UNIVERSITI PUTRA MALAYSIA

ULTRASTRUCTURAL CHANGES IN BOVINE OOCYTES INDUCED DURING IN VITRO PRODUCTION OF EMBRYOS

KIRON DEEP SINGH KANWAL

FPV 1999 4

ULTRASTRUCTURAL CHANGES IN BOVINE OOCYTES INDUCED DURING *IN VITRO* PRODUCTION OF EMBRYOS

By

KIRON DEEP SINGH KANWAL,

Dissertation submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in the Faculty of Veterinary Medicine and Animal Science Universiti Putra Malaysia

March 1999

DEDICATION

This thesis is dedicated to the loving memory of my father

Mr. Ajeet Singh Kanwal

and

my loving mother

Mrs. G. Kanwal

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to Dr. Abd. Wahid Haron, Chairman of my supervisory committee for his help, guidance, patience and encouragement through out the period of this study.

I am extremely grateful to Professor Dr. Tengku Azmi Tengku Ibrahim, the former chairman of my supervisory committee for helping me in designing this project and for providing moral support without which this study would not have been possible. His expertise in the field of electron microscopy, constructive criticism, inspiration and patience helped in bringing the thesis in its present form.

I am extremely thankful to Dr. Abas Mazni Othman for providing me with the necessary laboratory facilities, technical expertise, invaluable guidance during the course of this study. His informal and helpful attitude inspired me tremendously in completing this arduous journey.

I would be failing in my duty if I do not acknowledge the kind help and facilities provided by Dato' Professor Dr. Sheikh Omar Abdul Rahman, the Dean of the faculty in carrying out the current project. My sincere thanks to Dr. Abdul Aziz Saharee, Deputy Dean (Finance) for his kind help in financial matters and to Prof. Emer. M. R. Jainudeen for his invaluable comments and advice.

Thanks are due to Dr. Fauziah Othman, the member of my supervisory committee for her critical comments, to Dr. Jothi Malar Panandam for her kind assistance in statistical analysis of the data and to Dr. M. K. Menon for his valuable suggestions.

I am indebted to Pn. Munah Abdullah, Pengurus, Kompleks Rumah Sembelih, Senawang, Department of Veterinary Services, Malaysia for providing bovine ovaries for this study and also to Dr. Baljit Singh, Manager, Dairy Unit, UPM for providing dairy cows for experimental purposes.

Special thanks goes to Mr. Ho Oi Kuan, Mrs. Aminah Jusoh and Miss Azilah of the Electron Microscope Unit, UPM and to Dr. Vickyswerny and Mr. Raghunathan of the Electron Microscope Unit, Universiti Malaya, Kuala Lumpur for their help and co-operation at the units. Thanks are also due to Mr Mohd. Padzil A. Rahman, Mr Ahmad Aman, Mr Rozanor Ghani and Pn. Aini Rahman, staff at IVF laboratory, MARDI for their co-operation during the period of my research.

I am grateful to Dr. Laba Mahaputra and Dr. Herry Agoes of Fakultas Kedokteran Hewan, Universitas Airlangga, Surabaya, Indonesia for their co-operation to undertake part of my research in Indonesia.

I am also grateful to Dr. Mohd. Hair Bejo, Dr. Nazri bin Salim, Dr. Rosnina Yusoff for their advice and to Mr Yap Keng Chee, Mr. Abu Bakar, Mr. Zaid Othman and Mr. Fauzi Che Yusuf for their technical assistance.

Last but not the least, I would be failing in my mission if I do not acknowledge the sacrifices made by my loving mother and my near and dear ones who were always with me in my mind for giving me encouragement and guidance throughout my stay in Malaysia. My sincere thanks are due to my sister Dr. Roopinder, my brother Nayan and to all my friends particularly Inderbir, Gurmeet, Manjit Singh, Sukhdev, Bishwa, Karim, Damodar and Suharto for helping me in one way or the other to pursue and accomplish my studies at UPM.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Figure **Page**

LIST OF PLATES

LIST OF ABBREVIATIONS

AL	Annulate lamellae
ATP	Adenosine tri-phosphate
BO	Brackett and Oliphant
BSA	Bovine serum albumin
CCPE	Cumulus cell process endings
CG(s)	Cortical granule(s)
COC's	Cumulus-oocyte-complexes
D-PBS	Dulbecco's phosphate buffered saline
ER	Endoplasmic reticulum
ET	Embryo transfer
FCS	Foetal calf serum
FAF	Fatty acid free
FSH	Follicle stimulating hormone
g	gram (s)
GC	Golgi complex
GV	Germinal vesicle
GVBD	Germinal vesicle break down
h	hour(s)
hCG	Human chorionic gonadotrophin
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HF	Holstein Friesian
ICM	Inner cell mass

i.m.	intramuscular
IU	International units
IVC	*In vitro* culture
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
KK	Kedah Kelantan breed of cows
LH	Luteinizing hormone
MARDI	Malaysian Agricultural Research and Development Institute
mSOF	Modified synthetic oviduct fluid
PB	Polar body
pFSH	Porcine follicular stimulating hormone
PG $F_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PRID	Progesterone releasing intravaginal device
psi	Pound per square inch
PVS	Perivitelline space
RER	Rough endoplasmic reticulum
RO	Reverse osmosis
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SER	Smooth endoplasmic reticulum
SOF	Synthetic oviduct fluid
TEM	Transmission electron microscopy
UPM	Universiti Putra Malaysia
ZP	Zona pellucida

Abstract of dissertation submitted to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

ULTRASTRUCTURAL CHANGES IN BOVINE OOCYTES INDUCED DURING *IN VITRO* PRODUCTION OF EMBRYOS

By

KIRON DEEP SINGH KANWAL
March, 1999

Chairman : Dr. Abd. Wahid Haron, PhD.
Faculty : Veterinary Medicine and Animal Sciences
Universiti Putra Malaysia

Despite substantial progress in the development of procedures for *in vitro* maturation, *in vitro* fertilisation and *in vitro* culture, the production of viable embryos in most laboratories is around 20-30%. The objective of this study was to examine ultrastructural changes induced in bovine oocytes during various stages of *in vitro* embryo production as these changes could contribute towards the low viability of the embryos.

The oocytes recovered from slaughterhouse ovaries were transported in modified Dulbecco's phosphate buffered saline for various periods of time at two different temperatures, *in vitro* matured (IVM), *in vitro* fertilised (IVF) and *in vitro* cultured (IVC) to morulae stages. After submitting to various stages mentioned above the oocytes were processed for transmission electron microscopy and examined in Hitachi 7100 or Phillips CM 12 electron microscopes at 75 or 80 kV respectively.

Results exhibited that holding of oocytes in commonly used transport medium induced changes in the organelles such as rough endoplasmic reticulum, cortical granules, mitochondria and Golgi complexes both in the cumulus cells and those present in the oocytes. The severity of these changes was higher in oocytes transported at 2-4^0 C than those transported at 35-37^0 C the effect of which was reflected by the viability test. None of the cleaved oocytes (0/49) in the former while an average of 18.2% (10/55) of the oocytes in the latter developed to morulae. In the second experiment, the oocytes which were submitted to IVM, exhibited ultrastructural changes such as incomplete cumulus expansion, swelling of mitochondria, reduced incidence of cortical granules and accumulation of lipid droplets which were probably the factors affecting the viability of oocytes.

In the third experiment, examination of *in vitro* produced morulae (35/121, 28.9%) revealed the presence of a large number of lipid droplets, vacuoles and numerous mitochondria undergoing the process of degeneration which ultimately may affect the viability of embryos. It was concluded that the ultrastructural changes induced during various stages of *in vitro* embryo production contribute towards low viability of the *in vitro* produced embryos.

Abstrak disertasi yang dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi syarat untuk Ijazah Doktor Falsafah

PERUBAHAN-PERUBAHAN ULTRASTRUKTUR PADA OOSIT BOVIN TERCETUS SEMASA PENGHASILAN EMBRIO-EMBRIO *IN VITRO*

Oleh

KIRON DEEP SINGH KANWAL
Mac, 1999

Pengerusi : **Dr. Abd. Wahid Haron, PhD.**
Fakulti : **Kedoktoran Vetarinar dan Sains Peternakan**

Sungguhpun banyak kemajuan dalam perkembangan prosedur pematangan *in vitro*, persenyawaan *in vitro* dan kultur *in vitro*, penghasilan embrio-embrio hidup di kebanyakan makmal adalah sekitar 20-30%. Objektif kajian ini adalah untuk memeriksa perubahan-perubahan ultrastruktur pada oosit bovin yang tercetus semasa pelbagai peringkat penghasilan embrio secara *in vitro* di mana ini mungkin merupakan salah satu faktor penyumbang kepada rendahnya tahap hidup embrio tersebut.

Oosit-oosit yang dikumpul dari ovari rumah sembelih dipindah dalam larutan salina tertimbal Dulbecco's fosfat di pelbagai peringkat masa dalam dua suhu yang berbeza, dimatang, disenyawa dan dikultur secara *in vitro* sehingga peringkat morula. Selepas melalui beberapa peringkat, oosit ini ditetap dalam 2.5% gluteraldehid tertimbal dengan sodium cocodilat dan diproses untuk mikroskopi elektron penularan. Perubahan ultrastruktur tercetus di pelbagai peringkat diperiksa dengan menggunakan mikroskop elektron penularan Hitachi 7100 atau Phillips CM 12 pada 75kV atau 80 kV, masing-masing.

Keputusan menunjukkan bahawa menyimpan oosit dalam larutan pemindah mencetus perubahan-perubahan pada organel seperti retikulum endoplasmik kasar,

granul kortikal, mitokondria dan kompleks Golgi pada sel kumulus dan oosit. Perubahan ini meningkat menjadi teruk pada oosit yang dipindah pada 2-4^{0}C berbanding 35-37^{0}C. Tiada oosit yang membentuk morula (0/49) pada suhu terdahulu sementara suhu berikutnya menghasilkan sebanyak 18.2% (10/55) morula.

Dalam ujikaji kedua, oosit yang dilalukan pematangan *in vitro*, menunjukkan perubahan ultrastruktur seperti pengembangan kumulus tak lengkap, pengembungan mitokondria, pengurangan kortikal granul dan pengumpulan titisan lemak yang memberi kesan kepada tahap hidup oosit.

Dalam ujikaji ketiga, pemeriksaan morula (35/121, 28.9%) yang dihasilkan secara *in vitro* menunjukkan kehadiran banyak titisan lemak, vakuol dan mitokondria yang mengalami proses degenerasi di mana akhirnya memberi kesan kepada tahap hidup embrio. Kesimpulannya, perubahan-perubahan ultrastruktur yang tercetus semasa pelbagai peringkat penghasilan *in vitro* embrio merupakan salah satu faktor yang menyumbangkan kepada kadar konsepsi yang rendah embrio-embrio ini.

CHAPTER I

GENERAL INTRODUCTION

A cow normally produces single ovum at each ovulation and if fertilised it delivers a calf at the end of a long gestation period and hence the rate of genetic improvement is slow in this species. In her life time, a cow produces less than ten calves. The ovaries of a calf at birth contain a large pool of (75-160 x 10^3) primary oocytes, the majority of which are wasted in her life time. The availability of techniques to make greater use of these oocytes will facilitate rapid genetic progress of this animal and is one of the main objectives of research in animal reproduction.

Techniques are now available by which immature bovine oocytes recovered from the ovaries can be matured and fertilised *in vitro* to produce embryos for research and commercial production. Bovine *in vitro* fertilisation (IVF) has received greatest attention in the recent years and could serve as a model for the useful reproductive technologies. This technique can provide valuable source of low cost embryonic material and can be used as a replacement for conventional embryo transfer (ET) procedures. With this technique, *in vitro* produced embryos can be generated from oocytes collected from slaughterhouse or from live normally cycling females using intravaginal ultrasonographic puncture techniques (Pieterse *et al.*, 1991).