

Even though cumulus cell could easily be obtained and cultured, it only could be harvested from female, therefore, it had limited usage to be used to produce cloned male animals using cumulus cells. Hence, the fibroblast was the alternative cell where could be obtained from both male and female. However, different types of donor karyoplast had different levels of epigenetic modification (Santos *et al.,* 2002; 2003). Therefore, it was very important to choose the donor cell type in order to determine the successful cloning outcomes depending on the aims of the research (Oback and Wells, 2002a; 2002b).

University of Malaya

5.4 PRODUCTION OF CLONED EMBRYO THROUGH INTRA- AND INTERSPECIES SCNT APPROACHES USING CAPRINE EAR FIBROBLAST CELL (EXPERIMENT 4)

The aims of this experiment were to produce cloned caprine embryos from 2 different approaches of SCNT, namely, intraspecies (intraSCNT) and interspecies (interSCNT) which subsequently were transferred into recipient does. As a control, parthenogenetic activation (PA) embryos were used where the characteristics of parthenotes embryos structurally and morphologically similar to *in vitro* fertilised embryos. Additionally, parthenote could be a good model system to determine the effect of maternal factor (oocyte) on developmental embryos without involving male factor (sperm) (Hong *et al.,* 2005; Gupta *et al.,* 2008).

When the *in vitro* developmental competency was compared between caprine intraSCNT versus caprine parthenotes, there was no significant difference (P>0.05) in cleavage rate (76 vs. 73%). Similar pattern results in which no significant difference (P>0.05) was shown between interSCNT and bovine parthenotes (55 vs. 64%). Lu *et al.,* (2005) reported that the similarity of developmental competency in intra- or interSCNT versus parthenote embryos indicated that ear fibroblast cell (EFC) might be sufficient as donor karyoplast to be reprogrammed in the recipient cytoplast. In addition, high cleavage rate of cloned caprine indirectly showed that the manipulation technique and skill were reliable and no negative impact in developmental competence of embryos.

However, there was no blastocyst obtained in this experiment for both SCNT approaches. This could be due to the injection technique whereby whole cell intra-cytoplasmic injection (WCICI) was used in this experiment. Soh *et al.* (2012) reported that

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blastocyst rate (4%) of interspecies cloned caprine using intracytoplasmic injection procedure showed significantly lower than combination of sub-zonal injection and electrofusion (12%). This was described that the low efficiency of cloned caprine embryonic development using the WCICI technique might be caused by the damage of donor DNA induced in the isolated donor karyoplast before injection (Lee *et al.,* 2003; Chen *et al.,* 2007). WCICI technique needed the rupture of plasma membrane of the donor karyoplast by repeatedly pipetting in and out for a few times (circa 3 times) using a microinjection needle on the micromanipulator. Consequently, this WCICI technique could possibly cause injury to the donor karyoplast that would result in reprogramming failure by the recipient cytoplast after injection.

In maturation rates of 2 different species of the recipient cytoplast (caprine versus bovine) that were used in this experiments showed statistically no significant difference. High maturation reflected versatility of the oocytes culture in the *in vitro* maturation (IVM) medium in this experiment that could support *in vitro* development of oocyte for both species of ruminants (caprine and bovine) used in this experiments. Similarly, high enucleation rate and injection rate indicated that both oocytes from caprine and bovine species were suitable to be used as recipient cytoplasts. This suggested that the protocol as well as the skill of the personnel carrying out SCNT were reliable and standardised in this experiment.

In interspecies SCNT (interSCNT), bovine recipient cytoplast had high efficacy to support the developmental competency in embryos than the buffalo recipient cytoplast after injection of karyoplast either from bovine or buffalo (Lu *et al.,* 2005). Similarly, in intraspecies SCNT (intraSCNT), Kwong *et al.* (2011) reported that bovine-bovine intraSCNT showed high cleavage rate (2-cell to morula) compared to caprine-caprine

University of Malaya

intraSCNT). It showed that different species had their different developmental competency involving numerous different factors during the process of SCNT. Therefore, more studies should be conducted particularly on these 2 common ruminant species of recipient cytoplast in order to obtain high production of cloned embryos and subsequent live cloned offspring through intra- and interspecies SCNT. For example, the research should focus on chromosome compatibility between recipient cytoplast and donor karyoplast to be reprogrammed between the above-mentioned species.

In order to enhance the production of cloned caprine embryos and its subsequent live cloned offspring, either through intraSCNT or interSCNT, *in vitro* culture (IVC) medium and culture system was very important. Abdullah *et al.* (2011) reported that none of the caprine intraSCNT and interSCNT developed beyond morula stage. However, in the same laboratory, with increased glucose in IVC medium on culture Day 2, Kwong *et al.* (2012) successfully obtained blastocyst using similar cloning techniques. The same researchers, up to know, still unable to produce cloned caprine offspring.

In this experiment, attempts to transfer cloned embryos into the recipient does were made using both intraSCNT and interSCNT embryos, however, none of 8 attempts showed positive pregnancy. The actual reason this failure is not known. It could be due to numerous factors including quality and quantity of cloned embryos transferred, reproductive and body condition of the recipient does and embryo-recipient-synchrony. In the present experiment, 1 to 5 embryos were transferred to recipient does. Chen *et al.* (2007) and Liu *et al.* (2011) reported that large number of embryos (10 to 40) were transferred per recipient doe could increased pregnancy rate. On the contrary, there were some reports on healthy cloned offspring were obtained when small numbers of embryos (1 to 4) were transferred into the recipient does (Bagiusi *et al.,* 1999; Tang *et al.,* 2011).

University of Malaya

Therefore, issues regarding embryo transfer performance in the caprine still persist and need further detailed research including molecular and genetic bases as well as other intrinsic factors affecting cloned embryo and foetal development *in vivo*.

Even though the both SCNT approaches (intra- and interSCNT) in this study were successfully produce cloned caprine embryos, there were difficulties in getting cloned caprine blastocyst and its subsequent live offspring. Since this SCNT experiment was started in 2008 in our laboratory or even in Malaysia, further study on the factors that could be contributed in increasing the efficiency of production cloned embryos and subsequent live offspring were required.

5.5 GENERAL DISCUSSION

Generally, the *in vitro* production (IVP) of cloned animals by SCNT involves complex series of steps, oocyte collection, donor cell culture, oocyte maturation, enucleation, injection of donor karyoplast, activation, *in vitro* culture and embryo transfer. Each of these steps needs a perfect culture ecosystem both *in vitro* and *in vivo* environment involving the cloning protocol, biological materials used as well as intrinsic and extrinsic factors influencing success of producing viable and normal SCNT embryos, foetuses and offspring. The failure to sustain the process at any one step of SCNT protocol would invariable affect the developmental competence of the cloned products (embryos, foetuses and offspring). In other words, there are anomalies in donor karyoplast and/or recipient cytoplast reprogramming that resulted poor SCNT performance.

University of Malaya

Even though this research produced a significant number of original findings, there were shortcomings and weaknesses in designing and implementing this project due to physical, sources and time constraints. As a result, the author selected a few factors such as source of recipient cytoplast, time interval from hormonal treatment to LOPU and type of donor karyoplast with the aimed to optimise oocyte yield, oocyte maturation and oocyte developmental with the hope to provide new information and possible solution to produce cloned caprine embryos *in vitro* before this SCNT technique incorporated as an integral component of routine caprine management for the future industries.

5.1.1 Source of Oocyte (Recipient Cytoplast)

Embryos were produce conventionally via the union of a matured oocyte and a sperm during the process of fertilisation. Embryos also could be produced through assisted reproduction technologies (ART) whereby an oocyte is needed for DNA complement (IVF and ICSI) and for cytoplast (SCNT and PA). In SCNT, an embryo could be produced from a combination of donor karyoplast-recipient cytoplast, without involving male factor (sperm). Therefore, it is evident that a constant availability of oocytes was needed to produce large number of cloned embryos from different sources of oocytes such as LOPU and abattoir. In Malaysia, specifically in caprine embryo IVP studies, it was difficult to maintain the availability of oocyte, due to low population of goats and consequently fewer females were slaughtered in the country. In spite of this constraint, the author of this project managed to obtain satisfactory number of oocytes from abattoir and LOPU to conduct various experiments.

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Abattoir-derived oocytes provided cheaper cost and large number of oocyte, even though it was hard to determine the history of the slaughtered does. In some countries, this oocyte source had been the principle choice due to availability in unlimited quantity of ovaries (Martino *et al.,* 1994; Rho *et al.,* 2001). However, in Malaysia, low number of females was slaughtered due to the government policy and acute shortage of does in the country (Rajikin, 1996) as mentioned earlier. In addition, only few numbers of females were slaughtered and were often conducted in batches at only selected period of time in a year (Kwong, 2012). Thus, it worsened the availability of oocytes from this abattoir source in this country for various ART techniques including cloning experiments as experienced b the present author.

In the case of IVP cloned caprine embryo production through interspecies SCNT in this research, bovine oocyte was chosen to be the recipient cytoplast. The main reason of choosing bovine oocyte was due to the same number of chromosomes which is 60. It was believed that different chromosome number of the recipient cytoplast and donor karyoplast might affect the developmental of reconstructed oocytes after SCNT. However, Dominko *et al.* (1999) reported a finding that disparity of chromosome number between species has no negative effect on the development of embryos, which bovine recipient cytoplast (number of chromosome is 60) supported the developments of embryos after injection of donor karyoplast from various mammalian species [cattle (60); sheep (54); monkey (42); rat (42) and pig (38)]. In this study, bovine abattoir-derived ovaries were the only oocyte source that has been used in this experiment. In Malaysia, most of the slaughtered cattle were imported from other countries such as Australia, Thailand and Vietnam whereby the steers (castrated males) were preferred by the traders to be imported due to larger size of cattle compared to the female cattle for meat consumption. Furthermore, the fertile females

University of Malaya

were mainly retained in the exporter country for their own breeding programme. Once in a while older infertile females with unknown records were imported. In our study, we often faced with low quality oocytes obtained from abnormal and less developed ovaries resulted from the controlled management of these females for slaughtering purposes. It is worth noting that some of such females were without ovaries due to chemical sterilisation of the female so that there were infertile.

To overcome the problem of irregular and unpredictable slaughtering of goats in the country, LOPU was conducted as supplementary source in getting oocytes from live does for caprine embryo IVP (Phua, 2006; Kong, 2010; Kwong 2012). LOPU-derived oocyte was proved to be in efficient for oocyte retrieval (OR) in small ruminants, and high potential in producing good quality oocytes due to hormonal stimulation prior to LOPU (Baldassarre *et al.,* 1994; Gibbons *et al.,* 2007). LOPU may be used repeatedly (circa 5 times) without causing ovarian damage or decrease in the donor's fertility (Ablerio *et al.,* 2002; Peirson *et al.,* 2005).

In multiple ovulation and embryo transfer (MOET) programme, PMSG-treated does were shown to produce a high number of non-ovulated follicles, early regression of CL, short or irregular oestrous cycle and potentially risk in embryo expulsion (Amoah and Galaye, 1990). It was believed that no negative influence to the oocyte recovery (OR) through LOPU since the oocyte was aspirated from the growing follicle prior to ovulation. Tsunada and Sugie (1989) reported that PMSG-treated does (6) showed significantly lower in number of oocytes recovered from LOPU compared to FSH-treated does (10). However, Baldassarre *et al.* (2002) showed no significant between the PMSG -treated does (11) and FSH-treated does (14). This variation of results could be due to the differences in age, breeds, dosage and hormonal regime factors. In addition, the finding from this study

University of Malaya

showed that different range of time interval from hormonal treatment to LOPU played important role in which it influenced the superstimulation responses such as number of follicles, number of oocytes retrieved, number of ovulated follicles, survival rate during IVM and maturation rate. However, further study needs to be conducted to maximise the use the oocytes retrieved from different time interval from hormonal treatment to LOPU, for example, time interval of 36 to 40 hours may require longer IVM duration compared to time interval of 71 to75 hours which may require shorter IVM duration to enhance the maturation rate (metaphase II phase) of oocytes and subsequent embryo development competency.

Even though a large number of oocytes was obtained from abattoir-derived oocytes compared to LOPU-derived oocytes in this experiment, it showed that good oocyte quality, survival rate and maturation rate were comparatively better in LOPU than that of the abattoir-derived oocytes. Rahman *et al.* (2009) reported that high number of oocytes obtained from abattoir-derived oocytes were not survival during IVM which consequently gave low maturation rate of oocyte from the abattoir ovaries. In addition, Kwong (2012) reported that abattoir-derived oocytes were highly heterogenous in nature which the oocytes were came from different follicular stage and atresia of the oocyte that might contribute to the lower maturity of some oocytes that consequently decreased the overall maturation rate in abattoir-derived oocytes. In contrast, in LOPU, live hormonal-treated does were used for oocyte recovery, where only visible follicles (>2 to 3 mm) were aspirated by using OPU needle which have good oocyte quality and high chances to survive and matured for further use *in vitro* embryo production. Besides, in this experiment, time exposure of oocytes in room temperature environment (25ºC) of LOPU-derived oocytes was shorter (circa 30 minutes) than abattoir-derived oocytes (circa 2 to 3

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hours; considering the transportation from slaughterhouse to laboratory) might contribute to the *in vitro* competency.

It is very important to study the effect of oocyte source which could influence the distribution of oocyte quality, oocyte maturation and the developmental competence of embryos from various ART techniques. Hence, it could provide an idea to predict the outcomes of the experiment according to their aims of study.

5.1.2 Donor Karyoplast Types

Cloned animals have successfully been produced after nuclear transfer of somatic cells from cells/tissues/organs such as mammary gland, cumulus cell, oviduct, ear, skin, muscle, liver, tail and Sertoli cell (Kato and Tsunoda, 2010). However, it is still unclear which cell types or cell origins are most successful for mammalian cloning. In this experiment, the author compared the developmental rate of 2 different types of donor karyoplast. The results showed that the cleavage rate of ear fibroblast cell (EFC) has significantly higher compared to fresh cumulus cell (CC). This could be due to the fresh cumulus cell obtained after oocyte maturation was not synchronised at G phase state as the CC was not cultured and cell synchronised which consequently failed to be reprogrammed. Thus, it was suggested to evaluate the effect of different passages of cumulus cell in order to increase the production of cloned embryos, reflecting the efficiency of SCNT performance.

In calves SCNT, Kato *et al.* (2000) reported that no significant difference in blastocyst rate from each type of cell derived from adult, new born foetal male and female donor fibroblast cells. In addition, Kato *et al.* (1998) and Wells *et al.* (1999) demonstrated

that cell lines from cumulus and oviduct cells were suitable to be as the donor karyoplasts. The reasons for the differences in developmental potential among cell origin or cell lines were still unknown, which was no correlation between pregnancy success or prenatal death of young and the number of passages (2 to 7 passages) of donor cells through cell culture. In addition, even though the chromosome numbers in all cultured cell lines were apparently normal, it had the possibility that there might be submicroscopic genetic changes which could cause failure reprogramming that consequently failed to develop full-term.

There are many reports on intra- and interspecies SCNT using either cumulus cell or ear fibroblast cell as they were easily obtained and cultured that resulted in minor injury to the desired animal (Yang *et al.,* 2010). Donor karyoplast from foetal or new born fibroblast of cattle (Saikhun *et al.,* 2002), pig (Lee *et al.,* 2003) and buffalo (Shah *et al.,* 2009) where far higher in cloning efficiency when compared to the adult fibroblast (Selokar *et al.,* 2011). However, there was a limitation to produce male cloned offspring by using cumulus cell as the donor karyoplast could be only retrieved from females. Therefore, it is suggested to use ear fibroblast cell as donor karyoplast which was believed could be developed to full-term.

5.1.3 Summary of the Significant Findings

In summary, there were 4 new findings achieved in this laboratory which include: a) LOPU-derived oocytes was a better source of caprine recipient cytoplast in *in vitro* cloned embryo production compared to abattoir-derived oocyte; b) time interval of 66 to 70 hours from PMSG/hCG treatment to LOPU enhanced oocyte recovery rate, oocyte quality,

University of Malaya

oocyte survival rate and maturation rate at duration of 21 to 24 hours; c) donor karyoplast of ear fibroblast cell gave better SCNT performance compared to fresh cumulus cell; d) production of cloned caprine embryos was higher through intraspecies (caprine karyoplast-caprine cytoplast) SCNT approach compared to interspecies (caprine karyoplast-bovine cytoplast).

5.1.4 Constraint of Studies and Future Direction

Since the study was still new in this laboratory as well as in Malaysia, many fundamental issues and factors on the production of cloned caprine embryos through somatic cell nuclear transfer (SCNT) faced many challenges and needed improvement. As the time progressed in this research, there were some difficulties and constraints that had been faced such as:

a) ***Personnel skill:*** Longer time was needed during learning curve (circa 6 months) to posses the technical skill in preparation of microtools and handling of micromanipulator due to lack of facilities and expertise in this laboratory in the field of SCNT.

b) ***Inconstant of oocyte supply:*** Inconsistency of getting ovaries from abattoir was due to low number of slaughtered females which mainly slaughtered in different batches at the selected period of time. Even though LOPU was conducted for oocyte recovery, the number of oocytes retrieved per goat was not consistent. This could be described the difficulties to standardise the age, breed, health and physiological status of the donor oocyte due to low population of goats and acute shortage of does in Malaysia.

University of Malaya

c) ***Embryo developmental arrest:*** The major problem faced in this laboratory was a failure to obtain blastocysts, which the development of embryos was blocked at morula stage. Even though previous research members had overcome this problem by increasing the glucose in culture medium at Day 2, it seem not working in this research. This might be due to the different methods of donor karyoplast injection during SCNT procedure resulted in the failure of the supposedly cloned embryos to be reprogrammed.

Even though only some aspects of the factors influencing SCNT were evaluated in this research, the overall findings became the basis for further detailed studies to improve the production of cloned embryos and live-born cloned offspring. The following are some of the suggested aspects for further studies:

a) Application of this study to increase animal production especially in production of endangered animals using interspecies SCNT in order to prevent the extinction of the endangered species.

b) Optimisation of cumulus cell as donor karyoplast factors such as number of passages and cell cycle synchronisation.

c) Optimisation of time interval from hormonal treatment to LOPU in order to maximise the usage of retrieved oocytes such as duration of *in vitro* maturation at different time intervals.

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d) Increase in efficiency on production of cloned blastocyst by using whole cell intracytoplasmic injection (WCICI) of donor karyoplast in recipient cytoplast.

e) Optimisation of *in vitro* culture medium by increasing the glucose at Day 2 which could improve the developmental of cloned caprine embryos up to hatched blastocyst.

f) Improvement in efficiency of embryos transfer by increasing the number of cloned embryos transferred per recipient.

University of Malaya

Chapter 6

6.0 CONCLUSIONS

University of Malaya

Chapter 6

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This research was conducted to evaluate the effects of recipient cytoplast sources and donor karyoplast types on the efficiency developmental competence of embryos after somatic cell nuclear transfer (SCNT). For laparoscopic pick-up oocyte (LOPU)-derived oocytes, the evaluation on effect of time interval from PMSG/ hCG injection to LOPU on caprine stimulation response, oocyte recovery and maturation rate was investigated. The conclusion that can be made from this research as follows;

a) Oocyte (recipient cytoplast) derived from LOPU gave higher distribution of good oocyte quality (Grades A and B) and high maturation rate compared to abattoir-derived oocyte. Correspondingly, LOPU-derived oocytes gave better SCNT performance compared to abattoir-derived oocytes.
b) Time interval of 66 to 70 hours was the best time to performed oocyte retrieval from LOPU which gave high percentage of distribution of good oocyte quality (Grades A and B) (68%), oocyte survival rate (95%) and maturation rate (76%).
c) Donor karyoplast type of ear fibroblast cell has significantly improved the cleavage rate of cloned embryos compared to fresh cumulus cell.

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d) Both SCNT approaches (intra- and interSCNT) could be reprogrammed caprine ear fibroblast cell into both species of recipient cytoplast (caprine and bovine).

e) Intraspecies SCNT (caprine-caprine) was shown to be significantly higher in the production of cloned caprine embryos compared to interspecies SCNT (caprine-bovine)

f) Due to low population of goat in Malaysia, production of *in vitro* caprine embryos through assisted reproduction technique such as IVF, ICSI and SCNT was limited. Therefore, interspecies SCNT approach by using different species of recipient cytoplast (bovine oocyte) will be an alternative for the production of caprine embryos in future which could be transferred into surrogate mother to develop full-term.

g) In a nut-shell, high cleavage rate (up to 85%) of cloned caprine embryos was obtained from both SCNT approaches (intra- and interspecies).

University of Malaya

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University of Malaya

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University of Malaya

Appendix 1: LIST OF MATERIALS

University of Malaya

APPENDICES

APPENDIX 1: LIST OF MATERIALS

Appendix Table 1.1: List of equipment/instrument

Equipment/instrument	Model no.	Manufacturer
Abrasive stone or oilstone	-	Hall’s Arkansas Oilstones, USA
Autoclave	HA-300MII	Hirayama Hiclave, Japan
Centrifuge	D37520	Heracus, Germany
CIDR applicator	-	Pharmaciaand Upjohn, N.Zealand
CO2 incubator	HeraCell 240	Heracus, Germany
CO2 insufflator system	PG001	Aesculap, Germany
Digital analytical balance	AB104	Mettler Toledo, Switzerland
Digital camera(X-cam-a)	-	microLAMDA Sdn Bhd, Malaysia
Dissecting microscope	SZH10	Olympus, Japan
Heating stage (Thermoplate)	HATS-U55R30	Tokai Hit, Japan
Impulse sealer	KF-300H	Khind, Taiwan
Inverted Microscope	IX71	Olympus, Japan
Laminar flow cabinet	HLF-120	Gelman Sciences, Australia
Laparoscopic syatem:		Aesculap, Germany
a) Endoscopic camera system	PV431	
b) CCD camera	PV430	
c) Pediatric Storz laparoscope (7 mm)	PE688A	
d) Light probe with fibre optic Cable	PO913	
e) Light system (300 W)	OP927	
Liquid nitrogen tank	SC2/1V	MVE, USA
Microforge	-	Technical Products International, USA

University of Malaya

Micropipette grinder	EG-4	Narashige, Japan
Micropipette dispenser	-	Eppendorf, Germany
Micropipetter puller	P-97	Sutter Instrument Co, USA
Narishige hydraulic micromanipultors	ON3-99D	Narashige, Japan
Omnimeter	Vapro 5520	WESCOR Inc, USA
Oven	40050-IP20	Memmert GimbH, Germany
pH mater	HI-122	Hanna Instruments, Singapore
Refrigerator and freezer	SR-21NME	Samsung Electronics, Korea
Spirit burner	-	Shanghai Machinery
Stereomicroscope	SZH10	Olympus Opticacl, Japan
Surgical set	-	Aesculap, Germany
Surgical table	-	Syarikat Copens Enterprise, Malaysia
Trocar and canula (5.5 mm & 7.0 mm)	EJ456, EJ457	Aesculap, Germany
Ultrapure water purification system	Milli-Q PF Plus	Millipore, USA
Vapor pressure osmometer	5520	Vapro Wescor, USA
Equipment/instrument	Model no.	Manufacturer
Vortex mixer	VTX-3000L	LMS, Japan
Water bath	GMP-GC-19	Memmert GimbH, Germany

University of Malaya

Appendix Table 1.2: List of chemical, reagents and media

Chemical , reagents and media	Catalogue no.	Manufacturer
Penicillin G sodium salt	P3032	Sigma- Aldrich, USA
NaCl	S5886	Sigma- Aldrich, USA
KCl	P5405	Sigma- Aldrich, USA
$NaHCHO_3$	S5761	Sigma- Aldrich, USA
$NaH_2PO_4.H_2O$	S9638	Sigma- Aldrich, USA
$CaCl2.2H_2O$	C3881	Sigma- Aldrich, USA
$MgCl_2.6H2O$	M2393	Sigma- Aldrich, USA
Hepes: $C_8H_{17}N_2O_4SNa$	H3784	Sigma- Aldrich, USA
Hepes: $C_8H_{18}N_2O_4S$	H6147	Sigma- Aldrich, USA
6-DMAP	D2629	Sigma- Aldrich, USA
BME amino acid solution	B6766)	Gibco BRL,USA
$CaCl_2$	C5670	Sigma- Aldrich, USA
Calcium ionophore	C7522	Sigma- Aldrich, USA
D-Glucose	G6152	Sigma- Aldrich, USA
Dulbecco's modified eagle medium, DMEM	D5796	Gibco BRL,USA
EDTA	E9884	Sigma- Aldrich, USA
FBS	16000-044	Gibco BRL,USA
FSH	Folltrophin-V®	Intervet International, Holland
Hyaluronidase	H4272	Sigma- Aldrich, USA
KCl	P5405	Sigma- Aldrich, USA
KH_2PO_4	S5655	Sigma- Aldrich, USA
L-Cystein hydrochloride	C7477	Sigma- Aldrich, USA

University of Malaya

L-Glutamine	G3126	Sigma- Aldrich, USA
MEM non-essential amino acids solution	M7145	Gibco BRL,USA
Mineral oil	M8410	Sigma- Aldrich, USA
Na lactate, 60% syrup	L7900	Sigma- Aldrich, USA
Na_2HPO_4	S5136	Sigma- Aldrich, USA
NaCl	S5886	Sigma- Aldrich, USA
$NaHCO_3$	S5761	Sigma- Aldrich, USA
Oestradiol-17β	E8875	Sigma- Aldrich, USA
Phenol red powder	P3532	Sigma- Aldrich, USA
Streptomycin sulphate salt	S1277	Sigma- Aldrich, USA
TCM-199	11150-059	Sigma- Aldrich, USA
Trypsin	T4799	Sigma- Aldrich, USA
Penicillin G sodium salt	P3032	Sigma- Aldrich, USA
NaCl ()	S5886	Sigma- Aldrich, USA
KCl ()	P5405	Sigma- Aldrich, USA
$NaHCHO_3$	S5761	Sigma- Aldrich, USA
$NaH_2PO_4.H_2O$	S9638	Sigma- Aldrich, USA
$CaCl2.2H_2O$	C3881	Sigma- Aldrich, USA
$MgCl_2$.6H2O	M2393	Sigma- Aldrich, USA
Hepes: $C_8H_{17}N_2O_4SNa$	H3784	Sigma- Aldrich, USA

University of Malaya

Appendix Table 1.3: List of labwares and disposable

Labwares and disposable	Manufacturer
Aluminium foil	Reynolds Consumer Product, USA
Autoclave bag	Megalab Supplies, Malaysia
Blades	Gillette, USA
Borosilicate glass turbing	Drummond Scientific Company, USA
Choromic catgut and suture materials	Aesculap®
Culture dish	Nunc, Denmark
Disposable glass Pasture pipette	Hirschmann® Laborgerete, Germany
Disposable hand tissue	Megalab Supplies, Malaysia
FalconTM conical tube	Becton Dickinson, USA
Glassware (beaker, flask, measuring cylinder etc.)	Pyrex, Japan®
Microcentrifuge tube	Elkay, Costelleo
Micropipette tips without filter	Axygen Scientific, USA
Needle	Terumo Corporation, Japan
Parafilm	Pechiney Plastic Packaging, USA
Schott bottle	Duran, Germany
Serogocal pipette	LP Italian SPA, Italy
Sterile glove	Terumo Corporation, Japan
Syringe	Nunc, Denmark

University of Malaya

Appendix 2: STATISTICAL DATA

University of Malaya

APPENDIX 2: STATISTICAL DATA

Appendix Table 2.1: Distribution (%; mean±SEM) of oocyte quality obtained from different sources

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
GradeA	LOPU	22	32.4595	20.72455	4.41849	23.2708	41.6483	6.67	71.43
	Abattoir	18	19.7972	12.37108	2.91589	13.6452	25.9492	.00	40.00
	Total	40	26.7615	18.40339	2.90983	20.8758	32.6472	.00	71.43
GradeB	LOPU	22	36.2441	12.30700	2.62386	30.7875	41.7007	12.50	60.00
	Abattoir	18	33.2344	11.33502	2.67169	27.5977	38.8712	10.00	53.85
	Total	40	34.8898	11.82630	1.86990	31.1075	38.6720	10.00	60.00
GradeC	LOPU	22	24.5559	15.38084	3.27921	17.7364	31.3754	.00	44.44
	Abattoir	18	28.1111	12.47157	2.93958	21.9091	34.3131	12.50	50.00
	Total	40	26.1558	14.08517	2.22706	21.6511	30.6604	.00	50.00
GradeD	LOPU	22	6.7409	8.18241	1.74450	3.1130	10.3688	.00	22.22
	Abattoir	18	18.8567	8.46902	1.99617	14.6451	23.0682	7.69	40.00
	Total	40	12.1930	10.22633	1.61693	8.9225	15.4635	.00	40.00

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
GradeA	Between Groups	1587.311	1	1587.311	5.190	.028
	Within Groups	11621.392	38	305.826		
	Total	13208.703	39			
GradeB	Between Groups	89.674	1	89.674	.635	.430
	Within Groups	5364.916	38	141.182		
	Total	5454.590	39			
GradeC	Between Groups	125.131	1	125.131	.625	.434
	Within Groups	7612.154	38	200.320		
	Total	7737.285	39			
GradeD	Between Groups	1453.237	1	1453.237	21.035	.000
	Within Groups	2625.301	38	69.087		
	Total	4078.538	39			

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Appendix Table 2.2: Maturation rate of oocyte according to its grade obtained from different sources

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
LOPU	Grade a	11	76.8709	30.05341	9.06144	56.6808	97.0611	.00	100.00
	Grade B	11	75.4973	16.69792	5.03461	64.2795	86.7151	50.00	100.00
	Grade C	11	42.5736	28.56794	8.61356	23.3814	61.7658	.00	80.00
	Grade D	11	30.6064	41.28032	12.44648	2.8739	58.3389	.00	100.00
	Total	44	56.3870	35.78517	5.39482	45.5074	67.2667	.00	100.00
Abattoir	Grade a	9	46.9056	25.09012	8.36337	27.6196	66.1915	.00	75.00
	Grade B	9	41.4756	18.65215	6.21738	27.1382	55.8129	11.11	72.73
	Grade C	9	63.8778	27.81662	9.27221	42.4960	85.2595	14.29	100.00
	Grade D	9	57.4078	22.61026	7.53675	40.0280	74.7876	25.00	100.00
	Total	36	52.4167	24.41099	4.06850	44.1572	60.6762	.00	100.00

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
LOPU	Between Groups	18042.676	3	6014.225	6.498	.001
	Within Groups	37022.192	40	925.555		
	Total	55064.868	43			
Abattoir	Between Groups	2757.137	3	919.046	1.625	.203
	Within Groups	18099.239	32	565.601		
	Total	20856.375	35			

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Appendix Table 2.3: Cleavage rate of reconstructed oocyte after SCNT from different sources

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
twocell	LOPU	7	86.8371	18.54344	7.00876	69.6873	103.9870	50.00	100.00
	Abattoir	12	41.0450	39.01065	11.26140	16.2588	65.8312	.00	100.00
	Total	19	57.9158	39.49254	9.06021	38.8810	76.9506	.00	100.00
fourcell	LOPU	7	84.0314	22.49905	8.50384	63.2233	104.8396	42.86	100.00
	Abattoir	12	35.0550	37.45076	10.81110	11.2599	58.8501	.00	100.00
	Total	19	53.0989	40.18716	9.21957	33.7294	72.4685	.00	100.00
eightcell	LOPU	7	71.1229	17.86871	6.75374	54.5971	87.6487	42.86	100.00
	Abattoir	12	24.3017	34.30417	9.90276	2.5058	46.0975	.00	100.00
	Total	19	41.5516	36.93253	8.47290	23.7507	59.3525	.00	100.00
morula	LOPU	7	47.5857	19.06258	7.20498	29.9558	65.2157	21.43	70.00
	Abattoir	12	16.4842	24.79327	7.15720	.7313	32.2371	.00	80.00
	Total	19	27.9426	27.09917	6.21698	14.8812	41.0040	.00	80.00

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
twocell	Between Groups	9270.595	1	9270.595	8.382	.010
	Within Groups	18803.291	17	1106.076		
	Total	28073.886	18			
fourcell	Between Groups	10604.737	1	10604.737	9.763	.006
	Within Groups	18465.397	17	1086.200		
	Total	29070.134	18			
eightcell	Between Groups	9691.937	1	9691.937	11.087	.004
	Within Groups	14860.280	17	874.134		
	Total	24552.217	18			
morula	Between Groups	4276.512	1	4276.512	8.130	.011
	Within Groups	8942.058	17	526.003		
	Total	13218.570	18			

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Appendix Table 2.4: Cleavage rate of reconstructed oocyte after SCNT from different sources

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
LOPU	2 cell	7	86.8371	18.54344	7.00876	69.6873	103.9870	50.00	100.00
	4 cell	7	84.0314	22.49905	8.50384	63.2233	104.8396	42.86	100.00
	8 cell	7	71.1229	17.86871	6.75374	54.5971	87.6487	42.86	100.00
	morula	7	47.5857	19.06258	7.20498	29.9558	65.2157	21.43	70.00
	Total	28	72.3943	24.28545	4.58952	62.9774	81.8112	21.43	100.00
Abattoir	2 cell	12	41.0450	39.01065	11.26140	16.2588	65.8312	.00	100.00
	4 cell	12	35.0550	37.45076	10.81110	11.2599	58.8501	.00	100.00
	8 cell	12	24.3017	34.30417	9.90276	2.5058	46.0975	.00	100.00
	morula	12	16.4842	24.79327	7.15720	.7313	32.2371	.00	80.00
	Total	48	29.2215	34.57887	4.99103	19.1808	39.2621	.00	100.00

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
LOPU	Between Groups	6727.707	3	2242.569	5.852	.004
	Within Groups	9196.432	24	383.185		
	Total	15924.138	27			
Abattoir	Between Groups	4323.232	3	1441.077	1.222	.313
	Within Groups	51874.594	44	1178.968		
	Total	56197.826	47			

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Appendix Table 2.5: Distribution of oocyte quantity and quality retrieved from LOPU with different time interval of PMSG/ hCG treatment to LOPU

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
36-40	Grade a	12	34.0367	13.26589	3.82953	25.6079	42.4654	17.65	53.57
	Grade B	12	33.9758	13.19431	3.80887	25.5926	42.3591	17.86	61.11
	Grade C	12	15.6467	7.71002	2.22569	10.7480	20.5454	7.69	33.33
	Grade D	12	16.3392	7.46692	2.15551	11.5949	21.0834	5.56	28.57
	Total	48	24.9996	13.84924	1.99897	20.9782	29.0210	5.56	61.11
66-70	Grade a	12	34.0558	15.36524	4.43556	24.2932	43.8184	14.29	66.67
	Grade B	12	34.3717	10.93325	3.15616	27.4250	41.3183	11.11	55.56
	Grade C	12	17.5150	11.60296	3.34949	10.1428	24.8872	7.14	42.86
	Grade D	12	14.0558	10.48366	3.02637	7.3948	20.7168	6.67	44.44
	Total	48	24.9996	15.12320	2.18285	20.6083	29.3909	6.67	66.67
71-75	Grade a	14	29.1771	13.56325	3.62493	21.3460	37.0083	9.09	50.00
	Grade B	14	35.9993	17.65917	4.71961	25.8032	46.1954	9.09	62.50
	Grade C	14	17.7971	8.49138	2.26942	12.8944	22.6999	5.56	28.57
	Grade D	14	17.0279	13.03021	3.48247	9.5044	24.5513	4.76	54.55
	Total	56	25.0004	15.45918	2.06582	20.8604	29.1404	4.76	62.50

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
36-40	Between Groups	3896.662	3	1298.887	11.167	.000
	Within Groups	5118.003	44	116.318		
	Total	9014.665	47			
66-70	Between Groups	4147.635	3	1382.545	9.214	.000
	Within Groups	6601.786	44	150.041		
	Total	10749.421	47			
71-75	Between Groups	3554.166	3	1184.722	6.424	.001
	Within Groups	9590.074	52	184.424		
	Total	13144.240	55			

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Appendix Table 2.6: Cleavage rate of interspecies SCNT, caprine and bovine parthenotes embryo

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
twocell	interSCNT	8	55.0575	11.62956	4.11167	45.3349	64.7801	37.50	69.23
	PA caprine	10	73.1770	26.91721	8.51197	53.9216	92.4324	28.57	100.00
	PA bovine	13	64.2669	18.66843	5.17769	52.9857	75.5481	40.00	100.00
	Total	31	64.7645	20.90806	3.75520	57.0954	72.4337	28.57	100.00
fourcell	interSCNT	8	48.5800	12.72657	4.49952	37.9403	59.2197	31.25	69.23
	PA caprine	10	70.3300	26.65677	8.42961	51.2609	89.3991	28.57	100.00
	PA bovine	13	56.9292	20.16128	5.59173	44.7459	69.1126	30.00	100.00
	Total	31	59.0974	22.07146	3.96415	51.0015	67.1933	28.57	100.00
eightcell	interSCNT	8	34.2138	14.91751	5.27414	21.7424	46.6851	12.50	60.00
	PA caprine	10	54.6390	22.83598	7.22137	38.3031	70.9749	25.00	100.00
	PA bovine	13	46.7077	15.47062	4.29078	37.3589	56.0565	25.00	81.82
	Total	31	46.0419	19.13744	3.43719	39.0223	53.0616	12.50	100.00
morula	interSCNT	8	23.2350	11.14509	3.94039	13.9175	32.5525	6.25	40.00
	PA caprine	10	40.0990	17.72290	5.60447	27.4208	52.7772	12.50	66.67
	PA bovine	13	34.5923	19.17179	5.31730	23.0069	46.1777	.00	60.00
	Total	31	33.4377	17.70209	3.17939	26.9446	39.9309	.00	66.67
blastocyst	interSCNT	8	.0000	.00000	.00000	.0000	.0000	.00	.00
	PA caprine	10	7.3610	15.76860	4.98647	-3.9192	18.6412	.00	50.00
	PA bovine	13	11.6454	11.86164	3.28983	4.4775	18.8133	.00	33.33
	Total	31	7.2581	12.38008	2.22353	2.7170	11.7991	.00	50.00

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ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
twocell	Between Groups	1464.727	2	732.363	1.760	.191
	Within Groups	11649.676	28	416.060		
	Total	13114.403	30			
fourcell	Between Groups	2207.751	2	1103.876	2.491	.101
	Within Groups	12406.735	28	443.098		
	Total	14614.486	30			
eightcell	Between Groups	1864.105	2	932.052	2.861	.074
	Within Groups	9123.144	28	325.827		
	Total	10987.249	30			
morula	Between Groups	1293.820	2	646.910	2.234	.126
	Within Groups	8107.095	28	289.539		
	Total	9400.915	30			
blastocyst	Between Groups	671.773	2	335.887	2.395	.110
	Within Groups	3926.220	28	140.222		
	Total	4597.993	30			

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Appendix Table 2.7: Cleavage rate of reconstructed oocytes using different types of donor karyoplast

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
cumuluscell	2 cell	6	57.1683	13.67509	5.58283	42.8172	71.5195	33.33	75.00
	4 cell	6	46.3783	17.43343	7.11717	28.0831	64.6736	30.77	75.00
	8 cell	6	27.2533	21.33348	8.70936	4.8652	49.6415	7.69	60.00
	morula	6	15.5867	17.13832	6.99669	-2.3989	33.5722	.00	40.00
	Total	24	36.5967	23.29808	4.75570	26.7587	46.4346	.00	75.00
EFC	2 cell	7	82.7557	13.37390	5.05486	70.3869	95.1245	62.50	100.00
	4 cell	7	75.9700	19.92230	7.52992	57.5449	94.3951	37.50	100.00
	8 cell	7	64.4900	25.97275	9.81678	40.4692	88.5108	25.00	100.00
	morula	7	50.8171	27.56745	10.41952	25.3215	76.3128	12.50	100.00
	Total	28	68.5082	24.47703	4.62572	59.0170	77.9994	12.50	100.00

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
cumuluscell	Between Groups	6285.555	3	2095.185	6.760	.002
	Within Groups	6198.860	20	309.943		
	Total	12484.415	23			
EFC	Between Groups	4114.527	3	1371.509	2.729	.066
	Within Groups	12061.844	24	502.577		
	Total	16176.371	27			

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EFC

Duncan

Treatment	N	Subset for alpha = 0.05	
		1	2
morula	7	50.8171	
8 cell	7	64.4900	64.4900
4 cell	7	75.9700	75.9700
2 cell	7		82.7557
Sig.		.057	.162

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.000.

cumuluscell

Duncan

Treatment	N	Subset for alpha = 0.05		
		1	2	3
morula	6	15.5867		
8 cell	6	27.2533	27.2533	
4 cell	6		46.3783	46.3783
2 cell	6			57.1683
Sig.		.265	.075	.301

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

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Appendix 3: LIST OF PUBLICATIONS AND PRESENTATIONS

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Elsevier Editorial System(tm) for Livestock Science
Manuscript Draft

Manuscript Number:

Title: EFFECT OF TIME INTERVAL FROM PMSG-hCG TREATMENT TO LAPOSCOPIC OOCYTE PICK-UP (LOPU) ON STIMULATION RESPONSE, QUALITY OF OOCYTES AND MATURATION RATE IN GOAT

Article Type: Research Paper

Keywords: Laparoscopic oocytes pick-up (LOPU); PMSG-hCG; stimulation response; in vitro maturation

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Abstract: The objective was to investigate whether the time interval from PMSG-hCG treatment to LOPU affected the stimulation response, quality and maturation rate of oocytes. 39 does were used for oestrus synchronisation followed by administration of PMSG (1200 IU) and hCG (250 IU) (i.m.). LOPU was performed at 3 different time intervals: 36-40, 66-70 and 71-75 hr after hormonal treatment. The number of visible follicles, ovulated follicles and oocyte recovery were recorded. The retrieved oocytes were graded (Grade A, B, C and D) before cultured into IVM medium for 22-24 hr. The matured oocytes were evaluated by extrusion of first polar body (PBI). There were no significant differences on stimulation response and quality of oocytes retrieved at 3 different time intervals of hormonal treatment prior to LOPU. However, number of oocytes retrieved per goat was significantly different among 36-40 (15.25±2.4), 71-75 (11.00±1.4) and 66-70 hr (9.17±0.9). Group of 36-40 hr gave the lowest survival rate (78.73±4.1) followed by 71-75 (92.29±2.3) and 66-70 hr (95.07±2.3), which respectively corresponded to the maturation rate (53.92±2.1, 65.30±3.6 and 75.64±3.4). In conclusion, LOPU perform at 66-70 and 71-75 hr after PMSG-hCG treatment could increase the recovery and maturation rate of oocytes to be utilised for IVEP.

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1 EFFECT OF TIME INTERVAL FROM PMSG-hCG TREATMENT TO LAPOSCOPIC
2 OOCYTE PICK-UP (LOPU) ON STIMULATION RESPONSE, QUALITY OF OOCYTES
3 AND MATURATION RATE IN GOAT

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9

10 ABSTRACT

11 The objective was to investigate whether the time interval from PMSG-hCG treatment to
12 LOPU affected the stimulation response, quality and maturation rate of oocytes. 39 does
13 were used for oestrus synchronisation followed by administration of PMSG (1200 IU) and
14 hCG (250 IU) (i.m.). LOPU was performed at 3 different time intervals: 36-40, 66-70 and
15 71-75 hr after hormonal treatment. The number of visible follicles, ovulated follicles and
16 oocyte recovery were recorded. The retrieved oocytes were graded (Grade A, B, C and D)
17 before cultured into IVM medium for 22-24 hr. The matured oocytes were evaluated by
18 extrusion of first polar body (PBI). There were no significant differences on stimulation
19 response and quality of oocytes retrieved at 3 different time intervals of hormonal treatment
20 prior to LOPU. However, number of oocytes retrieved per goat was significantly different
21 among 36-40 (15.25±2.4), 71-75 (11.00±1.4) and 66-70 hr (9.17±0.9). Group of 36-40 hr
22 gave the lowest survival rate (78.73±4.1) followed by 71-75 (92.29±2.3) and 66-70 hr
23 (95.07±2.3), which respectively corresponded to the maturation rate (53.92±2.1, 65.30±3.6

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and 75.64±3.4). In conclusion, LOPU perform at 66-70 and 71-75 hr after PMSG-hCG treatment could increase the recovery and maturation rate of oocytes to be utilised for IVEP.

1. Introduction

In the last few decades, we have witnessed a rapid increase of animal farm using reproductive techniques such as *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT). Prior to *in vitro* embryo production (IVEP), oocytes were obtained from various sources such as ovariectomy, abattoir and laparoscopic oocyte pick-up (LOPU) (Kwong *et al*., 2012). LOPU is an efficient technique to be used for oocyte recovery from live animal which are medium size farm animal such as goat, sheep and pig. Moreover, oocyte recovery from LOPU has the appropriate timing of oocytes maturation as LOPU were subjected to oestrus synchronisation and superovulation (Baldasaree *et al*., 2002). In addition, LOPU gave less traumatic to the live animals which could be used for the repetition of LOPU for many times during the reproductive life of the desired and valuables genetics females (Baldasaree *et al*., 2007).

In goat superovulation programme, gonadotrophin treatment resulted in significant variation in quantity and quality of oocytes recovered from the donor does which consequently yielded low *in vitro* embryo production (IVEP) after *in vitro* maturation (IVM), IVF and *in vitro culture* (IVC). Pregnant mare's serum gonadotrophin (PMSG) and follicular stimulating hormone (FSH) are widely used for superovulation with the ultimate goal to maximise the number of oocytes or embryos obtained. Even though most successful superovulation protocols are using FSH, under unaccustomed to handling the animals,

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46 superovulation can be achieved with a single dose of PMSG compared to FSH which must
47 be injected at 12 hr intervals for 3 or 4 consecutive days (Armstrong *et al*., 1983; Pendleton
48 *et al.*, 1992).

49 A long half-life of PMSG is the main disadvantage for follicular growth which
50 maintaining stimulated and high blood oestradioal concentration after ovulation that
51 important in inducing the synthesis and release of prostaglandin $F_{2\alpha}$ and subsequently
52 resulting early luteal regression (Battye *et al*., 1988). Superovulation using PMSG in goats
53 normally resulted in stimulation and subsequent ovulation, respectively, at approximately 48
54 and 77 hr later (Cameron *et al*., 1988). Prior to LOPU, administration of PMSG at 36 and 48
55 hr showed no significant different in number of follicle and recovery rate in goat
56 (Baldasaree *et al*., 2002). In our previous study, we reported that a prolonged time interval
57 of LOPU at 60 and 72 hr after FSH-hCG can improve oocytes retrieval rate, oocyte quality,
58 maturation rate and goat embryo production (Abdullah *et al*., 2008). In order to reduce the
59 comparative economic expenses, animal handling and to obtain an optimal number of
60 oocytes using PMSG-hCG, the aim of this study was to evaluate the effect of time interval
61 of PMSG-hCG prior to LOPU on stimulation response, quality of oocytes and maturation
62 rate in goat.

63

64 **Materials and Methods**

65 *2.1 Animals*

66 A total of 39 does of various ages (2-4 years) of mixed local breeds were used in this
67 experiment. The goats were fed good quality of Napier grass and soya waste with free

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68 access to water. All animal used in experiment were in accordance to the guidelines of
69 Institute of Biological Sciences, University of Malaya.

70

71 *2.2 Oestrus synchronisation and ovarian stimulation*

72 The does were synchronised using intraviginal controlled internal drug release device
73 (CIDR, progesterone, 0.3 g; New Zealand) for a range 14 days combined with intramuscular
74 injection of 125 µg cloprostenol (Estrumate ®, Schering) 24 hours prior to CIDR removal.
75 During CIDR removal, the follicular development were stimulated by giving the does an
76 intramuscular injection of 1200 IU of PMSG (Folligon, Intervet) followed by 250 IU of
77 Ovidrel (hCG, Switzerland). Subsequently, the donor does were subjected to LOPU at
78 different ranges of designated time intervals (36-40, 66-70, 71-75 hr).

79

80 *2.3 Anaesthesia*

81 The treated does were isolated and off-fed and water in a pen for 24 hr prior to LOPU. The
82 does were given anaesthetic by intramuscular (i.m.) injection with mixed of Xylazine
83 hydrochloride and Ketamine hydrochloride (1:50) (1ml/30kg body weight).

84

85 *2.4 Laparoscopic oocytes pick-up (LOPU)*

86 Surgical sites of the abdominal and inguinal areas were cleaned with Hibiscrub before
87 shaved and was scrubbed with surgical iodine. Before oocyte collection, the aspiration and

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88 flushing system machine (V-MAR 5100 and V-MAR 4000, Cook) was prepared with 50 ml
89 syringe of flushing medium connected to the aspiration needle and collecting tube. The
90 trocar which connected to the plastic tubing that was connected to a CO_2 tank was inserted
91 through a small incision (3-5 cm) near the umbilicus for CO_2 gases insertion. After gas
92 insertion, the laparoscope which connected with the light source and video system was
93 inserted through the trocar sheath to facilitate the visualisation inside the abdomen. The
94 edge of the ovary was held using a pediatric grasper and the ovarian follicles were
95 punctured, flushed and aspirated by the oocyte pick-up (OPU) needle. The collecting tubes
96 were sent to the embryo room for oocyte collection and evaluation.

97

98 *2.5 Stimulation response*

99 Oestrus of goat was observed and recorded after 24 hr administration of PMSG until
100 initiation of LOPU. During LOPU, the size of the ovaries, number of follicles and number of
101 ovulated follicle were counted before the ovarian follicles were punctured.

102

103 *2.6 Oocytes retrieval and grading*

104 The collected cumulus-oocyte complexes (COCs) were washed in a flushing medium
105 supplemented with DPBS (10 tablets/l), penicillin-G (0.06 g/l), streptomycin (0.05 g/l) and
106 PVP-P360 (1 g/l) before washed again in IVM medium droplets for grading according to the
107 cumulus cell layer, as follows:

108 Grade A: COC more than 5 layers of cumulus cells

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Grade B: COC with 3-5 layers of cumulus cells
Grade C: COC with 1-2 of cumulus cells
Grade D: incomplete cumulus cells and cumulus-free oocyte (naked oocyte)

2.7 In vitro maturation (IVM)

The collected COCs from LOPU was cultured in IVM medium consist of TCM-199 as base medium supplemented with TCM-pyruvate, follicle stimulating hormone (FSH, Folltropin®-V), 1 mg/ml oestradiol-17β (Sigma), 50 ug/ml gentamicin sulphate salt (Sigma), 0.9 mg/ml L-cystein (Sigma) and 10% of FBS (Gibco) for 22-24 hr. The matured oocytes were confirmed by the extrusion of the first polar body (PBI).

2.8 Statistical analysis

All data were analysed using ANOVA test. All the statistical analysis was performed using the Statistical Package for Social Sciences for Windows version 16.0 (SPSS, IL, USA).

2. Results

Tables 1 and 2 showed that no significant differences were observed in goat stimulation response and the quality of oocytes at 3 different time interval from administration of PMSG-hCG prior to LOPU. However, number of oocytes retrieved per goat was significantly different between 36-40 hr group (15.25±2.4), 71-75hr (11.00±1.4)

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130 and 66-70 hr (9.17±0.9). The survival rate of oocyte after maturation showed that group of
131 36-40 hr gave the lowest (78.73±4.1) followed by 71-75 hr (92.29±2.3) and 66-70 hr
132 (95.07±2.3) corresponded with the maturation rate (53.92±2.1, 65.30±3.6 and 75.64±3.4,
133 respectively) [Table 3].

134

135 **3. Discussion**

136 In the present study, we have demonstrated that oocytes were successfully retrieved
137 through LOPU technique as early as 36 hr of time interval from gonadotrophin treatment
138 using PMSG/hCG to LOPU. During LOPU procedure, we observed that the ovulation rate
139 increased as the time interval increased 36 to 75 hr. In contrast, ovulation time and rate
140 could be observed at 56 hr after hormonal treatment when administered low dosage (200-
141 600 IU) of PMSG (Ritar *et al*., 1984).

142 High number of visible follicles per goat was obtained at 36-40 hr (16.4) time
143 interval from PMSG/hCG treatment to LOPU compared to previous reported when at 36 hr
144 (11.5) prior to LOPU (Baldassaree *et al*., 2002). This result could be due to high dosage of
145 administration of PMSG (1200 IU) were used in this experiment. However, compared to our
146 previous findings, the visible follicles were significantly higher when used FSH-hCG for
147 hormonal treatment prior to LOPU (Abdullah *et al*., 2008). Time interval group of 36-40 hr
148 gave the highest oocyte recovery rate followed by 71-75 hr and 66-70 hr. However, the
149 survival rate of the retrieved oocytes from group of 36-40 hr was significantly lower than
150 group 71-75 hr and 66-70 hr. These results could be due to the retrieved oocytes had not
151 undergone full meiotic competence, and cytoplasmic maturation failed to mature at early

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152 phase of follicular development. Further, *in vitro* environment has inadequacies to support
153 oocyte maturation consequently has lower developmental compared to *in vivo* matured
154 oocytes (Sutton *et al*., 2003). Besides, different stage of maturation of oocyte could be
155 observed if too long interval between stimulation and aspiration which is undesirable (De,
156 1992).

157 Moreover, the present study showed that the oocytes quantity and quality was not
158 improved at any time interval from PMSG-hCG treatment to LOPU which was in contrast to
159 the previous findings using FSH (Abdullah *et al*., 2008). This was probably due to FSH
160 treatment gives better results than PMSG prior to LOPU whereby it increased the diameter
161 of ovarian follicle (2-4 mm) that subsequently resulted in higher oocyte recovery
162 (Baldassarre *et al*., 1994). It is interesting to note that, in group of 36-40 hr, there was
163 occurrence of small follicular size and premature follicular regression with yellowish in
164 colour correlated with low oocyte survival rate after maturation. Small follicular size may
165 lead to less capacity to acquire developmental competence during follicular development as
166 described by Khatir *et al*., (2007). Besides, at started of follicular phase, less granulosa cells
167 present could give inappropriate microenviroment for the developing oocyte. Granulosa cell
168 monolayer also supports cytoplasmic maturation of growing oocytes to enhance the
169 maturation rate, activate fertilisation and subsequently improve cleavage rate (Teotia *et al*.,
170 2001).

171 There are still many factors could contribute in oocyte recovery through LOPU
172 method such as oestrus synchronisation, oocyte recovery (OR) cycle, ages, breeds and live
173 weight of goat (Anna *et al*., 2013). Besides, aspiration system is also important
174 consideration that affects oocyte recovery. The ability of laparoscopist, aspiration circuit,

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175 suction pressure, the diameter and length of the puncture needle could directly influence
176 success of oocyte recovery (Rodriguez *et al*., 2006).

177 *In vitro* maturation enabled the recovery oocytes to reach maturity at metaphase II
178 (MII) stage which was very important for further development after IVEP technique. Culture
179 medium for IVM also affected the maturation rate in which high maturation rate
180 (approximately 70%) was obtained by using TCM-199 based medium supplemented with
181 FSH and oestradiol-17β for IVM medium equivalent to that reported by Yadav *et al*. (2010)
182 but lower than Gall *et al*. (1996) and Samake *et al*. (2000). On the positive side, the ovulated
183 follicle [developed corpus luteum (CL)] of the donor does indicate that the does could be
184 used as recipients for production of offspring through embryo transfer (ET) after IVEP
185 following LOPU. The advantage of donor-cum-recipient concept is to maximise the usage of
186 a goat whereby in a LOPU cycle the donors with CL can be used as recipients for ET
187 programme.

188 In summary, considering the quality of oocytes, recovery, survival and maturation
189 rate of oocytes, 66-70 and 71-75 hr interval time are strongly suggested to perform LOPU in
190 goat compared to 35-40 hr time interval from PMSG-hCG treatment to LOPU. In spite of
191 occurrence of some ovulated follicles, this option provides a high number of oocytes for
192 various experiments as well as the same donors could be used as recipients in goat ET
193 programme.

194

195

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196 **Acknowledgements**

197 The authors thank the ABEL members for their assistance and UMRG grant (RG068-12BIO
198 and RG052-11BIO) for financial support.

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List of Tables:

Table 1: Stimulation response of different time interval from PMSG/hCG treatment to LOPU

Table 2: Distribution of oocyte quality retrieved from different time interval of PMSG-hCG treatment to LOPU

Table 3: Survival and maturation rate of goat oocyte according to time interval between PMSG/hCG and LOPU

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***Conflict of Interest Statement**

No conflict of interest in this study.

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Table

Tables

Table 1: Stimulation response of different time interval from PMSG/hCG treatment to LOPU

Time intervals (hr)	No. of goats	Stimulated follicle (SF)		Ovulated follicle (OF)		Oocyte recovery	
		Total (n)	No. of SF/ goat (Mean±SEM)	Total (n)	No. of OF/ goat (Mean±SEM)	Total (n)	No. of oocyte/ goat (Mean±SEM)
35 - 39	12	197	16.42±2.1[b]	9	0.75±0.3[a]	183	15.25±2.4[b]
65 - 69	12	132	11.00±1.4[a]	12	1.00±0.7[a]	110	9.17±0.9[a]
70 - 75	15	203	14.5±1.6[ab]	24	1.6±0.6[a]	165	11.00±1.4[ab]

[ab]Means with different superscript in same column were not significantly different (P<0.05)

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Table 2: Distribution of oocyte quality retrieved from different time interval of PMSG-hCG treatment to LOPU

Time interval (hour)	No. of oocytes retrieved	Distribution of oocytes quality (%)			
		Grade A (n)	Grade B (n)	Grade C (n)	Grade D (n)
35 - 39	183	34.04±3.8[a] (62)	33.98± 3.8[a] (63)	15.65±2.2[a] (26)	16.34±2.2[a] (32)
65 - 69	110	34.06±4.4[a] (44)	34.37±3.2[a] (44)	17.52±3.3[a] (21)	14.06±3.0[a] (17)
70 - 75	165	29.18±3.6[a] (56)	36.00± 4.7[a] (72)	17.80± 2.3[a] (32)	17.03± 3.5[a] (29)

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

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Table 3: Survival and maturation rate of goat oocyte according to time interval between PMSG/hCG and LOPU

Time interval (hr)	Total no. of IVM oocytes	No. of oocytes/ goat (Mean±SEM)	Survival oocytes/ goat (Mean±SEM)	Survival rate (n) (Mean±SEM)	Matured oocytes/ goat (Mean±SEM)	Maturation rate (n) (Mean±SEM)
35 - 39	183	15.25±2.4[b]	11.92±1.9 [a]	78.73±4.1[a] (143/183)	8.33±1.4[a]	53.92±2.1[a] (100/183)
65 - 69	110	9.17±0.9[a]	8.58±0.8 [a]	95.07±2.3[b] (103/110)	7.00±0.8[a]	75.64±3.4[c] (84/110)
70 - 75	165	11.00±1.4[ab]	10.57±1.0 [a]	92.29±2.3[b] (148/110)	8.0±1.2[a]	65.30±3.6[b] (112/165)

[abc]Means with different superscripts in same column were significantly different ($P<0.05$)

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In Vitro Cellular & Developmental Biology - Animal

Developmental Potential of Mouse Zona-Free Single Blastomere Derived from Isolated 2-, 4- and 8-Cell Embryos into Blastocyst and Inner Cell Mass (ICM) Outgrowths

--Manuscript Draft--

Manuscript Number:	IVAN-D-13-00252
Full Title:	Developmental Potential of Mouse Zona-Free Single Blastomere Derived from Isolated 2-, 4- and 8-Cell Embryos into Blastocyst and Inner Cell Mass (ICM) Outgrowths
Article Type:	Articles (full research papers)
Keywords:	mouse embryo; isolated single blastomere; inner cell mass; diameter of blastocyst; embryonic stem cell
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Abstract:	Cleavage efficiency of mouse embryos obtained from blastomere separation is still low, therefore the application of this technique to produce embryonic stem cell (ESC) from inner cell mass (ICM) is not yet perfected. The aims of this study were to evaluate the developmental potential of isolated single blastomere (SB) into blastocyst and ICM outgrowth at different stages of embryos. ICR strain females (age: 8-12 weeks; n=47) were superovulated using gonadotrophins and mated with ICR strain males (age: 10-14 weeks; n=20). The 2-cell embryos (n=366) were flushed from the oviduct of the treated females and cultured in vitro before assigned into the following groups: a) 2-cell, b) 4-cell, c) 8-cell and d) control prior blastomere separation. The SB were cultured in vitro until blastocyst stage for daily observation and record on cleavage rates. Developmental rate of SB into blastocyst at 2-cell (77.28±6.77) was greater than 4-cell (63.70±5.35) and 8-cell (55.73±3.35), corresponding to the results of ICM outgrowth at 2-cell (69.29±4.13), gave higher rate followed by 4-cell (55.73±7.81) and 8-cell (41.85±3.58). Diameter of blastocyst decreased as the SB parent embryo stage increased, with the respective ratio of 5:3 (2-cell: 92.55±1.59 vs. 56.48±0.40; 4-cell: 78.71±1.37 vs. 44.02±0.49 and 8-cell: 64.13±2.20 vs. 35.68±0.34) as well as total cell number in blastocyst (2-cell: 43.00±1.48; 4-cell: 28.33±1.15; 8-cell: 8.80±0.58). In conclusion, SB at different stages of mouse embryos is successful produce blastocysts in vitro that can be used as ICM source, which is a prerequisite for establishment of ESC outgrowth.

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Title: Developmental Potential of Mouse Zona-Free Single Blastomere Derived from Isolated 2-, 4- and 8-Cell Embryos into Blastocyst and Inner Cell Mass (ICM) Outgrowths

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Abstract

Cleavage efficiency of mouse embryos obtained from blastomere separation is still low, therefore the application of this technique to produce embryonic stem cell (ESC) from inner cell mass (ICM) is not yet perfected. The aims of this study were to evaluate the developmental potential of isolated single blastomere (SB) into blastocyst and ICM outgrowth at different stages of embryos. ICR strain females (age: 8-12 weeks; n=47) were superovulated using gonadotrophins and mated with ICR strain males (age: 10-14 weeks; n=20). The 2-cell embryos (n=366) were flushed from the oviduct of the treated females and cultured *in vitro* before assigned into the following groups: a) 2-cell, b) 4-cell, c) 8-cell and d) control prior blastomere separation. The SB were cultured *in vitro* until blastocyst stage for daily observation and record on cleavage rates. Developmental rate of SB into blastocyst at 2-cell (77.28±6.77) was greater than 4-cell (63.70±5.35) and 8-cell (55.73±3.35), corresponding to the results of ICM outgrowth at 2-cell (69.29±4.13), gave higher rate followed by 4-cell (55.73±7.81) and 8-cell (41.85±3.58). Diameter of blastocyst decreased as the SB parent embryo stage increased, with the respective ratio of 5:3 (2-cell: 92.55±1.59 vs. 56.48±0.40; 4-cell: 78.71±1.37 vs. 44.02±0.49 and 8-cell: 64.13±2.20 vs. 35.68±0.34) as well as total cell number in blastocyst (2-cell: 43.00±1.48; 4-cell: 28.33±1.15; 8-cell: 8.80±0.58). In conclusion, SB at different stages of mouse embryos is successful produce blastocysts *in vitro* that can be used as ICM source, which is a prerequisite for establishment of ESC outgrowth.

Keywords: mouse embryo, isolated single blastomere, inner cell mass, diameter of blastocyst, embryonic stem cell

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INTRODUCTION

The embryonic stem cell (ESC), derived from the inner cell mass (ICM) of the blastocyst, have special characteristics: self-renewal which is the cells can divide to duplicate themselves for a prolonged period of time without differentiating and pluripotent which is the cell can differentiate to different types of cells. The ESC research has the potential to be applied in regenerative medicine which essentially cures serious degenerative diseases such as heart disease, Parkinson's disease and leukaemia. However, derivations of ESC involve destruction of pre-implantation human embryos have raised many ethical issues.

Blastocysts derived from isolated single blastomere at early stage of embryos *inter alia* were commonly used for multiplication of genotypes and pre-implantation genetic diagnosis (PGD) (Bielanska *et al.* 2003; Katayama *et al.* 2010). Earlier studies reported that mouse single blastomere derives from 2-cell embryo could develop to term (Tarkowski, 1959). Subsequently, it led to the establishment of mouse ESC (Wakayama *et al.* 2007). Blastomere separation techniques had been successfully developed for various mammalian species. Developmental capacity of isolated rabbit blastomere was decreasing with increasing of embryo stage (Tao and Niemann, 2000). In monkey 35% of blastocysts was obtained after blastomere separation and led to pregnancy (Mitalipov *et al.* 2002). In pigs, the the number blastocysts derived from single blastomere of 4-cell was higher versus single blastomere of 8-cell with more total cell but lower ratio of ICM to total cell (Eckert *et al.* 1997).

Several reports had been described on the establishment of ESC from isolated single blastomere. Monolayer cell was obtained from isolated blastomere cultured *in vitro* on different extracellular matrix components (Wilton and Trounson, 1989). High percentage of blastocyst

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and subsequent ICM outgrowth derived from mouse 2-cell single blastomere was obtained after culture in KSOM and mES medium (Lorthongpanich *et al.* 2008) and high number of pluripotent ESC lines were obtained from single blastomere of 8-cell (Delhaise *et al.* 1996)

Therefore, this research was aimed to evaluate the potency of single blastomere separation from early stages of embryos for increasing the number of blastocysts to be used as a source for establishment of ESC line.

MATERIALS AND METHODS

Embryo flushing

Adult ICR females (8-12 weeks old) were superovulated using 10 IU injection of pregnant mare serum gonadotrophin (PMSG, Folligon; Intervet) followed by another 10 IU injection of human chorionic gonadotrophin (hCG, Chorulon; Intervet) 48 hr later and directly mated with ICR males (10-14 weeks old). The vaginal plug was examined on the following morning at 0800 hours. The 2-cell embryos were collected from mated females by cervical dislocation at 48 hr post-hCG injection. The embryos were flushed in the hepes buffered KSOM and cultured in 50 µl droplets KSOM supplemented with 4 mg/ml BSA, 5 µl/ml MEM non-essential amino acid and 10 µl/ml BME amino acid under silicone oil at $37^{o}C$ in CO_2 (5%) incubator until reaching 4- and 8-cell stages before blastomere isolation. The experiment with mice was conducted in accordance and approved by Institutional Animal Care and Use Committee, University of Malaya.

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Blastomere separation

The 2-, 4- and 8-cell embryos were transferred into a 20 µl droplet of pronase (0.5%) (Sigma) to digest the zona pellucida (ZP) before isolated mechanically by gently repeat pipetting in hepes KSOM medium.

Blastomere *in vitro* culture

All the zona free (ZF) single blastomeres and control group (ZF without separation at 2-cell) were washed and cultured *in vitro* in KSOM medium at 37°C in a humidified atmosphere with CO_2 (5%). The developments of the single blastomeres were observed daily under inverted microscope until blastocyst stage.

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Preparation of mouse embryonic fibroblast (MEF) as feeder cell layer

MEF were prepared from 14.5 days post-coitus mouse foetuses. All limbs, head, tail and internal organ of the foetuses were removed in phosphate buffered saline free-Ca/Mg, [PBS (-)]. Then, the foetuses were transferred into trypsin-EDTA (Sigma) solution and minced into small pieces. An optimal trypsin-EDTA medium was added into a beaker containing the small pieces of foetuses and stir on magnetic stirrer for 15 to 20 min. Culture medium supplemented with DMEM (Gibco), 10% of foetal bovine serum (FBS) (Gibco) and 1x penicillin/streptomycin (Sigma) was added into the conical tube with ratio 1.5-fold of the typsin-EDTA containing minced foetus and centrifuged at 3000 rpm for 5 minutes. The supernatant was removed and the

remaining pellet was pipetted with culture medium to make single cell which then seeded and cultured on a coated culture dish in CO_2 (5%) incubator at 37°C the cell reaching 80-90% confluency. The MEF was inactivated by using mitomycin C (Sigma) before being used as feeder cell layer for ICM outgrowth.

Diameter of blastocyst

Diameters of the isolated single blastomere and its subsequent blastocyst production were measured with XYClone measurement tools. Next, the blastocysts were stained with Hoechst staining for cell counting.

Blastocyst count cell

The blastocysts derived from isolated blastomere were stained for cell counting. The blastocysts were washed three times in PBS (-) and transfer in 10 µl droplet of fixative solution for 5 minute. Then, the blastocysts in a small volume of solution were placed onto the slides and covered by using a coverslip. About 5 µl solution of Hoechst 33342 (Sigma) was put at the edge of cover slip and let it spread into the blastocyst. The slides were visualised and number of cell were counted by using a fluorescent microscope.

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Statistical analysis

All the data were analysed using ANOVA test. All the statistical analysis was performed using the Statistical Package for Social Sciences for Windows version 16.0 (SPSS, IL, USA).

Results

Development potential of isolated single blastomere from different stages of parent embryos

Table 1 shows that development of isolated single blastomere decreased as the stage of embryos increased (2-, 4- and 8-cell) with cleavage rate of 80.88±6.53, 69.82±5.58 and 56.36±3.35, respectively. Control group gave the highest results (98.50±1.50). Single blastomere derived from different stages of embryos could develop until blastocyst as shown in Figure 1 (a-d).

Development of mouse ICM outgrowth on MEF feeder layer of blastocyst derived from ZF-single blastomere

Whole blastocyst derived-single blastomeres at different stages were cultured on MEF feeder layer for ICM outgrowth. Table 2 shows that blastocyst derived from control group gave the highest development (76.90±6.08) followed by 2-cell (69.29±4.13) 4-cell (55.73±7.81) and 8-cell (41.85±3.58) [Table 2]. Visible clump of ICM outgrowth derived from SB could be observed after attachment on MEF [Figure 1 (f)].

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Diameter of ZF-single blastomere and the subsequent blastocyst and the total number of cell

We were investigated the size of individual single blastomere and its subsequent blastocyst at different stage of parent embryos (Table 3). Figure 2 shows that the diameter of individual single blastomere was approximately 3/5 smaller than its subsequent blastocyst. The size of blastocysts derived from SB at different stages of parent embryos were corresponding to the results for total number of cell which were decreasing from 2- to 8-cell parent embryos (43.00±3.63 to 8.80±1.30). The blastocysts derived from SB and control group have been stained for cell counting (Figure 3).

Discussion

The aim of this study was to evaluate the developmental potential of single blastomere derived from different stages of embryo and its subsequent ICM outgrowth cultured on MEF feeder cell layer. We have demonstrated that all isolated single blastomeres from each different stages of embryo could develop until blastocyst stage. As increasing the age of parent embryos (2- to 8-cell) the developmental capacity of single blastomere into blastocyst was decreased. This was probably due to the intercellular junction between blastomeres where it becomes tighter with increasing embryo age; therefore, blastomere separation using mechanically gentle pipetting of the ZP-free embryo at later stage is more prone to blastomere injury. We also observed that high abnormal blastocysts formed from 8-cell parent embryos which developed into the characteristic

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of 'trophoblastic vesicle' [Figure 1 (e)] contained no embryonic inner cell mass (ICM) (Tarkowski and Wroblewska, 1967).

One explanation for the low blastocyst rate obtained in this study could be due to the absence of zona pellucida. It has important roles in order to protect the embryo, regulate cell division and cell allocation for the formation of ICM and trophectoderm in mouse (Suzuki *et al.* 1995) and pig embryos (Eckert *et al.* 1997; Tao *et al.* 1995).

In vitro culture (IVC) medium has been thought as one of the factors which can affect the development capacity of isolated single blastomere. KSOM medium has been used as IVC medium shows that single blastomere can develop up to blastocyst with relatively high developmental rate (77%), similar to result reported where high blastocyst formed when single blastomere derived from 2-cell cultured in KSOM than mES medium (Lorthongpanich *et al.,* 2008). In contrast, low blastocyst rate was obtained when blastomere cultured in T6 culture medium even tough growth factor was added into the medium (Sheikholslami *et al.* 2008). Therefore, we were suggested to culture the single blastomere up to blastocyst stage in KSOM medium before transferred the blastocyst derived-single blastomere on inactivate MEF feeder layer for ICM outgrowth using mESC medium which contain LIF for maintaining pluripotent state of ESC cell.

In our experiment, blastomeres were cultured in groups in order to do diverging observation on blastomere development. In addition, it is more effective culture system because growth factors were secreted during development of the blastomeres which have stimulatory effect on its development. It was suggest that single blastomere from 2- to 8-cell embryo can develop as normal embryo but the developmental potential was limited by non optimal culture

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system. The present results showed that isolated SB could develop to blastocyst with the size of the later was approximately 3/5 bigger than the former. This was in agreement that intact sister blastomere was capable of compensating for lost or damaged blastomere in an embryo and thus has the capacity to sustain development (Avis and Anderson, 1988).

Conclusion

It is one of aims to perform blastomere separation in order to increase the number of blastocyst. In other words, we are targeting that blastocyst derived from single blastomere could be used as a source of ICM for production of embryonic stem cell (ESC). From the results of our study, we suggest that single blastomeres from mouse embryos could be isolated at 2-, 4- and 8-cell stage to produce many blastocysts after *in vitro* culture, subsequently facilitating the use of ICM for production of mouse ESC.

Acknowledgement

The authors thank the ABEL members for their assistance and IPPP grant (PG149-2012B) for financial support.

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Tables

Table 1: Effect of different cell stage on development of isolated single blastomere

Stage of embryo	No. of isolated blastomere used	Development of isolated blastomere				
		No. of 2-cell (%)	No. of 4-cell (%)	No. of 8-cell (%)	No. of morula (%)	No. of blastocyst (%)
2-cell	172	80.88±6.53[b]	78.84±6.87[b]	78.08±6.94[b]	77.28±6.77[bc]	77.28±6.77[bc]
4-cell	252	69.82±5.58[ab]	69.83±5.58[ab]	69.83±5.58[ab]	69.83±5.58[ab]	63.70±5.35[ab]
8-cell	152	56.36±3.35[a]	56.36±3.35[a]	56.36±3.35[a]	55.73±3.35[a]	55.73±3.35[a]
Control*	198	98.50±1.50[c]	96.83±2.12[c]	95.72±2.22[c]	91.39±2.92[c]	91.39±2.92[c]

*ZF 2-cell without separation.
[abc]Means with different superscript in same column differ significantly ($P<0.05$)

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Table 2: Development of mouse ICM outgrowth on MEF after culturing blastocyst obtained from biopsied-SB of early stage embryos

Stage of embryos	No. of blastocysts derived-SB	No. of ICM outgrowth (%)
2-cell	145	69.29 ± 4.13^{b}
4-cell	73	55.73 ± 7.81^{ab}
8-cell	60	41.85 ± 3.58^{a}
Control*	157	76.90 ± 6.08^{b}

*ZF 2-cell without separation.
^{ab}Means with different superscript in same column differ significantly (P<0.05)

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Table 3: The diameter and cell number of blastocyst derived from isolated blastomere

Stage of embryo	Diameter of SB after isolation (µm) Mean±SE	Diameter of blastocyst derived-SB (µm) Mean±SE	No. of cell in blastocyst Mean±SE
2-cell	56.48±0.40[c]	92.55±1.59[c]	43.00±1.48[c]
4-cell	44.02±0.49[b]	78.71±1.37[b]	28.33±1.15[b]
8-cell	35.68±0.34[a]	64.13±2.20[a]	8.80±0.58[a]
Control*	-	108.28±2.13[d]	177.50±12.50[d]

*ZF 2-cell without separation.
[abcd]Means with different superscript in same column differ significantly (P<0.05)

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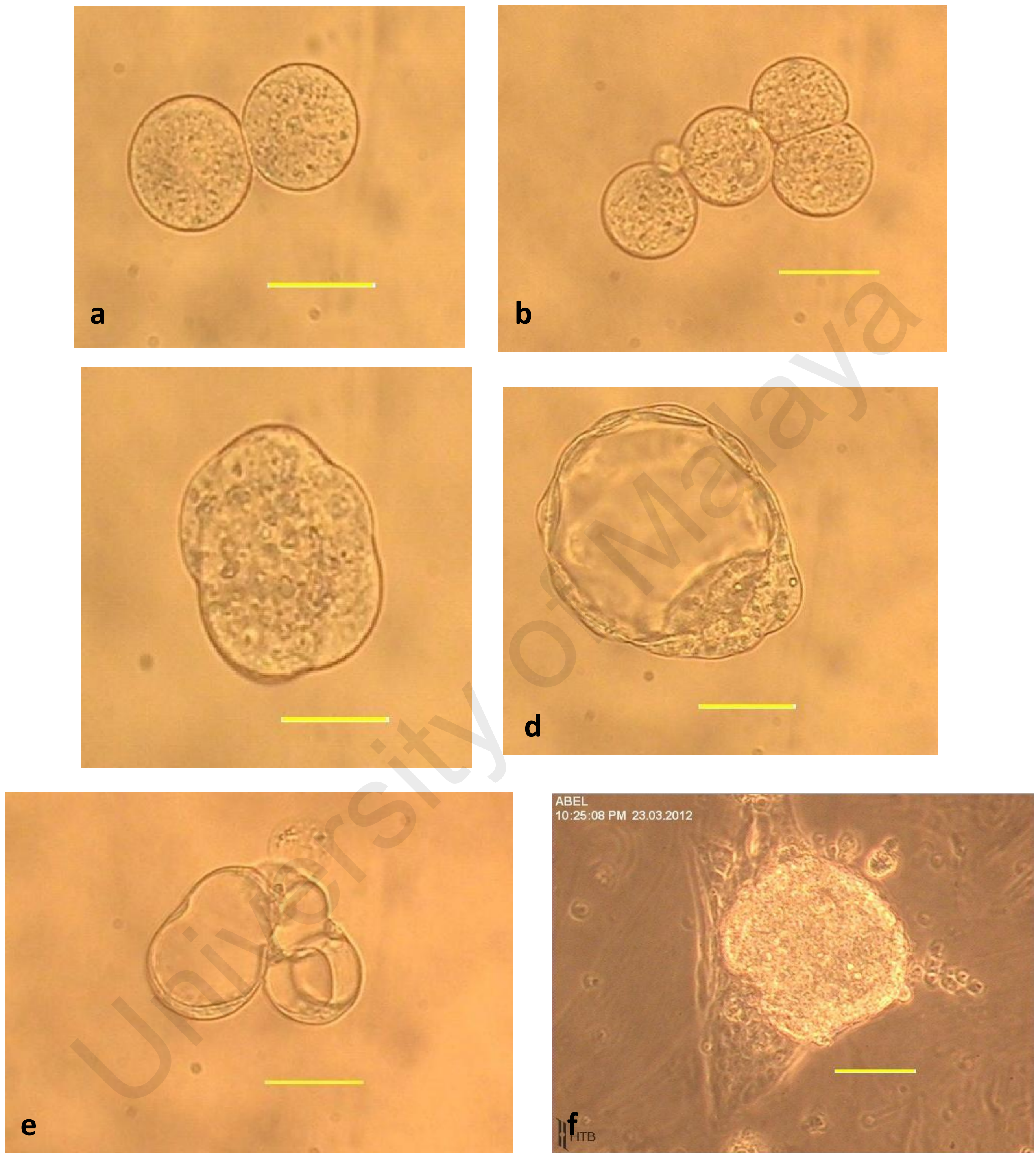

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Figure 1 (a-f): Development of isolated single blastomere from 2-cell stage embryos (a) 2-cell (b) 4-cell (c) morula (d) blastocyst (e) abnormal blastocyst and (f) ICM outgrowth; Bar, 60 µm.

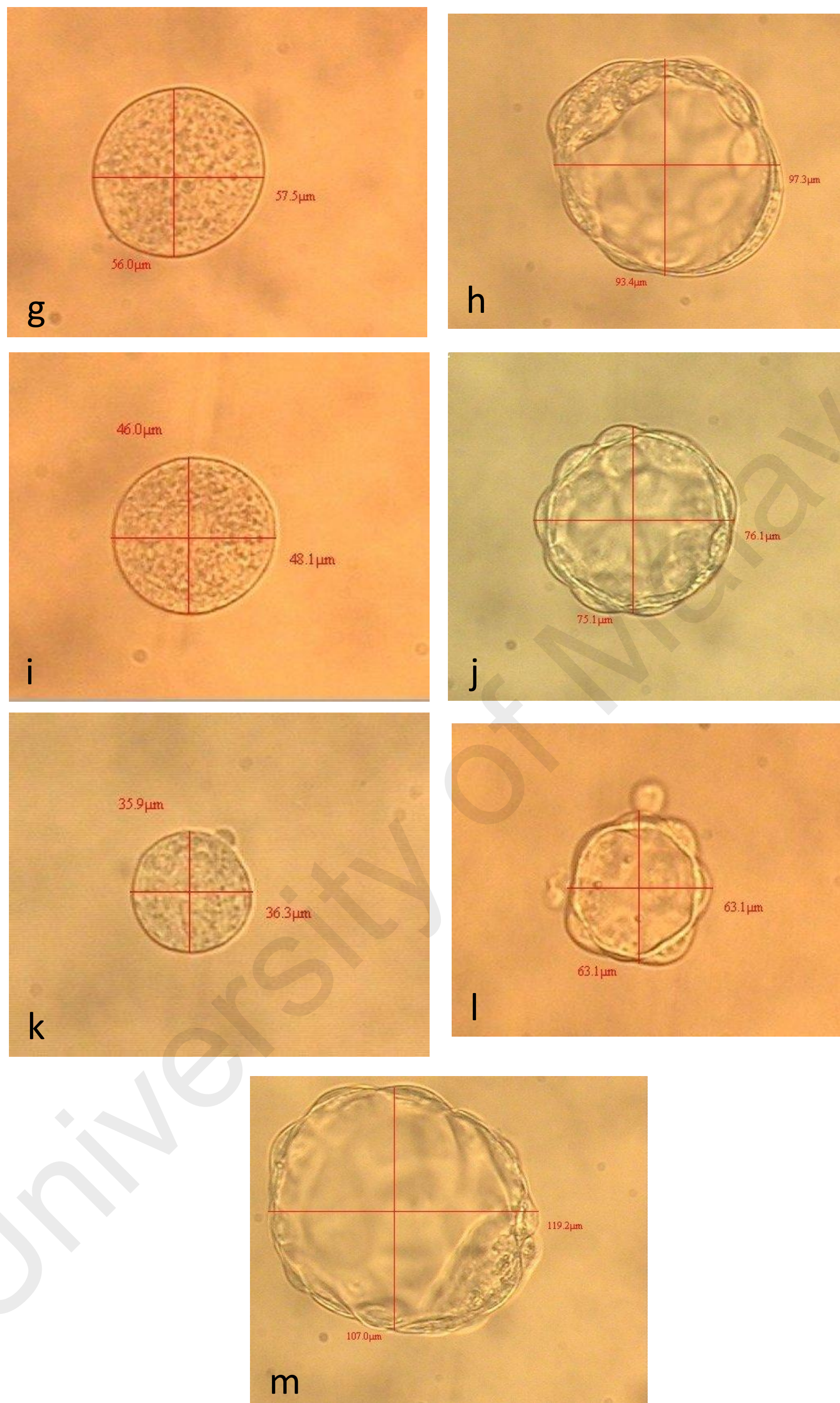

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Figure 2 (g-m): Diameter of isolated SB from different stage of embryos and its subsequent blastocyst; (g and h) 2-cell (i and j) 4-cell (k-l) 8-cell and (m) control.

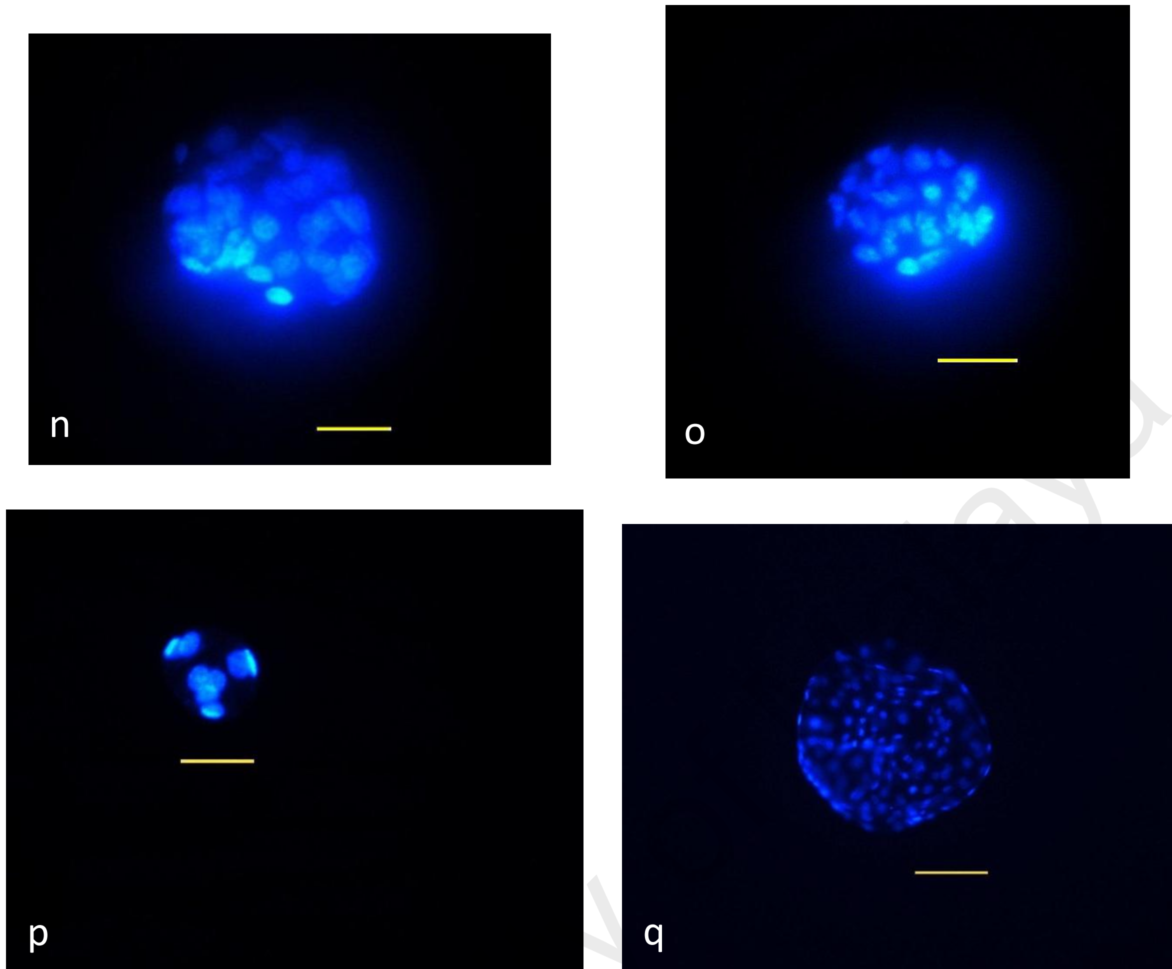

Figure 3 (n-q): Hoechst staining of blastocyst derived from SB at different stage of embryos; (n) 2-cell (o) 4-cell (p) 8-cell) and (q) control; Bar, 60 µm.

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1 **Production of cloned caprine embryos through somatic cell nuclear transfer influenced**
2 **by oocytes sources, pre-intracytoplasmic injection durations and enucleation techniques**
3

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13

14 **A short running title:** Production of cloned caprine embryos
15

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ABSTRACT

The aims of this investigation were to compare the effects of a) oocyte sources, b) pre-intracytoplasmic injection (pre-ICI) durations and c) enucleation techniques on success rate of somatic cell nuclear transfer cloning. Caprine oocytes were collected from laparoscopic ovum pick-up (LOPU), ovariectomy and abattoir sources. The matured oocytes were confirmed by extrusion of the first polar body. The first polar body and approximately 10–15% of the oocyte cytoplasm were squeezed out by laser or squeezing techniques. Fresh cumulus cells obtained from the matured oocytes were used for nuclear transfer using the whole-cell ICI technique. Oocytes from ovariectomy showed the highest maturation rate (70.5%) followed by LOPU (63.1%) and abattoir (51.7%) sources. However, oocytes from LOPU showed the highest enucleation rate (99.4%) followed by ovariectomy (98.1%) and abattoir (89.5%) sources. The 60 min pre-ICI duration yielded difference ($P < 0.05$) in blastocyst cleavage rate followed by the simultaneous and the 30 min pre-ICI durations. The laser enucleation technique yielded a greater cleavage rate compared to the squeezing enucleation technique in all the stages of embryo development. In conclusion, ovariectomy, 60 min pre-ICI duration and laser enucleation technique give the best result for successful production of cloned caprine embryos.

Key words: *abattoir, laparoscopic ovum pick-up, ovariectomy, oocyte sources, enucleation techniques*

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INTRODUCTION

The basic biology underlying nuclear transfer in cloning still remains unclear, although it began more than 20 years ago with the creation of Dolly the sheep (Baguisi *et al.* 1999), the first cloned mammal using somatic cell nuclear transfer (SCNT). Since then, success in cloning using adult somatic cells from other mammals has been reported, including murine (Bortvin *et al.* 2003), bovine (Genzyme 1990; Cognie 1999; Chen *et al.* 2007), caprine (Goh *et al.* 2012) and porcine species (Hill *et al.* 2000). A clone is described as the production of genetically identical animals (Kato *et al.* 1998). As noted previously (Keskintepe *et al.* 1994), clones are used for the selection of genetic traits or in research to reduce the number of experimental animals. The SCNT can mitigate the limited availability of high genetic values of research animals and enable optimum results while simultaneously reducing the number of animals for experiment. Cloning and transgenics can produce phenotypes of large animals to investigate devastating disease, but their contribution in research is limited. Cloned animals could also supersede genetically dissimilar animals where the investigated trait is highly heritable.

In the early 1990s, it was first attempted cloning to induce protein-based therapeutics in milk of transgenic caprine (Kubota *et al.* 2000). Using a microinjection technique, a genetic construct was inserted into the nucleus of embryos (1-cell stage). The first successful cloned caprine was produced from ear fibroblast cells (Goh *et al.* 2012). The first successful cloned caprine by nuclear transfer was produced in east China (Lamberson 1994). Improvements in cloning techniques would contribute new approaches that will help conserving and saving the endangered species. Successful nuclear transfer in caprines has also been reported (Lee *et al.* 2003). Cloning by nuclear transfer has great potential applications in pharmaceutical protein production, xenotransplanation and therapeutic cloning. It could be used as a valuable biochemical tool to yield important advances in

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1 pharmacology and medicine. The success rate of cloning is quite low due to many technical
2 factors such as the donor cell cycle, cytoplast of recipient, oocyte activation, reprogramming
3 of the donor nucleus and chemical agents (Martino *et al.* 1994). This experiment was
4 conducted to investigate the effects of the oocyte sources, durations of pre-intracytoplasmic
5 injection (pre-ICI) and enucleation techniques on caprine SCNT performance.
6

7 **MATERIALS AND METHODS**

8 All experimental procedures were performed according to the Guidelines for Animal
9 Experiment, University of Malaya, Malaysia. Three experiments were conducted for
10 evaluation of oocyte sources, pre-ICI durations and enucleation techniques for production of
11 cloned-caprine embryos. In Experiment 1 for oocyte sources, the matured oocytes were
12 enucleated by using laser technique and simultaneously insert the donor by using whole cell
13 intracytoplasmin injection (ICI). In Experiment 2 for pre-ICI durations, the source of oocyte
14 was randomly used to study the effect of pre-incubation before ICI. The matured oocytes
15 were subjected to DNA enucleation using laser techniques prior to ICI of donor cell. The
16 enucleated oocytes were grouped in 3 different times of pre-incubation before ICI; a)
17 simultaneously (without incubation) b) 30 min and c) 60 min. In Experiment 3 for
18 enucleation techniques, the matured oocytes from different sources were subjected randomly
19 to two different enucleation techniques: squeezing and laser. Briefly, a hole in the zona
20 pellucida was made above the first polar body with a cutting needle (squeezing) or a laser,
21 and 10–15% of the cytoplasm (containing metaphase II chromosomes) was gently squeezed
22 out beneath the first polar body with a cutting needle or pushed out with a laser. The
23 enucleated oocytes were fused with donor cell and activated simultaneously.
24

25 **Preparation of donor cells**

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1 Fresh caprine cumulus cells obtained from *in vitro* matured oocytes were harvested either
2 from laparoscopic ovum pick-up (LOPU), ovariectomy or abattoir ovaries. After 18–24 h of
3 *in vitro* maturation, cumulus cell was separated through pipetting in hyaluronidase (0.2%) for
4 5 min at 37°C. All the fresh cells were collected, washed in holding medium (TL-Hepes
5 working solution) and centrifuged for 5 min. A cumulus cell pellet was formed, and the
6 supernatant was removed. The TL-Hepes working solution (100 µL) was added to the pellet
7 and sucked up and down until the fresh cumulus cells became a single cell (7–8 µm) and
8 were ready for use.
9

10 **Oocyte retrieval and *in vitro* maturation**

11 The caprine ovaries were obtained from LOPU (aspiration-flushing system), ovariectomy
12 (ovaries were surgically removed) and abattoir sources [ovaries were transported to
13 laboratory in a thermos containing a 0.9% NaCl solution supplemented with penicillin-G (60
14 µg/mL) and streptomycin (50 µg/mL) at 30–35°C]. For the ovariectomy and the abattoir
15 sources, cumulus-oocyte complex (COC) was retrieved by ovarian slicing using a razor blade
16 in the culture dish containing the holding medium (TL-Hepes). The retrieved oocytes were
17 washed three times in maturation medium and cultured in *in vitro* (TCM-199 supplemented
18 with 10 µg/mL follicle-stimulating hormone, 1 µg/mL 17-β estradiol, 100 µM/mL
19 cysteamine, 0.2 mM sodium pyruvate, 10% foetal bovine serum) overlaid with mineral oil in
20 a CO_2 incubator (5%) at 38.5^{o}C for 18–24 h. After maturation was completed, the COC was
21 denuded with 0.2% hyaluronidase and then rinsed 5 times in the medium (TL-Hepes). Prior
22 to enucleation, all the mature oocytes were selected and moved to microdroplets containing
23 medium (TL-Hepes) with supplementation (5 µg cytochalasin B/mL) for 10 min.
24

25 **Nuclear transfer**

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1 Prior to injection, cytoplasm of an oocyte was drawn gently into the injection needle until a
2 sudden flux of cytoplasm was observed entering the needle. The flux ensured the breaking
3 cytoplasm's membrane that was necessary to facilitate the injection of the donor cell.
4 Cumulus cells were deposited into cytoplasm, washed in TL-Hepes medium and in KSOM
5 medium. Prior to activation, cells were incubated in droplet of KSOM medium at least 60
6 min.
7

8 **Activation and *in vitro* culture**

9 All the injected caprine oocytes were made active using calcium ionophore (5 μM) for 5 min
10 followed by 6-dimethylaminopurine (1.9 μM) for 4 h at 38.5°C under CO_2 (5%). After
11 activation, the reconstructed caprine oocytes were washed and cultured in the KSOM
12 medium in a CO_2 incubator (5%) at 38.5°C. The advancement of the cloned embryos was
13 observed every 2 days and recorded. The culture medium was refreshed every 2 days.
14

15 **Statistical analysis**

16 All statistical analyses were done with a standard statistical package (SPSS, version 16.0, IL,
17 USA).
18

19 **RESULTS**

20 Table 1 shows the cleavage rates of the caprine embryos via the SCNT of the 3 different
21 sources of oocytes—LOPU, ovariectomy and abattoir. Maturation rate was higher ($P < 0.05$)
22 when oocyte retrieved from ovariectomy (70.5%) and LOPU (63.1%) than oocyte retrieved
23 from abattoir (51.7%). The enucleation rate from LOPU source was higher (99.4%) followed
24 by ovariectomy (98.1%) and abattoir (89.5%) sources. These results are subsequent with
25 cleavage and developmental rates after SCNT (Figures 1 and 2).

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1 The developmental rate at blastocyst stage was higher ($P < 0.05$) with a pre-ICI
2 duration of 60 min (17.9%) than pre-ICI duration of simultaneous (1.4%) and 30 min (0.0%)
3 (Table 2). Table 3 shows the cleavage rates of the embryos from *in vitro* culture using
4 different enucleation techniques. No significant ($P > 0.05$) difference was observed in
5 enucleation rates between squeezing and laser. However, the embryo development at various
6 stages was higher ($P > 0.05$) with the laser enucleation technique compared to the squeezing
7 enucleation technique with values of 83.7 vs. 68.1%, 77.6 vs. 49.7%, 73.8 vs. 42.8%, 67.8 vs.
8 27.5%, 36.6 vs. 0.0% and 23.3 vs. 0.0% for the 2-cell, 4-cell, 8-cell, morula, blastocyst and
9 hatched blastocyst stages, respectively. For both enucleation techniques, the cleavage rates
10 decreased as the stages of development progresses. No blastocysts were obtained with the
11 squeezing enucleation technique.
12
13 **DISCUSSION**
14 Many factors influence the production of cloned caprine embryos via SCNT, including the
15 oocyte retrieval source, the duration of pre-ICI and the enucleation techniques used. The
16 findings from this study showed that the highest blastocyst and hatched blastocyst rates were
17 obtained with ovariectomy followed by LOPU and abattoir sources. The results could be due
18 to the surgically ovariectomised caprine ovaries consistently providing a greater follicle
19 surface exposure for oocyte retrieval. They could also be due to the ability to more clearly
20 visualise the follicles on the ovaries (Newport & Kirschner 1982). As reported previously
21 (Polejaeva *et al.* 2000), to obtain a greater number of oocytes with small follicles, the oocytes
22 have to be recovered by slicing the surface of the ovaries. Ovariectomy normally involves
23 ovarian slicing. More oocytes would be obtained via ovariectomy, and the opportunity to
24 develop and form blastocysts would be greater than with LOPU oocyte sources. It is
25 generally agreed that the ovarian slicing method yields a higher number of oocytes. The

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1 higher oocyte yield may be due to the presence of some follicles embedded deeply within the
2 ovarian cortex that are only released by slicing of the ovary. Unlike LOPU, the ovariectomy
3 by slicing protocol was done under visual observation, regardless of ovarian follicle size;
4 therefore, more oocytes were retrieved in this study. It was obtained more oocytes from
5 ovarian mincing or slicing than with follicular dissection or aspiration (Polejaeva *et al.* 2000).
6 It was also obtained a higher number of oocytes by surgically removing the ovaries of doe
7 (Smith 1989).

8 The highest cleavage rates were found with the 60 min pre-ICI followed by the
9 simultaneous injection and the pre-ICI of 30 min. This might be due to the enucleated caprine
10 oocytes needing time to restore the oocyte viability due to the sensitivity of the oocytes
11 following their placement in the CO_2 incubator before the injection of the donor cell. The
12 simultaneous injection immediately after enucleation could result in decreased viability of the
13 oocytes because they could easily lyse just after enucleation. The findings suggest that a pre-
14 ICI duration of 60 min seems to be more suitable for SCNT in caprine species.

15 Our results demonstrate that laser enucleation techniques can produce cloned caprine
16 to the hatched blastocyst stage. There are few reports on caprine SCNT research using laser
17 enucleation techniques (Martino *et al.* 1994). The laser is a new and advanced technique,
18 which has been used to enucleate recipient oocytes (cytoplast). Compared to the squeezing
19 technique, laser enucleation is easier and less time consuming in enucleating the caprine
20 oocytes. The time consuming nature of the former method may explain the low efficiency of
21 embryo development with this approach. The squeezing method took much longer to remove
22 DNA (metaphase II) from caprine oocytes compared to the aspiration technique (Wakayama
23 & Yanagimachi 1999). On the other hand, the operation of the laser enucleation technique in
24 the enucleation of porcine oocytes was shown to be easier (Wakayama and Yanagimachi

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1 1999). Although the laser enucleation technique is expensive, it yielded the highest rates of
2 SCNT in caprine species in the present study.

3 Reprogramming usually occurs in cloned embryos. Low efficiency of cloning may be
4 due to incomplete reprogramming. Therefore, correct epigenetic reprogramming is necessary
5 for successful and normal developmental of clones (Wakayama *et al.* 2000). According to
6 previous work, the incomplete reactivation of Oct-4 (pluripotency marker) may account for
7 some failures of nuclear transfer in embryo development (Wilmut *et al.* 1997; Wells *et al.*
8 1999). Reprogramming usually occurs within the short interval between transfer of the donor
9 nucleus into the enucleated oocyte and the time when zygotic transcription starts. It may also
10 occur due to a maternal zygotic transition. As reported earlier (Yang *et al.* 1991), the onset of
11 maternal zygotic transcription is species dependent. A better understanding of the processes
12 involved in nuclear reprogramming in cloned embryos would improve the overall success of
13 nuclear transfer.

14 In conclusion, cloned caprine embryos are successfully produced *in vitro* from LOPU,
15 ovariectomy and abattoir sources. Oocytes retrieved by ovariectomy seem to be a more
16 suitable source to produce cloned caprine embryos via SCNT. A pre-ICI duration of 60 min
17 yields the highest cleavage rates in the cloned caprine embryos produced. The laser
18 enucleation method shows higher cleavage rates up to the hatched blastocyst stage than the
19 squeezing method. The incorporation of laser enucleation technology will enhance the
20 efficiency of SCNT in the production of cloned caprine embryos.

21

22 **ACKNOWLEDGMENTS**

23 The authors wish to thank the Animal Biotechnology Embryo Laboratory members of the
24 University of Malaya for their assistance throughout this project. This project was funded by
25 IPPP (PS287, 2010A) of University of Malaya.

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