UNIVERSITI PUTRA MALAYSIA

IN VITRO PRODUCTION OF EMBRYOS FROM ABATTOIR-DERIVED CATTLE OOCYTES

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By

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**_IN VITRO_ PRODUCTION OF EMBRYOS
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Two studies involving some experiments were conducted to evaluate some factors affecting the *in vitro* production of cattle embryos from abattoir derived cattle oocytes.

In the first study, more oocytes per ovary were recovered by slicing with a surgical blade (29.3 oocytes) than by aspiration with a disposable syringe and needle (12.0 oocytes). Cumulus expansion rate and maturation rate were better in oocytes surrounded by cumulus cells than in denuded oocytes and fibrinated oocytes. To determine the influence of adding serum and hormones, cumulus oocyte complexes (COCs) were matured in four different maturation media and incubated for 22 h at 39°C with 5% CO_2 in humidified air. The addition of hormones to the maturation medium enhanced cumulus expansion rate and maturation rate. In the absence of

hormones, 20% serum level rendered better cumulus expansion than with 10% serum but had no effect on the maturation rate.

In the second study, factors affecting the IVF and the developmental competence of embryos were studied. *In vitro* matured oocytes were inseminated with swim-up separated sperm in IVF-TALP medium. At 18 or 44 h post insemination, the presumptive embryos were freed of cumulus and transferred into two culture media (IVC): modified synthetic oviductal fluid (mSOF) as cell-free culture system and M199 with bovine oviductal epithelial cell (BOEC) as co-culture system. At 6 hour after inseminaton, male pronucleus formation was first observed. There were no significant differences on the effect of serum level (10% or 20%) and hormones supplementation in the maturation medium on the cleavage rate and developmental competence of embryos. Cleavage and blastocyst rates were 71.2% and 6.2% for cumulus-intact oocytes whereas the rates were 47.2% and 1.9% for cumulus-free oocytes. Although the cleavage rate was not different, better morula and blastocyst rates were obtained from co-culture system.

The results indicate that hormones enhance cumulus cells expansion and maturation rates, cumulus cells facilitate fertilization while co-culture with BOEC rendered better developmental capacity of embryos. However, the failure of morula to develop to blastocysts i*n vitro* needs further study.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

PENGHASILAN EMBRIO SECARA *IN VITRO* DARI OOSIT LEMBU RUMAH SEMBELIH

Oleh

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Dua kajian yang melibatkan beberapa uji kaji telah dijalankan untuk menentukan faktor yang memberi kesan terhadap penghasilan embrio lembu secara *in vitro* dari oosit yang diperolehi dari lembu yang disembelih.

Dalam kajian pertama, lebih banyak oosit bagi setiap ovari diperolehi secara menghiris dengan menggunakan pisau pembedahan (29.3 oosit) berbanding dengan kaedah aspirasi menggunakan jarum dan picagari pakai buang (12.0 oosit). Kadar pengembangan kumulus dan kadar kematangan adalah lebih baik bagi oosit yang dikelilingi dengan sel kumulus berbanding oosit tanpa kumulus dan oosit berfibrin. Untuk menentukan pengaruh penambahan serum dan hormon, komplek oosit kumulus (COC) dimatangkan dalam empat bahantara pematangan dan dieram selama 22 jam pada 39°C dengan 5% CO_2 dalam udara lembap. Penambahan hormon dalam media pematangan meningkatkan kadar pengembangan kumulus dan kadar

pematangan. Dalam keadaan tanpa hormon, paras 20% serum memberikan pengembangan kumulus yang lebih baik berbanding dengan 10% serum tetapi tidak untuk kadar kematangan.

Dalam kajian kedua, kajian ditumpukan untuk menentukan faktor yang mempengaruhi persenyawaan *in vitro* dan keupayaan perkembangan embrio. Oosit yang dimatangkan secara *in vitro* diinseminasi dengan sperma yang diasingkan secara "swim-up" dalam media IVF-TALP. Pada 18 atau 44 jam selepas inseminasi, embrio tersebut dibuang kumulusnya dan dipindahkan ke dalam dua media kultur (IVC): cecair oviduk sintetik yang diubahsuai (mSOF) sebagai sistem kultur tanpa sel, dan M199 dengan sel epitelium oviduk bovin (BOEC) sebagai sistem kultur bersama. Selepas 6 jam diinseminasi, pembentukan pro-nukleus jantan dapat dilihat. Tidak terdapat sebarang perbezaan bererti bagi kesan paras serum (10% dan 20%) serta penambahan hormon dalam bahantara pematangan terhadap kadar pembelahan dan keupayaan perkembangan embrio. Kadar pembelahan dan blastosista adalah 71.2% dan 6.2% bagi oosit berkumulus manakala 47.2% dan 1.9% bagi oosit tanpa kumulus. Walaupun kadar pembelahan tidak berbeza, kadar morula dan blastosista didapati lebih baik untuk sistem kultur bersama.

Keputusan menunjukkan bahawa hormon meningkatkan pengembangan sel kumulus dan kadar kematangan, sel kumulus membantu persenyawaan sementara kultur bersama dengan BOEC menyebabkan keupayaan perkembangan embrio yang lebih baik. Namun demikian, kegagalan morula untuk berkembang ke blastosista secara *in vitro* memerlukan kajian selanjutnya.

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TABLE OF CONTENTS

	Page
ABSTRACT	ii
ABSTRAK	iv
ACKNOWLEDGEMENTS	vi
APPROVAL SHEET	viii
DECLARATION FORM	x
LIST OF TABLES	xiv
LIST OF PLATES	xv
LIST OF ABBREVIATIONS	xvii

CHAPTER

I	GENERAL INTRODUCTION	1
II	LITERATURE REVIEW	4
	2.1 Introduction	4
	2.2 *In Vivo* Development of Gametes	4
	2.2.1 Oogenesis	4
	2.2.2 Folliculogenesis	5
	2.2.3 Oocyte Maturation	6
	2.2.4 Sperm Maturation	8
	2.3 Sources of Oocytes	8
	2.3.1 *In Vivo* Collection of Oocytes	8
	2.3.2 *In Vitro* Collection of Oocytes	9
	2.3.3 Follicle Size and Quality	10
	2.3.4 Techniques of *In Vitro* Oocytes Recovery	11
	2.3.5 Oocytes Classification	12
	2.4 *In Vitro* Maturation	12
	2.4.1 Medium of *In Vitro* Maturation	12
	2.4.2 Serum	13
	2.4.3 Hormones	14
	2.4.4 Follicular Fluid	15
	2.4.5 Growth Factors	16
	2.4.6 Presence of Granulosa Cells	16
	2.4.7 Condition of IVM	17
	2.4.8 Assessment of Oocyte Maturation	19
	2.5 *In Vitro* Fertilization	20
	2.5.1 Sources of Spermatozoa	20
	2.5.2 Sperm Preparation	20
	2.5.3 Sperm Capacitation	22
	2.5.4 *In Vitro* Fertilization System	24
	2.5.5 Assessment of *In Vitro* Fertilization	25

2.6 *In Vitro* Culture 26
2.6.1 *In Vitro* Culture System 26
2.6.2 Co-culture System 27
2.6.3 Cell-free Culture System 28

III COLLECTION AND *IN VITRO* MATURATION OF CATTLE OOCYTES 29
3.1 Introduction 29
3.2 Objectives of The Experiments 30
3.3 Materials and Methods 31
3.3.1 Sterilization 31
3.3.2 Glass Micropipette Preparation 31
3.3.3 Collection of Ovaries 32
3.3.4 Recovery of Oocytes 32
3.3.5 Procedures of *In Vitro* Maturation 34
3.3.6 Evaluation of Oocyte Maturation 34
3.3.7 Experimental Design 36
3.3.8 Statistical Analyses 38
3.4 Results 39
3.4.1 Effect of Collection Method on The Recovery of Cattle Oocytes 39
3.4.2 Effect of Oocyte Quality on *In Vitro* Maturation of Cattle Oocytes 40
3.4.3 Effect of Serum and Hormones Supplementation to The Maturation Medium on *In Vitro* Maturation of Cattle Oocytes 41
3.5 Discussions 50

IV *IN VITRO* FERTILIZATION AND *IN VITRO* CULTURE OF CATTLE EMBRYOS 54
4.1. Introduction 54
4.2 Objectives of The Experiments 55
4.3. Materials and Methods 56
4.3.1 Procedures of *In Vitro* Maturation 56
4.3.2 Preparation of Sperm for *In Vitro* Fertilization 56
4.3.3 Procedures of *In Vitro* Fertilization 57
4.3.4 Evaluation of Fertilization 58
4.3.5 Procedures of *In Vitro* Culture 58
4.3.6 Evaluation of Embryos 61
4.3.7 Experimental Design 61
4.3.8 Statistical Analyses 63
4.4 Results 64
4.4.1 Time Sequence of *In Vitro* Penetration of Cattle Sperm 64
4.4.2 Effect of Serum and Hormones Supplementation to The Maturation Medium on *In Vitro* Fertilization and Developmental Capacity of Cattle Oocytes 65

4.4.3 Effect of Cumulus Cells Removal Prior to *In Vitro* Fertilization on Developmental Capacity of Cattle Oocytes..... 66
4.4.4 Developmental Capacity of *In Vitro* Fertlized of Cattle Oocytes in Two Different Culture Systems 67
4.5 Discussions 73

V GENERAL DISCUSSION 78

VI SUMMARY AND CONCLUSIONS 81

REFERENCES 85

APPENDICES 99
Appendix A 99
Appendix B 106

VITA 108

LIST OF TABLES

Table		Page
3.1	Effect of different collection methods on the recovery rates of the number and type of cattle follicular oocytes	39
3.2	Cumulus expansion and maturation rates of oocytes in the four different categories	40
3.3	Effect of serum and hormones supplementation to the maturation medium on cumulus expansion and maturation rates of cattle oocytes	42
3.4	Effect of serum and hormones (FSH + E_2) on nuclear maturation of cattle oocytes	43
4.1	The sequence of sperm penetration in cattle oocytes inseminated *in vitro*	64
4.2	Effect of serum and hormones supplementation to the maturation medium on fertilization rate and developmental capacity of cattle oocytes matured *in vitro*	65
4.3	Effect of cumulus cells removal prior to *in vitro* fertilization on cleavage rate and developmental capacity of cattle oocytes	66
4.4	Effect culture system of *in vitro* matured and fertilzed cattle oocytes on the cleavage rate and developmental capacity	67

LIST OF PLATES

Plate		Page
3.1	Category A oocyte (with ≥ 4 layers of cumulus cells) X 320	44
3.2	Category B oocytes (with 1-3 layers of cumulus cells) X 200	44
3.3	Category C oocytes (denuded (a) and partially denuded (b) oocyte) X 200	45
3.4	Category D oocyte, note the expansion of cumulus cells and the atretic appearance of the oopalsm (arrow), X 200	45
3.5	Acceptable oocytes for IVP embryos (A and B oocytes) X 40	46
3.6	Unacceptable oocytes for IVP of embryos (C oocytes), note the atretic appearance of oocplasm (arrow), X 40	46
3.7	Cumulus expansion of oocytes after IVM with hormones X 40	47
3.8	Cumulus expansion of oocytes after IVM without hormones X 40	47
3.9	Cumulus expansion of oocytes after IVM with hormones, note good expansion of the cumulus cells, X 200	48
3.10	Cumulus expansion of oocytes after IVM without hormones, note poor expansion of the cumulus cells, X 200	48
3.11	Matured oocyte, note the polar body (arrow) X 200 (unstained)	49
3.12	Metaphase II oocyte, note the metaphase II plate (a) and the polar body (b), X 400	49
4.1	Penetration of sperm into oocyte, note the sperm (arrow) and the metaphase palte) X 400	68
4.2	Two pronuclei, the male (a) and female (b) pronuclei, X 400	68
4.3	The cleaved oocytes at 48 h pi in mSOF medium X 40......................	69
4.4	The cleaved oocytes at 48 h pi after stripping the cumulus cells (note the sperm around the zona pellucida) X 200	69

Plate		Page

4.5 Eight-cell embryo co-culture with BOEC monolayer X 200 70

4.6 Morula at day 5 post insemination X 200 70

4.7 Expanded blastocysts at day 9 post insemination (arrow), the oviductal cell monolayer (background) 71

4.8 Expanded blastocyst (note the thinning of the zona) X 200 71

4.9 Hatching Blastocyst at day 10 post insemination X 200 72

4.10 Hatched blastocysts at day 10 post insemination X 200 72

LIST OF ABBREVIATIONS

AI	Anaphase I (First Anaphase)
BME	Basal Medium Eagle
BO	Brackett and Oliphant
boec	bovine oviductal epithelial cell
BSA	bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CL	Corpora lutea
COCs	Cumulus-oocytes complexes
CR1	Charles Rosenkrans 1 medium
E_2	Oestradiol
EGF	Epidermal growth factor
ET	Embryo transfer
FAF	Fatty acid free
FBS	Fetal bovine serum
FCS	Fetal calf serum
FF	Follicular fluid
FSH	Follicle stimulating hormone
g	Gram (s)
g	Gravities (relative centrifugal force)
G	Gauge (for needle size)
GAGs	Glycosaminoglycans
GV	Germinal vesicle
GVBD	Germinal vesicle break down

h	Hour (s)
hpi	Hour (s) post insemination
Hepes	N-2-Hydroxyethylpiperazine-N'-2- ethanesuphonic acid
IGF	Insulin-like growth factor
i.m.	Intramuscular
IU	International unit
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
LH	Luteinizing Hormone
MI	Metaphase I (First metaphase)
MII	Metaphase II (Second mataphase)
MEM	Minimum Essential Medium
mSOF	Modified synthetic oviduct fluid
OCS	Oestrous cow serum
OMI	Oocyte maturation inhibitor
PB	Polar body
PBS	Phosphate buffered saline
psi	pound per square inch
PDE	Posphodiesterase
PHE	Penicillamine, hypotaurine and epinephrine
PN	Pronucleus
RPMI	Rosewall Park Memorial Institute
SOF	Synthetic oviduct fluid

TI	Telophase I (First telophase)
TALP	Tyrode's albumin lactate pyruvate
TGF	Transforming growth factor
TL	Tyrode's lactate
TM	Transmigration process
ZP	Zona pellucida

CHAPTER I

GENERAL INTRODUCTION

Since the birth of the first calf from *in vitro* fertilization (IVF) of an ovulated oocyte (Brackett *et al.*, 1982), much research has been dedicated to the improvement of *in vitro* maturation, *in vitro* fertilization and embryo culture techniques. Hanada *et al.* (1986) reported the first calves born following *in vitro* fertilization of artificially matured oocytes cultured to the blastocysts stage in the rabbit oviduct. In another study, Lu and co-workers (1987) reported one of the first cattle pregnancies from totally *in vitro* procedures: maturation, fertilization and culture of the embryos.

Production of embryos *in vitro* represents a desirable option to enhance reproductive and genetic advances in cattle. Some commercial applications of *in vitro* fertilization technology have included efforts to upgrade beef cattle, to overcome infertility of valuable cows, to produce transgenic cows and to provide a source of sexed embryos. Greater utility can be anticipated with further advances predicted from ongoing efforts in research and development.

The technologies of *in vitro* embryo production, gene transfer, genetic analysis, genetic diagnosis and embryo cloning have the potential to be used synergistically in cattle breeding and improvement. Both gene transfer and cloning by nuclear transfer require the ability to culture embryos and preserve them *in vitro.*

During the past few decades, many live calves, kids, lambs and foals have been obtained and evidence have shown that it is possible to utilize the IVF technology on a commercial basis. *In vitro* production of embryos is constantly becoming a more useful tool for maximizing the number of offspring from valuable cows, producing calves from infertile cows and producing commercial beef cattle in program for beef production without brood cows.

However, the development of cattle embryos produced through *in vitro* techniques, thereby is still inferior to that of their counterparts *in vivo*. Generally, less than 30% of cattle oocytes can reach the morula and blastocyst stages through *in vitro* procedures. Development of procedures that will increase the number of viable embryos produced through *in vitro* maturation and fertilization procedures will economically benefit producers and commercial enterprises alike.

To produce embryos by *in vitro* techniques, it is necessary to recover the oocytes and to complete three biological phases: mature the oocytes, fertilize them and develop the resulting zygotes to the blastocyst stage, when they can be frozen or transferred freshly to the recipient. In recent years, the success of *in vitro* maturation and fertilization of oocytes of farm animals has been greatly improved: pregnancies and offspring being obtained after culture of oocytes *in vitro* and transfer of embryos to recipient animals. However, the percentage of oocytes reaching the blastocyst stage in a complete *in vitro* system (i.e maturation, fertilization and culture *in vitro*) still varies. The maximum rate of embryo production *in vitro* will depend on the optimization of the *in vitro* maturation, fertilization and culture components. This will require more attention to the essential requirements of the cells *in vitro*. Human

technical skills, biological variability in the quality of oocytes and sperm used as starting materials, and protocols are important components of *in vitro* production of cattle embryos.

Therefore, experiments were conducted to determine the factors contributing to every step of the process of *in vitro* production of cattle embryos.

The objectives of this study were:

1. to evaluate the effect of collection methods on the recovery rates of cattle oocytes
2. to investigate the effect of serum and hormone supplementation to the maturation medium, on *in vitro* maturation, fertilization and developmental rates of cattle oocytes
3. to determine the effect of cumulus removal prior to insemination on *in vitro* fertilization of cattle oocytes
4. to compare the effect of culture systems on developmental capacity of early cattle embryos.

CHAPTER II

LITERATURE REVIEW

2.1. Introduction

This review is divided into five parts. The first part reviews some fundamental processes of the male and female gametes, their development and events leading to fertilization *in vivo*. The second and the third parts review studies on sources of oocytes and maturation of oocytes *in vitro*. The fourth part describes the process of *in vitro* fertilization and the final part reviews the *in vitro* culture systems.

2.2 *In Vivo* Development of Gametes

2.2.1 Oogenesis

The formation and maturation of gametes must be completed in both the female and male species before the reproductive process can be initiated. Oogenesis is the formation, growth and maturation of the female gamete (Baker, 1982). The process begins in embryonic life, continues after birth (accelerating during puberty) and reaches a climax at the time of ovulation. The potential gamete associated with the primary follicle when first formed is the oogonium. Oogonia originate from an extension of the yolk sac that forms from the hind gut of the embryo (Bearden and Fuquay, 1980). Following initial formation, proliferation of oogonia by mitotic division, occurs within the parenchyma of ovary. This proliferation ceases before

birth so that the ovaries at birth contain a fixed number of potential ova or oocytes. It has been estimated that there may be more than 200,000 oocytes in primordial follicles in the ovaries of the heifer calf at birth, but less than 300 are likely to reach the ovulatory stage (Erickson, 1966). No oocytes will reach full maturity unless the female reaches puberty. Maturation of oocytes will continue in a cyclic manner after puberty. During each oestrous cycle a group of oocytes will start maturation while others remain dormant.

2.2.2 Folliculogenesis

Folliculogenesis or development of follicles (Baker, 1982) starts from the primordial follicle reverses developed during fetal life. The aim of folliculogenesis is to establish the appropriate environment in which oocytes can complete meiosis to produce a haploid gamete and ovulate from the follicle.

There are three basic types of follicles (Erickson 1966): (1) primordial follicles which consist of centrally located oocytes and one layer of granulosa cells; (2) growing (primary) follicles that consist of fully grown oocytes and several granulosa cell layers covered by the basal laminae in which oocytes grow and increase in follicular cell numbers and layers are occurring; (3) vesicular follicles, in which there is a fully grown oocyte with granulosa cells and a layer of differentiated thecal cell and antrum are present. At this stage both the gametogenic and steroidogenic functions of the ovary are developing. The oocytes of the cow is 120 – 160 µm in diameter at this stage and is surrounded by a capsule, the zona pellucida (Hafez and Hafez, 2000a).