



**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

	<b>Page</b>
<b>ACKNOWLEDGEMENTS</b> .....	iii
<b>LIST OF TABLES</b> .....	ix
<b>LIST OF FIGURES</b> .....	x
<b>LIST OF PLATES</b> .....	xi
<b>LIST OF ABBREVIATIONS</b> .....	xvii
<b>ABSTRACT</b> .....	xix
<b>ABSTRAK</b> .....	xxi
 <b>CHAPTER</b>	
<b>I. GENERAL INTRODUCTION</b> .....	<b>1</b>
 <b>II. LITERATURE REVIEW</b> .....	<b>5</b>
Introduction .....	5
Oogenesis .....	5
Oocyte .....	6
Cumulus Cells.....	7
Sources of Oocytes For IVF.....	8
Oocyte Quality .....	9
Ultrastructural Studies on Immature Oocytes .....	13
Corona-Cumulus Cells.....	14
Zona Pellucida.....	16
Perivitelline Space .....	17
Ooplasm .....	17
Microvilli .....	17
Mitochondria.....	18
Golgi Complex.....	22
Cortical Granules .....	23
Endoplasmic Reticulum .....	25
Annulate Lamellae .....	27
Cytoplasmic Lattices .....	28



Membrane Bound Vesicles .....	28
Lipids .....	29
Vacuoles.....	30
Nucleus .....	30
Temperature During Oocyte Transportation .....	34
Ultrastructural Changes During Oocyte Storage.....	35
Ultrastructural Studies on Matured Oocytes .....	36
<i>In Vitro</i> Culture .....	47
Ultrastructural Studies on Prehatched Embryos.....	47
<b>III. GENERAL MATERIALS AND METHODS.....</b>	<b>50</b>
Heat Sterilisation.....	50
Electron Microscopy .....	51
Maturation Droplets .....	54
Fertilisation Droplets.....	55
<i>In Vitro</i> Culture Droplets .....	57
<b>IV. ULTRASTRUCTURE OF A NORMAL IMMATURE BOVINE OOCYTE .....</b>	<b>58</b>
Introduction.....	58
Materials and Methods.....	59
Collection and Trimming of Ovaries .....	59
Oocyte Recovery .....	59
Selection of Oocytes .....	60
Electron Microscopy .....	60
Results.....	61
Discussion .....	75
<b>V. ULTRASTRUCTURAL CHANGES INDUCED DURING OOCYTE TRANSPORTATION .....</b>	<b>80</b>
Introduction.....	80
Materials and Methods.....	81
Preparation of Media.....	81
Collection of Ovaries .....	83
Processing of Ovaries.....	83
Recovery of Oocytes .....	84



Distribution of COC's.....	85
Electron Microscopy .....	86
Counting of Cortical Granules .....	86
<i>In Vitro</i> Maturation .....	87
<i>In Vitro</i> Fertilisation.....	87
<i>In Vitro</i> Culture .....	87
Results .....	88
Ultrastructural Changes at 2-4 <sup>o</sup> C .....	88
Ultrastructural Changes at 35-37 <sup>o</sup> C .....	108
Viability Test.....	122
Discussion .....	122
<b>VI. ULTRASTRUCTURAL CHANGES INDUCED DURING MATURATION OF OOCYTES <i>IN VITRO</i> .....</b>	<b>133</b>
Introduction .....	133
Materials and Methods.....	133
<i>In Vivo</i> Maturation .....	133
Maturation of Oocytes In Vitro.....	136
Ovary Collection.....	136
<i>In Vitro</i> Maturation .....	137
Electron Microscopy .....	137
Results.....	138
Light Microscopy .....	138
TEM Observations.....	139
Discussion .....	156
<b>VII. ULTRASTRUCTURAL CHANGES INDUCED DURING <i>IN VITRO</i> CULTURE OF BOVINE EMBRYOS.....</b>	<b>162</b>
Introduction .....	162
Materials and Methods.....	162
<i>In Vivo</i> Embryo .....	163
Selection of Embryos .....	164
<i>In Vitro</i> Embryos.....	165
Results.....	168
Discussion .....	176



<b>VIII. GENERAL DISCUSSION .....</b>	<b>179</b>
<b>IX. SUMMARY AND CONCLUSIONS .....</b>	<b>184</b>
<b>REFERENCES.....</b>	<b>187</b>
<b>APPENDIX - A : Preparation of Media.....</b>	<b>207</b>
<b>APPENDIX - B: Media for Electron Microscopy .....</b>	<b>214</b>
<b>APPENDIX - C: Result Tables.....</b>	<b>217</b>
<b>BIOGRAPHICAL SKETCH .....</b>	<b>218</b>



## LIST OF TABLES

<b>Table</b>		<b>Page</b>
1	Analysis of Variance of Comparison of Temperature Effect on No. of Cortical Granules When Time is 1 h.....	97
2	Analysis of Variance of Comparison of Temperature Effect on No. of Cortical Granules When Time is 3h.....	97
3	Analysis of Variance of Comparison of Temperature Effect on No. of Cortical Granules When Time is 6h.....	104
4	Analysis of Variance of Comparison of Temperature Effect on No. of Cortical Granules When Time is 12h.....	104
5	Analysis of Comparison of All Temperature-Time Combinations With Control .....	105
6	Comparison of Temperature Effect on Number of Cortical Granules Against Control for Different Transportation Time .....	105
7	Analysis of Variance of Comparison of Effect of Time When Temperature 2-4 <sup>0</sup> C .....	106
8	Analysis of Variance of Comparison of Effect of Time When Temperature 35-37 <sup>0</sup> C .....	107
9	Comparison of Time Effect Against Control for Two Temperatures.....	107
10	<i>In Vitro</i> Development of Bovine Oocytes Held in D-PBS at Various Temperatures and for Different Length of Time .....	122
11	Comparison of Transportation Time Effect on the Development of Oocytes to Blastocysts When Temperature is 35-37 <sup>0</sup> C .....	217
12	Presence of Dominant Follicles on the Ovaries of Superovulated Cows and the Number of Ovulated Ova relative to Time After PGF injection.....	138
13	Number of Mournalae / Blastocysts Produced <i>In Vivo</i> From Superovulated Donor Cows .....	169
14	Number of Morulae / Blastocysts Produced <i>In Vitro</i> Following IVM, IVF and IVC.....	169



## LIST OF FIGURES

Figure	Page
1. Upward Oviduct Flushing Method .....	135



## LIST OF PLATES

<b>Plate</b>		<b>Page</b>
1	Light Microscopic Appearance of a Normal Immature Bovine Oocyte	63
2	Cross-section of a Normal Immature Oocyte	63
3	Electron Micrograph of Corona-Cumulus Cells Containing Well Defined Large Nucleus	64
4	Adjacent Cumulus Cells in Communication Through Gap Junctions	64
5	Corona Radiata Cell Contains Elongated Mitochondria and Multivesicular Bodies	65
6	Rough Endoplasmic Reticulum and Lipid Droplets in Corona Cells	65
7	Numerous Golgi Complexes Associated with Vacuoles and Rough Endoplasmic Reticulum in Corona Radiata Cells	66
8	Cytoplasmic Processes from Corona Cells Traversing Through Zona Pellucida and Make Contact with Oolemma	66
9	Cytoplasmic Processes Contain Mitochondria and Rough Endoplasmic Reticulum	67
10	Cytoplasmic Processes Making Contact with Microvilli from Oolemma	67
11	Special Structure Specifically in Cumulus Cells -Concentric Lamellae Studded with Ribosome Known as Ribosome-Lamella Complex	68
12	Magnified Version of Ribosome-Lamella Complex	68
13	Hooded Mitochondria Associated with Endoplasmic Reticulum Present in Ooplasm of Immature Oocytes	71
14	Numerous Well Developed Golgi Complexes in Ooplasm	71
15	Aggregates of Cortical Granules Present in the Peripheral Ooplasm	72
16	Cortical Granules have Different Electron Densities	72
17	Large Sized Vesicles Uniformly Distributed in Ooplasm	73

18	Most of the Vesicles are Associated with Endoplasmic Reticulum	73
19	Vesicles Associated with Smooth Endoplasmic Reticulum and Many Vesicles have Broken Membranes	74
20	A Spherical Germinal Vesicle Containing Several Nucleoli Present in Peripheral Ooplasm	74
21	Oocyte from Control Group Showing Compact Arrangement of Cumulus Cells	89
22	Oocyte Transported at 2-4 <sup>0</sup> C for 1 h Shows no Notable Changes	89
23	Dilatation of Rough Endoplasmic Reticulum and Accumulation of Lipid Droplets in Corona-Cumulus Cells at the End of 3 h in D-PBS at 2-4 <sup>0</sup> C	90
24	Most of Corona-Cumulus Cells Have Pyknotic Nuclei and Extensive Dilatation of RER at the End of 12 h at 2-4 <sup>0</sup> C	90
25	Rough Endoplasmic Reticulum in Corona Cells in Control Group	91
26	Rough Endoplasmic Reticulum Present in Corona-Cumulus Cells Shows Dilatation at the End of 1h Held in D-PBS at 2-4 <sup>0</sup> C.	91
27	Increase in the Degree of Dilatation of Rough Endoplasmic Reticulum at the End of 6h in D-PBS at 2-4 <sup>0</sup> C	92
28	Dilatation in Rough Endoplasmic Reticulum is Severe at the End of 12 h in D-PBS at 2-4 <sup>0</sup> C and Accumulation of Lipid Droplets	92
29	Mitochondria Present in the Corona-Cumulus Cells of an Oocyte from Control Group	93
30	Mitochondria in Corona-Cumulus Cells Exhibit Swelling While Held in D-PBS at 2-4 <sup>0</sup> C for One Hour	93
31	Golgi Complex in Corona Cell in Association With Numerous Vesicles and Rough Endoplasmic Reticulum (Control Group)	94
32	Golgi Complex in Corona-Cumulus Cells Exhibit Swelling of its Sacules at the End of 1 h of Holding in D-PBS at 2-4 <sup>0</sup> C	94
33	Swelling in Golgi Complexes Present in Corona-Cumulus Cells is Relatively More at the End of 12 h in D-PBS at 2-4 <sup>0</sup> C	95
34	Accumulation of Lipid Droplets in Corona-Cumulus Cells at the End of 6 h in D-PBS at 2-4 <sup>0</sup> C	95
35	Compact Zona Pellucida of a COC from a Control Group	98



36	Zona Pellucida Appears Thicker in COC's Held in Transport Medium at 2-4 <sup>0</sup> C for One Hour	98
37	Zona Pellucida is Relatively Thicker in COC's Held in Transport Medium at 2-4 <sup>0</sup> C for 12 h	99
38	Different Size of Mitochondria Present in the Ooplasm of COC's from Control Group	99
39	Mitochondria in the Ooplasm Exhibit Swelling with Loss of Matrix and Cristae at the End of 1h in Transport Medium at 2-4 <sup>0</sup> C	100
40	Mitochondria in the Ooplasm Exhibit Ballooning of Cristae at the End of 6 h in Transport Medium at 2-4 <sup>0</sup> C	100
41	Golgi Complexes Present in the Peripheral Ooplasm of COC's from the Control Group Associated with Numerous Vesicles	101
42	Golgi Complexes in the Ooplasm Exhibit Extensive Swelling in COC's Held in Transport Medium at 2-4 <sup>0</sup> C for 12 h	101
43	Cortical Granules are Membrane Bound and Have Different Sizes and Electron Densities (Control Group)	102
44	Cortical Granules Appear Vacuolated at the End of 3h in Transport Medium at 2-4 <sup>0</sup> C	102
45	Cortical Granules Present in a Row Below the Oolemma Indicating a Reduction in Their Number at the End of 6h in D-PBS at 2-4 <sup>0</sup> C	103
46	Increase in Lipid Droplets in the Ooplasm of COC's Held in D-PBS at 2-4 <sup>0</sup> C for 6 h	103
47	Marked Intercellular Spaces Between Corona Cells and Zona and Organelle Free Area in Centre of Oocyte at the End of 3 h in D-PBS at 35-37 <sup>0</sup> C	110
48	Elongation of Corona Cells and Increase in Intercellular Spaces Between Corona-Cumulus Cells at the End of 6 h at 35-37 <sup>0</sup> C	110
49	Corona Radiata Cell of a COC Transported in D-PBS at 35-37 <sup>0</sup> C at the End of 3h Exhibit Vacuolation of its Cytoplasm	111
50	Corona Cell Undergoing the Process of Vacuolation of Cytoplasm at the End of 12 h	111
51	Rough Endoplasmic Reticulum Appears Blurred and Swollen in COC's Transported for 1h at 35-37 <sup>0</sup> C	112



52	Rough Endoplasmic Reticulum in the Corona-Cumulus Cells at the End of 12h is Better Preserved. Cytoplasmic Matrix Appears Granular	112
53	Mitochondria in Corona-Cumulus Cells Appear Normal And The Cytoplasmic Matrix Contains Dense Particles at the End of 1h at 35-37 <sup>0</sup> C	113
54	Reduction in the Size of Mitochondria and Increase in Lipid Droplets in Corona-Cumulus Cells at the End of 12h at 35-37 <sup>0</sup> C	113
55	Golgi Complexes in Corona-Cumulus Cells Appear Elongated Accompanied with Vesicles at the End of 3h at 35-37 <sup>0</sup> C	114
56	Stacks of Golgi Complexes Poorly Developed Associated with Vacuoles at the End of 12h at 35-37 <sup>0</sup> C	114
57	Zona Pellucida and Small Perivitelline Space of a COC Held in D-PBS at 35-37 <sup>0</sup> C for 1h	117
58	No Marked Changes in the Zona Pellucida at the End of 12h in Transport Medium at 35-37 <sup>0</sup> C. Perivitelline Space is Enlarged	117
59	Mitochondria in the Ooplasm Show No Significant Changes at the End of 1 h in D-PBS at 35-37 <sup>0</sup> C	118
60	Loss of Bounding Membrane and Cristae Apparent in Mitochondria in Ooplasm of COC's in D-PBS at 35-37 <sup>0</sup> C for 12h	118
61	Golgi Complex in the Ooplasm of COC's Held at 35-37 <sup>0</sup> C for 1h Exhibit Swelling and are Elongated. Vesicles are Present	119
62	The Stacks of Golgi Complexes Appear Straight, Elongated and Relatively More Organised at the End of 6h at 35-37 <sup>0</sup> C. Vesicles Contain Dense Material	119
63	Incidence of Cortical Granules at the End of 6h at 35-37 <sup>0</sup> C Seems To Be Reduced and Some of Them Form Vacuoles Around Them	120
64	Cortical Granules at the End of 12h Holding Time at 35-37 <sup>0</sup> C are Electron Lucent and Many Loosing Matrix	120
65	Few Cortical Granules Appear Fused with the Oolemma at the End of 12h at 35-37 <sup>0</sup> C. Zona Pellucida	121
66	Numerous Large Sized Lipid Droplets in the Ooplasm of COC's Held in Transport Medium at 35-37 <sup>0</sup> C for 6h	121
67	Light Micrograph of Ovulated Ova Devoid of Cumulus Cells	140

68	Semi-Thin Section of <i>In Vitro</i> Matured Oocyte Showing Cumulus Expansion	140
69	Electron Micrograph of an <i>In Vitro</i> Matured Oocyte Showing Increase in Intercellular Spaces Between Corona-Cumulus Cells, Elongation of Corona-Cumulus Cells	141
70	An Increase in the Incidence of Vacuoles Containing Electron Dense Material was Observed in the Corona Radiata Cells <i>In Vitro</i> Matured Oocytes	141
71	Incidence of Rough Endoplasmic Reticulum was Reduced in Corona-Cumulus Cells of <i>In Vitro</i> Matured Oocytes. Golgi Complex Associated with Numerous Vesicles	142
72	Most of Mitochondria in Corona-Cumulus Cells of <i>In Vitro</i> Matured Oocytes Appeared Swollen Accompanied with Loss of Matrix	142
73	Zona Pellucida in Ovulated Ova is Wider and Does Not Contain Any Remnants of Cytoplasmic Processes from the Corona Cells	146
74	Zona Pellucida of <i>In Vitro</i> Matured Oocytes Appears Compact, Less Wide and Contains Remnants of Cytoplasmic Processes	146
75	Part of Process from Corona Radiata Cells Can Still be Seen in the Zona Pellucida at the End of <i>In Vitro</i> Maturation	147
76	Higher Magnification of the Process of Corona Radiata Cells inside the Zona Contain Mitochondria and Vesicles	147
77	Wide Perivitelline Space in Ovulated Ova Contain Electron Lucent Material and Microvilli	148
78	Perivitelline Space <i>In Vitro</i> Matured Oocytes Contain Many Microvilli and the Ooplasm is Smoother than that Seen in Ovulated Ova	148
79	First Polar Body With One Half Chromatin in the Perivitelline Space of <i>In Vitro</i> Matured Oocyte	149
80	Numerous Cortical Granules are seen in a Row Immediately Below Oolemma in Ovulated Ova	149
81	Incidence of Cortical Granules <i>In Vitro</i> Matured Oocytes is Reduced Compared with Ovulated Ova. Large Cytoplasmic Body Present in the Perivitelline Space	150



82	Organelles in Ovulated Ovum Uniformly Distributed Except a Small Peripheral Zone which Lacks in General Organelles. A Large Perivitelline Space of Uniform Width Present.	150
83	Cytoplasmic Zone at the Periphery Lacking in Organelles is Wider <i>In Vitro</i> Matured Oocytes. Smaller and Uneven Perivitelline Space, Large Number of Lipid Droplets	151
84	Small Aggregates of Smooth Endoplasmic Reticulum are Seen in the Peripheral Ooplasm in Ovulated Ova	151
85	Aggregates of Smooth Endoplasmic Reticulum are Larger <i>In Vitro</i> Matured Oocytes and are Mostly Located at the Periphery	152
86	Mitochondria in the Ooplasm of Ovulated Ova are Relatively More Electron Dense and Contain Numerous Lamellae Like Cristae. Some of them are Hooded	152
87	Mitochondria <i>In Vitro</i> Matured Oocytes Appear Swollen, Less Electron Dense and Contain Fewer Cristae	153
88	Large Sized Vesicles Containing Granular Material, Associated with Smooth Endoplasmic Reticulum and Mitochondria are Seen in the Ooplasm of Ovulated Ova	153
89	Vesicles in the Ooplasm of <i>In Vitro</i> Matured Oocytes are Smaller in Size and Few are Associated with Smooth Endoplasmic Reticulum	154
90	Few Small Sized Lipid Droplets were Observed in the Ooplasm of Ovulated Ova	154
91	Numerous Large Sized Lipid Droplets were Found Distributed all Over the Ooplasm <i>In Vitro</i> Matured Oocytes	155
92	Light Microscopic Observations On <i>In Vitro</i> Produced Embryos Collected At 132 h Post-Insemination Showing Most of Them Are At Compacted Morula Stage	171
93	Light Microscopic Observations On An <i>In Vivo</i> Derived Morula (Compacting) Showing Some Blastomere Extrusion Collected From Superovulated Cow At Day 6 After Oestrus	171
94	Blastomeres of <i>In Vivo</i> Produced Morulae are in Communication Through Short Electron Dense Contact Areas. Nucleoli has Reticular Morphology	172
95	Electron Micrograph of <i>In Vitro</i> Morula Showing Asymmetrical Blastomeres, Large Sized Lipid Droplets, Numerous Vacuoles	172

96	Zona Pellucida of an <i>In Vivo</i> Produced Morula Showing Inner Surface Compact and Uniform While the Outer Surface is Irregular and has Spaces	173
97	Zona Pellucida of an <i>In Vitro</i> Produced Morula is Relatively Thinner and Appears to be Undergoing the Process of Degeneration. Cytoplasm of the Blastomeres is Electron Dense	173
98	Numerous Mitochondria of Various Shapes and Sizes are Present in the Blastomeres of <i>In Vivo</i> Produced Morula	174
99	Higher Magnification showing Immature Mitochondria having Translucent Matrix and Cristae have not Formed <i>In Vivo</i> Produced Morulae	174
100	Most of Mitochondria <i>In Vitro</i> Produced Morulae are Condensed, Vacuolated and Show Ballooning of Cristae	175
101	Numerous Multivesicular Bodies were Present in the Blastomeres of <i>In Vivo</i> Produced Morulae	175



## 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	<i>In vitro</i> culture
IVF	<i>In vitro</i> fertilisation
IVM	<i>In vitro</i> 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 <sub>2α</sub>	Prostaglandin F <sub>2α</sub>
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<sup>0</sup> C than those transported at 35-37<sup>0</sup> 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 tertimbang 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 tertimbang 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<sup>0</sup>C berbanding 35-37<sup>0</sup>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, pengembangan 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 menyumbang 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  $(75-160 \times 10^3)$  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).

