CRYOPRESERVATION OF OVINE OOCYTES

by

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TABLE OF CONTENTS

TABLE OF CONTENTS I

ABSTRACT VI

ACKNOWLEDGEMENTS VII

LIST OF PUBLICATIONS VIII

LIST OF FIGURES IX

LIST OF TABLES XI

LIST OF ABBREVIATIONS XIII

CHAPTER 1 1

LITERATURE REVIEW 1

1.1 INTRODUCTION 1

1.2 ASSISTED REPRODUCTIVE TECHNOLOGIES (ART) 4

1.2.1 Oocyte maturation 5

1.2.2 Fertilisation and polyspermy 7

1.3 CRYOPRESERVATION OF REPRODUCTIVE POTENTIAL IN ASSISTED REPRODUCTION 9

1.3.1 Sperm cryopreservation 9

1.3.2 Embryo cryopreservation 10

1.3.3 Oocyte cryopreservation 12

1.3.3.1 Rationale for oocyte cryopreservation in mammals 12

1.3.3.1.1 Oocyte cryopreservation in human reproduction 13

1.3.3.1.2 Application of oocyte cryopreservation in research 14

1.3.3.1.2 Application of oocyte cryopreservation in livestock production 15

1.4 CONVENTIONAL METHODS FOR CRYOPRESERVATION 16

1.4.1 Traditional slow freezing 18

1.4.2 Conventional vitrification 20

1.4.3 Ultrarapid vitrification 20

1.5 MECHANISMS OF CELL INJURY DURING CRYOPRESERVATION 22

1.5.1 Types of cell injuries during cryopreservation 22

1.5.1.1 Ice crystal formation 22

1.5.1.2 Osmotic shock 23

1.6 SLOW COOLING VS. VITRIFICATION 23

1.6.1 Variables in vitrification 25

1.6.1.1 Cooling and warming rates 26

1.6.1.2 Cryoprotectants 27

1.6.1.3 Cryodevices 28

1.7 OOCYTE STRUCTURE AND CRYOPRESERVATION DAMAGE. 31

1.8 CONCLUSIONS 32

1.9 GENERAL AIMS OF EXPERIMENTAL CHAPTERS 33
CHAPTER 2 36

GENERAL MATERIALS AND METHODS 36
2.1 ISOLATION OF OVINE CUMULUS OOCYTE COMPLEXES 36
2.2 VITRIFICATION OF OVINE COCs 37
2.2.1 Exposure of COCs to different vitrification solutions (VS) 37
2.2.2 Vitrification of COCs by solid surface vitrification (SSV) 37
2.2.2.1 Warming of SSV oocytes 38
2.2.3 Vitrification of COCs using cryoloop 39
2.2.3.1 Warming of cryoloop vitrified oocytes 39
2.2.4 Oocyte viability 40
2.3 IN VITRO MATURATION OF VITRIFIED-THAWED OVINE OOCYTES 41
2.3.1 Evaluation of cumulus cell expansion following IVM 41
2.3.2 Assessment of nuclear maturation using aceto orcein staining 41
2.4 IMMUNOSTAINING FOR EVALUATION OF CHROMOSOME AND SPINDLE CONFIGURATION 42
2.5 IN VITRO FERTILISATION OF OVINE OOCYTES 43
2.5.1 Sperm preparation 43
2.5.2 In vitro fertilisation 44
2.5.3 Evaluation of frequencies of in vitro fertilisation and polyspermy following IVF 44
2.6 PARTHENOGENETIC ACTIVATION 45
2.7 NUCLEAR TRANSFER 46
2.8 IN VITRO CULTURE OF OVINE EMBRYOS 47
2.8.1 Evaluation of the frequencies of cleavage and subsequent embryo development 48
2.8.2 Evaluation of total cell numbers 48
2.9 TUNEL ASSAY TO DETERMINE DNA FRAGMENTATION 48
2.10 KARYOTYPING OF IN VITRO PRODUCED BLASTOCYSTS 49
2.11 IN VITRO DOUBLE KINASE ASSAY 50
2.11.1 Preparation of oocyte lysate 50
2.11.2 In vitro double kinase assay 50
2.12 STATISTICAL ANALYSIS 51
CHAPTER 3 52

EFFECTS OF EXPOSURE OF IMMATURE OVINE OOCYTES TO DIFFERENT COMBINATIONS OF CRYOPROTECTANTS ON VIABILITY AND SUBSEQUENT DEVELOPMENT 52
3.1 INTRODUCTION 52
3.2 MATERIALS AND METHODS 54
3.3 RESULTS 55
3.3.1 Effects of different cryoprotectants on oocyte viability 55
3.3.2 Effects of different vitrification solutions on cumulus cell expansion following IVM 56

3.3.3 Effects of different cryoprotectants on meiotic maturation following IVM..... 57
3.4 DISSCUSION..... 59
CHAPTER 4..... 63
IN VITRO FERTILISATION OF OVINE OOCYTES VITRIFIED BY SOLID SURFACE VITRIFICATION (SSV) AT GERMINAL VESICLE STAGE..... 63
4.1 INTRODUCTION..... 63
4.2 MATERIALS AND METHODS..... 65
4.3 RESULTS..... 66
4.3.1 Recovery rates and oocyte viability following SSV..... 66
4.3.2 Cumulus cell expansion and meiotic maturation following IVM 67
4.3.3 Frequencies of fertilisation and polyspermy following IVF..... 70
4.3.4 Kinetics of first cleavage following IVF..... 72
4.3.5 In vitro embryo development following IVF..... 73
4.4 DISCUSSION..... 75
CHAPTER 5..... 81
VITRIFICATION OF IMMATURE OVINE OOCYTES USING THE CRYOLOOP: EFFECTS ON VIABILITY, IN VITRO MATURATION, FERTILISATION AND SUBSEQUENT DEVELOPMENT..... 81
5.1 INTRODUCTION..... 81
5.2 MATERIALS AND METHODS..... 83
5.3 RESULTS..... 84
5.3.1 Recovery rates and oocyte survival..... 84
5.3.2Cumulus cell expansion and nuclear maturation following IVM. 86
5.3.3Spindle and chromosome organization..... 88
5.3.4 In vitro fertilisation and polyspermy..... 91
5.3.5 In vitro embryo development following IVM/IVF..... 93
5.3.6 Total cell numbers and apoptotic nuclei of in vitro produced blastocysts..... 96
5.3.7 Chromosomal analysis of in vitro produced embryos..... 99
5.4 Discussion..... 101
CHAPTER 6..... 106
MPF/MAPK, PARTHENOGENETIC ACTIVATION, AND SOMATIC CELL NUCLEAR TRANSFER (SCNT) OF OVINE OOCYTES VITRIFIED AT GV-STAGE..... 106
6.1 INTRODUCTION..... 106
6.2 MATERIALS AND METHODS..... 108
6.3 RESULTS..... 109
6.3.1 MPF/MAPK activities..... 109
6.3.2 Frequencies of cleavage following parthenogenetic activation.. 110
6.3.3 In vitro embryo development following parthenogenetic activation..... 111
6.3.4 Apoptosis in blastocysts produced following parthenogenetic

activation..... 113
6.3.5 Frequencies of enucleation and fusion..... 114
6.3.6 Cleavage and subsequent development of SCNT embryos..... 115
6.3.7 Total cell numbers and apoptosis in blastocysts produced by SCNT..... 115
6.3.8 Karyotyping of SCNT embryos..... 117
6.4 DISCUSSION..... 118
CHAPTER 7..... 122

VITRIFICATION OF IMMATURE OVINE OOCYTES WITH CRYOLOOP: EFFECT OF CYTOCHALASIN B PRE-TREATMENT ON VIABILITY AND SUBSEQUENT DEVELOPMENT..... 122
7.1 INTRODUCTION..... 122
7.2 MATERIALS AND METHODS..... 124
7.3 RESULTS..... 125
7.3.1 Effect of CB pre-treatment on viability of vitrified COCs..... 125
7.3.2 Effect of CB treatment on fertilisation events following IVF... 126
7.3.3Effect of CB treatment on in vitro embryo development following IVF..... 129
7.4 DISCUSSION..... 133
CHAPTER 8..... 136

EFFECT OF CAFFEINE TREATMENT ON SPINDLE CONFIGURATION, MPF/MAPK ACTIVITIES AND SUBSEQUENT DEVELOPMENT OF OVINE OOCYTES VITRIFIED AT GERMINAL VESICLE (GV) STAGE..... 136
8.1 INTRODUCTION..... 136
8.2 MATERIALS AND METHODS..... 138
8.3 RESULTS..... 139
8.3.1 Effect of caffeine treatment on MPF/MAP kinases activities. 139
8.3.2Effect of caffeine treatment on spindle and chromatin configuration..... 141
8.3.3 Effect of caffeine treatment on frequency of cleavage following IVF..... 144
8.3.4 Effect of caffeine treatment on subsequent development following IVF..... 145
8.4 DISCUSSION..... 148
CHAPTER 9..... 152

EFFECT OF DEMECOLCINE PRE-TREATMENT ON VIABILITY, TIMING OF FIRST POLAR BODY EXTRUSION, SPINDLE CONFIGURATION, AND SUBSEQUENT DEVELOPMENT OF OVINE OOCYTES VITRIFIED AT GERMINAL VESICLE (GV) STAGE..... 152
9.1 INTRODUCTION..... 152
9.2 MATERIALS AND METHODS..... 153
9.3 RESULTS..... 154

9.3.1 Effect of Deme pre-treatment on viability of vitrified oocytes.... 154
9.3.2 Timing of first polar body (PBI) extrusion..... 154
9.3.3 Effect of Deme pre-treatment on spindle and chromosome organization of ovine oocytes vitrified at GV stage..... 156
9.3.4 Effect of Deme pre-treatment on in vitro embryo development following IVM/IVF/IVC of ovine oocytes vitrified at GV stage.. 158
9.3.5 Effect of Deme pre-treatment on parthenogenetic development of ovine oocytes vitrified at GV stage..... 160
9.4 DISCUSSION..... 162

CHAPTER 10..... 164

GENERAL DISCUSSION..... 164

REFERENCES..... 172

APPENDIX A..... 201

MEDIA AND STOCK SOLUTIONS..... 201

APPENDIX B..... 216

CRYOPRESERVATION OF OOCYTES IN DIFFERENT SPECIES... 216

APPENDIX C..... 222

VITRIFICATION OF BOVINE BLASTOCYSTS AND ESTABLISHMENT OF PREGNANCY..... 222

ABSTRACT

Oocyte cryopreservation represents one of the most recent developments in the field of reproductive technologies. However, despite of significant progress, the efficiency of oocyte cryopreservation is still very low. Cryopreservation of mature metaphase II (MII) oocytes has been reported to induce disorganization of the meiotic spindle and chromosome damage. However, cryopreservation of immature oocyte at germinal vesicle (GV) stage may provide an alternative which avoids these problems. Slow freezing protocols have more recently been replaced by vitrification approaches. In this thesis, recovery, viability and subsequent developmental potential following in vitro fertilisation (IVF), parthenogenetic activation or somatic cell nuclear transfer (SCNT) of ovine oocytes vitrified at GV stage and matured in vitro were studied. Solid surface vitrification (SSV) and cryoloop technologies share the advantages of using a containerless system and small volumes of solution (less than 1 µl) which favours rapid cooling. Maturation, fertilisation, cleavage and blastocyst development were significantly decreased in SSV vitrified oocytes as compared to controls. Following cryoloop vitrification, frequencies of in vitro maturation (43.4 *vs* 63.2%), oocytes with normal spindle and chromosome configuration (50.0 *vs* 70.4%) and fertilisation (54.0 *vs* 74.1%) did not differ significantly between vitrified and control oocytes. Numbers of cleaved embryos that developed to the blastocyst stage following IVM/IVF/IVC did not differ significantly between vitrified and control groups (29.4 *vs* 45.1%). In vitro matured ovine oocytes vitrified at GV stage using cryoloop were activated by two different protocols (1) a combination of calcium ionophore (A 23187), cycloheximide and cytochalasin B (CA+CHX/CB), (2) strontium and CB (Sr/CB). No blastocysts developed in vitrified oocytes activated by CA+CHX/CB; however, 3.8% were obtained following Sr/CB activation. Developmental competence of ovine oocytes vitrified at GV stage and used as cytoplast recipients for SCNT was evaluated. Although the frequencies of cleaved embryos were significantly decreased in vitrified oocytes as compared to control, development to morula and blastocyst stage embryos was not significantly different. No significant differences were observed in total cell numbers, number of apoptotic nuclei as detected by Hoechst and TUNEL assay and proportions of diploid embryos in day 7 blastocysts produced following IVF or SCNT of vitrified oocytes as compared to control. Pre-treatment of ovine GV-oocytes with cytochalasin B (7.5 µg/ml for 60 min) or demecolcine (0.1 µg/ml for 20 min) prior to vitrification improved frequencies of maturation, fertilisation and subsequent development following IVF or parthenogenetic activation. Caffeine treatment during IVM (10 mM for 6 h) increased the frequencies of blastocyst development in vitrified/thawed GV ovine oocytes.

Taken together, these studies suggest that, ovine oocytes vitrified at GV stage can be matured, fertilised and develop in vitro to blastocyst stage embryos. Cryoloop vitrification resulted in higher maturation, fertilisation and subsequent development as compared to SSV. Strontium can be used effectively for parthenogenetic activation of vitrified/thawed ovine GV oocytes. Ovine oocytes vitrified at GV stage can be used effectively as cytoplast recipients for SCNT.

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List OF FIGURES

Figure 1.1 Developmental events in mammals 7
Figure 1.2 Schematic representation of an oocyte/embryo (circle) during slow freezing, conventional vitrification, and ultrarapid vitrification..... 17
Figure 2.1 Vitrification of COCs using SSV..... 38
Figure 2.2 Cryoloop vitrification of immature ovine oocytes 40
Figure 2.3 Production of oocyte somatic cell couplets from ovine oocyte vitrified at GV-stage..... 47
Figure 3.1 Effects of exposure of immature ovine oocytes to different vitrification solutions on survival rates 55
Figure 3.2 Cumulus cell expansion following IVM of ovine oocytes exposed to different vitrification solutions at GV-stage..... 56
Figure 3.3 Nuclear maturation of ovine oocytes..... 58
Figure 4.1 Recovery rates of ovine oocytes vitrified at GV-stage by Solid Surface Vitrification (SSV)..... 66
Figure 4.2 Viability of ovine oocytes vitrified at GV-stage by Solid Surface Vitrification (SSV)..... 67
Figure 4.3 Degenerated oocyte recovered following SSV of ovine COCs.... 67
Figure 4.4 Cumulus cell expansion following IVM of ovine oocytes vitrified at GV-stage using SSV 68
Figure 4.5 Oocytes with expanded cumulus cells..... 69
Figure 4.6 Meiotic maturation of ovine oocytes..... 69
Figure 4.7 Fertilisation events in ovine oocytes..... 71
Figure4.8 Developmental stages of ovine embryos produced by IVF of ovine oocytes vitrified at GV-stage using SSV..... 75
Figure 5.1 Recovery rates of ovine oocytes vitrified at GV-stage using the cryoloop 85
Figure 5.2 Survival of ovine oocytes vitrified at GV-stage by the cryoloop..... 85
Figure 5.3 Damaged oocytes recovered following cryoloop vitrification 85
Figure 5.4 Cumulus cell expansion following IVM of ovine oocytes vitrified at GV-stage using cryoloop 86
Figure 5.5 Oocytes with expanded cumulus cells 87
Figure 5.6 Meiotic maturation of ovine oocytes 88
Figure5.7 Examples of spindle and chromosome organization in ovine oocytes 90
Figure 5.8 Fertilisation status of ovine oocytes following IVF..... 92
Figure 5.9 Developmental stages of ovine embryos produced by IVF of vitrified/ thawed GV-stage oocytes..... 96
Figure 5.10 Total cell numbers and apoptotic nuclei in day 7 embryos determined by Hoechst and TUNEL assay..... 98
Figure 5.11 Chromosomal analysis of day 7 in vitro produced blastocysts..... 100
Figure 6.1 Maturation promoting factor (MPF) and mitogen-activated protein kinase (MPAK) activities 109

Figure 6.2 Representative blastocyst stage embryos stained with Hoechst and TUNEL assay 113
Figure 6.3 Enucleation of ovine oocytes vitrified at GV-stage..... 114
Figure 6.4 Day 7 blastocysts produced by SCNT..... 115
Figure 7.1 Effects of CB pre-treatment on viability of vitrified/thawed ovine GV oocytes..... 125
Figure 7.2 In vitro fertilisation of ovine oocytes..... 128
Figure 7.3 Day 7 blastocysts produced by IVF of ovine oocytes vitrified at GV stage and pre-treated with CB..... 131
Figure 7.4 Total cell numbers in day 7 in vitro produced blastocysts..... 132
Figure 8.1 Activation of MPF..... 137
Figure 8.2 Effects of caffeine treatment on maturation promoting factor (MPF) and mitogen-activated protein kinase (MPAK) activities..... 140
Figure 8.3 Spindle and chromatin configuration..... 142
Figure 8.4 Day 7 ovine blastocysts produced by IVF..... 146
Figure 9.1 Effect of demecolcine (Deme) pre-treatment on viability of ovine oocytes vitrified at GV-stage..... 154
Figure 9.2 Effect of demecolcine (Deme) pre-treatment on timing of first polar body extrusion (PBI) in ovine oocytes vitrified at GV-stage.... 155
Figure 9.3 Ovine oocyte showing first polar body (PBI) following 24 h IVM.. 155
Figure 9.4 Spindle and chromatin configuration of ovine oocytes following 24 IVM..... 156
Figure 9.5 Day 7 in vitro produced blastocysts..... 160
Figure 10.1 The best conditions used for vitrification of immature ovine oocytes applied in this thesis..... 171

List OF TABLES

Table1.1 Mammalian species yielding normal offspring following the transfer of cryopreserved embryos to foster mother..... 11
Table 1.2 Applications of oocyte cryopreservation..... 13
Table 1.3 Comparison of the three approaches for cryopreservation of oocytes 18
Table 1.4 A comparison of vitrification with slow-cooling procedures..... 25
Table1.5Variables in vitrification that can profoundly influence its effectiveness..... 25
Table 3.1Effects of exposure of immature ovine oocytes to different vitrification solutions on survival rates..... 55
Table 3.2Effect of exposure to different vitrification solutions on subsequent nuclear maturation of immature ovine oocytes..... 58
Table 4.1 Recovery rates and viability of ovine oocytes vitrified at GV-stage by Solid Surface Vitrification (SSV)..... 66
Table 4.2 Nuclear maturation of ovine oocytes vitrified at GV-stage by SSV. 69
Table 4.3 Frequencies of in vitro fertilisation and polyspermy following IVM/IVF of ovine oocytes vitrified at GV-stage by SSV..... 70
Table 4.4 First cleavage following IVM/IVF of ovine oocytes vitrified at GV stage..... 72
Table 4.5 In vitro embryo development following IVF of vitrified/thawed ovine COCs using SSV..... 74
Table 5.1 Recovery rates and viability of ovine oocytes vitrified at GV-stage using cryoloop..... 84
Table 5.2 Nuclear maturation of ovine oocytes vitrified at GV-stage using cryoloop..... 87
Table 5.3 Effect of vitrification of immature ovine oocytes on spindle and chromosome configuration following IVM..... 89
Table 5.4 In vitro fertilisation of ovine oocytes vitrified at GV-stage using cryoloop..... 91
Table 5.5 Effects of cryoloop vitrification on survival rates after IVF..... 94
Table 5.6 Developmental potential of ovine oocytes vitrified at GV-stage using cryoloop..... 95
Table 5.7 Total cell numbers and apoptotic nuclei in day 7 blastocyst embryos..... 97
Table 5.8 Chromosomal composition of day 7 blastocysts produced by IVF of ovine oocytes vitrified at GV-stage..... 100
Table 6.1 Cleavage rates following parthenogenetic activation of ovine oocytes vitrified at GV stage..... 110
Table 6.2 In vitro embryo development following parthenogenetic activation of ovine oocytes vitrified at GV stage..... 112
Table 6.3 Numbers of apoptotic nuclei and apoptotic index in day 7 blastocysts produced by Sr/CB parthenogenetic activation..... 113
Table 6.4 In vitro embryo development of SCNT embryos produced using ovine oocytes vitrified at GV stage as cytoplast recipients..... 116

Table 6.5 Total cell numbers and apoptotic nuclei in day 7 blastocysts produced by SCNT of ovine oocytes vitrified at GV stage..... 117
Table 6.6 Cytogenetic analysis of day 7 blastocysts produced by SCNT of ovine oocytes vitrified at GV stage..... 117
Table 7.1 Effect of cytochalasin B (CB) pre-treatment on viability of vitrified/thawed ovine GV-oocytes..... 125
Table 7.2 Effect of cytochalasin B (CB) pre-treatment on in vitro fertilisation of ovine oocytes vitrified at GV-stage..... 127
Table 7.3 Effect of cytochalasin B (CB) pre-treatment on development of ovine oocytes vitrified at GV stage..... 130
Table 8.1 Effect of caffeine treatment on spindle and chromatin configuration of ovine oocytes vitrified at GV-stage..... 143
Table 8.2 Effect of caffeine treatment on cleavage following IVF of ovine oocytes vitrified at GV stage..... 144
Table 8.3 Effect of caffeine treatment on subsequent development of ovine oocytes vitrified at GV stage..... 147
Table 9.1Effect of demecolcine (Deme) pre-treatment on spindle and chromatin configuration of ovine oocytes vitrified at GV-stage..... 157
Table 9.2 Effect of demecolcine (Deme) pre-treatment on in vitro embryo development following IVM/IVF/IVC of ovine oocytes vitrified at GV-stage..... 159
Table 9.3 Effect of demecolcine (Deme) pre-treatment on parthenogenetic development of vitrified/thawed ovine immature oocytes..... 161

LIST OF ABBREVIATIONS

A23187	Calcium ionophore
AI	Artificial insemination
AI/TI	Anaphase/telophase of the first meiotic division
ART	Assisted reproductive technologies
BSA	Bovine serum albumin
CA	Calcium ionophore A23187
CA+CHX/CB	Calcium ionophore/Cycloheximide/Cytochalasin B
Ca_i^{++}	Intracellular calcium
cAMP	Cyclic adenosine monophosphate
CB	Cytochalasin B
CD	Cytochalasin D
CG	Cortical granules
CHX	Cycloheximide
CO_2	Carbon dioxide
COCs	Cumulus oocyte complexes
CPAs	Cryoprotectants
CSF	Cytostatic factor
D.C.	Direct current
DAPI	4',6-diamidino-2-phenylindole
Deme	Demecolcine
DMSO	Dimethylsulfoxide
DNA	Deoxy ribonucleic acid
6-DMAP	6-Dimethylaminopurine
EG	Ethylene glycol
EM	Electron microscope
ER	Endoplasmic reticulum
ET	Embryo transfer
FAF	Fatty acid free

FBS	Foetal bovine serum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanatem
FP	Female pronucleus
FSH	Follicle stimulating hormone
G2	Gap 2 phase
Gly	Glycerol
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
h	Hour
hpa	Hours post activation
hpf	Hours post fusion
hpm	Hours post onset of maturation
H-SOF	HEPES-buffered synthetic oviduct fluid
H-TCM	HEPES-Tissue culture medium 199
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IP3	Inositol 1,4,5,- triphosphate
IVC	In vitro culture
IVF	In vitro fertilisation
IVM	In vitro maturation
IVP	In vitro embryo production
L	Litre
LH	Luteinizing hormone
LN_2	Liquid nitrogen
M	Mol
µg	Microgram
µl	Microliter
µm	Micrometer
MAPKs	Mitogen-activated protein kinase
MDS	Minimum drop size

MI	Metaphase of the first meiotic division
MII	Metaphase of the second meiotic division
Min	Minute
mm	millimeter
mM	Millimolar
MP	Male pronucleus
MPF	Maturation promoting factor
mRNA	Messenger ribonucleic acid
mSOFaaci	Modified synthetic oviduct fluid medium containing essential and non essential amino acid
MVC	Minimum volume cooling
MW	Molecular weight
NEBD	Nuclear envelop breakdown
NT	Nuclear transfer
OPS	Open pulled straw
PBI	First polar body
PBS	Phosphate buffer saline
PBS/FBS	Phosphate buffer saline/foetal bovine serum
PBS/PVP	Phosphate buffer saline/polyvinylpyrrolidone
PFA	Paraformaldehyde
pi	Post insemination
PROH	1,2-propanediol
PVA	Polyvinyl alcohol
PVP	polyvinylpyrrolidone
rpm	Rotation per minute
RT	Room temperature
SCNT	Somatic cell nuclear transfer
Sec	second
SEM	Standard error of mean
SOF	Synthetic oviduct fluid
SOPS	Super open pulled straw

Sr	Strontium
Sr/CB	Strontium/cytochalasin B
$SrCl_2$	Strontium chloride
SSV	Solid surface vitrification
T	Trehalose
T14	Threonine 14
TCM-199	Tissue culture medium 199
TE	Trophoectoderm
TUNEL	Terminal Transferase dUTP Nick End Labelling
UV	Ultraviolet
Y15	Tyrosine 15
ZP	Zona pellucida

CHAPTER 1

LITERATURE REVIEW:

Cryobiology

1.1 INTRODUCTION

Cryobiology is the branch of biology that studies the effect of low temperatures on living things. The word cryobiology is derived from the Greek "Cryo = Cold", "Bios = Life", and "logos = science". In practice, cryobiology is the study of biological material or systems at temperatures below physiological conditions. Materials or systems studied include proteins, cells, tissues, organs, or whole organisms. There are six major areas that can be identified in the study of cryobiology: 1) study of cold-adaptation of microorganisms, plants, and animals, 2) cryopreservation of cells, tissues, gametes, and embryos of animal and human origin, 3) storage of organs under hypothermic conditions for transplantation, 4) lyophilization (freeze-drying), 5) cryosurgery, and 6) physics of supercooling, ice nucleation/growth and mechanical engineering aspects of heat transfer during cooling and warming. Cryopreservation as an applied aspect of cryobiology is the process where cells or whole tissues are preserved by cooling to low sub-zero temperatures such as –196°C (the boiling point of liquid nitrogen, LN_2). At these low temperatures, any biological activity including the biochemical reactions that would lead to cell death are effectively stopped. People have long believed that very low temperatures would only be detrimental to cells and tissues. They could not possibly imagine the advances in cryobiology which have been achieved and the possibilities that this has opened. The history of cryobiology can be traced back to antiquity, as early as in 2500 BC low temperatures were used in Egypt in medicine. The use of cold was recommended by Hippocrates to stop bleeding and swelling. In the late 1600s, Henry Power froze a jar of vinegar eels in salt water

and after thawing, he found that they were still as active as they were prior to freezing. Power was the first to theorize that, cold did not have so-called "killing properties" that are possessed by heat (Sittig 1963). With the emergence of modern science in 1683, Robert Boyle wrote a monograph "New Experiments and Observations Touching Cold" in which he described the effects of freezing on living animals (Parkes 1960). Another pioneer in cryobiology, Lazzaro Spallanzani conducted extensive studies on tissues of several species and their reaction to low temperatures in late 1700s (Sittig 1963). In 1913, Maksimov described the beneficial effects of glycerol and non-permeable cryoprotectants including sucrose on plant cryostability (Maksimov 1913). In 1940, Luyet and Gehenio published a book entitled "Life and Death at Low Temperatures" in this book, they outlined the basic components for the study of cryobiology (Luyet and Gehenio 1940). In the late 1940s, Cristopher Polge and his colleagues at the University of Cambridge accidentally discovered the protective capabilities of glycerol when they used bottles of chemicals that had been inadvertently miss labelled. This accidental discovery enabled them to successfully cryopreserve spermatozoa of chickens and cattle (Polge *et al.* 1949). Therefore, a spermatozoon was the first reproductive cell to be cryopreserved in LN_2. The discovery of the ability of glycerol to protect the cells against freezing damage led to the derivation of the science of low temperature biology. In 1951, the first calf produced by artificial insemination (AI) with frozen thawed spermatozoa was born (Stewart 1951). In 1960, Peter Mazur conducted extensive experiments to model the response of microorganisms when subjected to low temperatures and freezing. These early studies resulted in the development of the discipline that is now known as cryobiology (Mazur 1963; 2004). The cryopreservation of reproductive cells and embryos embraces a science in which there is a considerable background of theory. Since the discovery of the protective action of glycerol (Polge *et al.* 1949), a wealth of experience of freezing and thawing of a great variety of cells and tissues has been accumulated, not least that associated with cryostorage of spermatozoa and embryos. The oocyte, on the other hand, has so far largely evaded a satisfactory solution, presenting as it does a large cell which is highly

differentiated and arrested part-away through the reduction divisions of meiosis in which the genetic information is mixed and the chromosome complement is halved. Its specialization implies that the unique features peculiar to its state must be preserved in order to carry out its purpose. While this can also be true of spermatozoa, their large numbers allow less than optimal procedures to be acceptable. Oocytes, however, are available in strictly limited numbers; thus, cryopreservation protocols have to be very successful before they can become useful (Watson and Holt 2001). The last decades have witnessed a revival of interest in reproductive biology, owing in part to successful application of gamete and embryo culture to medical, veterinary and biotechnology practice, and in part to pressing needs of today's society. In medical science, assisted reproductive technologies (ART) have been developed primarily to alleviate sterility, while in agricultural sciences the growing needs of the booming world population has provided the impetus to improve the efficiency of livestock production (Elder and Dale 2000). There have been an extraordinary series of exciting developments in knowledge of and technology applied to animal reproduction at the gamete, embryo and whole animal level during the past fifty years. These developments include AI, freezing and sexing of sperm, superovulation, oestrus cycle regulation, recovery of embryos developed in vivo, embryo freezing and sexing, and embryo transfer. In addition, recovery of oocytes for in vitro maturation (IVM), in vitro fertilisation (IVF) and in vitro culture (IVC) have led to new experimental approaches, advancing knowledge and ability to assist with problems in reproduction. All of these technologies utilize sperm, oocytes and/or the product of fertilisation at some stage. Thus, all involve sexual reproduction which helped to achieve asexual reproduction in mammals by cloning (Wolf and Zelinski-Wootn 2001). In this chapter, I will present a brief discussion on some aspects of assisted reproductive technologies (ART) in both humans and animals. In addition, I will give a detailed overview on oocyte cryopreservation, its application and consequent cryoinjuries as well as different approaches involved in oocyte cryopreservation in different mammalian species.

1.2 ASSISTED REPRODUCTIVE TECHNOLOGIES (ART)

Infertility can be considered as an international health problem. It considers one of the most frequently occurring chronic health problems, affecting young and adult people. It is time-consuming and costly; it concerns all social classes and races. Infertility has high prevalence; it affects at least 10% of married or cohabiting women of 18–44 years (VanSteirteghem 2004). A quarter of all married or cohabiting women in that age range are confronted at least once in their life time with infertility. The infertile male and female partners represent a substantial part of the population in developed countries. Different aetiologies have been reported to induce infertility such as ovulation disorders, sperm abnormalities, tubule causes, idiopathic infertility and endometriosis (VanSteirteghem 2004). Over the past decades, several developments have increased the knowledge about reproductive medicine, such as developments in reproductive technologies and microsurgery which played a role in treatment of infertility in women and men. A major milestone has been in vitro fertilisation; the first baby born from IVF was reported in 1978 (Steptoe *et al.* 1980). After that, the technique was applied worldwide in 1980's. IVF has proved to be the treatment of choice to alleviate infertility associated with women causes (VanSteirteghem 2004). A similar solution for treating male factor infertility was the development of intracytoplasmic sperm injection (ICSI) (Palermo *et al.* 1992). Since the first birth in 1992, ICSI has proved to be a choice for treatment of infertility caused by male factor (VanSteirteghem 2004). Historically, progress in cellular and molecular embryology in domestic animals had been difficult due to the limited availability of suitable experimental materials at an acceptable cost. Gametes and embryos were obtained in vivo usually from superovulated donors and collected at required developmental stage. In addition to cost; animal welfare concerns have more and more limited the use of animals in such research. For these and many other reasons development of in vitro techniques for the production of mammalian embryos has received great attention in the past twenty years. In vitro embryo production (IVP) in mammalian species provides an excellent source of low-cost embryos for basic research on developmental biology and physiology as well as for commercial

application of the emerging technologies such as nuclear transfer and transgenesis (Baldassare *et al.* 2002). In addition, the application of IVP has been proposed as a strategy for the rescue of some endangered species (Ptak *et al.* 2002). From a health point of view, embryo transfer in domestic animals has the potential to facilitate safe worldwide movement of germplasm (Cognie *et al.* 2003). IVP technology is well established in cattle, but has limited application in other domestic animals (Galli and Lazzari 2003). IVP involves three main steps; oocyte maturation, fertilisation and culture of putative embryos for up to 7 days until formation of the blastocysts (Cognie *et al.* 2003).

1.2.1 Oocyte maturation

In the dominant follicles, oocytes remain arrested at the diplotene stage of the meiotic prophase which is comparable with the G2 phase of the cell cycle. In vivo, resumption of meiosis is initiated by a preovulatory Luteinizing hormone (LH) surge, disruption of the gap junctions between oocytes and cumulus cells occurs shortly after this surge. During the period between the LH surge and ovulation, the oocytes undergo nuclear and cytoplasmic maturation. Nuclear maturation, lasting for 24 h in cattle and sheep, 44 h in pigs and 36 h in horses, involves two consecutive meiotic divisions (M-phase) in the absence of DNA replication (S-phase). The 1st meiotic division reduces the chromosome content of the oocyte by 50%, the discarded DNA being extruded as the 1st polar body (PBI). The oocyte then initiates but does not complete the second meiotic division, becoming arrested at metaphase (MII) until fertilisation. Ooplasmic maturation involves the ability of oocytes to block polyspermy, decondense penetrated spermatozoa and form pronuclei after fertilisation in addition to redistribution of organelles and accumulation of granules along the oolemma (van den Hurk and Zhao 2005). Following oocyte maturation along with the LH surge and reaching the maximal size of the follicle, ovulation takes place by rupture of follicular wall at one side-the stigma. At this time the follicles respond to the LH surge with a shift in the granulosa cells estrogenic environment to a progesterogenic one and with the production of hyaluran by the cumulus cells which is responsible for the

mucification and expansion of the cumulus cells and termination of oocyte-cumulus junctions (Picton *et al.* 1998). The changes associated with the above mentioned steps for in vivo maturation, can be replicated in vitro, but the cryopreservation properties of most oocytes matured from GV to MII stage in vitro differ from those matured in vivo (Leibo and Loskutoff 1993; Leibfried-Rutledge *et al.* 1997). Developmental events in mammals including oocyte formation, growth, maturation and sperm penetration are presented in **Fig 1.1**.

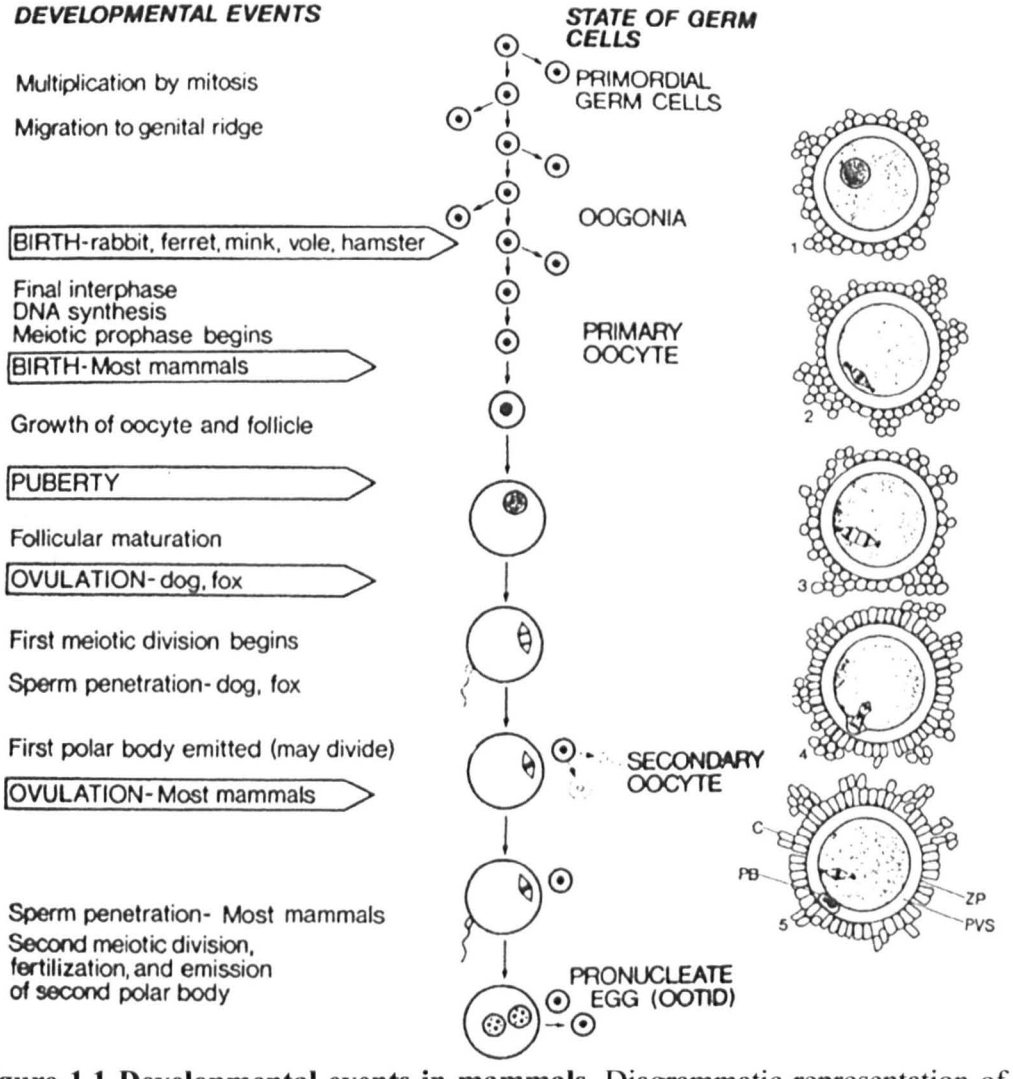


Figure 1.1 Developmental events in mammals. Diagrammatic representation of the different stages of oocyte formation, growth, maturation and sperm penetration in different mammalian species (**Adapted from Baker 1972**).

1.2.2 Fertilisation and polyspermy

Fertilisation is the union of a single sperm with an egg (Coy *et al.* 2008). This is a multistep process which includes interaction of the sperm with the outer vestments of the oocytes, penetration of the zona pellucida (ZP), fusion of the sperm and oocyte plasma membranes, pronuclear formation followed by DNA synthesis, then pronuclear fusion (syngamy) and alignment of their respective chromosomes on the first cleavage spindle (Raymond *et al.* 1981; Williams 2002). Before

fertilisation, mammalian spermatozoa undergo a series of biochemical and biophysical changes called capacitation (Wani 2002), by which the sperm acquire the ability to fertilise. Capacitation involves the removal of sperm coating materials that are acquired during epididymal transit or during exposure to seminal plasma and cholesterol depletion resulting in an increased membrane permeability to calcium (Wani 2002). Fertilisation results in increased intracellular calcium (Ca_i^{++}) and intracellular insitol triphosphate (IP_3) in the egg. As a consequence of increase in Ca_i^{++}, exocytosis of cortical granules, hardening of the ZP, blocking of polyspermy and decondensation of sperm chromatin occur. After fertilisation, sperm mitochondria dissociate and remain aggregated in the cytoplasm of the oocytes; however, they do not persist beyond the pre-implantation stages and only maternal mitochondria are inherited (Williams 2002). The sperm tail also penetrates the oocyte at fertilisation, in most mammals the sperm centriole is responsible for nucleation of a microtubule network for pronuclear migration (Williams 2002), but most of the remaining structural elements of the sperm tail rapidly disappear after penetration (Williams 2002). Several hours after sperm-oocyte fusion, calcium oscillations cease at the time of pronuclear formation but reappear during the first mitosis which is important in the first cleavage (Ozil 1998). Calcium causes inactivation of cytostatic factor (CSF) which stabilises maturation promoting factor (MPF), and then cyclin degradation occurs (Williams 2002). Loss of active cytostatic factor and inactivation MPF allows entry of the female chromatin into anaphase II (Williams 2002). Following DNA synthesis, male and female pronuclei migrate to the centre of the newly formed cell zygote, the pronuclear membranes breakdown and the chromosomes undergo syngamy (Williams 2002). Polyspermy refers to the fertilisation of an oocyte by two or more spermatozoa, this is a common problem following IVF in cow (Wang *et al.* 1997; Coy *et al.* 2005), sheep (Slavik *et al.* 2005), goat (Mogas *et al.* 1997), human (Aoki *et al.* 2005) and is particularly frequent in pigs (Hao *et al.* 2006). In most mammals, initiation of calcium oscillations induces the fusion of the cortical granules (CG) with the oolema thus releasing their content into the perivitelline space (cortical reaction). CG enzymes change the ability of the ZP to bind sperm

or induce acrosome reaction, so preventing further sperm penetration. Therefore, polyspermy can be caused by incomplete migration of CG, low Ca_i^{++} transients, less endoplasmic reticulum (ER) containing Ca_i^{++} (Wang *et al.* 2003). However, incomplete or delayed ZP reaction could be a major cause of polyspermy (Coy *et al.* 2002; Wang *et al.* 2003).

1.3 CRYOPRESERVATION OF REPRODUCTIVE POTENTIAL IN ASSISTED REPRODUCTION

During the last few years, cryopreservation has become a relevant addition to therapeutic concepts in reproductive medicine (Nawroth *et al.* 2005). Valuable genetics can be preserved through the cryopreservation of sperm, oocytes or embryos. Methods to cryopreserve sperm and embryos have been successful (Mazur *et al.* 2008). However, despite advances in cryobiology, cryopreservation of oocytes from most species is inefficient, and the results are inconsistent (Yavin and Arav 2007).

1.3.1 Sperm cryopreservation

Sperm were among the first vertebrate cells to be successfully cryopreserved in frogs, roosters, cattle and humans (Mazur *et al.* 2008). The success in the latter three species stemmed primarily from the discovery of the protective action of glycerol (Polge *et al.* 1949). In agriculture, over 250, 000, 000 doses of semen from genetically superior bulls were preserved worldwide in 1998, and over 100, 000, 000 cows received their first insemination from that frozen semen (Thibier and Wanger 2002). Frozen sperm allows dissemination of genetics and this results in the increase in milk production over the last several decades (Mazur *et al.* 2008). In human, the first birth following the use of cryopreserved sperm were reported in the 1950's (Bunge *et al.* 1954) and this approach is now in widespread use for storage and quarantine of donated sperm and storage of reproductive potential when loss of fertility is imminent (Gook and Edgar 2007). Recently, cryopreservation of spermatozoa has become a standard practice for all semen

donors until tests demonstrate that it is free from HIV and other pathogens (Mazur *et al.* 2008). In endangered species, AI with frozen thawed spermatozoa helps in genetic management through the distribution of genes among geographical dispersed breeding centres and zoos (Dinnyes *et al.* 2007). Sperm cryopreservation would also allow international transport of genetic materials without compromising political and legal restrictions. The shape and size of the sperm head could be factors that determine the cryosensitivity of spermatozoa (Liebermann *et al.* 2002). Comparative studies between different species such as boar, bull, ram, rabbit, cat, dog, horse and human revealed a negative correlation between the size of the sperm head and cryostability (Nauk 1991). Among the above mentioned species, human spermatozoa have the smallest size with maximal cryostability (Gao *et al*, 1997).

1.3.2 Embryo cryopreservation

Birth of the first live offspring from cryopreserved embryos (Whittingham *et al.* 1972) opened the door to the possible application of this technology in the context of animal breeding and clinical assisted reproduction (Gook and Edgar 2007). Since the birth of first mice from cryopreserved embryos (Whittingham *et al.* 1972), live births have been reported in over 20 mammalian species (**Table 1.1**). In 2004, approximately 550,000 bovine embryos were transferred into recipients (Thibier 2004); about half of them were cryopreserved (Walker *et al.* 2006). Successful cryopreservation of embryos have been reported in several species including cattle (Nedambale *et al.* 2004; Walker *et al.* 2006), sheep (Green *et al.* 2009; Shirazi *et al.* 2010), pigs (Dobrinsky 1997), horses (Campos–Chillon *et al.* 2009), mice (Kasai *et al.* 1990), and humans (Edgar *et al.* 2005).

Table 1.1 Mammalian species yielding normal offspring following the transfer of cryopreserved embryos to foster mother

Species	*Years*	*Reference*
Mouse	1972	Whittingham *et al.* (1972)
Cattle	1973	Wilmut and Rawson (1973)
Rabbit	1974	Bank and Maurer (1974)
Rat	1975	Whittingham (1975)
Sheep	1976	Willadsen *et al.* (1976)
Goat	1976	Bilton and Moore (1976)
Horse	1982	Yamamoto *et al.* (1982)
Human	1984	Zeilmaker *et al.* (1984)
Baboon	1984	Pope *et al.* (1984)
Marmoset	1986	Summers *et al.* (1986)
Cynomologus macaque	1986	Balmaceda *et al.* (1986)
Cat	1988	Dresser *et al.* (1988)
Rhesus macaque	1989	Wolf *et al.* (1989)
Pig	1989	Hayashi *et al.* (1989)
Red deer	1991	Dixon *et al.* (1991)
Wapiti	1991	Wenkoff and Bringans (1991)
Hybrid macaque	1992	Cranfield *et al.* (1992)
Swamp buffalo	1993	Kasiraj *et al.* (1993)
Fallow deer	1994	Morrow *et al.* (1994)
Mongolian gerbil	1999	Mochida *et al.* (1999)
Hamster	1999	Lane *et al.* (1999a)

1.3.3 Oocyte Cryopreservation

Oocyte competence can be defined as the ability of an oocyte to be fertilised develop into an embryo, establish a pregnancy and produce healthy offspring (Anguita *et al.* 2007). The availability of viable developmentally competent oocytes has been a limiting factor preventing progress in the development of IVF, embryo culture and related reproductive technologies in many species. Additionally, the relatively short fertilisable life span of mammalian oocytes limits implementation of many in vitro technologies. This limitation could be overcome by the ability to cryopreserve oocytes. However, to date the developmental potential of cryopreserved mammalian oocytes is typically highly compromised. Therefore, research on the cryopreservation of oocytes has become a priority. Success in this area could have important practical implications in both animal and human assisted reproductive technologies. Cryopreservation of oocytes from several mammalian species at different developmental stage is summarized in (***Appendix B***).

1.3.3.1 Rationale for oocyte cryopreservation in mammals

The cryopreservation of female gametes is an important technology that is applied in both human and animal reproduction. Cryopreservation permits the long-term storage of cells and tissues such that acceptable numbers of cells are viable upon subsequent warming. Oocyte cryopreservation in mammals has a series of potential applications (**Table 1.2**) aimed at the maintenance of biodiversity (Ambrosini *et al.* 2006).

Table 1.2 Applications of oocyte cryopreservation

Human reproductive medicine
• Improve the efficiency of IVF
• Alternative to embryo freezing
• Oocyte banking
• Oocyte donation program
• Oocyte preservation for patient with ovarian hyperstimulation syndrome
• Treatment of premature ovarian failure
• Preserve future fertility
• Treatment of congenital infertility disorders
Research applications
• Preserve germplasm of genetically- modified animals
• Nuclear transfer program
Livestock production
• Preserve valuable genetic blood lines
• Improve breeding programs
• Avoid seasonal variations and sanitary constraints
• Transportation
• Preservation of endangered species

1.3.3.1.1 Oocyte cryopreservation in human reproduction

Infertility is considered one of the most serious problem facing human (Alesi 2005). As mentioned earlier, IVF is considered the treatment of choice for women infertility. In IVF programs, ovarian response is artificially stimulated through hormonal administration which subsequently increases the number of ovarian follicles. As is common practice in IVF clinics, oocytes are collected from all stimulated follicles, however, due to the lack of suitable methods for oocyte cryopreservation, all of them are subjected to IVF and only two or three of the best quality embryos selected for transfer. The majority of the remaining embryos are frozen for future use (Gomes *et al.* 2008). This will produce supernumerary embryos (Hoffman *et al.* 2003). Therefore; cryopreservation of oocytes would be helpful to solve this issue and permits the production of only required embryos.

Oocyte cryopreservation could also be an alternative to embryo storage due to ethical issues and legal restrictions in some countries.

Additionally, oocyte cryopreservation would offer the possibility for the establishment of oocyte banks (Gomes *et al.* 2008). An oocyte bank is important in oocyte donation program (Karow 1997; Chang *et al.* 2009). Also, oocyte banking provides women with the option to delay childbearing plan until their professional careers are established (Leibo 2004). Establishment of oocyte banks could eliminate the problems associated with synchronization of donor and recipient cycles; it will also allow more effective screening of donors for infectious disease (Karow 1997). Oocyte cryopreservation could be useful in treatment of congenital infertility disorders, prevention of post- operative fertility loss and treatment of ovarian failures (Gomes *et al.* 2008). Moreover, it may also offer alternatives for infertile patients who are subject to ovarian hyper-stimulation syndrome or polycystic ovarian syndrome (Fabbri *et al.* 2001; Fabbri 2006; Lucena *et al.* 2006). In addition, oocyte cryopreservation may be helpful in cases of male factor infertility or problems associated with difficulty of sperm collection, inadequate seminal samples or nonviable spermatozoa at the time of oocyte retrieval (Lucena *et al.* 2006).

Also, oocyte cryopreservation could be used to conserve fertility in women diagnosed with cancer who subsequently loose their gonadal function as a consequence of chemo-and radiotherapy (Leibo 2004; Gomes *et al.* 2008).

1.3.3.1.2 Application of oocyte cryopreservation in research

Since the successful isolation of mouse embryonic stem cells (Evans and Kaufman 1981) and the development of targeted gene disruption strategies (Koller *et al.* 1989), hundreds of genetically-modified mouse strains have been produced (Critser and Russell 2000, Mazur *et al.* 2008). Keeping these strains is important for future research, due to their value as models for biological systems. By increasing the numbers of strains, it has become impossible in terms of cost and

space to maintain all of them as breeding colonies (Mazur *et al.* 2008). Cryopreservation of germplasm offers an alternative way by allowing the preservation of frozen colonies as a backup in case of disease outbreak. Genetic modifications of domestic animals such as sheep, cattle, and pigs also can produce animals for the production of pharmaceuticals which are important for the treatment of some human diseases (Lindsay *et al.* 2004). Moreover, genetically modified animals may be used for organ xenotransplantation (Lai *et al.* 2002) or disease modeling. Thus, preservation of genetic materials from these animals is useful for biomedical applications (Turk and Laughlin 2004).

Due to the absences of embryonic stem cells in livestock species and generation lines, the use of nuclear transfer (NT) technology is an important technique for genetic modification (Ward and Brown 1998). However, the efficiency of NT technology is relatively low and depends primarily on freshly collected and matured oocytes. Therefore, oocyte cryopreservation would provide an alternative option for a stable supply of oocytes for NT. This would also help to avoid changes in oocyte quality which are associated with seasonal variations (*et al.* Dinnyes *et al.* 2000; Hou *et al.* 2005; Sung *et al.* 2010).

1.3.3.1.3 Application of oocyte cryopreservation in livestock production

From a commercial point of view, in livestock farming there is a need for cryostoring oocytes from domestic animals (e.g. cattle, pigs and sheep) with high economic value in order to enhance the development of improved breeding program. Additionally, producers could preserve valuable genetic blood lines from females and could market oocytes rather than embryos whose sire had already been chosen. In livestock, where large numbers of ovaries can be easily obtained from slaughterhouse, many oocytes can be collected, preserved and then used for IVP when re-warmed, thus diminishing seasonal variations or sanitary constraints. Also, oocyte cryopreservation could be used to rescue gametes when females die unexpectedly or accidentally (Ledda *et al.* 2007; Checura and Siedel 2007). In the future, cryopreserved oocytes could be used in genetic

recombination through fertilisation and production of cross-bred animals. Oocyte banking also permits international exchange of oocytes, avoiding animal transportation and its sanitary risks and injuries (Pereira and Marques 2008). Moreover, oocyte cryopreservation and banking may be important to preserve endangered species (Watson and Holt 2001). Cryopreservation of the oocytes from these rare species would allow the application of assisted reproductive technologies. Also, oocyte and embryo banks as an interface between in situ and ex situ conservation programs allow the maintenance of biodiversity of endangered species from domestic and wild animals (Woelders *et al.* 2006; Andrabi and Maxwell 2007).

1.4 CONVENTIONAL METHODS FOR CRYOPRESERVATION

While the ability to freeze embryos has become standard practice in assisted reproductive technologies, cryopreservation of oocytes remains a challenge and has not been widely used despite significant research (***Appendix B***). The principles of cryopreservation are believed to be the same for all living cells; the most important consideration being the removal of most of the water from cells before they freeze intracellulary. Almost all cryopreservation strategies are based on two main factors: cryoprotectants (CPAs) and cooling-warming rates. Currently there are three strategies for oocyte and embryo storage; slow freezing, traditional (conventional) vitrification (vitrification in-straw), and ultra-rapid vitrification (Kasai 2002; Dinnyes *et al.* 2007; **Fig 1.2 and Table 1.3**). Storage, warming and re-hydration (i.e. removal of CPAs) differ only slightly between the three protocols. The main differences between the three methods of cryopreservation exist in the addition of CPAs and cooling rates (Vajta and Kuwayama 2006).

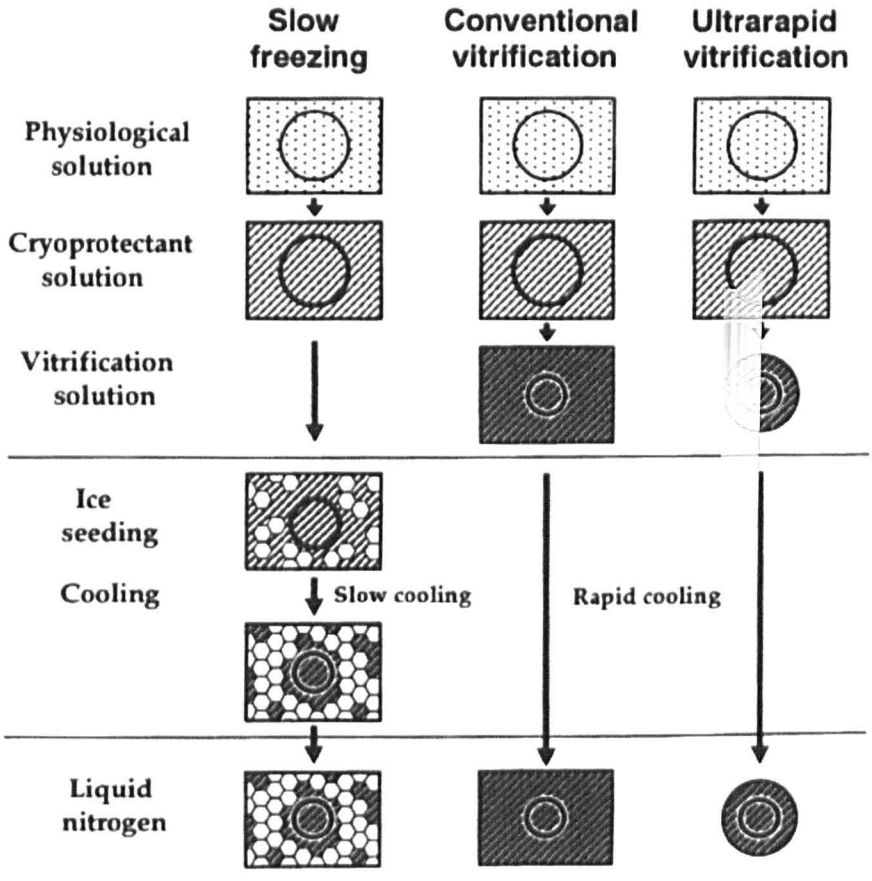


Figure 1.2 Schematic representation of an oocyte/embryo (circle) during slow freezing, conventional vitrification, and ultrarapid vitrification. White hexagons represent ice crystals. The concentration of cryoprotectant is shown by the darkness of shading (**Adapted from Kasai 2002**).

Table 1.3 Comparison of the three approaches for cryopreservation of oocytes (Adapted from Dinnyes *et al.* 2007)

Parameters	Freezing	In-straw vitrification	Ultrarapid vitrification
Oocyte storage container	Straw	Straw	Cryovial, straw, special device or no container
CPA concentration	1.3 – 1.5 M	5.5 – 7.5 M	3.5 – 5.5 M
Time in CPA (concentration for equilibration	NA	2 – 5 min	2 – 5 min
Time in CPA (concentration for cooling	15 – 20 min	> 1 min	> 10 sec
Time required for cooling	90 – 120 min	2 – 3 min	<0.1 sec
Ice crystal formation	Yes	No	No
Osmotic injury	Low risk	High risk	High risk
Toxic injury	Low risk	High risk	High risk
Chilling injury	High risk	Low risk	Low risk
Warming rate	Low to moderate	Moderate to high	High
Cost	High	Low	Low
Commercial application	Extensive	Limited	Limited

1.4.1 Traditional slow rate freezing

Slow freezing was the first system used for embryo cryopreservation and this method has subsequently become highly standardized with considerable industrial and commercial background (Ledda *et al.* 2007). In this system, controlled cooling rates allow extra and intra-cellular fluid exchange without serious osmotic effects

and changes in cell shape. This fact is reflected in its alternative name; equilibrium freezing (Vajta 2000). Conventional slow freezing procedures have the following distinctive steps (**Fig. 1.2**); 1) oocytes exposed at room temperature (RT) to molar concentrations of low molecular weight permeating CPAs, such as ethylene glycol (EG), dimethylsulphoxide (DMSO, Me_2SO) or propylene glycol until equilibrium is reached between CPAs and embryos; 2) induction of ice crystals (seeding) at –5 to –7°C and holding at this temperature for 5–10 min; 3) controlled slow cooling around 0.2 to 2.0°C/min; 4) plunging at –30 to –70°C and storage in LN_2 at –196°C; 5) controlled thawing at around 250°C/min (e.g. 25°C water bath without stirring); 6) removal of CPAs at RT prior to culture or transfer (Palasz and Mapletoft 1996). In slow freezing, oocytes are immersed in hypertonic CPA solutions, and an outward flux of water is generated by osmosis because the cell membranes are more permeable to water than to the CPA. Cell volume is restored, at equilibration, however, cooling the cell to subzero temperatures causes a second volume variation due to the fact that, firstly water freezes extracellulary and salt concentration rises outside the oocytes (Ambrosini *et al.*, 2006); water therefore leaves the cell again by osmosis (Jondet *et al.* 1984). Induction of ice crystals "seeding" is usually induced at –6°C, so the unstable state of supercooling of the water inside the cell, which will lead to sudden ice nucleation and consequently cell death, can be prevented (Ambrosini *et al.* 2006; Jain and Paulson 2006). Once ice crystals are induced, there is a tendency of smaller crystals to grow into larger ones, which is lethal to the cells and must be avoided (Knight and Duman 1986). The risk of intracellular ice formation increases when the temperature is changing either by cooling or re-warming. Hence, during thawing the temperature is usually raised very rapidly through the critical range (Todorw *et al.* 1989). The slow freezing technique has been used successfully to freeze oocytes and embryos of various mammalian species, especially those that are less sensitive to chilling such as mouse and cat (Ruppert-Lingham 2003; Luvoni *et al.* 1997, Luvoni 2006) and has also been used extensively to freeze human oocytes and embryos (Fabbri 2006). Conversely, poor results have been reported for species more sensitive to chilling injuries including cattle, sheep, pigs, and horses (Martino *et al.* 1996b;

Isachenko *et al.* 1998; LeGall and Massip, 1999; Vajta *et al* 1998). Furthermore, an important constraint to the use of conventional slow freezing is the critical period of oocyte exposure to the CPAs and the necessity to perform only in a laboratory equipped with an automated freezing system (Ledda *et al.* 2007) so that the cooling rate can be closely controlled (Kasai 2002).

1.4.2 Conventional vitrification

In 1985, Rall and Fahy devised an innovative method called vitrification (ice-free rapid solidification), in which oocytes/embryos suspended in a highly concentrated solution (20.5% DMSO, 15.5% acetamide, 10% propylene glycol and 6% poly-ethylene glycol) were loaded in a straw and directly plunged into LN_2 from a temperature above 0°C (Rall and Fahy 1985). Significantly, higher levels of oocyte and embryo viability can be maintained through this technique. This is primarily attributed to the smaller chance of intracellular ice formation, although intracellular ice can be formed even in vitrification if the concentration of the CPAs in the cell is not high enough. The biggest obstacle to this approach is the toxicity of the CPAs, because high concentrations 5–8 mol/L are used to prevent ice formation (Kasai 2002). However, various improvements to this technique have been achieved, and many embryos in different species including mice (Rall and Fahy 1985; Kasai *et al.* 1990); rabbits (Kasai *et al.* 1992), cattle (Vajta *et al.* 1998; Sommerfeld and Niemann 1999; Nedambale *et al.* 2004; Vieira *et al.* 2007); and sheep (Naitana *et al.* 1997; Leoni *et al.* 2003) have been preserved with minimal loss of viability.

1.4.3 Ultrarapid vitrification

In some species, consistently high survival of oocytes and embryos has not been obtained by either slow freezing or conventional vitrification (Kasai 2002). Therefore, improvement or development of the cryopreservation methods is necessary (Kasai 2002). The first reason for the low survival would be the sensitivity of oocytes and embryos to chilling, including porcine embryos, bovine oocytes and bovine embryos at early cleavage stages (Leibo *et al.* 1996). The

second reason would be lower permeability of the cell membrane, which would lead to the formation of intracellular ice and osmotic swelling. The third reason would be the toxicity of the cryoprotectant during exposure of the cells to concentrated vitrification solution (Kasai 2002). For instance, in blastocysts with a large fluid filled blastocoels cavity, a longer exposure would be necessary to make this space concentrated (Kasai 2002). Moreover, in some species (e.g. hamster embryos) oocytes and embryos are quite sensitive to an exposure just in an in vitro environment (Kasai 2002). A new strategy aiming to overcome these injuries is to markedly increase both cooling and warming rates. By this approach, critical temperatures at which the cells are injured could be passed quickly, so the formation of intracellular ice might be avoided even in less concentrated cells and the use of lower concentrations of CPAs could be possible (Kasai 2002). In fact, the concentration of permeating CPA in ultrarapid vitrification is lower than that adopted for conventional vitrification. In conventional vitrifictation, solutions with 40% (or more) concentration of permeating CPA were widely used (Rall and Fahy 1985; Kasai 1990; Nakagata 1989), whereas, in ultrarapid vitrification, solutions of 30% CPA were frequently used (Martino *et al.* 1996a). In ultrarapid methods, oocytes and embryos are pre-treated in a solution containing low concentration of permeating CPA before being suspended in a vitrification solution **(Fig 1.2)**. This pre-treatment is effective to promote permeation of the CPA in less toxic conditions (Kasai 2002). The concentration of the permeating cryoprotectant used in the pre-treatment ranges from 2 to 3% (Papis *et al.* 2000) to 20% (Vajta *et al.* 1997). The exposure time in vitrification solution should be the minimal required as long as intracellular ice formation is prevented and the toxic effect of the vitrification solution is avoided. The optimal time depends not only on the vitrification solution and the type of cell, but also on the temperature. In many cases, oocytes and embryos were exposed for only 25–30 sec at high temperature (35°C, Martino *et al.* 1996a; Vajta *et al.* 1998; Mukaida *et al.* 2001). In all ultrarapid methods oocytes and embryos are warmed by immersing the sample as quickly as possible in a hypertonic solution containing sucrose or trehalose (Kasai 2002). It has been reported that, by decreasing the volume of the solution and the

container, cooling and warming rates become much higher (Kasai 2002, Vajta and Kuwayama 2006). Various methods have been devised for handling oocytes and embryos with minimal volumes of solutions, these methods will be discussed later in detail.

1.5 MECHANISMS OF CELL INJURY DURING CRYOPRESERVATION

Cells must be preserved at temperatures below the glass transition temperature of the cytoplasm and the suspending solution, which is approximately –130°C, at which effective cryopreservation without a decrease in survival could be obtained. In practice, LN_2 (–196°C) is used to maintain the temperature. Not only during cooling but also during processing before cooling, during warming, and at recovery the cells are at risk of various types of injury. Therefore, all of these injuries must be prevented in order to minimize cell damage during cryopreservation.

1.5.1 Types of cell injuries during cryopreservation

1.5.1.1 Ice crystal formation

Mammalian oocytes and embryos are large cell masses, a high proportion of which is occupied by water, when they are cooled to low temperatures intracellular ice is liable to form. This phenomenon is fatal because even a small amount of ice is likely to re-crystallize, become larger and have a destructive effect on the cellular structure. To avoid ice crystal formation, the cells must be concentrated and storage must occur below the glass transition temperature (Kasai 2002; Jain and Paulson 2006). In slow freezing, cells are concentrated gradually during slow cooling, whereas in vitrification, cells are concentrated upon direct suspension in a concentrated solution (Kasai 2002, **Fig 1.2**). Extra-cellular ice can be formed by slow cooling of oocytes or embryos, as the unfrozen fraction in which cells are located becomes smaller because the cells are large (Mazur 1977). On the other hand, when water is cooled to below its freezing point, it solidifies into a

crystalline structure "ice" and these ice crystals occupy a greater volume than water and subsequently causes damage to the cells (Jain and Paulson 2006). Therefore, avoidance of ice crystal formation is one of the principal goals of successful cryopreservation. As the water transitions from liquid to ice, any solutes in the liquid phase are excluded from the solid. This will decrease the freezing point of unfrozen solutions, and result in an increase in the concentration of electrolytes and other solutes (Lovelock 1954). These high concentrations are toxic to the frozen oocytes and embryos, and the avoidance of these solution effects is a second major goal of successful cryopreservation (Jain and Paulson 2006).

1.5.1.2 Osmotic shock

During re-warming the solid ice melts and releases free water resulting in a reduction of osmolarity of the surrounding solution (Jian and Paulson 2006). Slow re-warming can cause further cell damage by thawing and re-crystallization of free water. On the other hand, rapid re-warming leads to sudden drops in extracellular osmotic pressure and consequently rapid shifts of the water into the cell, which results in swelling and cell injury (Mazur 1980). This is called osmotic shock, and its avoidance is the third major goal of successful cryopreservation. Addition of CPAs into the solution could prevent these injuries (Kasai 2002; Jian and Paulson 2006), but, higher concentrations of CPAs are toxic to the cells (Liebermann *et al.* 2002).

1.6 SLOW COOLING VS. VITRIFICATION

Cryopreservation without ice formation (vitrification) could be beneficial in comparison to freezing methods for a number of reasons, firstly not least being cost as it does not require any expensive equipment. Secondly is much faster taking only few seconds for cooling and warming (Dinnyes *et al.* 2007). Vitrification is considered an alternative to conventional freezing and has been successfully used for the cryopreservation of spermatozoa (Saki *et al.* 2009), embryos (Vieira *et al.* 2007), oocytes (Vieira *et al.* 2002; Vieira *et al.* 2008) and

stem cells (Stachecki *et al.* 2008). Freezing includes the precipitation of water as ice; the resulting separation of the water from the dissolved substances poses problems. The physical definition of vitrification is the solidification of a solution in which water is rapidly cooled and formed into a glassy, vitrified state from the liquid phase at low temperature, not by ice crystallization but by extreme elevation in viscosity (MacFarlane 1978; Fahy *et al.* 1984). This phenomenon was described by Fahy (1986) as the viscosity of the sample becomes greater and greater until the molecules become immobilized and the sample is no longer a liquid, but rather has the properties of a solid. However, vitrification is a result of high cooling rates (2000–25000°C/min) associated with high concentrations of cryoprotectants (5–7 M) which depress ice crystal formation and increase the viscosity at low temperature. The strategy of vitrification is basically different from that of slow cooling, a slow rate of cooling attempts to maintain a delicate balance between the various factors, which may result in damage, such as ice crystal formation, osmotic injury, cryoprotectant toxicity, concentrated intracellular electrolytes, chilling injury, zona and embryo fracture, and alterations of intracellular organelles, cytoskeleton and cell-to-cell contacts (Massip *et al.* 1995; Dobrinsky 1996; Kasai 1996), whereas, vitrification totally eliminates ice crystal formation (Vajta 2000). Furthermore, the increased cooling rate during vitrification decreases chilling injury, i.e. damage of intracellular lipid droplets, lipid-containing membranes and the cytoskeleton, passing rapidly through the dangerous zone from +15°C to –5°C (Dobrinsky 1996; Martino *et al.* 1996 a; Isachenko *et al.* 1998). Vitrification as an ultra-rapid cooling technique is based on direct contact between the vitrification solutions containing the cryoprotectant agents and the liquid nitrogen. A comparison of the advantages and disadvantages of conventional freezing and vitrification is presented in **Table 1.4.**

Table 1.4 A comparison of vitrification with slow-cooling procedures (Adapted from Kuleshova and Lopata 2002)

Accessibility and regulation	Vitrification	Slow cooling
Can be observed	Yes	no
Can be analysed	yes	no
Interaction with oocytes and embryos	yes	no
Control of solute penetration	yes	no
Control of dehydration rate	yes	no
Maintenance of physiological temperature during equilibration procedure	yes	no
Duration of incubation	~10 min	~3 h
Prolonged temperature shock	no	yes
Interference with oocyte or embryo	low	high
Fracture of zona pellucida	no	possible
Formation of ice crystal	no	possible

1.6.1 Variables in Vitrification

Many variables have been reported to affect the efficiency of vitrification and survival rates of vitrified cells **Table 1.5.**

Table 1.5 Variables in vitrification that can profoundly influence its effectiveness (Adapted from Liebermann *et al.* 2002)

1. Type and concentrations of cryoprotectant
2. Media used as base media (holding media)
3. Témperature of the vitrification solution at exposure
4. Time of exposure to cryoprotectants
5. Volume of cryoprotectant solution
6. Device used for vitrification
7. Technical proficiency of the embryologist
8. Quality and developmental stage of the tested cells/tissue
9. Contamination of the sample by direct contact with LN_2

1.6.1.1 Cooling and warming rates

The success of cryopreservation depends on two main parameters: 1) the speed of freezing (i.e. cooling rate) and 2) the effects of the dissolved substances (i.e. concentration of the cryoprotectants). Cooling rate is one of the principle determinants in the success and cell survival during vitrification. If the cooling rate is low, cytoplasmic water will flow out of the cell and freeze extracellulary and if the rate is too rapid, cytoplasm will not have sufficient time to dehydrate, resulting in the supercooling and eventual freezing (Rall 1987). In fact, cooling too slowly may kill the cells by exposing them to high concentrations of CPA for long time, whereas rapid cooling can cause cell death due to ice crystal formation. Mazur (1977) reported that the cell survival decreases at low cooling rates, increases to maximum at an optimal cooling rate which permits most of the water to move out from the cells and to vitrify extracellularly, and finally declines at high cooling rates. High cooling rates (from 15,000 to 30,000°C/min) can be achieved by using a small volume (< 1μL) of highly concentrated CPAs (Martino *et al.* 1996a). Moreover, the use of high cooling rates combined with small sample volumes during vitrification reduces the CPA concentrations, exposure time and subsequent CPAs toxicity (Yavin and Arav 2007). Cooling the cells to subzero temperatures results in formation of extracellular ice crystals and super-cooling of the cytoplasm, once cytoplasm is cooled to a temperature below –10 or –15°C, intra-cellular ice abruptly forms. This phenomenon is called intracellular nucleation and is detrimental to the cell (Mazur 1970). However, if the cells are warmed very rapidly, this damage may be rescued. In addition to cooling, the warming rate is also very important for successful cryopreservation of mammalian cells. Following cryopreservation, the cell has to be re-hydrated and the cryoprotectant removed. The optimum warming rate for a given type of cell is highly dependent on the optimum cooling rate that preceded it. Warming of vitrified oocytes is critical, because transient formation of ice crystals might occur during de-vitrification, if the speed of warming is not adequate (Rall 1987). Early investigators assumed that rapid warming of mammalian cells after cryopreservation was always better because cells had a shorter time to re-crystallize and were exposed to

cryoprotectants for less time. High warming rates could be achieved when vitrified oocytes were directly plunged into a warming solution (i.e. from –196°C to 37°C), during this time, the warming rate reaches 4460°C/min (Liebermann *et al.* 2002). The rapid warming process allowed a very rapid dispersion of intracellular ice crystals, if any; the extracellular ice melts and permeated the cellular membrane in a liquid state to re-hydrate the oocytes (Friedler *et al.* 1988).

1.6.1.2 Cryoprotectants

Cryoprotectants are additional chemicals used in cryopreservation to avoid ice formation and shock effects (Pereira and Marques 2008). They are divided into two categories, permeating and non-permeating. Permeating cryoprotectants are small molecules that have the ability to penetrate the cell membrane, form hydrogen bonds with intracellular water molecules, lower the freezing temperature of the resulting mixture and consequently prevent ice crystallization (Pereira and Marques 2008). At high concentrations, they inhibit the formation of ice crystals and lead to the development of a solid, glass like, so-called vitrified state in which water is solidified, but not expanded (Jain and Paulson 2006). Also, permeating CPAs protect the cells against solution effects by remaining in the solution and thus effectively diluting the residual electrolytes (Jain and Paulson 2006). Propylene glycol (1, 2-propanediol, PROH, Molecular weight MW- 76.1, Renard and Babinet 1984) is the most commonly used permeating CPA in oocyte and embryo freezing at concentration of 1.5 M (Jain and Paulson, 2006; Pereira and Marques 2008). At this concentration the toxicity is low, but its ability to prevent ice formation is limited (Pereira and Marques 2008). Other permeating cryoprotectants were commonly used, ethylene glycol (EG, MW- 62.07; Miyamoto and Ishibashi, 1977); glycerol (MW-92.1; Polge *et al.* 1949), dimethylsulfoxide (DMSO, Me_2SO, MW-78.13; Wilmut and Rowson 1973). EG is currently the most commonly used permeating CPA for oocyte vitrification, because its characteristic features of low molecular weight, high permeation ability, and low toxicity (Kuwayama *et al.* 2005). In contrast to the permeating cryoprotectants, non-permeating cryoprotectants remain extracellular. They act by

drawing free water from the cell, and subsequently causing dehydration of the intracellular space. They are used in combination with permeating cryoprotectant to increase the net concentration of permeating CPA inside the cell and also prevent ice crystal formation (Jain and Paulson 2006). Two categories of non permeating CPAs were reported 1) low molecular weight non-permeating CPAs such as galactose (Mw- 180.2; Leibo and Oda 1993), glucose (Mw-181.1; Storey and Storey 1988), sucrose (Mw-342.3; Palasz and Mapletoft 1982); trehalose (Mw-378.3; Rudolph and Crowe 1985); 2) high molecular weight (> 50000 Daltons) non-permeating CPAs like polyvinylpyrrolidone (PVP; Leibo and Oda 1993), polyvinyl alcohol (PVA; Seidel *et al.* 1990); polyethylene glycol (PEG; O'Neil 1997), Ficoll (Shaw *et al.* 1997). Vitrification solutions are aqueous CPA solutions that do not freeze when cooled at high cooling rates to very low temperature (Liebermann *et al.* 2002). Therefore, CPA solutions are usually prepared in buffered media with a stable pH between 7.2–7.4. Although, Dulbecco's phosphate buffered saline (PBS) is most commonly used, other HEPES-buffered culture media such as TCM-199 have been used successfully.

1.6.1.3 Cryodevices

Various new techniques and types of carriers have been used with the overall aim of improving the survival of oocytes/embryos following cryopreservation. Until recently, 0.25 mL standard insemination straws were widely used for vitrification of oocytes and embryos. Relatively high cooling rate 2500°C/min could be achieved by direct plunging of the straw into LN_2 (Palasz and Mapletoft 1996). However, using this method, oocytes suffered significantly from cryodamage (Martino *et al.* 1996a).

Martino *et al.* (1996a) reported that bovine oocytes could be vitrified within a thin film (< 1 µL) of vitrification solution placed on an electron microscope (EM) grid. Kasai (2002) reported that by using the EM grid the sample could be cooled instantly when plunged into LN_2, however, because the grid is small in size (3.05 mm in diameter and 0.037 mm thick), few oocytes or embryos could be loaded.

Therefore, Matsumoto *et al.* (2001) suggested the use of a triangular piece of nylon mesh (1.0 cm base and 1.3 cm height, with 60 μm meshes) so that many oocytes (65 bovine cumulus oocyte complexes) could be loaded.

In 1998, Vajta *et al.* devised the open-pulled straw (OPS) method by heating and softening insemination straws (0.25 mL) and then shortening them manually until the inner diameter and the thickness of the wall decreased from 1.7 to 0.8 mm and from 0.15 to 0.07 mm, respectively. The straws were then, cut at the narrowest point with a razor blade. Embryos were loaded by means of the capillary effect with approximately 1 μL of vitrification solution; the estimated cooling rate in OPS was around 20000°C/min (Vajta *et al.* 1998).

Lane *et al.* (1999a) then developed a refined system for embryo cryopreservation "the cryoloop", which consisted of a small nylon loop (20 μm wide and 0.5–0.7 mm in diameter) mounted on a stainless steel pipe inserted into the lid of a cryovial. The oocytes or embryos were loaded over a thin film of vitrification solution into the loop. The loop containing the oocytes or embryos was then directly immersed into LN_2.

Another rapid approach which was used to achieve high cooling rate was completely containerless called "microdrops" (or "microdroplets"). This method was first demonstrated by Landa and Tepla in 1990, without intention to increase cooling/warming rates (Landa and Tepla 1990). Papis *et al.* (1999) reported that this method was more effective than conventional straw for vitrification of bovine embryos. In this approach, oocytes in small volumes of vitrification solution are dropped directly into LN_2; this successfully eliminates the insulation effects of the container wall. An alternative way to avoid the vapour formation around the sample was the solid surface vitrification (SSV) method (Dinnyes *et al.* 2000). This technique makes a shortcut to exclude entirely the vapour formation by dropping the vitrification solution containing the oocytes onto a pre-cooled metal

surface in the LN_2. SSV provided an efficient method of heat transfer in conjunction with containerless vitrification in microdrops.

Alternative modification of the vitrification technique depends on the use of partially solidified liquid nitrogen (nitrogen slush); this is prepared by placing LN_2 in glass vacuum desiccators. The temperature of LN_2 then decreases below the boiling point to almost –210°C, increasing the cooling rate to 24000°C/min (Martino *et al.* 1996a).

Arav and Zeron (1997) developed another technique called minimum drop size (MDS), in which 0.1 to 0.5 μL of vitrification solutions were cooled and warmed. This method was successfully used for vitrification of bovine oocytes (Arav and Zeron 1997). Another device was developed by the same group Arav *et al.* (2000), the VitMaster, this device producing vacuum in a reinforced LN_2 container, so that a considerable part of LN_2 evaporates but the remaining solution cools and even starts to solidify to form LN_2 slush.

The most recent development of ultrarapid vitrification methods was based on the concept of minimum volume cooling (MVC, Kuwayama and Kato 2000). In the origin of the procedure, very small volumes of vitrification solution containing oocytes or embryos were loaded onto the inner wall of 0.25 mL standard insemination straws, after sealing, the straw was immersed directly into LN_2. In the cryotop procedure, a thin plastic film strip attached to a plastic handle was the carrier tool; loading was performed by the use of a glass capillary under the control of a stereomicroscope. Before cooling almost all liquid was removed so that the embryos or the oocytes were only surrounded by a very thin layer of vitrification solution. Although all these methods result in high cooling and warming rates by using a minimal volume of freezing medium coupled with direct contact with LN_2, most of these approaches have introduced a problem due to contamination by direct contact with LN_2. To solve this problem some approaches such as VitSet technique performed the cooling in factory derived, and/or filtered

or UV-sterilized LN_2, followed by wrapping the sample and the carrier tool into a sealable container before storage (Vajta *et al.* 1998). Recent alternative solutions to the problem of LN_2 were the cryotip (Kuwayama *et al.* 2005) and super open pulled straw (SOPS, Isachenko *et al.* 2005). In fact, any technology in reproductive biology must ensure and guarantee the full protection of biological objects from microorganisms (Bielanski *et al.* 2000). Liquid nitrogen, which is used for storage of frozen material, can be a source of contamination by these microorganisms (Bielanski *et al.* 2000). Different types of viruses, which are simple and very cryo-stable structures, may increase their virulence after direct plunging and storage in liquid nitrogen, such as hepatitis virus (Hawkins *et al.* 1996) and herpes virus (Jones and Darville 1989). So, the critical requirement in vitrification is a combination of high cooling rate and a safe procedure.

1.7 OOCYTE STRUCTURE AND CRYOPRESERVATION DAMAGE

Fundamental knowledge of cryobiological characteristics of each cell type is needed in order to develop an effective cryopreservation protocol (Critser *et al.* 1997). Diverse strategies have been tested to minimize cooling induced cell injury. Irrespective of the efficiency of the above mentioned freezing strategies, it appears that oocytes pose specific problems because of their unique physical and biological proprieties that also vary between species. The oocyte is one of the largest mammalian cells ranging from 80 to 120 μm in diameter depending on species. Immature oocytes at the germinal vesicle (GV) stage are characterised by a large diploid nucleus (prophase I), a dense band of filamentous actin subjacent to the oolemma, and other organelles scattered throughout the ooplasm, including mitochondria, endoplasmic reticulum, and Golgi apparatus. The Golgi of the oocytes produces lysosome-like vesicles, the cortical granules that are randomly distributed throughout the cytoplasm. The mature oocyte (metaphase of the second meiotic division, MII) is characterized by a large peripheral spindle apparatus with microtubules extending from pole to pole and from each pole to kinetochores of the chromosomes aligned along the metaphase plate. Abundant actin containing

microfilaments are distributed in the perinuclear and cortical ooplasm, where they direct organelle distribution and polar body extrusion during meiosis. In MII oocytes cortical granules migrate to the periphery of the ooplasm just beneath the actin band, where they are ready to undergo exocytosis at the time of fertilisation. As first proposed by Mazur in 1963, one of the major factors that influences the response of a cell to freezing is the ratio of its surface area to volume. In general the larger the cell, the slower it must be cooled to survive freezing (Mazur 1963). For example in humans the flattened paddle shaped sperm cell has a surface area to volume ratio of 4.3, meanwhile the spherical oocyte has a surface area to volume ratio of only 0.05. Consequently, human oocytes require much longer to reach osmotic equilibrium when exposed to CPAs than do spermatozoa (Gilmore *et al.* 1997). Additionally, the lower surface area to volume ratio of the oocytes has an adverse effect on the transport of water and CPAs through plasma lemma (Leibo 1980). Apart from the size, the shape of the oocyte is problematic. The almost perfect sphere slows down formation of an equal distribution of any substance including permeable CPAs coming from outside or released from the oocyte (Kuwayama 2007). The third major factor responsible for oocyte cryodamage is the lowest possible cell number; from this point of view multicellular embryos can survive and compensate for as much as 50% loss of their cells as demonstrated by biopsies or bisection of the embryos, or even suboptimal culture conditions (Kuwayama 2007). Mammalian oocytes have a complex of organelles and subcellular structures, which are particularly sensitive to temperature and osmotic pressure. The effects of cryopreservation on oocyte structures and subsequent development will be discussed in later experimental chapters.

1.8 CONCLUSIONS

Despite considerable interest surrounding the application of oocyte cryopreservation as a tool for the treatment of infertility in women, which can overcome some of the ethical problems associated with embryo freezing, and

provide storage for future use as well as for preservation of genetic material from rare or valuable animals and use in NT, this technique cannot yet be considered as well-established. Low survival rates, the absence of comprehensive information of the status of essential biological attributes of oocytes after thawing and insufficient developmental rates, all contribute to the need for further improvement. The unique physical and biological properties of oocytes render them more sensitive to chilling injuries. As previously discussed, many problems are associated with MII oocyte cryopreservation including chilling injuries and toxicity to the CPAs which lead to considerable morphological and functional damage. Cryopreservation of GV-stage may provide an alternative approach for preservation of female gametes, due to the lack of the meiotic spindle which is one of the main targets for chilling injuries and cryodamage. More realistic advances could come from the optimization of freezing protocols for immature and matured oocytes using vitrification (ice-free cryopreservation) techniques which result in better preservation of viability and developmental potential, however many variables need to be considered in order to achieve effective vitrification.

1.9 GENERAL AIMS OF EXPERIMENTAL CHAPTERS

In this thesis, the recovery, viability and subsequent developmental potential of cryopreserved oocytes were studied. The overall aim for these studies was to establish a technique for oocyte cryopreservation which would support subsequent development following different in vitro techniques. Sheep were chosen as an experimental model due to their agricultural importance in Egypt and the availability of biological materials from mature ewes in Nottingham. To achieve this aim, the experimental chapters in this thesis were designed.

Firstly, to evaluate viability and subsequent in vitro maturation of ovine GV-stage oocytes following exposure to different combinations of cryoprotectants. Oocyte viability was based on morphological evaluation, cumulus cell expansion and nuclear maturation following exposure of GV-stage oocytes to different

combinations of ethylene glycol, glycerol and DMSO (**Chapter 3, Effect of exposure of immature ovine oocytes to different combinations of cryoprotectants on viability and subsequent in vitro maturation**).

Secondly, to evaluate the effects of vitrification using solid surface vitrification (SSV) technique on recovery, viability, cumulus cell expansion, nuclear maturation following IVM, frequencies of in vitro fertilisation, polyspermy and subsequent in vitro embryo development of vitrified/thawed ovine GV-oocytes (**Chapter 4, In vitro fertilisation of ovine oocytes vitrified by solid surface vitrification (SSV) at germinal vesicle stage**).

Thirdly, to investigate the effects of Cryoloop vitrification on developmental potential of ovine GV-stage oocytes following vitrification and warming. The effects of cryoloop vitrification on recovery, viability, cumulus cell expansion and meiotic status following IVM, spindle and chromatin configuration, frequencies of in vitro fertilisation and polyspermy, first cleavage following IVF and subsequent development. Total cell numbers, numbers of apoptotic nuclei, and ploidy of day 7 in vitro produced blastocysts were also evaluated (**Chapter 5, Vitrification of immature ovine oocytes using the cryoloop: Effects on viability, in vitro maturation, in vitro fertilisation, and subsequent development**).

Fourthly, to elucidate the maturation promoting factor (MPF) and mitogen-activated protein kinas (MAPK), in addition to in vitro embryo development following parthenogenetic activation (PA) and somatic cell nuclear transfer (SCNT) of ovine oocyte vitrified at GV-stage. Embryo quality based on morphological evaluation and detection of apoptotic nuclei and apoptotic index in day 7 in vitro produced blastocysts following PA or SCNT using TUNEL assay. Also, the ploidy of day 7 blastocysts was evaluated by karyotyping (**Chapter 6, MPF/MAPK activities, parthenogenetic activation, and somatic cell nuclear transfer of ovine oocytes vitrified at germinal vesicle (GV) stage**).

Fifthly, the effect of cytochalasin B pre-treatment as a cytoskeletal stabilizer on viability, frequencies of IVF and polyspermy, and subsequent development of ovine oocytes vitrified at GV-stage (**Chapter 7, Vitrification of immature ovine oocytes with cryoloop: Effect of cytochalasin B pre-treatment on viability and subsequent development**).

The effect of caffeine treatment during IVM on spindle configuration, MPF/MAPK activities and subsequent in vitro embryo development following IVF of ovine oocytes vitrified at GV-stage (**Chapter 8, Effect of caffeine treatment on spindle configuration, MPF/MAPK activities, and subsequent development of ovine oocytes vitrified at germinal vesicle stage (GV) stage**).

Finally, the effect of demecolcine pre-treatment on viability, timing of first polar body extrusion, spindle configuration and subsequent development following IVF and parthenogenetic activation of ovine oocytes vitrified at GV-stage (**Chapter 9, Effect of demecolcine pre-treatment on viability, timing of first polar body extrusion, spindle configuration, and subsequent development of ovine oocytes vitrified at germinal vesicle (GV) stage**).

CHAPTER 2

GENERAL MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich, Dorset, United Kingdom, unless otherwise stated. Recipes of media and solutions not mentioned here are presented in *Appendix A.*

2.1 ISOLATION OF OVINE CUMULUS OOCYTE COMPLEXES

Fresh ovaries from mature ewes were collected at a local slaughterhouse, immediately placed into pre-warmed phosphate-buffered saline (PBS) in a vacuum flask to maintain the temperature and then transported to the laboratory within 2–3 hours (h) of collection at 25°C. In the laboratory, the ovaries were washed twice with fresh pre-warmed sterile PBS (37°C) in a sieve and then placed into a flask with 100–200 ml of warm PBS. In a laminar flow hood, antral follicles (2 to 3 mm diameter) were aspirated from individual ovaries using 21-gauge needle attached to a 10 ml syringe. The point of the needle was inserted into the ovary 2–3 mm away from the selected follicle; the follicle was then carefully penetrated from the underside to avoid loss of the oocyte. Aspirated follicular fluid was regularly transferred into a 50 ml conical polystyrene tube containing 20 ml of dissection medium, HEPES-buffered tissue culture medium (H-TCM 199 with Earle's salts; Gibco Life Technologies Inc., Paisley, UK) containing 10% heat inactivated foetal bovine serum (FBS; Gibco) in a hot box at 39°C, and allowed to settle for 10 min. After settling, ¾ of the volume was removed and the remaining fluid transferred into a 92 x 10 mm Petri dish (Nunclon, Roskilde, Denmark). Cumulus oocyte complexes (COCs) were identified and collected on a warm stage at 39°C under a stereomicroscope (Leica MZ 12.5, Leica, Microsystems, Germany). Oocyte quality was determined on the basis of morphological features. Briefly, only good quality COCs showing an even granulated cytoplasm with at least three layers of cumulus

cells was selected. The selected COCs were washed three times in dissection medium at 39°C, and then transferred into 2 ml dissection medium in 35 mm Petri dish (Nunc, Rosklide, Denmark) at 39°C in a humidified atmosphere of 5% CO_2 in air until use.

2.2 VITRIFICATION OF OVINE COCs

2.2.1 Exposure of COCs to different vitrification solutions (VS)

Selected COCs were exposed for 10 min to equilibration solution, 10% ethylene glycol (EG) and 0.25 mol (M) trehalose (T), this solution was prepared in PBS supplemented with 10% heat inactivated FBS. After that, oocytes were exposed for 60 sec to one of three different vitrification solutions (VS), all vitrification solutions were composed of PBS and 10% FBS in addition to different combinations of cryoprotectants, VSI (20% EG and 20% dimethylsulfoxide (DMSO), VSII (25% EG plus 25% DMSO) or VSIII (25% EG and 25% glycerol). After exposure, dilution of the cryoprotectants was performed by transfer of the oocytes to 10% EG and 1 M T, 0.5 M T in PBS and 10% FBS, and then finally to PBS/FBS for 3 min each at 39°C. After cryoprotectant dilution, oocyte viability was determined by morphological examination. COCs with normal appearance were washed twice in maturation medium and then matured in vitro for 24 h.

2.2.2 Vitrification of COCs by solid surface vitrification (SSV)

Selected COCs were vitrified by solid surface vitrification (SSV) as previously described (Dinnyes *et al.* 2000) with minor modifications. Briefly, COCs were handled for 2–3 min in a base medium (SSV BM) consisting of H-TCM 199 supplemented with 20% heat inactivated FBS, and then suspended in the SSV equilibration medium (4% EG in SSV BM) for 6–10 min at room temperature (22–24°C). Following equilibration, groups of 5–10 oocytes were rinsed three times in small drops (approximately 25 µl) of SSV solution consisting of 35% EG, 5%

polyvinylpyrrolidone (PVP), 0.4 M T in BM on a warming plate at 39°C for less than 60 sec. The oocytes were then either processed through the warming solutions (toxicity control) or submitted to the complete vitrification process (**Fig. 2.1**). For vitrification, groups of 5–10 oocytes with 1–2 µl of vitrification solution were aspirated into a glass pipette, then the solution containing the oocytes was expelled from the tip of the pipette onto the dry surface of a hollow metal cube pre-cooled by partial immersion in liquid nitrogen (LN_2) maintaining its temperature between –150 and –180°C.

2.2.2.1 Warming of SSV oocytes

Following vitrification, the vitrified droplets containing the oocytes were moved immediately using nitrogen-cooled forceps into a 35 mm Petri dish (Nunc, Rosklide, Denmark) containing 2 ml of 0.3 M trehalose solution in SSV BM for 1 min at 39°C. After that, oocytes were consecutively transferred for 3 min intervals into 500 µl of 0.15 M, 0.075 M, 0.0375 M trehalose solution in SSV BM, and finally into SSV BM at 39°C in a four-well dish (Nunc, Denmark, **Fig. 2.1**). After warming, COCs were morphologically examined to evaluate their survival. Surviving vitrified-thawed oocytes were washed twice in maturation medium and matured in vitro for 24 h.

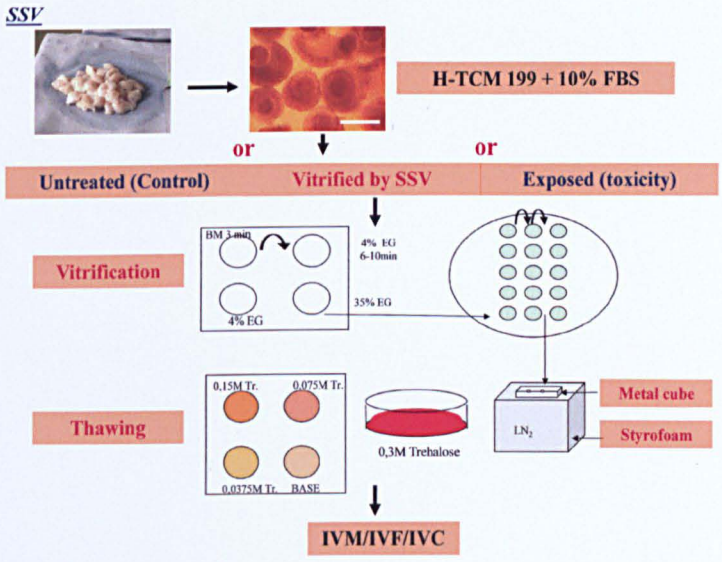


Figure 2.1 Vitrification of COCs using SSV. Diagram shows procedures of solid surface vitrification (SSV) of ovine oocytes. H-TCM 199: HEPES-Tissue Culture 199 medium, FBS: Foetal Bovine Serum, BM: Base Medium, EG: Ethylene Glycol, Tr: Trehalose, LN_2: Liquid Nitrogen, IVM: In vitro Maturation, IVF: In vitro Fertilisation, IVC: In vitro Culture.

2.2.3 Vitrification of COCs using Cryoloop

Following oocyte recovery, selected COCs were vitrified using the Cryoloop technique previously described by Lane *et al.* (1999a). Cryoloops used for vitrification consisted of a nylon loop (20 μm wide; 0.5–0.7 mm diameter) mounted on a stainless steel tube inserted into the lid of a cryovial. The loops were purchased mounted with epoxide into vials (Hampton Research, Aliso Viejo, CA, USA). After washing three times in a base medium (cryoloop BM), H-TCM 199 supplemented with 10% heat inactivated FBS, COCs were transferred to 500 μl-droplets of equilibration solution consisting of 10% EG plus 0.25 M trehalose in cryoloop BM for 3 min on a warm stage at 39°C (**Fig. 2.2, A**). The oocytes were then moved to a small drop (20 μl) of vitrification solution which composed of 20 % EG and 20 % DMSO in BM at 39°C on a warming plate (**Fig 2.2, B**). After that, the oocytes were either processed through the warming solutions (toxicity control) or vitrified. For vitrification, the cryoloop was dipped into the vitrification solution to create a thin film by surface tension. A total of 3–5 oocytes were suspended on the film using a fine pulled glass mouth pipette. The cryoloop containing the oocytes was plunged into a cryovial previously submerged and filled with LN_2. The transfer of oocytes into the vitrification solution and the vitrification process were performed within 1 min.

2.2.3.1 Warming of cryoloop vitrified oocytes

To warm the cryoloop vitrified oocytes, the cryovial submerged under the LN_2 was opened and the cryoloop containing the oocytes was removed from LN_2 and placed

directly into a well of 500 µl base medium containing 10% EG and 1 M trehalose. The oocytes immediately fell from the loop into the warming solution. The oocytes were then moved from this solution after 3 min and transferred to 500 µl drop of 0.5 M trehalose solution in BM and then to cryoloop BM for another 3 min. All the thawing procedures were conducted at 39°C. After thawing, COCs were examined microscopically to assess their viability. Viable oocytes were washed twice in maturation medium and then matured in vitro for 24 h.

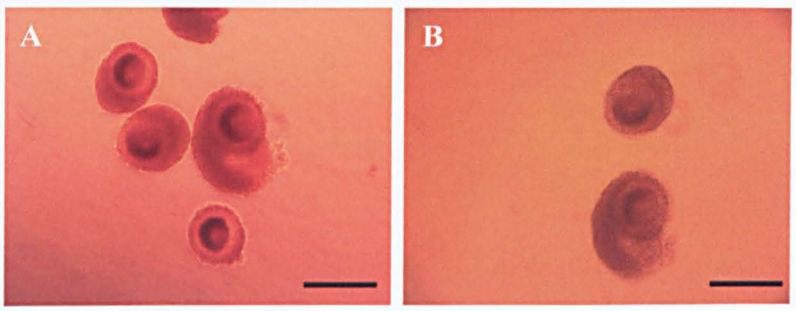


Figure 2.2 Cryoloop vitrification of immature ovine oocytes. (**A**) immature ovine oocytes exposed to equilibration solutions before cryoloop vitrification. (**B**) COCs in vitrification solutions. Scale bar = 50 µm.

2.2.4 Oocyte viability

After exposure of COCs to different vitrification solutions, SSV, and cryoloop vitrification, oocyte survival was morphologically evaluated immediately after thawing and cryoprotectant dilution under a stereomicroscope (Leica MZ 12.5, Leica Microsystems, Germany). Oocytes with a spherical and symmetrical shape, evenly granulated cytoplasm and no signs of lysis, membrane damage, swelling, vacuolization, degeneration or leakage of the cellular content were recorded as viable (normal oocytes). Oocytes with ruptured zona pellucida, ruptured vitelline membrane or having dark or faint fragmented cytoplasm with signs of degeneration were assigned as non-viable (abnormal oocytes).

2.3 IN VITRO MATURATION OF VITRIFIED/THAWED OVINE OOCYTES

After thawing, morphologically normal COCs were washed twice in maturation medium, bicarbonate-buffered Tissue Culture Medium 199 (TCM 199 with Earle's salts, Gibco BRL/Life Technologies) supplemented with 10% FBS, 5 μg/ml FSH (Vetropharm, Ireland), 5 μg/ml LH (Vetropharm, Ireland), 1 μg/ml oestradiol-17β, 0.3 mM sodium pyruvate, 100 μM cysteamine and 50 μg/ml gentamycin. Dissection and maturation media were sterilised by filtration through a 0.22 μm Millipore filter (Corning Inc, Germany) and equilibrated in a humidified atmosphere of 5% CO_2 at 39°C for at least 2 h prior to use. For maturation, groups of 40–45 oocytes were transferred into four-well dishes (Nunc, Denmark) containing 500 μl of maturation medium overlaid with 300 μl mineral oil to avoid evaporation, these dishes were pre-warmed at 39°C in a humidified atmosphere of 5% CO_2 in air before use. In most experiments except for nuclear transfer **(Chapter 6)** and caffeine treatment **(Chapter 8)**, oocytes were matured in vitro (IVM) for 24 h at 39°C in a humidified atmosphere of 5% CO_2 in air as previously described (Lee and Campbell 2006).

2.3.1 Evaluation of cumulus cell expansion following IVM

After 24 h IVM, cumulus expansion was evaluated under a stereomicroscope (Leica, MZ 12.5, Leica Microsystems, Germany) the percentage of oocytes with loosened layers of cumulus cells was noted.

2.3.2 Assessment of nuclear maturation using aceto orcein staining

24 h post onset of maturation (hpm), cumulus cells were removed by repeated pipetting in H–TCM 199/PVP containing 300 IU/ml hyaluronidase. Groups of 10–15 completely denuded oocytes were mounted on a clean glass slide in a small drop of medium. The slide was prepared by placing four spots of Vaseline (96%) and

paraffin wax mixture (4%) in a position equal to the four corners of a cover slip to be used for holding the oocytes (18 mm x 18 mm, No. 1 BDH.). The cover slip was placed onto the Vaseline wax spots and gently pressed down until the oocytes were slightly compressed and not able to roll but remained intact. Slides were placed into a jar containing fixative (ethanol: acetic acid 3:1) for at least 24 h. Fixed oocytes were stained with 1.0% orcein by drawing the solution under the cover slips using a piece of filter paper (Whatman No. 1). The solution was prepared by boiling 1 gm of orcein in 45 ml acetic acid for two hours, after cooling, the solution was filtrated. The filtrate was then diluted with 55 ml of distilled water to give a 100 ml of a 45% of solution. The solution was stored at room temperature and only upper clear area of the stain was used. Excess orcein was removed by drawing ethanol under the cover slip again using filter paper. The slides were examined under phase contrast (Leica, Germany) to assess the state of nuclear maturation. Oocytes were classified into four stages based on nuclear chromatin configuration: germinal vesicle (GV), metaphase-I (MI), anaphase-I + telophase-I (AI/TI), and metaphase II (MII). Oocytes at MII were considered as matured.

2.4 IMMUNOSTAINING FOR EVALUATION OF CHROMOSOME AND SPINDLE CONFIGURATION

Meiotic spindle and chromatin status were evaluated by immunostaining of in vitro matured oocytes for tubulin and chromatin according to Bogliolo and colleagues (2007a) with some modifications. Following removal of cumulus cells as described (**Section, 2.3.2**); oocytes were fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature. Fixed oocytes were then washed three times in phosphate-buffered saline supplemented with 20% FBS (PBS/FBS) for 10 min each. The oocytes were then transferred into PBS/FBS containing 0.5% Triton X-100 as a permeabilizing agent for 30 min at room temperature. Subsequently, the oocytes were incubated overnight at 4°C with primary antibody; mouse monoclonal anti - α - tubulin antibody (1:200) in PBS/FBS plus 0.5% Triton X-100. After three washes in PBS/FBS, the oocytes were incubated with fluorescein isothiocyanate (FITC)-

goat labelled secondary antibody (1:200) at room temperature for 60 min. Oocytes were then washed three times in 500 µL PBS/FBS and finally stained with a solution of 10 µg/ml bisbenzimide (Hoechst 33342) in PBS/FBS for chromatin labelling. Oocytes were then mounted on diagnostic microscope slides (Erie Scientific CO. Portsmouth, UK) in Vectashield mounting medium containing DAPI (Vector Laboratories, Inc. Burlingham, CA) under a cover slip. The slides were examined by epifluorescence (Leica DMR, Germany), images were captured using a digital camera (Hammamatsu, Japan) and analysed using Simple PCI software (Compix, CA, USA). The oocytes were assigned a quality score based on the morphological normality of the meiotic spindle and chromatin (Succu *et al.* 2007a). The oocytes were classified as normal when bearing the classical symmetrical barrel shape meiotic spindle or abnormal if oocytes showed disorganized, clumped or dispersed spindle elements, multiple spindle-like structures or absent (missing) not visible meiotic spindle. In addition, normal chromatin identified by two sets of chromatin aligned regularly along the equatorial plane of the meiotic spindle, abnormal showed clumping or dispersed chromatin from the centre of the spindle.

2.5 IN VITRO FERTILISATION OF OVINE OOCYTES

2.5.1 Sperm preparation

Cryopreserved semen pellets from a Texel ram were purchased (Britbreed, UK) and stored under LN_2. Approximately 1 h before fertilisation, a single 0.2 ml frozen semen pellet containing 400 x 10^6/ml progressively motile spermatozoa was thawed in a dry glass tube pre-warmed at 37°C in a hot box and then shaken in a beaker filled with warm water (30°C) for 30–60 sec. The thawed semen was prepared for in vitro fertilisation (IVF) by swim up technique, 80 µl of thawed semen was placed under 3 ml of sperm wash medium *(Appendix A)* in 15 ml centrifuge tubes pre-warmed at 37°C. Spermatozoa were allowed to swim up by incubation of centrifuge tubes at 45° angle for 1 h at 37°C. Motile spermatozoa were collected by aspirating

and mixing the supernatant from each tube (1/3 volume) which was then centrifuged at 1700 rpm for 5 min. Following centrifugation, the supernatant was discarded leaving around 200 µl of the medium above the pellet level. The sperm concentration was calculated by adding 10 µl of sperm suspension to 990 µl of distilled water in small test tube and mixing well. 10 µl of sperm mix were loaded into an improved bright-line Neubauer counting chamber (Marienfield, Germany), sperm were allowed to settle for two minutes and then counted microscopically. The sperm concentration was adjusted to 2.0 x 10^6 sperm /ml. The motility of the spermatozoa was also checked following swim up under phase contrast microscope (Leica).

2.5.2 In vitro fertilisation

Mature COCs (24 hpm) were washed once in pre-warmed fertilisation medium at 39°C *(Appendix A)* containing 2% sheep serum. Groups of 40–50 oocytes were transferred into four-well dishes (Nunc) containing 500 µl of fertilisation medium and 2.0 x 10^6 sperm /ml. Oocytes and sperm were co-incubated for 18 h at 39°C in a humidified atmosphere of 5% CO_2 in air.

2.5.3 Evaluation of frequencies of in vitro fertilisation and polyspermy following IVF

At 18 h post-insemination (hpi), presumptive zygotes were washed thoroughly and repeatedly pipetted in PBS supplemented with 4 mg/ml PVP (PBS/PVP) to remove all cumulus cells and attached spermatozoa. They were then fixed in 4% PFA in PBS/PVP for 20 min at room temperature and then washed three times in 500 µl PBS/PVP. After that, presumptive zygotes were transferred to 500 µl PBS/PVP containing 10 µg/ml Hoechst 33342 for 10 min at room temperature. Oocytes were washed three times in 500 µl PBS/PVP, then they were mounted on a clean glass slide in Vectashield mounting medium with DAPI (Vector Laboratories, USA) and then covered with a thin cover slip. Samples were examined using a fluorescence

microscope (Leica DMR, Heidelberg, Germany) fitted with a digital camera (Hamamatsu, Japan) and image analysis software (Simple PCI, Compix Inc., USA). The inseminated oocytes were categorized as fertilised when they have sperm/swollen sperm head in ooplasm, or a male and/or female pronuclus, polyspermic fertilisation was characterized by presence of more than one sperm in ooplasm or more than two pronuclei, oocytes with no signs of sperm penetration were considered unfertilised.

2.6 PARTHENOGENETIC ACTIVATION

After removal of cumulus cells from in vitro matured oocytes at 24 hpm, oocytes were washed three times in HEPES-buffered synthetic oviduct fluid (H-SOF) medium supplemented with bovine serum albumin (BSA, 4mg/ml), non-essential amino acids, and sodium citrate. Oocytes at MII stage with a visible first polar body (PBI) were selected and activated for 5 min in 5 μM calcium ionophore (A 23187) in H-SOF-BSA at 39°C. Oocytes were then washed twice in H-SOF-BSA and once in embryo culture medium (mSOFaaci, modified SOF medium supplemented with amino acids and fatty acid free-BSA 4 mg/ml), followed by incubation in embryo culture medium supplemented with 10 μg/ml cycloheximide (CHX) and 7.5 μg/ml cytochalasin B (CB) for 4–5 h at 39°C in a humidified atmosphere of 5% CO_2. Alternatively, oocytes were activated using a second method, selected MII oocytes were washed 3 times in 500 μl of calcium free CZB (Chatot, Ziomet, and Bavister) medium, oocytes were then activated by 10 mM $SrCl_2$ (strontium chloride) and 7.5 μg/ml CB in calcium free CZB medium for 4–5 h at 39°C in a humidified atmosphere of 5% CO_2. The calcium free CZB was pre-warmed in the incubator at least 1 h before adding 100x strontium solution (10 μl/ml), this was to avoid strontium precipitation during activation.

2.7 NUCLEAR TRANSFER

At 15-16 hpm, enucleation was performed according to the method previously described (Lee and Campbell 2006). Briefly, maturing oocytes at anaphase I - telophase I (AI-TI) were stripped of cumulus cells as previously described (**Section, 2.3.2**). Denuded oocytes with an extruding 1st polar body were selected and cultured in manipulation medium (HEPES-buffered synthetic oviduct fluid [H-SOF] containing non essential amino acids, sodium citrate and 4 mg/ml bovine serum albumin [BSA], pH 7.4) supplemented with Hoechst 33342 (5 µg/ml) for 15 min at 39°C. Laser assisted enucleation (XY clone laser, Hamilton Thorne, USA) was carried out in manipulation medium containing 7.5 µg/ml CB on a heated stage at 39°C by aspirating a small amount of cytoplasm protruding from the protruding AI-TI containing the maternal chromosomes. Enucleation was checked by exposing the aspirated cytoplasm to U.V. light, whilst in the pipette, for the presence of the chromosomes. Enucleated oocytes were then returned to maturation medium under the same conditions (**Section, 2.3**) until transfer of the donor nuclei.

For transfer of donor nuclei, enucleated oocytes were transferred from the maturation medium to manipulation medium H-SOF-BSA. A quiescent primary ovine foetal fibroblast cells used as nuclear donor (passage 3-5) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1.0% (v/v) ß-mercaptoethanol, 2.0 mM L-glutamine, 1.0% (v/v) penicillin/streptomycin and 10% FBS until approximately 80–90% confluent, quiescence was then induced by reducing the concentration of FBS to 0.1% for at least 3 days before to use. Donor cell was inserted under the zona pellucida of each enucleated oocyte, allowing the membrane of the donor cell to have contact with the oolema (**Fig 2.3**). The couplets (2–3) were placed and manually aligned in the fusion chamber containing 0.3 M mannitol without calcium ions. Fusion was induced by two D.C. pulses of 1.25 kV/cm for 30 µsec using a multiporator (Eppendorf, Germany) between 22 and 24 h after onset of maturation. Couplets were then returned to culture medium (C.SOF, mSOFaaci containing BME-essential amino acid and MEM-non essential amino

acids and FAF-BSA 4 mg/ml) until activated. For activation, reconstructed embryos approximately 1 h post fusion (hpf) were washed three times in 500 µl calcium-free CZB medium, then they were activated using 10 mM $SrCl_2$ in calcium-free CZB with CB (7.5 µg/ml) for 4–5 h at 39°C in a humidified atmosphere of 5% CO_2 in air.

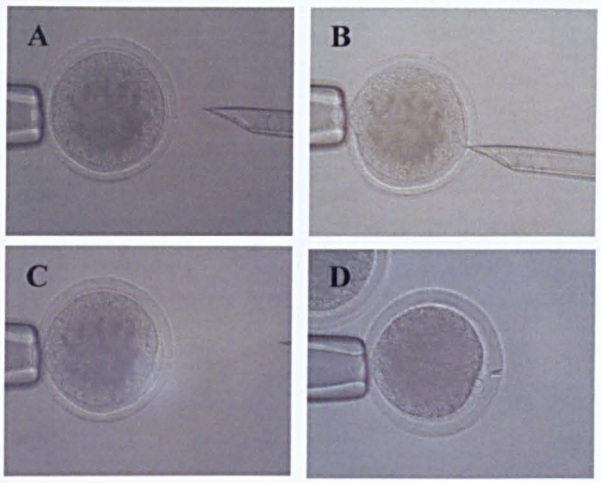


Figure 2.3 Production of oocyte somatic cell couplets from ovine oocyte vitrified at GV-stage. (**A**) Donor cell is positioned at the tip of the pipette. (**B**) The nuclear transfer pipette is inserted through the hole made during enucleation and the donor cell deposited in the perivitelline space. (**C and D**) Cells for transfer are pushed to ensure close contact with the oolema.

2.8 IN VITRO CULTURE OF OVINE EMBRYOS

At 18 hpi or 4–5 h post activation (hpa), presumptive zygotes were washed three times in 500 µl H-SOF containing non essential amino acids and 4 mg/ml BSA at 39°C. IVF embryos were repeatedly pipetted to remove any attached cumulus cells and adhered spermatozoa). After that, they were washed twice in embryo culture medium (C.SOF, mSOFaaci containing BME-essential amino acid and MEM-non essential amino acids and FAF-BSA 4 mg/ml). Following washing, the embryos (15–20) were transferred into 50 µl drops of pre-warmed and equilibrated C.SOF-BSA culture medium covered with mineral oil and incubated at 39°C in a

humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 until day 7 (Day 0 = Day of insemination or day of activation).

2.8.1 Evaluation of the frequency of cleavage and subsequent embryo development

The numbers of cleaved embryos were assessed at 24 and 48 hpi or hpa and cleaved embryos were transferred into fresh culture medium. Development to morula stages was evaluated on day 5. On day 7, development to blastocyst was recorded and morphologically evaluated under a stereomicroscope and divided into early, expanded or hatched blastocyst.

2.8.2 Evaluation of total cell numbers

Total cell numbers for day 7 in vitro produced blastocyts were evaluated using Hoechst stain. After washing in PBS supplemented with 4 mg/ml PVP, embryos were fixed in 4% PFA in PBS/PVB for 10 min at room temperature. After thoroughly washing in PBS/PVB, blastocyst embryos were incubated in PBS/PVP containing 10 µg/ml Hoechst 33342 for 5 min at room temperature. After washing in PBS/PVP, they were mounted on clean glass slides in Vectashield mounting medium with DAPI (Vector Laboratories, Inc. Burlingham, CA.) and covered with a cover slip. The samples were examined under epifluorescence (Leica DMR, Germany) fitted with a digital camera (Hamamatsu, Japan) and image analysis software (Simple PCI, Compix Inc., USA).

2.9 TUNEL ASSAY TO DETERMINE DNA FRAGMENTATION

Day 7 embryos were removed from embryo culture medium (mSOFaaci containing 4 mg/ml FAF-BSA [C-SOF]) and washed three times in 200 µl drops of PBS/PVP at 39°C by passing the embryos through the drops. After washing, embryos were incubated in 4% PFA containing 10 µg/ml bisbenzimide (Hoechst 33342) for 30

min at room temperature, followed by two washes in 200 µl PBS/PVP. Embryos were incubated in 500 µl of freshly prepared permeabilization solution (0.1% v/v Triton X-100 in PBS/PVP) for 5 min at room temperature, and then washed twice in 200 µl PBS/PVP. To detect DNA degradation in apoptotic cells, Terminal Transferase dUTP Nick End Labelling (TUNEL) was performed using an in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instruction. The embryos were incubated in 50 µl of the TUNEL reaction mixture (1:10; dilution of enzyme, terminal deoxynucleotidyl transferase from calf thymus, in label solution, nucleotide mixture) at 39°C for 45 min in a humidified chamber to avoid evaporation of the reaction mixture (Choi 2008). Embryos were washed in 200 µl PBS/PVP, and then mounted on glass slides in Vectashield mounting medium with DAPI (Vector Laboratories, Inc. Burlingham, CA.) under a cover slip. DNA fragmentation was visualised by epifluorescence microscope (Leica DMR, Germany) fitted with a digital camera (Hamamatsu, Japan) and image analysis software (Simple PCI, Compix Inc., USA).

2.10 KARYOTYPING OF IN VITRO PRODUCED BLASTOCYSTS

Day 7 blastocysts were prepared and examined for their cytogenetic compositions as described by King *et al.* (1979) with minor modifications. Blastomeres of the embryos were synchronised at metaphase by incubation of blastocysts in culture medium containing 0.05 µg/ml demecolcine solution for 3h at 39°C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. After washing in PBS/PVP, blastocysts were transferred to hypotonic solution (0.8% sodium citrate) for 10 min at room temperature, and followed by 10 min incubation in a solution of 0.56% potassium chloride. The blastocysts were then fixed in a solution consisting of 1:1.2 sodium citrate (0.8%) and methanol: acetic acid (2:1) for 15 min at room temperature. Subsequently, blastocysts were mounted individually in a marked frame onto a freshly cleaned glass microscope slide in a minimal volume of fixative. By observing the slides under stereomicroscope (Leica) and before evaporation of the

fixative drop, a freshly prepared solution of methanol: acetic acid (1:1) was dropped onto the embryo until complete cell spreads were obtained. After air drying, the slides were stained with 4% (v/v) Giemsa solution for 5 min. Slides were then washed with water and left to dry in air at room temperature. The ploidy was evaluated under phase contrast microscope (Leica, Germany) at x 100 under oil immersion. Embryos were classified as being normal when diploid chromosomes (54, 2 n) or abnormal, when haploid (n) or polyploidy (≥3 n).

2.11 IN VITRO DOUBLE KINASE ASSAY

2.11.1 Preparation of oocyte lysate

The preparation of oocyte lysate and analysis of maturation promoting factor (MPF) and mitogen activated protein kinase (MAPK) activities were performed as previously described (Ye *et al*, 2003) with some modifications. Briefly, groups of 10 cumulus stripped oocytes were thoroughly washed in DPBS containing 0.1% polyvinyl alcohol (PVA) at 39°C and then placed into 5 µl of ice-cold lysis buffer containing 45 mM *β*-glycerophosphate (pH 7.3), 12 mM *ρ*-nitrophenylphosphate, 2 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS), 12 mM $MgCl_2$, 12 mM ethyleneglycol bis (2-aminoethyl-ether) tetraacetic acid (EGTA), 0.1 mM EDTA, 20 mM Na_3VO_4, 10 mM NaF, 2 mM dithiothreitol (DTT), 2 mM phenylmethylsulphonyl fluoride, 2 mM benzamidine, 20 µg/ml leupeptin, 20 µg/ml pepstatin A and 19.5 µg/ml aprotinin. The samples were frozen in LN_2 and stored at –80°C until analysed.

2.11.2 In vitro double kinase assay

The activities of MPF and MAPK kinases were measured simultaneously using histone H1 and bovine myelin basic protein (MBP) as their in vitro substrates, respectively. The oocyte lysate was thawed and then refrozen in liquid nitrogen (−196 °C) once. The kinase reaction was started by adding the oocyte lysate to 5 µl

kinase assay buffer containing 45 mM β-glycerophosphate (pH 7.3), 12 mM ρ-nitrophenylphosphate, 20 mM MOPS, 12 mM $MgCl_2$, 12 mM EGTA, 0.1 mM EDTA, 2 mM Na_3VO_4, 10 mM NaF, 4 mg/ml H1, 6 mg/ml MBP, 40 μM protein kinase A (PKA) inhibiting peptide (Santa Cruz Biotechnology; Autogen Bioclear, Clane), 43 μM protein kinase C (PKC) inhibiting peptide (Promega, Southampton) and 10 Ci/mmol [γ-^{32}P] ATP (PerkinElmer). The mixtures were incubated at 37°C for 30 min with gentle shaking. The reaction was stopped by adding 10 μl ice-cold 2×SDS sample buffer which was made from [125 mM Tris-Cl (pH 6.8) (Fisher Scientific), 200 mM DTT, 4% (w/v) SDS (Fisher Scientific), 0.01% (w/v) bromophenol blue and 20% (w/v) glycerol]. After boiling for 5 min, the substrates were separated by standard polyacrylamide gel electrophoresis (SDS-PAGE, 15% gels) using a Mini-Protean II dual slab cell (Bio-Rad, Hercules, CA) at 140 V for 1.5 h. Gels were dried on 3 mm filters and exposed to phosphor-screens (Fuji film). The phosphor images of gels (screens) were captured and the kinase activities were quantified using an FX phosphor image analysis system (Bio-Rad).

2.12 STATISTICAL ANALYSIS

At least three replicates were carried out for each experimental group. Data were analysed using Chi square test and un-paired student's *t*-test. All results are considered to be statistically significant at $P< 0.05$, unless otherwise stated. The statistical analysis was performed using Graph Pad Software (http://www.graphpad.com).

CHAPTER 3

EFFECTS OF EXPOSURE OF IMMATURE OVINE OOCYTES TO DIFFERENT COMBINATIONS OF CRYOPROTECTANTS ON VIABILITY AND SUBSEQUENT IN VITRO MATURATION

3.1 INTRODUCTION

Maintaining the viability and potential developmental ability of living reproductive cells and tissues of several mammalian species after long-term storage represents an important tool in human and animal reproduction (Ledda *et al.* 2007). Although the viability of cryopreserved mammalian oocytes was first demonstrated about 30 years ago (Whittingham 1977) and the first domestic offspring was reported nearly 20 years ago (Hamano *et al.* 1992), this technology is still considered to be at an early stage. Slow freezing protocols have been replaced by the most recent vitrification approaches. Vitrification is a physical process in which cryoprotectant solutions form a glassy solid state, this can be achieved by elevation in the viscosity of the solution, through using high cooling rates in association with high concentrations of cryoprotectant (Rall and Fahy 1985; Vajta *et al.* 1998). So, the vitrification solution must be sufficiently concentrated to avoid crystallization during cooling (Kasai *et al.* 2002). However, high concentrations of cryoprotectant are toxic and may cause osmotic injury to the oocytes (Liebermann *et al.* 2002) which is responsible for low developmental potential of cryopreserved oocytes (Fahy *et al.* 1984). Many suggestions have been reported to minimize the toxicity of vitrification solutions such as use of less toxic cryoprotectants, combinations of different cryoprotectants, prior exposure to less

concentrated cryoprotectants and reduction of exposure time to vitrification solution (Vajta *et al.* 2000).

Different cryoprotectants like ethylene glycol (EG), glycerol (GLY), dimethylsulfoxide (DMSO), propylene glycol and 1, 2-propanediol (PROH) have been used in different combinations for vitrification of mammalian oocytes and embryos (Liebermann and Tucker 2002; Yamada *et al.* 2007). Several studies demonstrated that EG would be an ideal cryoprotectant (Shaw *et al.* 1997; Cetin and Bastan 2006), because of its ability to penetrate cell membranes faster than other cryoprotectants (Shaw *et al.* 1997), also EG was proven to be less toxic than other permeable cryoprotectants (Martino *et al.* 1996a; Dinnyes *et al.* 2000). DMSO was reported to have toxic effects on bovine oocytes (Arav and Zeron 1997) causing disassembly of the spindle microtubules and movement of pericentrioler material to the centre of the oocytes. However, other studies reported successful oocyte vitrification with DMSO alone (Wani *et al.* 2004a, b) or in combination with other cryoprotectants (Vieira *et al.* 2002; Yamada *et al.* 2007). It has been reported that, vitrification solution containing DMSO and EG could parthenogenetically activate in vitro matured ovine oocytes, also it caused zona pellucida hardening and subsequent decreased sperm penetration (Tian *et al.* 2007). Few studies have reported the use of glycerol as a permeable cryoprotectant in oocyte vitrification. Wani and colleagues obtained 23.5% maturation rates following vitrification of immature buffalo oocytes with 7 M glycerol (Wani *et al.* 2004a). Moreover, lower maturation rates (54.3%) were reported following exposure of immature bovine oocytes to vitrification solutions containing 25% EG in combination with 25% DMSO (Yamada *et al.* 2007). In buffalo, a severe reduction in the frequency of in vitro maturation was reported following exposure of germinal vesicle (GV) oocytes to a combination of 3 M EG and 3 M glycerol (Mahmoud *et al.* 2009). However, evaluation of the effects of different cryoprotectants with varying concentrations on survival of immature ovine oocytes is lacking.

Thus, the present study of cryoprotectant combinations is important to improve the efficiency of ovine oocyte vitrification, aiming to minimize the toxic effect of cryoprotectant solutions. The objectives of these studies were to evaluate the survival rates and subsequent in vitro maturation of ovine GV-stage oocytes following exposure to different vitrification solutions containing three different combinations of EG, DMSO, and GLY.

3.2 MATERIALS AND METHODS

3.2.1 Exposure of COCs to different vitrification solutions

Recovery of ovine oocytes was carried out as previously described (**Chapter 2.1**). Selected oocytes were washed three times in washing medium and randomly assigned into four groups, 1) control groups (directly subjected to in vitro maturation), 2) VSI group (exposed to vitrification solution I), 3) VSII group (exposed to vitrification solution II), and 4) VSIII group (exposed to VIII). Exposure of COCs to different combinations of cryoprotectants was carried out as described (**Chapter 2.2.1**). After cryoprotectant dilution, oocyte viability was determined as described in (**Chapter 2.2.4**).

3.2.2 Evaluation of cumulus cell expansion and nuclear maturation

COCs with morphologically normal appearance were washed twice in maturation medium and then matured in vitro as demonstrated (**Chapter 2.3**). Following 24 h post onset of maturation (hpm), cumulus cell expansion and meiotic maturation were evaluated as previously described (**Chapter 2.3.1** and **2.3.2**).

3.2.3 Statistical Analysis

Data were analysed by using Chi square test. All results are considered to be statistically significant at $P< 0.05$, unless otherwise stated.

3.3 RESULTS

3.3.1 Effects of different cryoprotectants on oocyte viability

The effects of exposure of immature ovine oocytes to different vitrification solutions containing different combinations of cryoprotectants (VSI: 20% EG + 20% DMSO; VSII: 25% EG + 25% DMSO; VSIII: 25% EG + 25% glycerol) on oocyte viability based on morphological evaluation are illustrated in **Table 3.1** and **Fig 3.1**. No significant differences ($P > 0.05$) were observed in the proportions of oocytes with normal morphology (viable oocytes) between three different vitrification solutions (81.9, 75.3, and 81.8% for VSI, VSII, and VSIII, respectively, **Fig. 3.1**).

Table 3.1 Effects of exposure of immature ovine oocytes to different vitrification solutions on survival rates.

Treatment	*No. Oocytes*	*No. Normal*	*%*
VSI	215	176	81.9%
VSII	162	122	75.3%
VSIII	214	175	81.8%

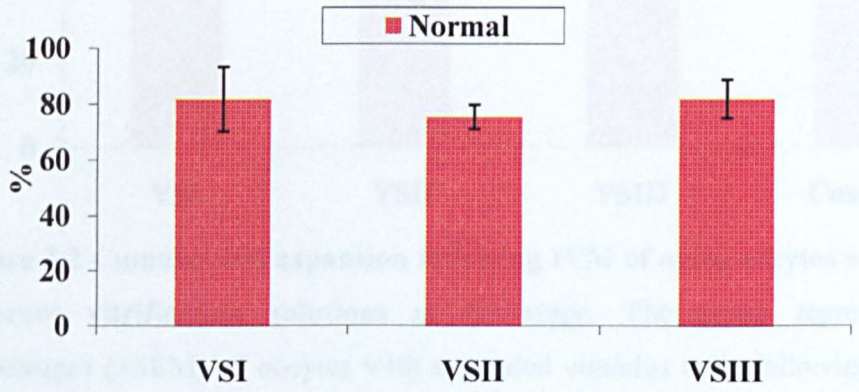


Figure 3.1 Effects of exposure of immature ovine oocytes to different vitrification solutions on survival rates. The graph represents the proportions (±SEM) of viable (with normal morphology) ovine COCs following exposure to different combinations of cryoprotectants: VSI (20% EG + 20% DMSO), VSII

(25% EG + 25% DMSO), VSIII (25% EG + 25% Gly). Four replicates were performed.

3.3.2 Effects of different vitrification solutions on cumulus cell expansion following IVM

The effects of different cryoprotectants on the numbers of oocyte with expanded cumulus cells following IVM are presented in **Fig 3.2**. The percentages of cumulus cell expansion following IVM were significantly decreased ($P< 0.01$) in oocytes exposed to VSI (20% EG + 20% DMSO) and VSII (25% EG + 25% DMSO) as compared to the control group (66.2 and 66.5 *vs* 93.3%, respectively). However, these values were not significantly different ($P> 0.05$) from oocytes exposed to VSIII (25% EG + 25% glycerol, 77.7%, **Fig. 3.2**).

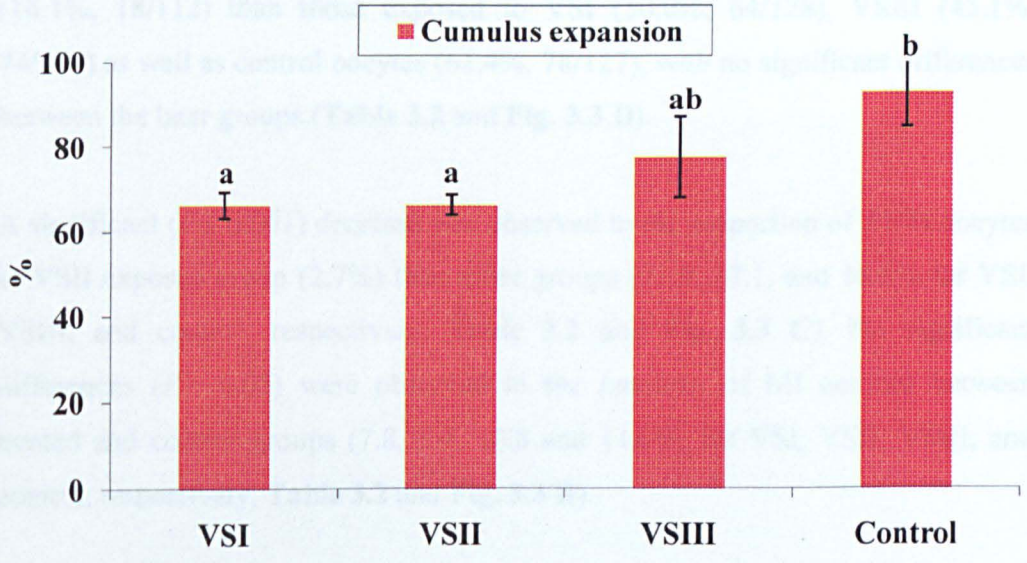


Figure 3.2 Cumulus cell expansion following IVM of ovine oocytes exposed to different vitrification solutions at GV-stage. The graph represents the percentages (±SEM) of oocytes with expanded cumulus cells following IVM of COCs exposed to different vitrification solutions. Different letters are significantly different ($P< 0.01$). Four replicates were performed.

3.3.3 Effects of different cryoprotectants on meiotic maturation following IVM

Nuclear status of ovine oocytes from different groups was evaluated 24 hpm. Oocytes were categorized into four groups according to nuclear appearance; GV, MI, AI/TI, and MII stages. Oocytes at MII were recorded as matured. The results are presented in **Table 3.2** and **Fig 3.3.**

Numbers of oocytes remaining at the GV-stage (75.9%, 85/112) were significantly higher ($P< 0.001$) in oocytes exposed to VSII (25% EG + 25% DMSO) than other groups (20.3, 26.8, and 11.0% for VSI, VSIII and control, respectively, **Table 3.2** and **Fig. 3.3 A**). Also, a significant difference ($P< 0.05$) was observed between VSIII and control oocytes. On the other hand, the frequency of nuclear maturation (oocytes at MII-stage) was the lowest ($P< 0.001$) for oocytes exposed to VSII (16.1%, 18/112) than those exposed to VSI (50.0%, 64/128), VSIII (45.1%, 74/164) as well as control oocytes (61.4%, 78/127), with no significant differences between the later groups (**Table 3.2** and **Fig. 3.3 D**).

A significant ($P< 0.001$) decrease was observed in the proportion of AI/TI oocytes in VSII exposed group (2.7%) than other groups (21.9, 17.1, and 16.5% for VSI, VSIII, and control, respectively, **Table 3.2** and **Fig. 3.3 C**). No significant differences ($P> 0.05$) were observed in the numbers of MI oocytes between treated and control groups (7.8, 5.4, 10.8 and 11.0%, for VSI, VSII, VSIII, and control, respectively, **Table 3.2** and **Fig. 3.3 B**).

Table 3.2 Effect of exposure to different vitrification solutions on subsequent nuclear maturation of immature ovine oocytes

Treatment	*No.*	*Meiotic maturation No. (%)*			
	Oocytes	*GV*	*MI*	*AI/TI*	*MII*
VSI	128	26 (20.3%)^{ad}	10 (7.8%)^{a}	28 (21.9%)^{a}	64 (50.0%)^{a}
VSII	112	85 (75.9%)^{c}	6 (5.4%)^{a}	3 (2.7%)^{b}	18 (16.1%)^{b}
VSIII	164	44 (26.8%)^{d}	18 (10.8%)^{a}	28 (17.1%)^{a}	74 (45.1%)^{a}
Control	127	14 (11.0%)^{a}	14 (11.0%)^{a}	21 (16.5%)^{a}	78 (61.4%)^{a}

Values with different superscripts in the same column are significantly different (a, c, b $P< 0.001$; a, d $P < 0.05$). GV (Germinal Vesicles), MI (Metaphase-I), AI/TI (Anaphase-1 + Telophase-I), MII (Metaphase-II), VSI (20% EG + 20% DMSO), VSII (25% EG + 25% DMSO), VSIII (25% EG + 25% Gly).

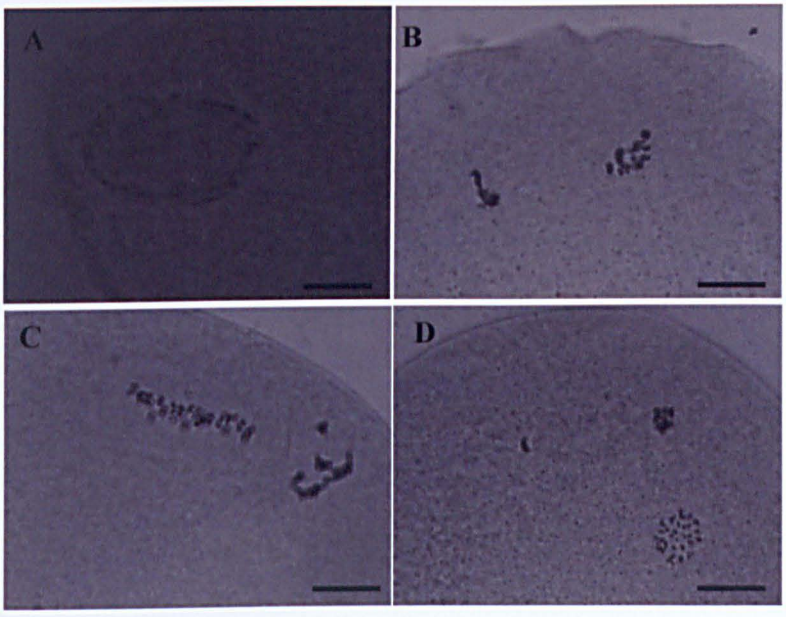


Figure 3.3 Nuclear maturation of ovine oocytes. The figure shows different stages of nuclear maturation of ovine oocytes following 24 h IVM and orcein staining, (**A**) Oocyte at GV-stage, (**B**) MI stage, (**C**) AI/TI stage, (**D**) MII stage, scale bar = 50 μm.

3.4 DISCUSSION

The major problem associated with cooling and cryopreservation of oocytes is the low percentage of oocytes retaining the ability to undergo normal maturation and fertilisation (LeGal and Massip 1999). This may be attributable to the exposure to cryoprotectants and cooling process (Mahmoud *et al.* 2009). Therefore, research on different cryoprotectants and combinations here is important for successful vitrification, since greater concentrations of vitrification solution may be toxic to the oocytes (Yamada *et al.* 2007). In this study the effects of different vitrification solutions with various combinations of cryoprotectants on viability and subsequent in vitro maturation of GV-stage ovine oocytes were determined.

Until recently, attention has been focused on the cryopreservation of mature oocytes, however, at this stage; the microtubular spindles are highly sensitive to cooling and cryoprotectant agents resulting in damage which may include chromosomal abnormalities (Men *et al.* 2003b). Therefore, the development of an efficient protocol for the cryopreservation of immature oocytes with their cumulus cells would offer an alternative to the storage of mature oocytes (Silvestre *et al.* 2006). In this study, the viability (based on morphological observation) of immature ovine oocytes exposed to different vitrification solutions was not affected by the types and concentrations of the cryoprotectants ranging from 75 to 82%. However, following in vitro maturation, significantly lower maturation rates (16.1%) were observed in oocytes exposed to VSII (25% EG + 25% DMSO) as compared with other groups. In similar studies in bovine oocytes Yamada and colleagues (2007), reported that exposure of immature oocytes to the same vitrification solutions with the same concentrations for 30 sec did not affect the frequencies of in vitro maturation as compared to control, indicating that this exposure time, irrespective of vitrification solution had no effect on oocyte maturation ability. However, when they increased the time of exposure to 60 sec, they found a significant decrease in the frequency of in vitro maturation of oocytes exposed to VSII and VSIII. Based on these observations, the results suggest that

for this exposure period (60 sec) these cryoprotectants (VSII: 25% EG 25% DMSO) have toxic effects on the oocytes (Yamada *et al.* 2007). In sheep, Silvestre *et al.* (2006) demonstrated that, exposure of immature oocytes obtained from pre-pubertal ewes to 20% EG plus 20% DMSO significantly decreased the maturation rates as compared to control (41.1% *vs* 79.9%). In buffalo, Mahmoud *et al.* (2009) reported that maturation rates were severely affected by exposure of GV-oocytes to different cryoprotectants. They reported highest maturation rates after exposure to EG (54.3%) and EG plus DMSO (47.5%), but the lowest maturation rates were observed in EG with glycerol (36.8%) and DMSO with glycerol (29.9%). In agreement with the results reported in this study, Albarracin *et al.* (2005a) reported that bovine oocytes exposed to 20% EG and DMSO showed a similar maturation frequency as control. Moreover, Wani *et al.* (2004a) observed that exposure of immature buffalo oocytes to 7 M concentrations of DMSO, EG or PROH followed by removal of cryoprotectants in descending concentrations of sucrose did not affect the survival and maturation rates as compared to control. In cattle, Cetin and Bastan (2006) reported very low maturation rates (14.9%) following vitrification of immature oocytes using 40% DMSO.

COCs have some characteristics that are adverse to cryoprotectant equilibrium between intra and extracellular environments. Factors that influence the passage velocity of cryoprotectants through the cell membrane and entrance into the ooplasm are important in oocyte vitrification (Yamada *et al.* 2007). The oocyte is a large single cell, with a small surface/volume ratio, and surrounded by compact cumulus cell layers. The role of cumulus cells during oocyte vitrification is still controversial. It has been reported that the presence of compact layers of cumulus cells surrounding immature mouse oocytes blocked the permeation of the high toxic cryoprotectant and might help to prevent swelling of oocytes during removal of the cryoprotectant (Miyake *et al.* 1993). Vajta *et al.* (1998) showed that, the cumulus layers and accumulated glycoproteins may reduce the speed of cryoprotectant penetration. Bogliolo *et al.* (2007a) reported that denudation of immature ovine oocytes before IVM and prior to vitrification increased the

frequencies of oocyte viability and subsequent maturation. Dhali *et al.* (2000) reported that, the presence of compact cumulus cell mass could reduce the rate of EG or DMSO passage into the oocytes.

The maturation rate obtained in the present study (50%, in VSI group) was similar to that reported by Silvestre *et al.* (2006) for pre-pubertal ovine immature oocytes following exposure to the same concentrations of cryoprotectants (20% EG + 20% DMSO). Although, the maturation rate was similar in oocytes recovered from pre-pubertal or adult sheep, a previous report showed that oocytes recovered from pre-pubertal sheep had significant biological differences compared with oocytes obtained from adult animals (Ledda *et al.* 2001). The higher viability and maturation reported here following exposure to VSI could be ascribed to multiple step-wise exposures of immature oocytes to cryoprotectants which resulted in less harmful effects than a single step exposure (Isachenko *et al.* 1998; Abe *et al.* 2005).

It has been reported that exposure of mouse oocytes to the three commonly used cryoprotectants (propanediol, EG, and DMSO) induces an immediate increase in intracellular calcium (Ca_i^{++}). The magnitude and duration of (Ca_i^{++}) transient induced varied between the cryoprotectants with EG giving the smallest and shortest increase, and propanediol giving the highest level. However, DMSO affects (Ca_i^{++}) stores directly, possibly by permeabilizing mitochondria and/or the endoplasmic reticulum (ER) (Larman *et al.* 2006; Gardner *et al.* 2007). In these studies, very low maturation rate (16.1%) was reported after exposure of oocytes to vitrification solution (VSII) containing higher concentrations of EG (25%) and DMSO (25%). This lower value seems to be associated with the use of this concentration of DMSO, because, the maturation rate (45.1%) was not affected in oocytes exposed to VSIII (25% EG + 25% glycerol) as compared to control. Glycerol in combination with either EG or DMSO has been used as cryoprotectant for oocyte vitrification in buffalo (Wani *et al.* 2004a, b; Mahmoud *et al.* 2009), cattle (LeGal and Massip 1999; Yamada *et al.* 2007) but with lower maturation

rates. Glycerol has been widely used for freezing of bovine embryos because of its low cytotoxicity. However, glycerol can induce severe osmotic damage to the cytoplasm due to its low membrane permeability (Szell *et al.* 1989). The low permeability of the cells to glycerol may increase the risk of osmotic stress during thawing and dilution as the water enters the cells more quickly than the glycerol. This may explain the poor results obtained following vitrification of oocytes by glycerol (Wani *et al.* 2004a).

In the present study, a combination of 20% EG and 20% DMSO can be used effectively for the cryopreservation of oocytes. Because of their low molecular weight, these components can easily permeate through cell membranes rapidly to achieve concentration equilibrium across the membranes. Moreover, EG, because of its high permeability and low toxicity has been found to be convenient for vitrification of immature human (Liebermann and Tucker 2002), cattle (Cetin and Bastan 2006), horses (Hurtt *et al.* 2000), pigs (Somfai *et al.* 2007), buffalo (Mahmoud *et al.* 2009) and sheep (Bogliolo et al. 2007a) oocytes.

In conclusion, exposure of immature ovine oocytes to vitrification solution containing higher concentrations of the permeating cryoprotectant DMSO has an adverse effect on subsequent in vitro maturation. However, a combination of EG plus DMSO at a concentration of 20% each can be used effectively as a vitrification solution for ovine GV-oocytes. Further studies are required to evaluate the effects of different exposure times to various cryoprotectants on viability and subsequent development of immature ovine oocytes. Also, further studies are required to test the effects of different vitrification solutions on development of immature oocytes during vitrification by different techniques.

CHAPTER 4

IN VITRO FERTILISATION OF OVINE OOCYTES VITRIFIED BY SOLID SURFACE VITRIFICATION (SSV) AT GERMINAL VESICLE STAGE

4.1 INTRODUCTION

Oocyte cryopreservation has become one of the most challenging approaches to restore fertility of chemo-and radiation therapy treated women with compromised ovarian function (Fabbri *et al.* 2001; Fabbri 2006). In livestock, where large numbers of ovaries can be easily obtained from a slaughterhouse, many oocytes can be collected, preserved and then incorporated in an in vitro production (IVP) system when considered appropriate, diminishing seasonal variations or sanitary constraints. Increasing cooling and warming rates as well as high concentrations of cryoprotectant are the critical factors which determine the success of vitrification. To achieve high cooling rates, the sample size and the volume of vitrification solution should be decreased. So, the sample has chance to be surrounded by liquid phase not vapour. Special carriers have been used to minimize the volume of vitrification solutions (see **Chapter 1.6.1.3**). In an alternative technique called Solid Surface Vitrification (SSV), oocytes in a small droplet of solution are vitrified upon contact with a metal surface partially immersed into liquid nitrogen (LN_2) and pre-cooled to –180°C (Dinnyes *et al.* 2000). SSV was used effectively for the cryopreservation of in vitro matured cattle (Dinnyes *et al.* 2000; Sripunya *et al.* 2010), goat (Begin *et al.* 2003), pig (Somfai *et al.* 2006), buffalo (Boonkusol *et al.*2007; Gasparrini *et al.* 2007), sheep (Zhang *et al.* 2009) oocytes. Moreover, this method was used for vitrification of immature pig oocytes (Gupta *et al.* 2007).

It has been previously described that the meiotic stage of oocyte influences its ability to survive cryopreservation. Until recently, attention has been focused on the cryopreservation of mature MII oocytes, despite, the fact that various kinds of cryoinjury and severe malfunctions can be caused to the oocytes at this stage, such as spindle disorganization (Mandelbaum *et al.* 2004), loss or clumping of microtubules resulting in some scattering of chromosomes (Sathananthan *et al.* 1988), increased polyploidy after fertilisation (Al-Hasani *et al.* 1987; Carroll *et al.* 1990) and subsequent decrease in fertilisation rates (Mandelbaum *et al.* 2004; Somfai *et al.* 2007). Conversely, vitrification of immature oocytes at the germinal vesicle (GV) stage might circumvent these problems due to fact that the genetic material is held within the contours of a nuclear envelope. However, there are few studies that have reported the development of immature oocytes following vitrification (Somfai *et al.* 2010).

The objectives of this study were to evaluate the effects of SSV and exposure to SSV solutions (35% EG, 5% PVP, 0.4 M trehalose for less than 60 sec) without freezing (Toxicity control) on the viability and subsequent development of ovine oocytes vitrified at GV-stage. Following parameters were assessed 1) recovery and survival rates following SSV, 2) cumulus cell expansion and nuclear maturation following in vitro maturation (IVM), 3) frequencies of in vitro fertilisation (IVF) and polyspermy, 4) kinetics of first cleavage following IVF, 5) in vitro embryo development following IVM/IVF.

4.2 MATERIALS AND METHODS

4.2.1 Vitrification of COCs by solid surface vitrification (SSV)

COCs obtained from slaughtered sheep ovaries were washed three times in washing medium (see **Chapter 2.1**) and randomly assigned into three groups, 1) control group (directly subjected to in vitro maturation, IVM), 2) Exposed group (EXP, exposed to SSV vitrification solution without plunging into LN_2), 3) SSV group (vitrified by SSV, **Chapter 2.2.2**). Following vitrification and warming, viable oocytes were matured in vitro for 24 h (**Chapter 2.3**). 24 h post onset of maturation (hpm), cumulus cell expansion and meiotic maturation were evaluated as previously described (**Chapter 2.3.1** and **2.3.2**).

4.2.2 In vitro fertilisation (IVF) and Culture (IVC)

Matured oocytes were fertilised in vitro using frozen thawed ram semen as previously described (**Chapter 2.5**). 18 hpi (hours post insemination); frequencies of in vitro fertilisation and polyspermy were evaluated as previously described (**Chapter 2.5.3**). Presumptive zygotes were cultured in vitro as described in (**Chapter 2.8**). Frequencies of cleaved embryos were assessed at 24, 30 and 48 hpi. On day 7, developmental rates to blastocyst stage embryos were recorded and morphologically evaluated under stereomicroscope and divided into early, expanded and hatched blastocysts.

4.2.3 Statistical Analysis

At least three replicates were carried out for each experimental group. Data were analysed using Chi square test. All results are considered to be statistically significant at $P< 0.05$, unless otherwise stated.

4.3 RESULTS

4.3.1 Recovery rates and oocyte viability following SSV

The effects of SSV of immature ovine oocytes on recovery rates and viability based on morphological evaluation are illustrated in **Table 4.1** and **Figs 4.1, 4.2** and **4.3**. No significant differences ($P > 0.05$) were observed in the proportions of recovered oocytes following SSV (75.7%, 143/189) or exposure to SSV vitrification solution (EXP, 90%, 108/120, **Fig 4.1**). Of those oocytes recovered 74.8%, 107/143 and 82.4%, 89/108 were recorded as morphologically normal (viable) in SSV and EXP groups, respectively (**Fig 4.2**), the difference between the two groups was not significant. Also, no significant differences were observed in the numbers of recovered oocytes with abnormal morphology (damaged, non viable) following SSV and EXP (25.2 *vs* 17.6%, respectively, **Figs 4.2** and **4.3**).

Table 4.1 Recovery rates and viability of ovine oocytes vitrified at GV-stage by Solid Surface Vitrification (SSV).

Treatment	*Total No. Oocytes*	*No. Recovered (% of total oocytes)*	*No. Normal (% of recovered oocytes)*
SSV	189	143 (75.7%)	107 (74.8%)
EXP	120	108 (90.0%)	89 (82.4%)

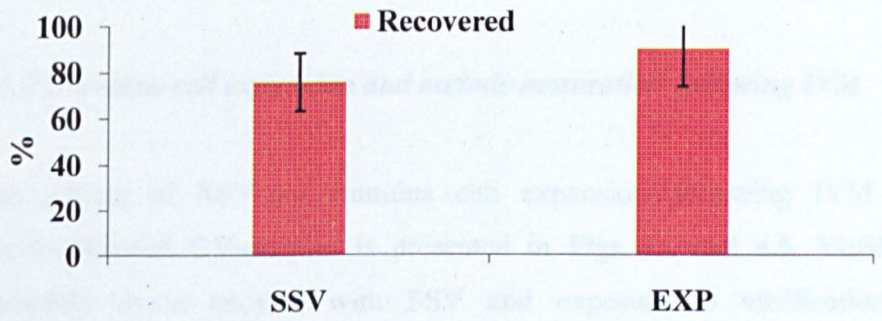


Figure 4.1 Recovery rates of ovine oocytes vitrified at GV-stage by Solid Surface Vitrification (SSV). The graph represents the proportions (±SEM) of recovered oocytes following SSV of ovine COCs and exposure to vitrification solution (EXP).

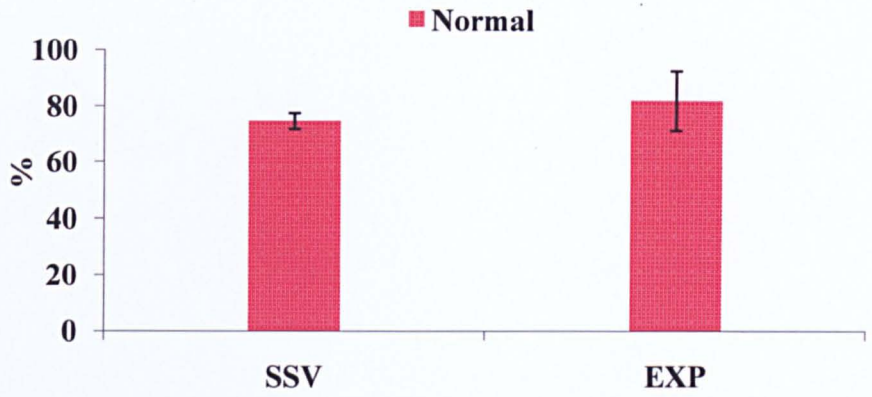


Figure 4.2 Viability of ovine oocytes vitrified at GV-stage by Solid Surface Vitrification (SSV). The figure represents the percentages (±SEM) of recovered oocytes with normal (viable) morphology following SSV and exposure to vitrification solution (EXP). Three replicates were carried out.

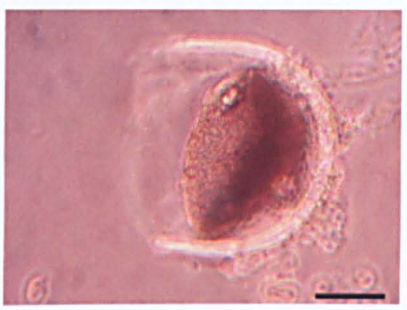


Figure 4.3 Degenerated oocyte recovered following SSV of ovine COCs. The figure shows an oocyte with damaged zona pellucida and degenerate cytoplasm following SSV. Scale bar = 50 µm.

4.3.2 Cumulus cell expansion and meiotic maturation following IVM

The effects of SSV on cumulus cell expansion following IVM of ovine vitrified/thawed GV-oocytes is presented in **Figs 4.4** and **4.5.** Vitrification of immature ovine oocytes with SSV and exposure to vitrification solution significantly ($P< 0.01$) decreased the proportions of oocytes with expanded cumulus cells as compared with control (41.3 and 58.3 *vs* 80.9% for SSV, EXP, and control, respectively, **Figs 4.4** and **4.5**).

The nuclear maturation of ovine oocytes vitrified at GV-stage using SSV and exposed to SSV vitrification solution is presented in **Table 4.2** and **Fig 4.6.** Frequencies of in vitro maturation (oocytes at MII stage) were significantly decreased ($P< 0.01$) in both SSV and EXP groups as compared to control (23.7 and 35.6 *vs* 71.9%, respectively). In contrast, at 24 hpm, numbers of oocytes that remained at the GV-stage were significantly higher ($P< 0.01$) in both SSV and EXP groups than in control (62.9 and 51.7 *vs* 9.6% respectively). No significant differences were observed between treated and control groups in terms of MI and AI/TI stages. These values ranged from 4.6 to 10.2% **Table 4.2** and **Fig 4.6.**

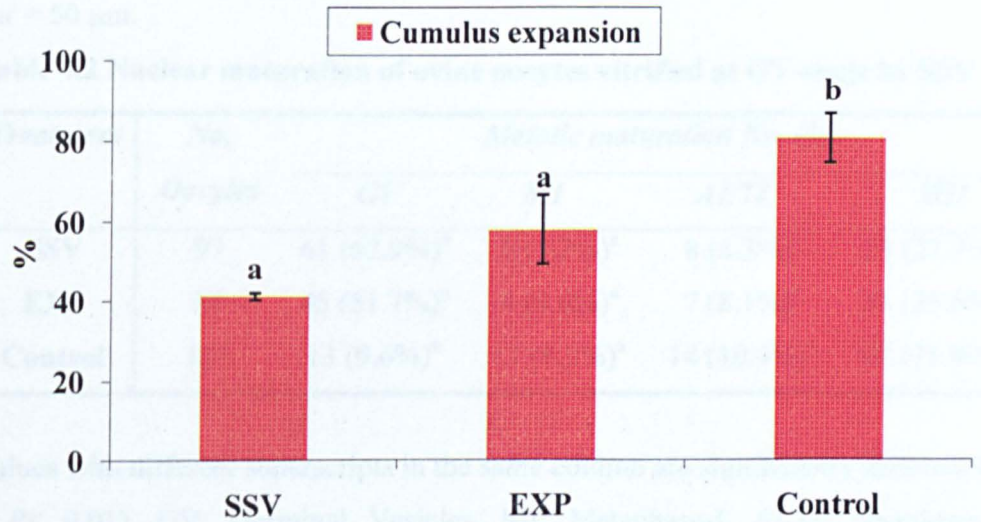


Figure 4.4 Cumulus cell expansion following IVM of ovine oocytes vitrified at GV-stage using SSV. The figure represents the percentage (±SEM) of oocytes with expanded cumulus cells following IVM of COCs vitrified by SSV or exposed to SSV solution (EXP). Different letters indicate significant differences ($P< 0.01$). Three replicates were performed.

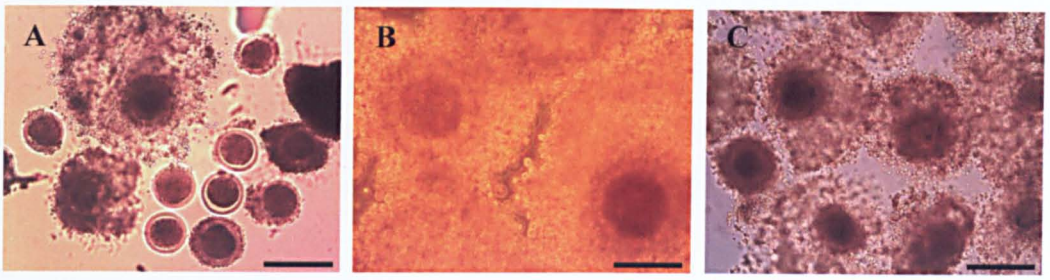


Figure 4.5 Oocytes with expanded cumulus cells. The figure reveals cumulus cell expansion following 24 h IVM of ovine oocytes (**A**) vitrified at GV-stage using SSV, (**B**) exposed to SSV solution or (**C**) non treated (control) group, scale bar = 50 µm.

Table 4.2 Nuclear maturation of ovine oocytes vitrified at GV-stage by SSV

Treatment	*No. Oocytes*	*Meiotic maturation No. (%)*			
		GV	*MI*	*AI/TI*	*MII*
SSV	97	61 (62.9%)[a]	5 (5.2%)[a]	8 (8.3%)[a]	23 (23.7%)[a]
EXP	87	45 (51.7%)[a]	4 (4.6%)[a]	7 (8.1%)[a]	31 (35.6%)[a]
Control	135	13 (9.6%)[b]	11 (8.2%)[a]	14 (10.4%)[a]	97 (71.9%)[b]

Values with different superscripts in the same column are significantly different (a, b $P< 0.01$). GV: Germinal Vesicles, MI: Metaphase-I, AI/TI: Anaphase-1/ Telophase-I, MII: Metaphase-II, SSV: Solid Surface Vitrification, EXP: Exposed to vitrification solution without freezing. Three replicates were performed.

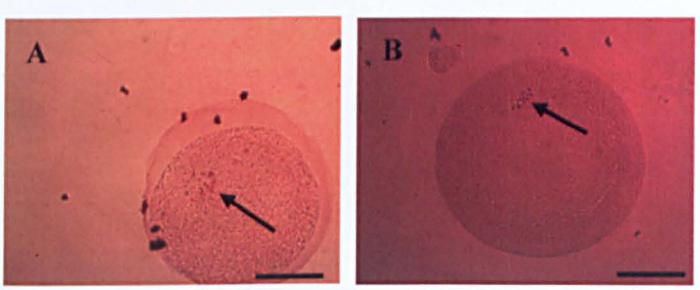


Figure 4.6 Meiotic maturation of ovine oocytes. The figure shows stages of nuclear maturation following 24 h IVM of ovine oocytes vitrified at GV-stage using SSV. Oocytes were examined after orcein staining (**A**) oocyte at GV-stage, and (**B**) MII stage. Scale bar = 50 µm.

4.3.3 Frequencies of in vitro fertilisation and polyspermy following IVF

The effects of SSV or exposure to vitrification solution on in vitro fertilisation following IVF of ovine oocytes vitrified at GV-stage are presented in **Table 4.3** and **Fig 4.7.** Vitrification of immature ovine oocytes with SSV significantly ($P<$ 0.01) decreased the in vitro fertilisation rates (39.3%) as compared to non treated control group (64.7%). However, exposure to SSV solution alone did not significantly affect fertilisation (56.4%) as compared to control. In terms of polyspermy (presence of more than one sperm in ooplasm or more than two pronuclei), SSV resulted in lower percentages (5.4%) than EXP (11.7%) or control (15.3%), however the differences were not significant between three groups. Significantly higher ($P<$ 0.01) percentages of oocytes remained unfertilised in SSV group (55.4%) compared to EXP (31.9%) and control (20.0%, **Table 4.3** and **Fig 4.7**).

Table 4.3 Frequencies of in vitro fertilisation and polyspermy following IVM/IVF of ovine oocytes vitrified at GV-stage by SSV.

Treatment	*No. Oocytes*	*Fertilisation No. (%)*		
		Fertilised	*Polyspermic*	*Unfertilised*
SSV	56	22 (39.3%)[a]	3 (5.4%)[a]	31 (55.4%)[a]
EXP	94	53 (56.4%)[b]	11 (11.7%)[a]	30 (31.9%)[b]
Control	85	55 (64.7%)[b]	13 (15.3%)[a]	17 (20.0%)[b]

Values with different superscripts in the same column are significantly different (a, b $P<$ 0.01). Fertilised oocytes (characterised by presences of sperm/swollen sperm head in ooplasm, or a male and/or female pronuclus), polyspermy (oocytes have more than one sperm in ooplasm or more than two pronuclei), unfertilised oocytes (with no signs of sperm penetration), SSV (Solid Surface Vitrification), EXP (Exposed to vitrification solutions without freezing). Three replicates were carried out.

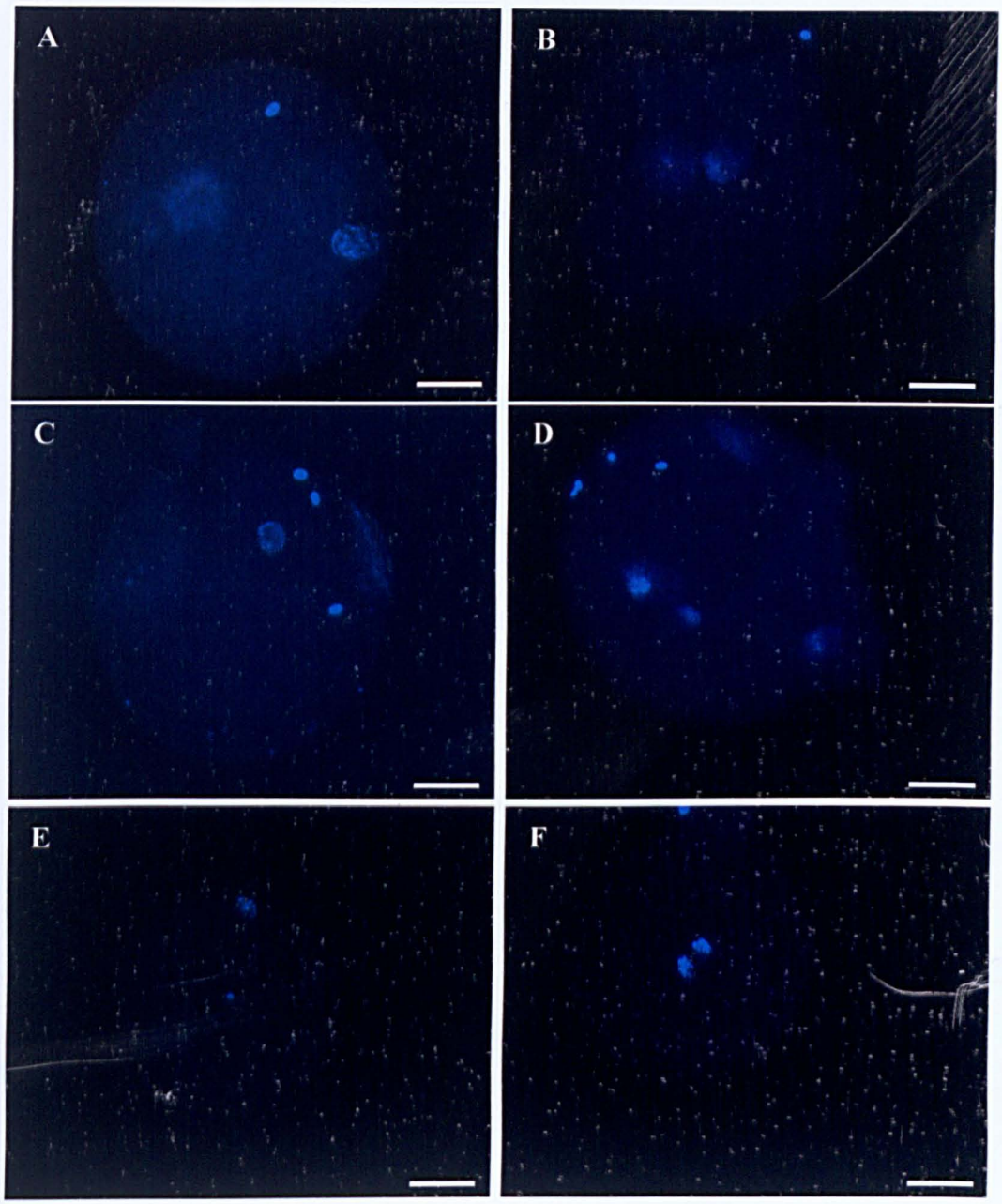


Figure 4.7 Fertilisation events in ovine oocytes. The figure illustrates IVM/IVF oocytes at 18 hpi stained with Hoechst. **(A** and **B)** fertilised oocytes with male and female pronuclei, **(C** and **D)** oocytes with polyspermic fertilisation, and **(E** and **F)** unfertilised oocytes with no signs of sperm penetration. Scale bar = 50 µm.

4.3.4 Kinetics of first cleavage following IVF

Timing of first cleavage following IVF of ovine oocytes vitrified at GV-stage using SSV was evaluated at 24 and 30 hpi. The results of this experiment are presented in **Table 4.4.** The total cleavage was calculated from total oocytes used for IVF, while kinetics of first cleavage was recorded based on total cleavage.

Significantly ($P< 0.001$) lower frequencies of cleaved embryos were observed in vitrified group compared to EXP and control (4.1 *vs* 50 and 52.5%, respectively). Assessment between 24 and 30 hpi indicates that most of vitrified oocytes (75%) cleaved late as compared to EXP (58.6%) and control (43.8%) groups. At 24 h, only 25% of cleaved oocytes were recorded in SSV groups, while 41.4 and 56.3% were observed for EXP and control oocytes, respectively (**Table 4.4**).

Table 4.4 First cleavage following IVM/IVF of ovine oocytes vitrified at GV stage

Treatment	*No. Oocytes*	*No. Cleaved (%) hpi*		*No. Total cleaved (%)*
		24	*30*	
SSV	97	1 (25.0 %)[a]	3 (75.0 %)[a]	4 (4.12%)[a]
EXP	58	12 (41.4%)[a]	17 (58.6 %)[a]	29 (50.0%)[b]
Control	61	18 (56.3 %)[a]	14 (43.8 %)[a]	32 (52.5%)[b]

Values with different superscripts in the same column are significantly different (a, b $P< 0.001$). Total cleavage rate is calculated on IVF oocytes while kinetic cleavage rates are based on total cleaved oocytes, hpi (hours post insemination). Three replicates were performed.

4.3.5 In vitro embryo development following IVF

The effects of SSV and exposure to vitrification solution on the frequencies of oocyte cleavage at 48 hpi and subsequent in vitro embryo development up to blastocyst stages are presented in **Table 4.5** and **Fig 4.8**.

Vitrification of immature ovine oocytes using SSV significantly ($P< 0.001$) decreased the frequency of cleavage at 48 hpi as compared to EXP and control groups (9.6 *vs* 55.9 and 76.6%, respectively). Also, exposure to vitrification solution alone (EXP) significantly reduced cleavage.

A similar trend was observed for day 7 blastocyst development. Significantly ($P< 0.001$) lower percentages of oocytes developed to blastocyst stage embryos following SSV (0.5%) as compared to EXP (20%) and control (45.3%) groups (**Table 4.5** and **Fig 4.8**). The difference between EXP and control groups was also significant at ($P< 0.01$). Based on the numbers of cleaved oocytes, 5.4% of the cleaved embryos had developed to the blastocyst stage at day 7 following IVF in SSV group compared to 35.2 and 59.2% for EXP and control groups (**Table 4.5**). Morphological evaluation of in vitro produced blastocysts revealed that, no blastocysts at early and expanded stages were observed in SSV groups, but 6.2 *vs* 13.1% were recorded as early and 6.6 *vs* 13.1% as expanded blastocysts for EXP and control (**Table 4.5** and **Fig 4.8**). In terms of hatched blastocysts, all in vitro produce blastocysts from SSV were hatched and represented 0.5% of the total oocytes vitrified. This value was significantly lower ($P< 0.001$) than those observed in EXP (7.2%) and control (18.7%) groups.

Table 4.5 In vitro embryo development following IVF of vitrified/thawed ovine COCs using SSV

Treatment	*No. Oocytes*	*No. Cleaved 48 hpi (%)*	*No. Blastocysts (% of oocytes)*				*Total Blastocysts (% of cleaved)*
			Early	*Expanded*	*Hatched*	*Total*	
SSV	385	37 (9.6%)[a]	0	0	2 (0.5%)[a]	2 (0.5%)[a]	(5.4%)[a]
EXP	290	165 (55.9%)[b]	18 (6.2%)[a]	19 (6.6%)[a]	21 (7.2%)[b]	58 (20.0%)[b]	(35.2%)[b]
Control	278	213 (76.6%)[c]	37 (13.1%)[b]	37 (13.1%)[b]	52 (18.7%)[c]	126 (45.3%)[c]	(59.2%)[c]

Values with different superscripts in the same column differ significantly (a *vs* b and a *vs* c $P< 0.001$ while b *vs* c $P< 0.01$). hpi (hours post insemination). Seven replicates were carried out.

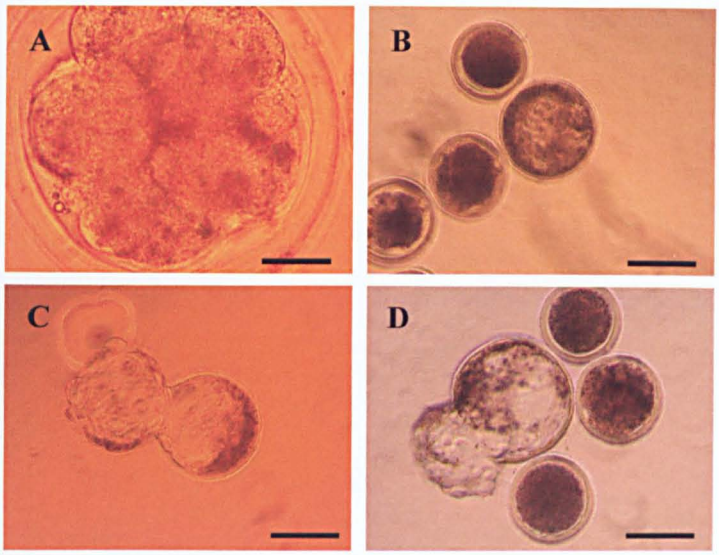


Figure 4.8 Developmental stages of ovine embryos produced by IVF of ovine oocytes vitrified at GV-stage using SSV. Examples of embryos produced following IVF (**A**) SSV. (**B**) Day 7 early blastocyst produced following IVM/IVF of ovine oocytes exposed (EXP) to SSV solution without freezing. (**C**) Day 7 hatched blastocysts produced by IVF of ovine oocytes exposed to SSV solution, and (**D**) vitrified using SSV technique. Scale bar = 50 μm.

4.4 DISCUSSION

Vitrification, as a simple cryopreservation method of directly submerging a cell in liquid nitrogen after brief exposure to a cryoprotectant agent, is substantially less harmful to cells such as oocytes and embryos that have high chilling sensitivities than are conventional cryopreservation methods using traditional slow-freezing (Martino *et al.* 1996a; Vajta *et al.* 1998). The advantage to the cells of vitrification over conventional cryopreservation procedures is that the 'open system' lacks an insulating layer around the sample. The vapour surrounding the cells can create effective insulation that reduces temperature transfer, resulting in a decreased cooling rate and possibly crystallization (Liebermann and Tucker 2002). The volume of the vitrification solution should be minimized as much as is practical to

ensure that the cells are surrounded with liquid nitrogen and not vapour. Thus the duration of any coating vapour is reduced and the rate of cooling is increased. Combination of direct contact with liquid nitrogen and small volume of vitrification solution allows higher cooling rates to be achieved, because heat conduction in liquid is much faster than in vapour. SSV as a containerless system which favours rapid heat exchange during cooling was effectively used for cryopreservation of in vitro matured bovine oocytes (Dinnyes *et al.* 2000), however its application in vitrification of IVM ovine oocytes resulted in very low (2%) blastocyst rates following IVF (Zhang *et al.* 2009). The present study investigated the effects of SSV on viability and subsequent developmental potential after IVM/IVF of vitrified/thawed ovine GV-oocytes.

SSV is a very cheap method that enables preservation of several microdrops of vitrification solution within a short period of time, each containing a large number of oocytes (Somfai *et al.* 2010). On the other hand, controlling the droplet size by this method requires extensive practice and there is a constant chance of oocyte loss through sticking of oocytes to the inner surface of the glass capillary or due to random drop dispersion caused by the flipping movement (Begin *et al.* 2003). The recovery rate is the number of remaining oocytes after expulsion of solution from the container during warming. Its analysis is important because cells must tolerate a sequence of volumetric concentrations and expansions which are related to different concentrations of the solution, and they can lose or rupture during this process (Gomes *et al.* 2008). The recovery rate following SSV (75.7%) reported here was slightly lower than that obtained by (Begin *et al.* 2003, 83%) and (Gupta *et al.* 2007, 91%) following SSV of IVM goat and pig oocytes. These variations might be ascribed to differences in protocols and technical approaches. In the goat study, the authors used a hollow metal cube covered with aluminium foil immersed in liquid nitrogen, however, in pig experiments; a piece of aluminium foil floating on liquid nitrogen was used as a cooling surface. Somfai *et al.* (2007) compared the two methods for vitrification of pig oocytes and they reported higher survival rates with aluminium foil than metal cube. Nevertheless, it must also be

emphasized that use of aluminium foil might add a technical variable and would require technical skill in controlling the size of the droplets formed on the cold surface, to keep the metal surface dry and to avoid setting of liquid nitrogen vapour on the surface of the droplet whilst being placed onto the metal surface.

The frequencies of viable oocytes based on morphological evaluation reported in this study (74.8%) were slightly lower than those reported by Al-aghbari and Menino (2002, 88.9%) and Bogliolo *et al.* (2007a, 84.3%). The differences between studies may be due to the cryodevices used for vitrification (cryotops in Bogliolo *et al.* 2007a versus SSV). However, Al-aghbari and Menino (2002) used the same method (SSV), so the differences in the results were not clear. But, the objective of Al-aghbari and Menino study was to develop an effective technique for vitrification of sheep ovarian tissues and they collected ovine COCs by slashing of the ovaries with a scalpel. Moreover, they evaluated oocytes survival using hematoxylin and eosin staining. SSV was used successfully for cryopreservation of IVM sheep oocytes with high survival rates (84.6%, Zhang *et al.* 2009). In pig, high survival (80%) was also reported following SSV of GV and MII oocytes (Gupta *et al.* 2007). In contrast, Somfai *et al.* (2010) reported lower viability (27.7%) following SSV of pig GV oocytes. Following exposure to SSV solution (EXP, toxicity test), ~80% of oocytes were viable. Similarly, Dinneys *et al.* (2000) and Somfai *et al.* (2010) reported 90–94% survival following exposure of cattle MII and pig GV oocytes to the same cryoprotectants.

Oocyte maturation provides an indication of the impairment of oocytes following exposure to cryoprotectant or vitrification. In the present study, the degree of cumulus cell expansion was significantly decreased in both treatment groups (SSV and EXP, 41.3 and 58.3%, respectively) as compared to control (80.9%). In contrast, a study by Isachenko *et al.* (2001) reported excellent expansion of cumulus cells following IVM of vitrified/thawed ovine GV oocytes. They suggested that this could be due to the presence of light unsaturated lipids in cumulus cells. Also, Gupta *et al.* (2007) observed significantly decreased (31%) in

cumulus cell expansion after IVM of porcine oocytes vitrified by SSV at GV-stage using DMSO as a cryoprotectant. This observation could be attributed to the damage and interruption that occur to the cumulus cells during vitrification. Bogliolo *et al.* (2007a) observed that most of the cumulus cells surrounding vitrified/thawed immature ovine oocytes had numerous cytoplasmic vacuoles and damaged cell projections. In addition, exposure to the cryoprotectants has been found to disorganize the actin filaments within transzonal processes through which cumulus cells establish physical contact with the oocytes (Younis *et al.* 1996). In the present study, vitrification of ovine GV oocytes and exposure to cryoprotectants significantly decreased the frequencies of in vitro maturation (oocytes at MII stage, 23.7 and 35.6%) as compared to the control group (71.9%). Lower maturation rates were also reported by Isachenko *et al.* (2001, 27.5%), Bogliolo *et al.* (2007a, 31.2%) following vitrification of ovine GV-oocytes. Moreover, vitrification of immature oocytes resulted in lower maturation rates in other species such as goats (Kharche *et al.* 2005, 27.5%); cattle (Kim *et al.* 2007, 41.1%); buffalo (Wani *et al.* 2004b, 29.2%; Sharma and Purohit 2008, 39.2%), and horses (Tharasanit *et al.* 2006a, 27.6%) as compared to the control groups. Following IVM of ovine vitrified/thawed GV-oocytes, many oocytes (62.9%) did not resume meiosis and remained at GV stage. This block in oocyte development may be due to aberrant protein synthesis after freezing (Kharche *et al.* 2005). Different lines of evidence clearly indicate that surrounding cumulus cells play a fundamental role in the maturation process (Li *et al.* 2006). These cells and the oocyte are functionally and physically connected, establishing a sophisticated network of mutual interaction, which ultimately confers full developmental competence to the oocyte (Bogliolo *et al.* 2007a).

It has been reported that cooling affects spindle fibre integrity (Chen *et al.* 2003) and cortical granule vesicles (Vincent and Johnson 1992). Depolymerization of the spindle fibres is likely to lead to aneuploidy (Al-Hasani *et al.* 1987) and premature release of the cortical granules is likely to lead to zona hardening (Vincent and Johnson 1992). Alterations of zona pellucida glycoproteins, especially ZP2, are

reported to be responsible for zona hardening in mouse oocytes (Moller and Wassarman 1989). The frequencies of in vitro fertilisation reported here were significantly lower in vitrified oocytes as compared to EXP and control groups and higher numbers of oocytes remained unfertilised (with no signs of sperm penetration) in vitrified groups as compared to EXP and controls (**Table 4.3**). In cattle, lower fertilisation rates were also reported following IVF of vitrified/thawed GV-oocytes (Kim *et al.* 2007, 78.6%). In contrast to these results, Somfai *et al.* (2010) reported high fertilisation rates (91.7%) after IVF of vitrified/thawed porcine GV oocytes, suggesting that normal cytoplasmic maturation had occurred.

The deleterious consequences arising from cryodamage may also appear during cleavage or pre-implantation development (Coticchio *et al.* 2004). In fact, it has been well documented that, vitrified-warmed oocytes cleave and reach blastocyst stage in significantly lower proportions as compared to control (Kelly *et al.* 2006; Kim *et al.* 2007; Succu *et al.* 2008,). This study investigated the effect of SSV on kinetics of first cleavage and subsequent in vitro embryo development following IVM/IVF/IVC of ovine oocytes vitrified at GV stage. Vitrified warmed oocytes displayed a delay in cleavage compared to control, but the differences were not significant. Similarly, Sucuu *et al.* (2008) reported a delay in cleavage following IVF of vitrified/warmed ovine MII oocytes. The delay in cleavage rates observed in vitrified oocytes, may suggest a decrease in gamete competence caused by cryopreservation procedures (Succu *et al.* 2008). In the present study, the frequencies of cleaved embryos (48 hpi) and blastocyst development (Day 7 pi) were significantly lower in vitrified oocytes as compared to EXP and control groups (**Table 4.5**). Cleavage (9.6%) and blastocyst development (5.4%) reported here were lower than those obtained by Succu *et al.* (2007a, 12.5%) and Succu *et al.* (2008, 17%) following IVF of vitrified/thawed ovine MII oocytes. These differences may be due to the cryodevices used (cryoloop and cryotop versus SSV); or to differences in the meiotic stage (MII versus GV) of oocytes when vitrified. Higher percentage of blastocysts development (35.2%) was reported

following exposure of immature ovine oocytes to vitrification solutions alone indicating that the use of a mixture of a relatively low molecular weight and highly penetrated cryoprotectant (i.e. EG), a viscosity-increasing compound (PVP), and a membrane-protective sugar (trehalose) is efficient for oocyte vitrification (Dinnyes *et al.* 2000). Although lower rates of blastocyst development were obtained from the vitrified oocytes, morphological evaluation of those blastocysts suggested them to be of good quality (hatched), however further studies are required to evaluate total cell numbers in these blastocysts. The low frequency of blastocyst development reported here may be associated with the changes in lipid content (mainly phospholipids) and fatty acid composition in frozen-thawed oocytes (Kim *et al.* 2001). Also, oocyte vitrification has been reported to be accompanied with alterations in mitochondrial activity such as loss of activity or abnormal distribution (Abe *et al.* 2005). More recently, it has been speculated that oocyte vitrification may also affect embryo development on a molecular level by decreasing the levels of maternal mRNAs in ovine oocytes (Succu *et al.* 2008).

In conclusion, these results indicate that immature ovine oocytes vitrified with SSV as a simple and rapid procedure can survive and subsequently be matured, fertilised and develop in vitro up to the blastocyst stage, although the frequency of development is low. The results also show that the high concentrations of cryoprotectants *per se* applied in SSV were not detrimental for in vitro development. The vitrification protocol described here is not a definitive protocol; rather, it provides direction for further investigations into the cryopreservation of ovine oocytes, which may lead to more effective techniques. Therefore, further studies are required to improve the survival and developmental potential of vitrified/thawed ovine oocytes.

CHAPTER 5

VITRIFICATION OF IMMATURE OVINE OOCYTES USING THE CRYOLOOP: EFFECTS ON VIABILITY, IN VITRO MATURATION, FERTILISATION AND SUBSEQUENT DEVELOPMENT

5.1 INTRODUCTION

Oocyte cryopreservation represents one of the most recent developments in the field of reproductive technologies and will become an important tool for the creation of genetic resources banks in domestic animals (Ledda *et al.* 2007). Despite some success with different species and the publications in the past years of several fundamental cryobiology studies, oocyte cryopreservation as an established procedure, able to compete with the efficiency of sperm and embryo freezing, remains largely unaccomplished (Shaw *et al.* 2000). The cryopreserved /warmed oocytes normally show several ultrastructural (Abe *et al.* 2005) and structural alterations, such as phase change of lipid bilayers, lysis of cytoplasmic membrane (Agca *et al.* 1998) and nuclear fragmentation (Men *et al.* 2003a). As early as 1985, ice-free cryopreservation of mouse embryos at –196°C by vitrification was reported (Rall and Fahy 1985) as an alternative approach to cryostorage. The key aim of any vitrification protocol is to achieve high cooling rates in association with high concentrations of cryoprotectant. High cooling rates allow reduction in the concentration of the cryoprotectants used and subsequent reduction in their toxic and osmotic effects. Special carriers have been used to minimize the volume of vitrification solution (see **Chapter 1.6.1.3**). Vitrification of oocytes using the cryoloop has advantages over conventional vitrification in

that, the open system lacking any thermo insulating layer, coupled with the small volume of less than 1 µl, results in both rapid and uniform heat exchange during cooling (Lane *et al.* 1999a, b; Saki and Dezfuly 2005). So far, the cryoloop has been used effectively for vitrification of human, mouse (Lane *et al.* 1999a, b) blastocysts with high survival rates. Furthermore, Mavrides and Morroll (2002) obtained high survival and cleavage rates similar to fresh oocytes following intracytoplasmic sperm injection (ICSI) of vitrified bovine oocytes using cryoloop. Cryopreservation of germinal vesicle (GV) oocytes would generate a readily available source of oocytes for research and allow experiments to be performed at a convenient time. However, data obtained to date, indicate that immature oocytes are more susceptible to cryoinjury and the survival and subsequent developmental potential are greatly impaired in comparison to fresh oocytes (Hochi *et al.* 1998). Structural modifications of cytoskeleton, mitochondria, cortical granules and nucleoli have been described to be associated lower efficiency of vitrified/warmed GV oocytes.

The aims of these studies were to investigate the effects of Cryoloop vitrification on viability and subsequent developmental competence of ovine oocytes vitrified at GV-stage. Following categories were examined, 1) recovery and survival rates following vitrification and warming, 2) cumulus expansion and nuclear maturation following in vitro maturation (IVM), 3) Spindle and chromosome configuration, 4) fertilisation events following in vitro fertilisation (IVF), 5) in vitro embryo development following IVM/IVF/IVC. Total cell numbers, numbers of apoptotic nuclei, and chromosomal constituents of in vitro produced blastocysts were evaluated.

5.2 MATERIALS AND METHODS

5.2.1 Vitrification of cumulus oocyte complexes (COCs) using cryoloop

COCs obtained from slaughtered sheep ovaries were washed three times in washing medium (see **Chapter 2.1**) and then randomly divided into three groups, 1) control (directly matured in vitro), 2) toxicity (COCs exposed to cryoloop vitrification solutions without freezing), 3) vitrified (COCs were vitrified using cryoloop, **Chapter 2.2.3**) groups. Following vitrification and warming, oocyte viability was determined as previously described (**Chapter 2.2.4**). Viable oocytes were matured in vitro for 24 h (**Chapter 2.3**). At 24 hpm (hours post onset of maturation), maturation status, spindle and chromatin configuration were evaluated as described in (**Chapter 2.3.1, 2.3.2** and **2.4**).

5.2.2 In vitro fertilisation (IVF) and culture (IVC)

In vitro matured oocytes were fertilised using 2.0 x 10^6 frozen/thawed ram semen as previously described (**Chapter 2.5**). 18 hpi (hours post insemination), fertilisation events were examined by staining with 10 µg/ml Hoechst 33342 (**Chapter 2.5.3**). In vitro culture of presumptive zygotes and embryo evaluation were carried out as previously described (**Chapter 2.8** and **2.8.1**). Total cell numbers and number of apoptotic nuclei were determined using Hoechst and TUNEL assay as described in (**Chapter 2.9**). Cytogenetic analysis of day 7 blastocyst embryos was evaluated according to the method described in (**Chapter 2.10**).

5.2.3 Statistical Analysis

Data were analysed by Chi square test. Total cell numbers and apoptotic cells were analysed using un-paired student's *t*-test. All results were considered to be statistically significant at $P< 0.05$, unless otherwise stated.

5.3 RESULTS

5.3.1 Recovery rates and oocyte survival

The recovery rates and subsequent oocyte viability following cryoloop vitrification or exposure to vitrification solutions are presented in **Figs 5.1, 5.2,** and **5.3**. After cryoloop vitrification (82.6%, 161/195) of vitrified/warmed oocytes were recovered. These proportions did not differ significantly ($P > 0.05$) from those obtained following exposure to vitrification solutions without cooling (Toxicity, 96.6%, 172/178, **Fig 5.1**). However, numbers of lost oocytes were significantly ($P < 0.05$) higher in vitrified group than toxicity (17.4 *vs* 3.4%, respectively). Based on morphological evaluation, the percentages of normal (viable) oocytes did not differ significantly ($P > 0.05$) between vitrified (72.6%, 61/84) and toxicity groups (93.2%, 96/103, **Fig 5.2**). However, the proportions of recovered oocytes with abnormal morphology (non viable) were significantly ($P < 0.01$) higher in vitrified group as compared to toxicity controls (27.4 *vs* 6.8%, respectively) as illustrated in **Fig 5.3**.

Table 5.1 Recovery rates and viability of ovine oocytes vitrified at GV-stage using the cryoloop.

Treatment	*No. Recovered (%)*	*No. Normal (%)*
Vitrified	161/195 (82.6%)	61/84 (72.6%)
Toxicity	172/178 (96.6%)	96/103 (93.2%)

No significant differences were observed in the frequencies of recovered and viable oocytes (morphologically normal) between vitrified and toxicity groups.

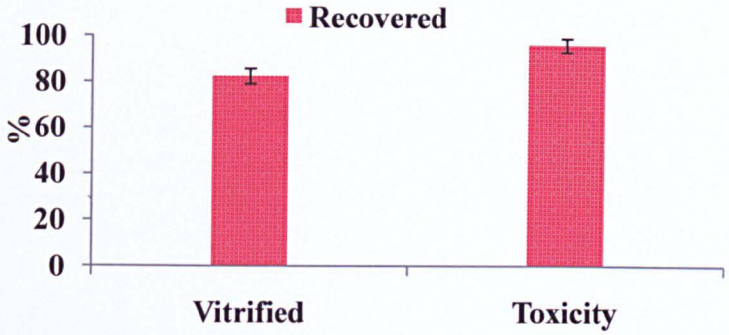


Figure 5.1 Recovery rates of ovine oocytes vitrified at GV-stage using the cryoloop. The graph represents the percentages (±SEM) of oocytes recovered following vitrification of COCs using cryoloop or exposure to vitrification solution (Toxicity). Four replicates were performed.

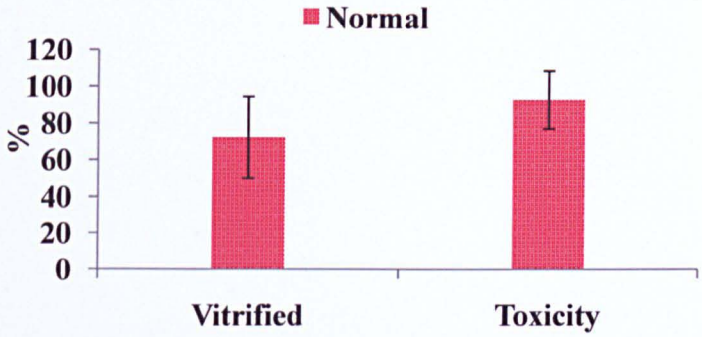


Figure 5.2 Survival of ovine oocytes vitrified at GV-stage by the cryoloop. The graph represents the percentages (±SEM) of recovered oocytes with normal (viable) morphology following cryoloop vitrification (vitrified) or exposure to vitrification solution (Toxicity). Four replicates were performed.

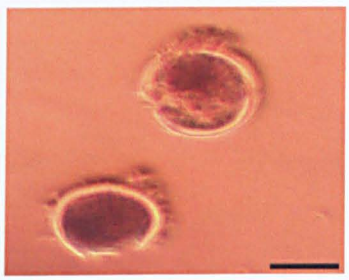


Figure 5.3 Damaged oocytes recovered following cryoloop vitrification. The figure shows oocytes with cracks in zona pellucida and degenerated cytoplasm obtained following vitrification of ovine COCs. Scale bar = 50 μm.

5.3.2 Cumulus cell expansion and nuclear maturation following IVM

The effect of cryoloop vitrification on cumulus cell expansion following IVM of vitrified/thawed ovine GV-oocytes is presented in **Figs 5.4** and **5.5**. The percentage of oocytes with expanded cumulus cells was significantly ($P< 0.01$) decreased following vitrification (70.4%) as compared to control group (95.2%). However, these values did not differ significantly ($P> 0.05$) from toxicity group (91.8%, **Figs 5.4** and **5.5**). The meiotic maturation of ovine oocytes vitrified at GV-stage using cryoloop is presented in **Table 5.2** and **Fig 5.6**. No significant differences were observed in the frequencies of in vitro maturation (oocytes at MII stage) between treatment and control groups (43.4 and 60.0% *vs* 63.2%, in vitrified, toxicity, and control, respectively). Similar trends were observed in the proportions of oocytes reaching MI and AI/TI stages (values ranged 7.5 to 10.3% and 5.7 to 13.8%, respectively, **Table 5.2**). The numbers of oocytes remaining at GV-stage were significantly higher ($P< 0.01$) in vitrified group as compared to toxicity and control (34.4 *vs* 18.5 and 12.6%, respectively **Table 5.2** and **Fig 5.6**).

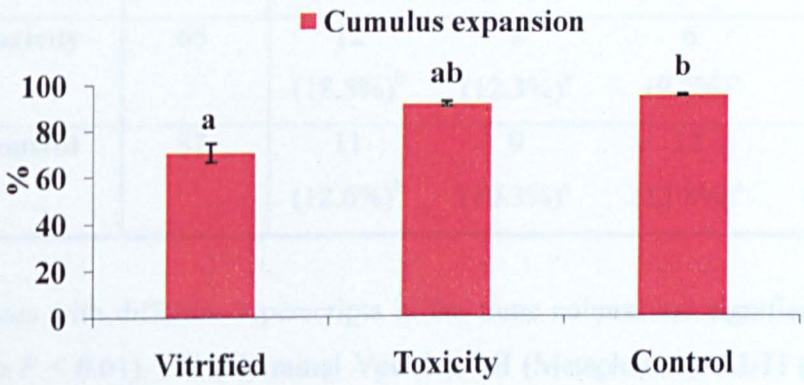


Figure 5.4 Cumulus cell expansion following IVM of ovine oocytes vitrified at GV-stage using cryoloop. The graph represents the percentages (±SEM) of oocytes with expanded cumulus cells following IVM of COCs vitrified by cryoloop or exposed to vitrification solutions without freezing (Toxicity). Different letters are significant different ($P< 0.01$). Four replicates were carried out

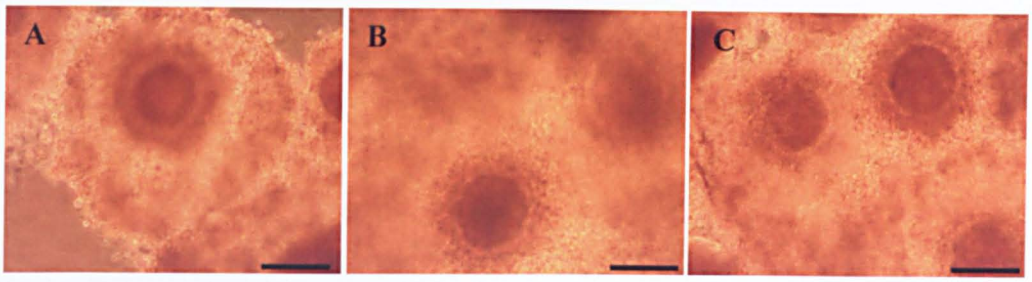


Figure 5.5 Oocytes with expanded cumulus cells. The figure shows comparative cumulus cell expansion following 24 h IVM of ovine oocytes (**A**) vitrified at GV-stage using cryoloop, (**B**) exposed to vitrification solution (toxicity) or (**C**) non-treated (control) group. Scale bar = 50 µm.

Table 5.2 Nuclear maturation of ovine oocytes vitrified at GV-stage using cryoloop

Treatment	*No. Oocytes*	*Meiotic maturation No. (%)*			
		GV	*MI*	*AI/TI*	*MII*
Vitrified	53	23 (43.4%)[a]	4 (7.5%)[a]	3 (5.7%)[a]	23 (43.4%)[a]
Toxicity	65	12 (18.5%)[b]	8 (12.3%)[a]	6 (9.2%)[a]	39 (60.0%)[a]
Control	87	11 (12.6%)[b]	9 (10.3%)[a]	12 (13.8%)[a]	55 (63.2%)[a]

Values with different superscripts in the same column are significantly different (a, b $P < 0.01$). GV (Germinal Vesicle), MI (Metaphase-I), AI/TI (Anaphase-I + Telophase-I), MII (Metaphase-II). Four replicates were performed for each experimental group.

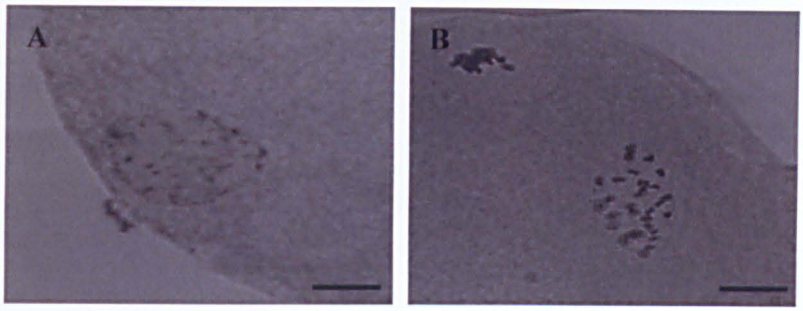


Figure 5.6 Meiotic maturation of ovine oocytes. The figure shows chromatin status in orcein stained oocytes following 24 h IVM after vitrification at GV-stage using cryoloop. (**A**) GV-stage, and (**B**) MII stage. Scale bar = 50 μm.

5.3.3 Spindle and chromosome organization

Spindle configuration and chromosome organization was examined in control, vitrified and toxicity control oocytes at 24 hpm (**Table 5.3** and **Fig 5.7**). No significant differences ($P > 0.05$, χ^2) were observed in the frequencies of oocytes with normal spindle and chromatin configuration between treated (vitrified and toxicity) and control groups (50.0, 54.9 and 70.4%, respectively). Also, similar trends were noticed in the percentage of oocytes with missing spindle and the values ranging (from 22 to 29%). However, although the proportion of oocytes with abnormal spindle morphology was higher in both vitrified and toxicity groups as compared to control, the differences were not significant (20.8, 19.6 *vs* 7.4%, $P = 0.067$). Similarly the proportions of oocytes that exhibited abnormal chromosome configuration between these groups (50.0, 45.1 and 29.6%) were not significant (**Table 5.3** and **Fig 5.7**).

Table 5.3 Effect of vitrification of immature ovine oocytes on spindle and chromosome configuration following IVM

Treatment	*No. Oocytes*	*Spindle configuration*			*Chromosome configuration*	
		No. Normal (%)	*No. Abnormal (%)*	*No. Missing (%)*	*No. Normal (%)*	*No. Abnormal (%)*
Vitrified	48	24 (50.0%)	10 (20.8%)	14 (29.2%)	24 (50.0%)	24 (50.0%)
Toxicity	51	28 (54.9%)	10 (19.6%)	13 (25.5%)	28 (54.9%)	23 (45.1%)
Control	54	38 (70.4%)	4 (7.4%)	12 (22.2%)	38 (70.4%)	16 (29.6%)

Oocytes were immunostained using mouse monoclonal anti - α - tubulin antibody (1:200) at 24 hpm to evaluate spindle and chromosome configuration in three different groups (vitrified, toxicity (oocytes exposed to vitrification solutions without freezing) and fresh control). No significant differences were observed in the frequencies of oocytes with normal spindle and chromosome configuration between three groups. Four replicates were carried out for each experimental group.

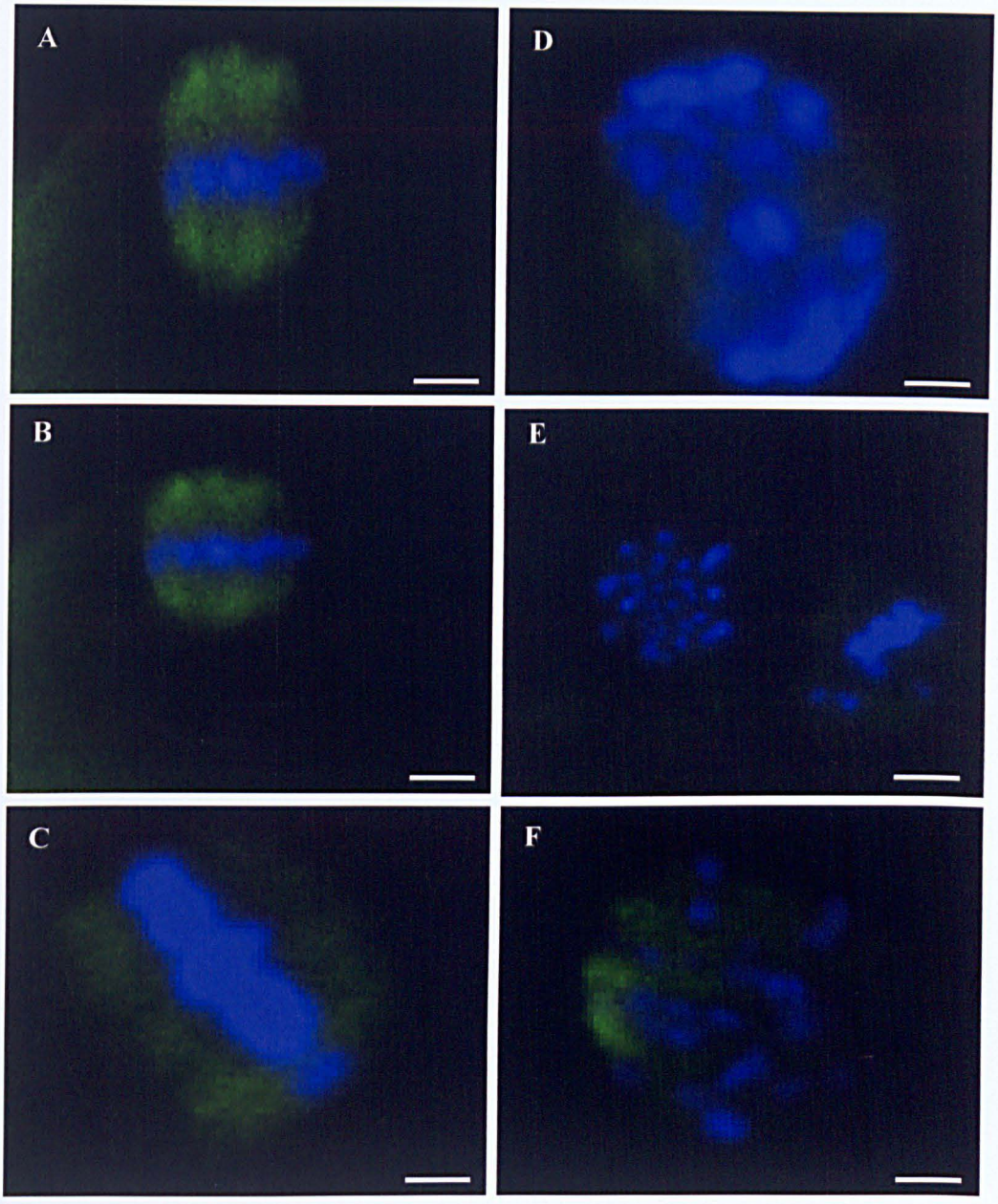


Figure 5.7 Examples of spindle and chromosome organization in ovine oocytes. The figure shows spindle (green) and chromatin (blue) morphology in ovine oocytes following immunostaining at 24 hpm. (**A, B,** and **C**) show normal morphology (symmetrical barrel shape meiotic spindle with chromatin aligned regularly along the equatorial plane of spindle), while (**D, E,** and **F**) illustrates abnormal morphology in vitrified, toxicity and control oocytes, respectively. Scale bar = 50 μm.

5.3.4 In vitro fertilisation and polyspermy

The frequencies of in vitro fertilisation and polyspermy following IVM/IVF of ovine oocytes vitrified at GV-stage using cryoloop are presented in **Table 5.4** and **Fig 5.8**. Interestingly, no significant differences were observed in the percentages of fertilised oocytes among three groups (54.0, 75.0, and 74.1%, in vitrified, toxicity and control, respectively). Moreover, no differences were detected in the frequency of unfertilised oocytes (17.5 to 29.0%). However, cryoloop vitrification resulted in significantly ($P< 0.001$) higher percentages of oocytes with polyspermic fertilisation as compared to controls (17.0 *vs* 3.5%). These values did not differ significantly from those reported in toxicity oocytes (7.5%, **Table 5.4** and **Fig 5.8**).

Table 5.4 In vitro fertilisation of ovine oocytes vitrified at GV-stage using cryoloop

Treatment	*No. Oocytes*	*Fertilisation No. (%)*		
		Fertilised	*Polyspermic*	*Unfertilised*
Vitrified	100	54 (54.0%)[a]	17 (17.0%)[a]	29 (29.0%)[a]
Toxicity	80	60 (75.0%)[a]	6 (7.5%)[ab]	14 (17.5%)[a]
Control	143	106 (74.1%)[a]	5 (3.5%)[b]	32 (22.4%)[a]

Values with different superscripts in the same column are significantly different (a, b $P< 0.001$). Four replicates were carried out.

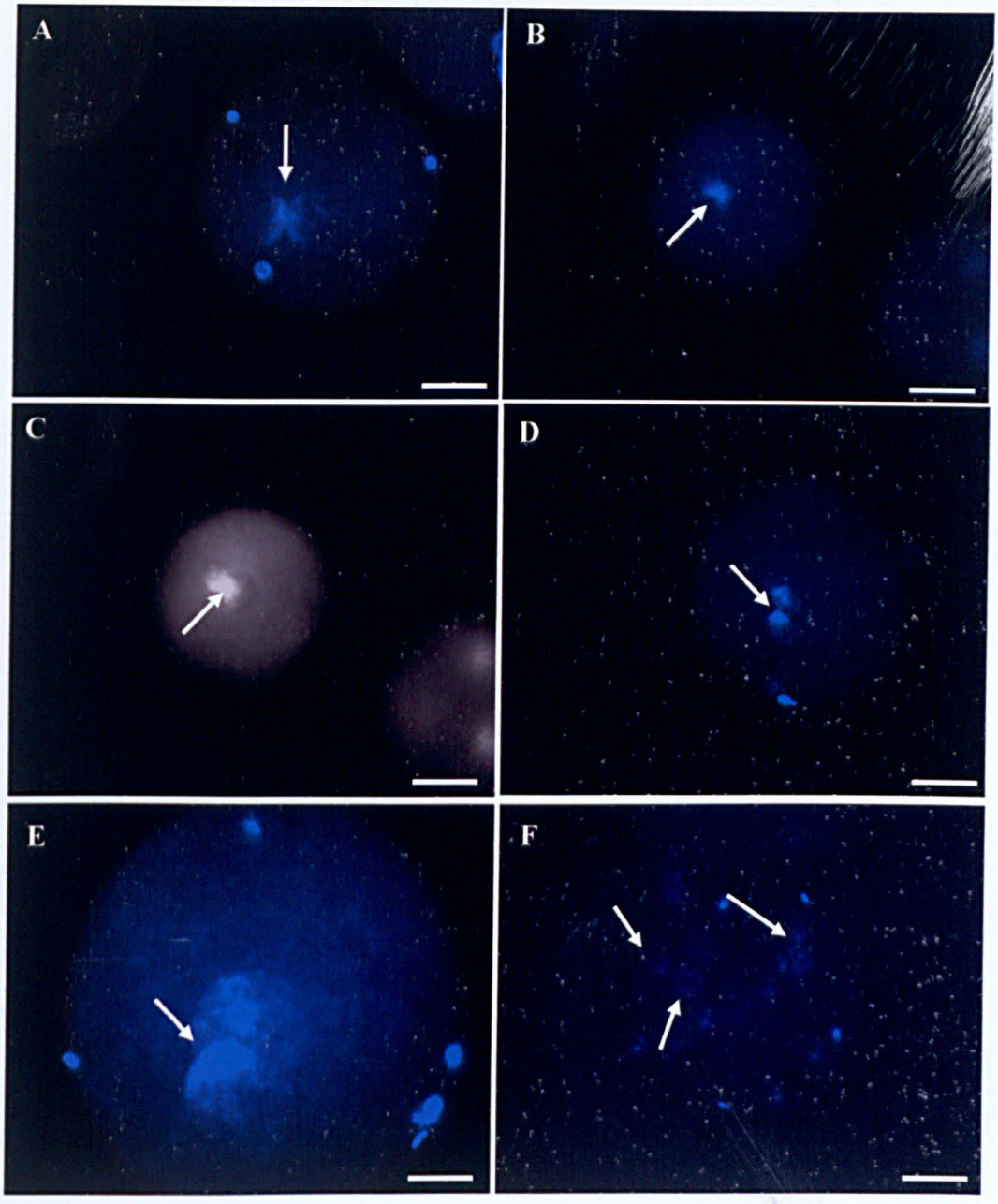


Figure 5.8 Fertilisation status of ovine oocytes following IVF. The figure shows IVM/IVF oocytes at 18 hpi stained with Hoechst 33342 and examined under a fluorescence microscope (Leica, Germany). (**A**) Fertilised oocytes with male and female pronuclei (arrows) obtained in control group, (**B** and **C**) fusion of male and female pronuclei in control oocytes (syngamy), (**D** and **E**) fertilised oocytes in toxicity and vitrified group, with male and female pronuclei, (**F**) polyspermic fertilisation in vitrified oocytes. Scale bar = 50 μm.

5.3.5 In vitro embryo development following IVM/IVF

The effects of cryoloop vitrification on the viability of ovine oocytes subsequently matured and fertilised in vitro are presented in **Table 5.5.**, and the frequencies of cleavage and subsequent development in **Table 5.6** and **Fig 5.9**. At 18 hpi, in vitro fertilised oocytes were examined for signs of degeneration such as damage of zona pellucida and/or cytoplasm; damaged oocytes were discarded and not used for IVC. Vitrification of ovine oocytes significantly ($P< 0.05$) decreased the percentage of viable oocytes following IVF compared to the control group (72.3 *vs* 88.6%, respectively). However, these values did not differ significantly from those in toxicity group (83.6%). In contrast, the proportions of degenerate oocytes following IVF were significantly higher ($P< 0.05$) in vitrified group than toxicity and control (27.7 *vs* 16.4 and 11.4%, respectively, **Table 5.5**).

The frequencies of cleaved oocytes at 24 and 48 hpi were significantly lower ($P< 0.01$) in vitrified group as compared to both toxicity and control groups (17.3 *vs* 47.0 and 68.9% at 24 hpi and 41.9 *vs* 85.5 and 89.7% at 48 hpi, respectively, **Table 5.6** and **Fig 5.9**). Significant differences were observed in the percentage of cleaved oocytes at 24 hpi between toxicity and control groups ($P< 0.05$). Development to the morula stage (day 5 pi) was significantly lower in the vitrified group as compared to toxicity and control (36.4 *vs* 60.7 and 62.6%). Similar trends were reported for the frequencies of development to blastocyst (day 7 pi). Significantly ($P< 0.01$) lower percentages of oocytes developed to the blastocyst stage in the vitrified group (12.3%) as compared to toxicity (42.7%) and control (40.4%) groups (**Table 5.6** and **Fig 5.9**). Interestingly, the proportions of cleaved embryos that developed to blastocyst stage did not differ significantly between vitrified and control groups (29.4 *vs* 45.1%, respectively). No significant differences were observed in the frequencies of early blastocysts between groups (3.7, 5.9, and 7.4%, in vitrified, toxicity and control, respectively). However, significantly ($P< 0.05$) lower numbers of expanded blastocysts were obtained from vitrified oocytes (4.9%) as compared to non treated control (12.3%), but

these values did not differ significantly from those obtained in toxicity group (7.7 %). Hatched blastocysts were significantly ($P< 0.01$) decreased in vitrified groups than in toxicity and control (3.7 *vs* 29.1 and 20.7%, respectively, **Table 5.6** and **Fig 5.9**).

Table 5.5 Effects of cryoloop vitrification on survival rates following in vitro maturation and fertilisation of ovine oocytes.

Treatment	*No. oocytes*	*Viability following IVF No. (%)*	
		Viable	*Degenerated*
Vitrified	224	162 (72.3%)[a]	62 (27.7%)[a]
Toxicity	140	117 (83.6%)[ab]	23 (16.4%)[b]
Control	229	203 (88.6%)[b]	26 (11.4%)[b]

Values with different superscripts in the same column (a *vs* b) are significantly different at $P< 0.05$.

Table 5.6 Developmental potential of ovine oocytes vitrified at GV-stage using cryoloop

Treatment	*No. Oocytes*	*No. Cleaved (%) hpi*		*No. Morula (%)*	*No. Blastocysts (% of oocytes)*				*Total blastocysts (% of cleaved 48 hpi)*
		24	*48*		*Early*	*Expanded*	*Hatched*	*Total*	
Vitrified	162	28 (17.3%)^{a}	68 (42.9%)^{a}	59 (36.4%)^{a}	6 (3.7%)^{a}	8 (4.9%)^{b}	6 (3.7%)^{a}	20 (12.3%)^{a}	29.4%^{b}
Toxicity	117	55 (47.0%)^{b}	100 (85.3%)^{b}	71 (60.7%)^{b}	7 (5.9%)^{a}	9 (7.7%)^{bc}	34 (29.1%)^{b}	50 (42.7%)^{b}	50.0%^{c}
Control	203	140 (68.9%)^{c}	182 (89.7%)^{b}	127 (62.6%)^{b}	15 (7.4%)^{a}	25 (12.3%)^{c}	42 (20.7%)^{b}	82 (40.4%)^{b}	45.1%^{bc}

Cleavage rates were evaluated at 24 and 48 hpi (hour post insemination). Values with different superscripts in the same columns are significantly different a *vs* b and a *vs* c at $P < 0.01$ while b *vs* c at $P < 0.05$. Four replicates were carried out.

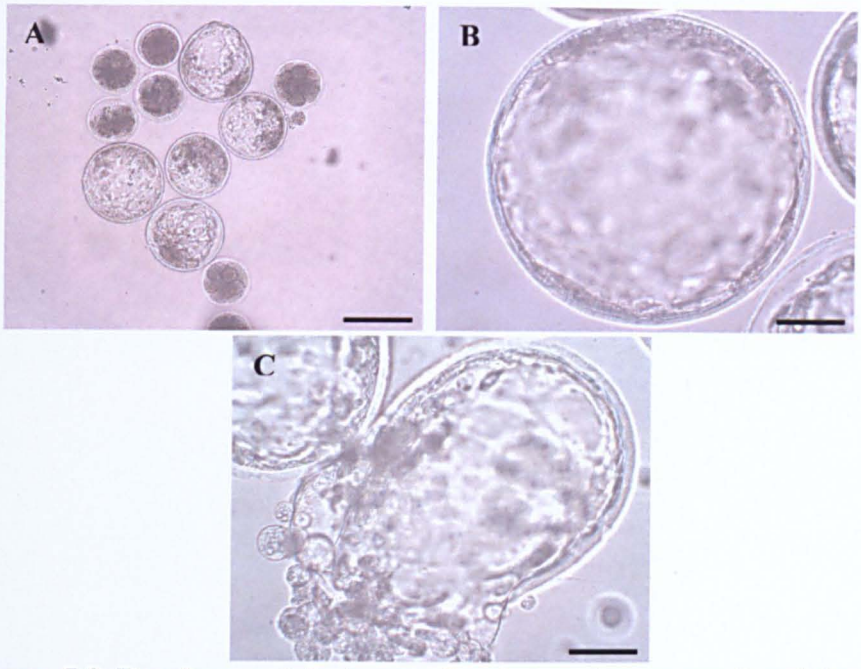


Figure 5.9 Developmental stages of ovine embryos produced by IVF of vitrified/thawed GV-stage oocytes. The figure shows **(A)** different developmental stages at day 7 of embryos produced following IVM/IVF/IVC of vitrified/thawed ovine oocytes using cryoloop, **(B)** expanded and **(C)** hatched blastocysts. Scale bar = 50 μm.

5.3.6 Total cell numbers and apoptotic nuclei of in vitro produced blastocysts

Total cell numbers and apoptotic nuclei in day 7 in vitro produced blastocysts were detected by Hoechst staining and TUNEL assay (Choi 2008) and compared between three groups (vitrified, toxicity and control, **Table 5.7**). The total cell numbers and numbers of apoptotic nuclei did not differ significantly between treatment and control groups (87.4 ± 7.6 and 87.2 ± 8.7 *vs* 101 ± 8.4, for total cell numbers) and (8.6 ± 1.5 and 8.1 ± 0.6 *vs* 6.5 ± 0.8, for apoptotic nuclei) in vitrified, toxicity, and control, respectively. Also, the apoptotic index (number of apoptotic nuclei/number of total cells in blastocyst) did not differ significantly between vitrified and control groups (9.4 ± 1.2 *vs* 6.8 ± 0.8, **Table 5.7** and **Fig 5.10**).

Table 5.7 Total cell numbers and apoptotic nuclei in day 7 blastocyst embryos produced following IVM/IVF/IVC of vitrified/thawed ovine GV oocytes.

Treatment	No. Blastocysts	Total cell numbers/ blastocyst (mean ± SEM)	No. Apoptotic nuclei (mean ± SEM)	Apoptotic index (%) (mean ± SEM)
Vitrified	13	87.4 ± 7.6^{a}	8.6 ± 1.5^{a}	9.4 ± 1.2^{ab}
Toxicity	11	87.2 ± 8.7^{a}	8.1 ± 0.6^{a}	10.2 ± 1.3^{b}
Control	11	101.0 ± 8.4^{a}	6.5 ± 0.8^{a}	6.8 ± 0.8^{a}

Values with different superscripts (a *vs* b) in the same column are significantly different at ($P< 0.05$).

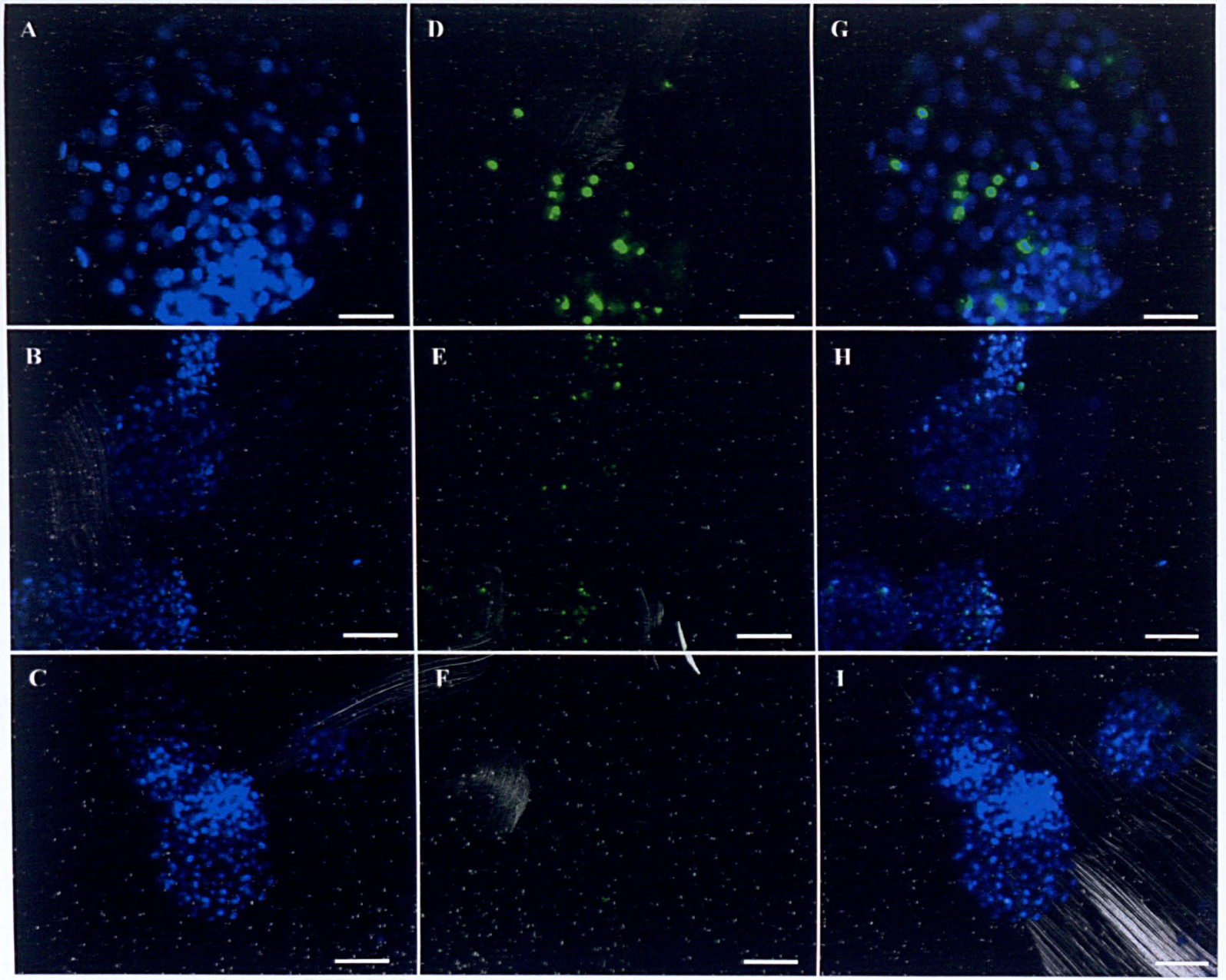

A
D
G
B
E
H
C
F
I

Figure 5.10 Total cell numbers and apoptotic nuclei in day 7 embryos determined by Hoechst staining and TUNEL assay. The figure shows **(A-C)** all nuclei appear blue, **(D-F)** apoptotic nuclei appear green, and **(G-I)** merge between total cells and apoptotic nuclei in day 7 embryos produced following IVM/IVF/IVC of ovine oocytes in vitrified, toxicity, and control groups, respectively. Scale bar = 50 μm.

5.3.7 Chromosomal analysis of in vitro produced embryos

The ploidy of day 7 blastocysts was evaluated **Fig 5.11**, and the results are summarised in **Table 5.8**. The embryos were categorized as diploid (2n), haploid (n), or polyploidy (≥ 3n); the diploid embryos were considered normal, others reported as abnormal. The proportion of embryos with normal diploid chromosomal complements (54, 2n, **Fig 5.11, A**) was higher in blastocysts obtained from control and toxicity groups as compared to vitrified (88.0 and 81.8 *vs* 71.4%, respectively). However, the differences between three groups were not significant. Also, no significant differences were observed in the percentages of haploid embryos between three groups (14.3, 13.6, and 8.0%, in vitrified, toxicity, and control, respectively). Although, the frequency of polyploidy was higher in embryos produced from vitrified oocytes (14.3%) than those obtained from other groups (~ 4%), the differences were not significant (**Table 5.8** and **Fig 5.11 B**).

Table 5.8 Chromosomal composition of day 7 blastocysts produced by IVM/IVF/IVC of ovine oocytes vitrified at GV-stage

Treatment	*No. Blastocysts*	*Ploidy No. (%)*		
		Diploid	*Haploid*	*Polyploid*
Vitrified	7	5 (71.4%)	1 (14.3%)	1 (14.3%)
Toxicity	22	18 (81.8%)	3 (13.6%)	1 (4.5%)
Control	25	22 (88.0%)	2 (8.0%)	1 (4.0%)

No significant differences were observed in the frequencies of diploid embryos as detected by karyotyping of day 7 blastocysts between vitrified, toxicity and control groups.

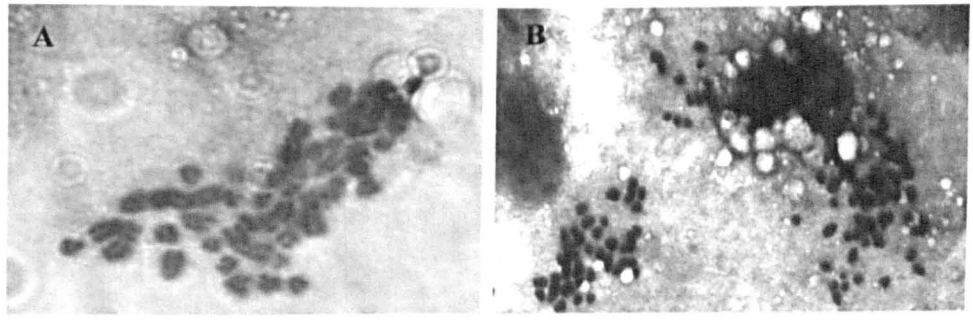


Figure 5.11 Chromosomal analysis of day 7 in vitro produced blastocysts. The figure reveals cytogenetic analysis of ovine embryos produced by IVM/IVF/IVC of oocytes vitrified at GV-stage using cryoloop. (**A**) normal diploid (54, 2n) chromosomes, and (**B**) abnormal polyploid embryos (≥ 3n).

5.4 DISCUSSION

Cryopreservation of embryos using a slow cooling protocol is an established method; however, when applied to oocytes slow cooling resulted in very low survival rates (Martino *et al.* 1996b). Therefore, new methods of cryopreservation have been developed, including vitrification. High cooling rates could be achieved by decreasing the volume of vitrification solutions in a combination with direct and rapid contact with LN_2. Vitrification of oocytes and embryos using cryoloop has advantages over conventional procedures in that the containerless system lacking any insulating layer, coupled with a small volume of less than 1 µl, result in both rapid and uniform heat exchange during cooling. Also, containerless vitrification is an open system enabling visualization of the sample during manipulation. Moreover, cooling and warming are straightforward as the loop is plunged directly into the LN_2 or in the warming solutions (Lane *et al.* 199a, b). Loss of oocytes after vitrification and warming has been reported in several species including goats (Begin *et al.* 2003), and mice (Gomes *et al.* 2008). This loss can be influenced by the number of oocytes per cryodevice, as increased the numbers enhances the time required for movement of the cells through solutions, making over exposure or loss of the cells are possible. The post-thaw recovery rate (82.6%) reported in the present study was slightly higher than that reported by (Begin *et al.* 2003, 74%) following cryoloop vitrification of IVM goat oocytes. This loss can occur, if the cryoloop touches the inner wall of the storage tube (Begin *et al.* 2003). During cryopreservation, oocytes suffer severe adverse physiological conditions and this results in various cytological injuries to the oocytes (Men *et al.* 2003a). The survival rates reported here (72.6%) were slightly lower than that reported by Bogliolo *et al.* (2007a, 84.3%) following vitrification of ovine GV oocytes. These differences may be due to the cryodevices used in vitrification (cryotops in Bogliolo *et al.*). Following exposure of oocytes to cryoprotectant solutions alone (toxicity test) over 90% recovered oocytes were viable, suggesting that these combinations of cryoprotectant are efficient for vitrification of GV-ovine oocytes.

In the present study, the percentage of oocytes with expanded cumulus cells following IVM was significantly lower in vitrified group as compared to controls; however, this percentage did not affected in toxicity group. These results suggest that, the damage of the cumulus cells resulted during vitrification/thawing procedures and not from exposure to the cryoprotectants. Interestingly, the results in the present study demonstrate that, no significant differences observed in the frequencies of oocytes reaching MII stage in vitrified group as compared to control (43.4 *vs* 63.2%, **Table 5.2**). This value was higher than those reported by Isachenko *et al.* (2001, 27.5%), Silvestre *et al.* (2006, 12.7%), and Bogliolo *et al.* (2007a, 31.2%) following IVM of vitrified/thawed ovine GV-oocytes. The differences between the results may be due to several factors including, differences in vitrification protocols, OPS in Isachenco *et al.*, and Silvestre *et al.* and cryotop in Bogliolo *et al.* studies. The effects of the three cryodevices (OPS, cryoloop, and cryotop) on viability and subsequent development of vitrified/thawed IVM ovine oocytes were evaluated by Succu *et al.* (2007a). Higher percentages of damaged oocytes were reported following OPS (45%) as compared to other methods (20 and 22.8% in cryoloop and cryotop groups, respectively); however, spontaneous activation was increased in cryotop group (54.5%) as compared to OPS (28.8%) and cryoloop (22.4%) groups. Higher maturation rates reported in the present study as compared to other studies may be also due to the differences in vitrification solutions used and equilibration time (time at which oocytes are equilibrated in lower concentrations of cryoprotectants before incubated in vitrification solutions with higher concentrations of cryoprotectants). Isachenko *et al.* (2001) used 40% EG with one step vitrification (without equilibration), however, Silvestre *et al.* (2006) and Bogliolo *et al.* (2007a) used a combination of 20% EG and 20% DMSO with stepwise vitrification and 30 sec equilibration time. In the present study, oocytes were equilibrated in 10% EG plus 0.25 M trehalose for 3 min before vitrification and then vitrified in a solution of 20% EG plus 20% DMSO for less than 1 min. Several studies reported that, combinations between tow permeable cryoprotectants at lower concentrations decrease the toxicity of vitrification

solution (Liebermann and Tucker 2002). Also, shorter equilibration time applied in Bogliolo *et al* studies seems to be not sufficient for the cryoprotectants to penetrate the oocytes.

Searching for indicators of oocyte quality, investigators have reported that normal fertilisation and embryo development are more likely in oocytes with normal spindles, which may partially serve as an indicator of adequate oocyte cytoplasmic maturation (Wang and Keefe 2002; Keefe *et al.* 2003). A spindle apparatus is a dynamic collection of microtubules, comprised of α- and β- tubulin and associated structural proteins, which act to coordinate cyto- and karyokinetic events essential for normal chromosome segregation (Chen *et al.* 2003). Disorganization and disruption of the spindles can result in dispersal of chromosomes and subsequent chromosomal anomalies after fertilisation such as aneuploidy and polyploidy (Coticchio *et al.* 2005). Temperature fluctuations are known to directly affect cytoskeletal organization and chromosome configuration in mouse (Gomes *et al.* 2008), human (Almeida and Bolton 1995; Wang *et al.* 2001), bovine (Wu *et al.* 1999; Albarracin *et al.* 2005a, b; Morato *et al.* 2008a, b), porcine (Liu *et al.* 2003; Rojas *et al.* 2004), equine (Tharasanit *et al.* 2006a, b) and ovine (Succu *et al.* 2007a) oocytes. Succu *et al.* (2007a) reported that spindle configuration in vitrified/thawed ovine MII oocytes is affected by the cryodevices used, abnormal configuration was increased in OPS (65.2%) and cryoloop (59.2%) vitrified oocytes than in cryotop (55.2%). Also, they reported that exposure of oocytes to cryoprotectants alone exerts major toxic effects on spindle organization. In the present study, no significant differences were observed in the percentage of oocytes with normal spindle and chromatin configuration between vitrified, toxicity, and control groups. Alterations that occur to the meiotic spindle following IVM of vitrified immature oocytes may be due to cryodamage in the key regulatory factors such as MPF and MAPK which are responsible for spindle formation and microtubule organization (Wu *et al.* 1999; Albarracin *et al.* 2005b).

Cryopreservation of oocytes has been reported to induce premature release of cortical granules (Carroll *et al.* 1990), which subsequently affect frequencies of fertilisation. In cattle and pigs, lower fertilisation rates have been reported following IVF of vitrified/thawed GV and MII oocytes respectively (Kim *et al.* 2007, 65.6%; Somfai *et al.* 2007, 51.9%) as compared to control groups. In sheep, Tian *et al.* (2007) reported lower penetration rates (28.2%) after IVF of vitrified/thawed MII oocytes, they suggested that exposure of MII ovine oocytes to vitrification solution containing DMSO resulted in parthenogenetic activation and subsequent premature zona hardening. Interestingly, in the present study, no significant difference in fertilisation rates was observed between vitrified and control groups indicating normal cytoplasmic maturation in vitrified oocytes. These results suggest that vitrification of ovine GV oocytes using cryoloop can be an alternative to avoid cooling-induced zona hardening. Similarly, previous studies in pigs (Somfai *et al.* 2010) and mice (Eroglu *et al.* 1998) reported that vitrified/thawed GV oocyte can be matured and fertilised in vitro with similar trends as fresh controls.

In the present study, higher rates of degenerated oocytes were reported following IVF of vitrified oocytes. Also, lower frequencies of cleavage, morula, and blastocyst development were observed in vitrified oocytes as compared to control and toxicity groups. However, numbers of cleaved embryos that developed to blastocysts were not significantly different between vitrified and control groups (**Table 5.6**). The frequencies of blastocyst development reported in this study (29.4%) were much higher than those reported by Succu *et al.* (2007 a, b, 12.5% and 0%, respectively), Succu *et al.* (2008, 17.0%), and Zhang *et al.* (2009, 2.6%) following IVF of vitrified/thawed MII oocytes. The lower developmental rates reported in these studies may be due to structural and biochemical damage caused by vitrification of MII oocytes (Succu *et al.* 2007 a, b). Also, Succu *et al.* (2008) reported that vitrification can produce changes in mRNA contents in ovine MII. Herein, no significant differences were observed in total cell numbers and numbers of apoptotic nuclei in blastocysts between treatment and control groups.

Cryopreserved oocytes often degenerated via apoptosis in subsequent culture (Men *et al.* 2003b). Insulin-like growth factor I has been reported to inhibit apoptosis in subsequent culture (Makarevich and Markkula 2002). FBS contains many substances, among of them is growth factors, therefore, the developmental advantage of embryo produced from oocytes vitrified and handled in FBS containing medium may be because of the anti apoptotic effects of growth factors in FBS (Horvath and Seidle 2008). In the present studies, the same batch of FBS was used throughout all experiments. Abnormal chromosomal constituents in embryos produced by IVF of frozen/thawed oocytes may be due to the stage of oocytes, the polyploidy was reported to be higher following IVF of vitrified immature oocytes. Also, damage to the cortical granules during oocyte cryopreservation may be incorporated in this phenomenon (Al- Hasani *et al.* 1987). In the present study, no significant differences were reported in the frequencies of normal diploid embryos between treatment and control groups, however, polyploid embryos increased in vitrified group, which may be due to the higher frequencies of polyspermic fertilisation reported in vitrified oocytes. Taken together, the results suggest that, vitrification of immature COCs using cryoloop may be advantageous in preserving or restoring developmental competence in cryopreserved ovine oocytes.

In conclusion, immature ovine oocytes vitrified at GV-stage using the cryoloop can be matured, fertilised and develop in vitro with high developmental potential similar to non vitrified control. Moreover, the results suggest that, good quality blastocysts with normal ploidy can be produced from vitrified immature ovine oocytes. The vitrification solutions applied in this study did not impair in vitro embryo production in sheep. The vitrification method presented here may serve as a basic platform for further development of a standard protocol for cryopreservation of immature ovine oocytes. However, further studies are needed to assess in vivo development of vitrified/thawed immature ovine oocytes and the ability to establish pregnancy and produce live offspring.

CHAPTER 6

MPF/MAPK ACTIVITIES, PARTHENOGENETIC ACTIVATION AND SOMATIC CELL NUCLEAR TRANSFER (SCNT) OF OVINE OOCYTES VITRIFIED AT GV-STAGE

6.1 INTRODUCTION

Cryopreserved oocyte can, in theory, be used to produce offspring via assisted reproductive techniques such as in vitro embryo production (Vajta *et al.* 1998), and can also serve as host cytoplasts for cloning by nuclear transfer (NT) (Dinnyes *et al.* 2000). Vitrification of sheep oocytes is a challenge, and much more difficult than in other mammals, such as mice (Gomes *et al.* 2008) and cattle (Vieira *et al.* 2002, Vieira *et al.* 2008). Various vitrification methods have been used to cryopreserve ovine oocytes (Sucuu *et al.* 2007a, b; Bogliolo *et al.* 2007a), but live births from vitrified ovine oocytes have not been reported. Although the extent and exact cause of reduced developmental competence following cryopreservation may vary between species, factors shown to contribute to reduced oocyte quality include damage to the mitochondria in equine and bovine oocytes (Hochi *et al.* 1996; Abe *et al.* 2005), and enzymes critical to oocyte maturation and subsequent development in sheep (mitogen activated protein kinase: MAPK and maturation promoting factor; MPF; Bogliolo *et al.* 2007a).

The efficiency of cryopreserved gametes and embryos is usually assessed by the ability of the resultant embryos to develop into blastocysts or term offspring. However, cryopreservation was reported to have side effects on zona quality by

cracking the zona or inducing precocious cortical granules exocytosis (Fuku *et al.* 1995; Tian *et al.* 2007) that may compromise fertilisation outcomes (Somfai *et al.* 2006). Therefore, an alternative strategy, parthenogenetic activation, could be used to assess the developmental potential of cryopreserved oocytes. Parthenogenetic activation of oocytes represents a valid tool to investigate the comparative roles of paternal and maternal genomes in controlling early embryo development, hence, indirectly to assess the quality of the oocytes matured in vitro. Furthermore, an optimal protocol for oocyte activation is required for the success of advanced technologies such as cloning by NT. Parthenogenetic activation of mammalian oocytes can be induced by a D.C. electrical pulse (Zhu *et al.* 2002) or by chemicals that promote intracellular calcium increase, such as, ethanol (Hou *et al.* 2009); calcium ionophore A23187 (CA, Liu *et al.* 1998), ionomycin (Wells *et al.* 1999) or strontium (Sr, Meo *et al.* 2005). These may be used alone or in combination with protein synthesis inhibitor such as cycloheximide (CHX) or kinase inhibitors such as 6-Dimethylaminopurine (6-DMAP) (Loi *et al.* 1998). Parthenogenetic activation of vitrified oocytes has been reported in cattle (Dinnyes *et al.* 2000, Hou *et al.* 2005; Yang *et al.* 2008; Hou *et al.* 2009), pig (Somfai *et al.* 2006; Du *et al.* 2008), goat (Begin *et al.* 2003), mouse (Endoh *et al.* 2007), and human (Morbeck *et al.* 2009). Incorporation of cryopreserved oocyte in NT programme as a cytoplast could be an additional way to evaluate its developmental potential.

The aims of this study were to assess 1) MPF/MAPK activities following IVM of ovine oocytes vitrified at GV stage, 2) developmental competence following parthenogenetic activation and somatic cell nuclear transfer (SCNT) of vitrified/ thawed ovine GV oocytes. In parthenogenetic activation, the effectiveness of two activation protocols was compared (1) a combination of calcium ionophore (A 23187), cycloheximide, and cytochalasin B (CA+CHX/CB) and (2) strontium and CB (Sr/CB).

6.2 MATERIALS AND METHODS

6.2.1 Vitrification and warming of COCs

Recovery of ovine oocytes was carried out as previously described (**Chapter 2.1**). COCs were vitrified using cryoloop as described in (**Chapter 2.2.3**). Vitrified/thawed oocytes were matured in vitro for 24 h (**Chapter 2.3**). At 24 hpm (hours post maturation) MPF/MAPK kinases activities were evaluated as described in (**Chapter 2.11**).

6.2.2 Parthenogenetic activation and IVC

At 24 hpm, oocytes showing first polar body and homogenous cytoplasm were selected and activated using two different protocols, CA+CHX/CB or Sr/CB as described in (**Chapter 2.6**). 4–5 hpa (hours post activation), presumptive zygotes were transferred into culture medium (**Chapter 2.8**). Embryo development, total cell numbers, and numbers of apoptotic nuclei in day 7 blastocysts were evaluated as described in (**Chapter 2.8.1, 2.8.2 and 2.9**).

6.2.3 Somatic cell nuclear transfer (SCNT)

At 15–16 hpm, SCNT was performed as described in (**Chapter 2.7**). Reconstructed embryos were activated using Sr/CB protocol as described earlier (**Chapter 2.7**). Presumptive zygotes were cultured for 7 days in C.SOF at 39°C (**Chapter 2.8**). Total cell numbers, apoptotic nuclei, and chromosome numbers in day 7 blastocyst stage embryos were evaluated.

6.3 RESULTS

6.3.1 MPF and MAPK activities

MPF and MAPK activities were measured at 24 hpm in ovine oocytes vitrified at GV stage versus fresh control (**Fig 6.1**). Vitrification of ovine GV oocytes resulted in lower activities of both MPF and MAPK as compared to control group; however, the difference was not statistically significant (**Fig 6.1**).

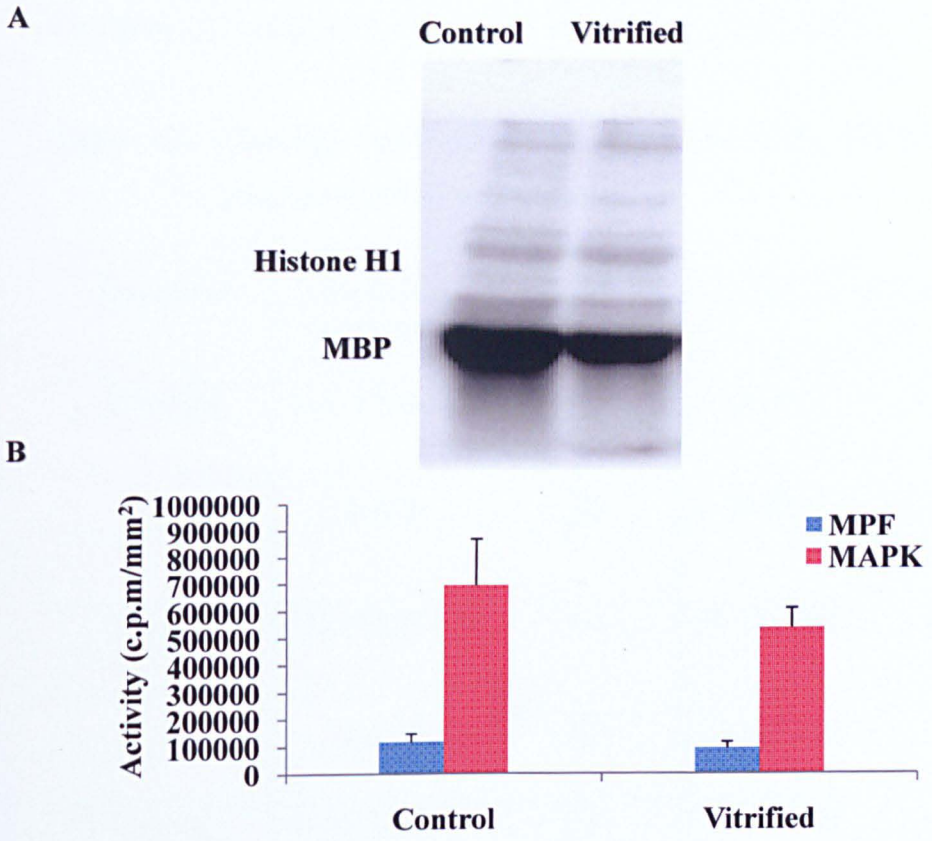


Figure 6.1 Maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activities. The figure shows MPF and MAPK activities in vitrified GV and control ovine oocytes at 24 hpm detected with an in vitro double-kinase by phosphorylation of histone H1 and myelin basic protein (MBP), respectively. (**A**) Phospho-image of PAGE, ^{32}P radioactivity representing kinase activities. (**B**) Relative kinase activities (cpm/mm^2). Ten oocytes were assayed at each treatment, and three replicates were performed.

6.3.2 Frequencies of cleavage following parthenogenetic activation

The effects of vitrification and exposure to vitrification solution on cleavage rates following IVM and parthenogenetic activation of ovine oocytes by two different methods (CA+CHX/CB versus Sr/CB) are summarised in **Table 6.1**. Cleavage was significantly ($P< 0.05$) lower in vitrified oocytes activated by two protocols (CA+CHX/CB and Sr/CB) at both 24 (6.2 and 3.8%) and 48 hpa (28.4 and 27.5%) as compared to toxicity and control groups. Cleavage rates were significantly increased ($P< 0.05$) at 24 and 48 hpa in toxicity and control oocytes activated by CA+CHX/CB as compared to Sr/CB activated oocytes (71.4, 85.7, 74.5, and 92.7% *vs* 21.7, 40.0, 43.2, and 63.3%, respectively, **Table 6.1**).

Table 6.1 Cleavage rates following parthenogenetic activation of ovine oocytes vitrified at GV stage

Treatment	*Method of activation*	*No. Oocytes*	*No. Cleaved oocytes (%) hpa*	
			24	*48*
Vitrified	**CA+CHX/CB**	81	5 (6.2%)[a]	23 (28.4%)[a]
	Sr/CB	80	3 (3.8%)[a]	22 (27.5%)[a]
Toxicity	**CA+CHX/CB**	56	40 (71.4%)[c]	48 (85.7%)[bc]
	Sr/CB	60	13 (21.7%)[b]	24 (40.0%)[a]
Control	**CA+CHX/CB**	55	41 (74.5%)[c]	51 (92.7%)[b]
	Sr/CB	139	60 (43.2%)[d]	88 (63.3%)[c]

Values with different superscripts in the same column (a, b, c) are significantly different ($P< 0.05$). Ca+CHX/CB: calcium ionophore (A 23187) + cycloheximide and cytochalasin B. Sr/CB: strontium + CB. hpa: hours post activation.

6.3.3 In vitro embryo development following parthenogenetic activation

Day 5 morula and frequencies of blastocyst development (day 7 pa) as well as total cell numbers following parthenogenetic activation of ovine oocytes vitrified at GV stage are presented in **Table 6.2.** Irrespective of activation method no significant differences were observed in the frequencies of development to morula between vitrified and toxicity groups (range 18.8 to 35.7%). However, vitrification of oocytes resulted in significantly ($P< 0.05$) lower frequency of development to morula stages in two activation protocols as compared to control groups (39.6 to 49.1%)

As presented in **Table 6.2**, no blastocysts developed in vitrified oocytes activated by CA+CHX/CB; however, 3.8% of oocytes developed following activation by Sr/CB. This frequency was significantly lower ($P< 0.05$) than that obtained in toxicity and control oocytes activated by two methods (20 to 27.3%). Based on the numbers of cleaved embryos, no significant differences were observed in the frequencies of blastocyst development between vitrified (13.6%); or toxicity and control oocytes activated by CA+CHX/CB (25 and 21.6%). However, numbers of cleaved embryos reaching blastocyst stages were significantly lower in control oocytes activated by CA+CHX/CB than those activated by Sr/CB (43.2%).

No significant differences were observed in the proportions of hatched blastocysts produced from vitrified oocytes (1.3%) as compared to toxicity and control groups (4.3 to 7.1%). In addition, total cell numbers in blastocyst stage embryos did not differ significantly between vitrified oocytes (85 ± 13.9) and other groups (103 ± 9.9 , 89.6 ± 8.4, 104.9 ± 6.3, and 105.6 ± 6.7, in toxicity and control oocytes activated by CA+CHX/CB or Sr/CB, respectively, **Table 6.2**).

Table 6.2 In vitro embryo development following parthenogenetic activation of ovine oocytes vitrified at GV stage

Treatment	*Method of activation*	*No. Oocytes*	*No. Morula (%)*	*No. Blastocysts (% of oocytes)*				*Total No. Blastocysts (% of cleaved 48 hpa)*	*Total cell numbers/ blastocyst (mean ±SEM)*
				Early	*Expanded*	*Hatched*	*Total*		
Vitrified	**CA+CHX/CB**	81	18 (22.2%)^{a}	0 (0.0)^{a}	0 (0.0)^{a}	0 (0.0)^{a}	0 (0.0)^{a}	N/A	N/A
	Sr/CB	80	15 (18.8%)^{a}	2 (2.5%)^{ac}	0 (0.0)^{a}	1 (1.25%)^{ab}	3 (3.8%)^{a}	13.6%^{a}	85.0± 13.9
Toxicity	**CA+CHX/CB**	56	20 (35.7%)^{ab}	4 (7.1%)^{bc}	4 (7.1%)^{bc}	4 (7.1%)^{b}	12 (21.4%)^{b}	25.0%^{ab}	103± 9.9
	Sr/CB	60	17 (28.3%)^{ab}	5 (8.3%)^{bc}	4 (6.7%)^{bc}	3 (5.0%)^{b}	12 (20.0%)^{b}	50.0%^{b}	89.6± 8.4
Control	**CA+CHX/CB**	55	27 (49.1%)^{b}	7 (12.7%)^{b}	1 (1.8%)^{ac}	3 (5.8%)^{b}	11 (20.0%)^{b}	21.6%^{a}	104.9± 6.3
	Sr/CB	139	55 (39.6%)^{a}	16 (11.5%)^{b}	16 (11.5%)^{b}	6 (4.3%)^{ab}	38 (27.3%)^{b}	43.2%^{b}	105.6± 6.7

Values with different superscripts in the same column (a, b, c) are significantly different ($P< 0.05$).Ca+CHX/CB: calcium ionophore (A 23187) plus cycloheximide and cytochalasin B, Sr/CB: strontium plus CB. hpa: hours post activation. N/A: not applied. Three replicates were carried out.

6.3.4 Apoptosis in blastocysts produced following parthenogenetic activation

Apoptotic cell numbers and apoptotic index of day 7 blastocysts produced by Sr/CB activation of vitrified, toxicity, and control oocytes are presented in **Table 6.3** and **Fig 6.2.** No significant differences were observed in the numbers of apoptotic nuclei (determined by TUNEL assay) between vitrified, toxicity, and control groups (15.8 ± 2.8, 10.6 ± 1.3, and 12.9 ± 0.7, respectively). The same trend was observed in terms of apoptotic index (number of apoptotic nuclei/total cell numbers in blastocysts), with values of 17.8 ± 5.2, 12.1 ± 0.9, and 15.1 ± 1.2 for vitrified, toxicity, and control groups, respectively.

Table 6.3 Numbers of apoptotic nuclei and apoptotic index in day 7 blastocysts produced by Sr/CB parthenogenetic activation

Treatment	*No. Blastocysts*	*No. Apoptotic nuclei (mean ± SEM)*	*Apoptotic index (mean ± SEM)*
Vitrified	4	15.8 ± 2.8	17.8 ± 5.2
Toxicity	8	10.6 ± 1.3	12.1 ± 0.9
Control	15	12.9 ± 0.7	15.1 ± 1.2

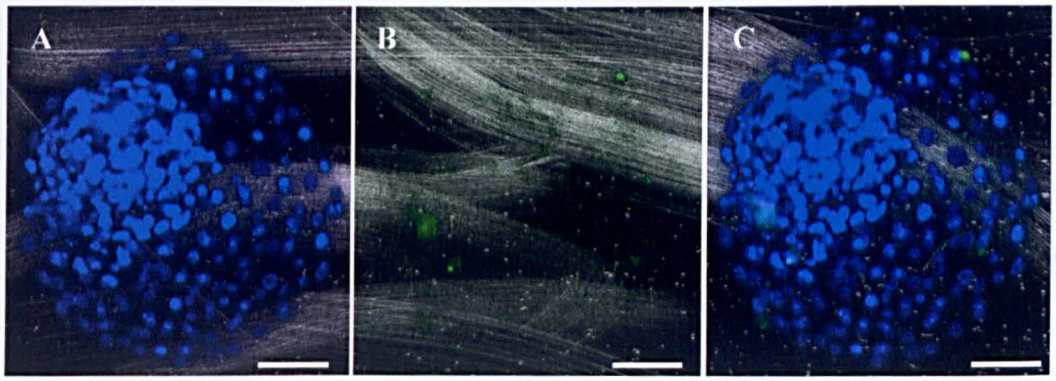


Figure 6.2 Representative blastocyst stage embryos stained with Hoechst and TUNEL assay. (**A**) total cell numbers appear blue, (**B**) apoptotic nuclei appear green, and (**C**) merge between total cells and apoptotic nuclei in day 7 blastocysts produced following IVM and parthenogenetic activation of vitrified/thawed ovine GV oocytes. Scale bar = 50 μm.

6.3.5 Frequencies of enucleation and fusion

Enucleation and fusion of ovine oocytes vitrified at GV stage and used as cytoplast recipients for SCNT is presented in **Fig 6.3**. High frequencies of enucleation (~99%) and fusion (~98%) were achieved in both vitrified and control oocytes.

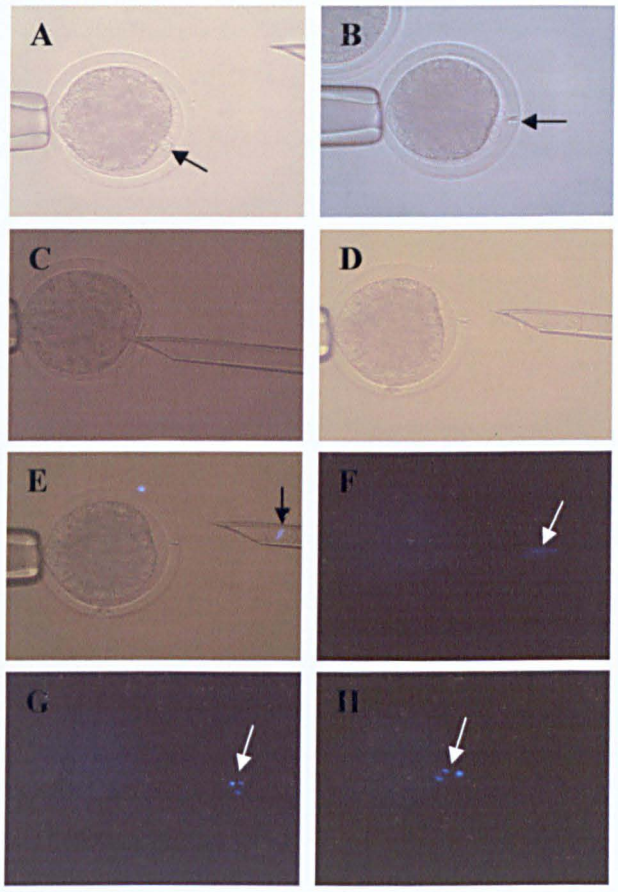


Figure 6.3 Enucleation of ovine oocytes vitrified at GV-stage. (**A**) Holding the Anaphase-I-Telophase-I (AI/TI) oocyte before enucleation (AI/TI spindle is showed by: black arrow). (**B**) Hole in the zona pellucida made by laser to assist in enucleation indicating by black arrow. (**C, D** and **E**) penetration of the zona pellucida by enucleation pipette. (**F, G** and **H**) confirmed by epiflourescence light (AI/TI spindle marked by white arrow).

6.3.6 Cleavage and subsequent development of SCNT embryos

The developmental potential of SCNT embryos produced using vitrified/thawed GV oocytes as cytoplast recipients is presented **Table 6.4.** The frequencies of cleaved embryos 24 and 48 hpa were significantly lower ($P< 0.05$) in vitrified/thawed group (31 and 48%) than in control group (55.1 and 85.0%). However, no significant difference was observed in the frequencies of development to morula (38.0 *vs* 46.7%) and blastocyst stages (13.0 *vs* 23.4%). Based on 48 hpa cleavage, the same percentage of blastocyst development was obtained in both groups (~27%). A similar trend was observed in the proportions of early, expanded, and hatched blastocysts (4.0, 2.0, and 7.0 *vs* 6.5, 6.5, 10.3%, in vitrified and control groups, respectively, **Fig 6.4 A**).

6.3.7 Total cell numbers and apoptosis in blastocysts produced by SCNT

As shown in **Table 6.5** and **Fig 6.4 B**, no significant differences were observed in total cell numbers, numbers of apoptotic nuclei or apoptotic index as detected by Hoechst and TUNEL assay in blastocysts produced by SCNT of vitrified versus control oocytes (90.3 ± 4.9, 13.1 ± 0.9, and 14.9 ± 1.4 *vs* 97.6 ± 4.6, 13.2 ± 1.4, 13.6 ± 1.1, respectively).

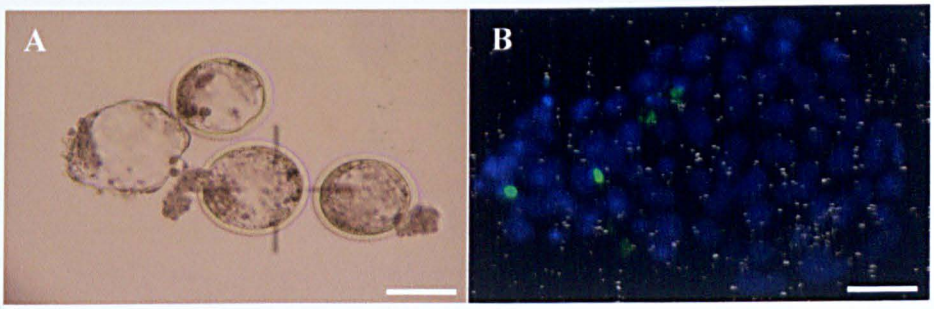


Figure 6.4 Day 7 blastocysts produced by SCNT. The figure shows **(A)** phase contrast day 7 hatched blastocysts **(B)** stained with Hoechst produced by SCNT using ovine oocytes vitrified at GV stage as cytoplast recipients. Scale bar = 50μm.

Table 6.4 In vitro embryo development of SCNT embryos produced using ovine oocytes vitrified at GV stage as cytoplast recipients

Treatment	*No. fused oocytes*	*No. Cleaved (%) hpa*		*No. Morula (%)*	*No. Blastocysts (% of fused oocytes)*				*Total No. Blastocysts (% of cleaved 48hpa)*
		24	*48*		*Early*	*Expanded*	*Hatched*	*Total*	
Vitrified	100	31 (31.0%)[a]	48 (48.0%)[a]	38 (38.0%)[a]	4 (4.0%)[a]	2 (2.0%)[a]	7 (7.0%)[a]	13 (13.0%)[a]	27.1%[a]
Control	107	59 (55.1%)[b]	91 (85.0%)[b]	50 (46.7%)[a]	7 (6.5%)[a]	7 (6.5%)[a]	11 (10.3%)[a]	25 (23.4%)[a]	27.5%[a]

Values with different superscripts in the same column are significantly different ($P< 0.05$). Four replicates were performed.

Table 6.5 Total cell numbers and apoptotic nuclei in day 7 blastocysts produced by SCNT of ovine oocytes vitrified at GV stage

Treatment	*No. Blastocysts*	*Total cell numbers (mean ± SEM)*	*No. Apoptotic nuclei (mean ± SEM)*	*Apoptotic index (mean ± SEM)*
Vitrified	8	90.3 ± 4.9	13.1 ± 0.9	14.9 ± 1.4
Control	21	97.6 ± 4.6	13.2 ± 1.4	13.6 ±1.1

6.3.8 Karyotyping of SCNT blastocysts

The ploidy of SCNT derived blastocysts was evaluated and the results are summarized in **Table 6.6**. Embryos were categorized as diploid (54, 2n), haploid (n), or polyploid (≥ 3n); diploid embryos were considered as normal, others were reported as abnormal. The proportion of embryos with diploid chromosomal complements was lower in blastocysts obtained by SCNT of vitrified oocytes as compared to control (60.0 *vs* 75.0%, respectively). But, the difference between two groups was not significant. Also, no significant differences were observed between two groups in terms of haploid (20 *vs* 10.0%) and polyploid (20 *vs* 15%) embryos.

Table 6.6 Cytogenetic analysis of day 7 blastocysts produced by SCNT of ovine oocytes vitrified at GV stage

Treatment	*No. Blastocysts*	*Ploidy No. (%)*		
		Diploid	*Haploid*	*Polyploid*
Vitrified	5	3 (60.0%)	1 (20.0%)	1 (20.0%)
Control	20	15 (75.0%)	2 (10.0%)	3 (15.0%)

Chromosomal constituents of day 7 blastocyst embryos produced by SCNT of ovine oocytes vitrified at GV-stage were by karyotyping. No significant differences were observed between vitrified and control groups.

6.4 DISCUSSION

Little information is available on the biochemical and molecular changes that occur after oocyte cryopreservation. Concerning this point, the activities of MPF and MAPK following 24 h IVM of vitrified/thawed GV oocytes were compared to fresh control oocytes. The activities of both kinases were decreased in vitrified oocytes as compared to control; however, the difference was not significant (**Fig 6.1**). These two factors are known to play a pivotal role in meiotic and mitotic cell cycles (Nurse 1990). Previously, it has been reported that, the activity of MPF was significantly lower in OPS, cryoloop, and cryotop vitrified MII ovine oocytes (Succu *et al.* 2007a), however, the activity was restored after 2 h culture in OPS, cryoloop vitrified oocytes but not in cryotop group. Bogliolo *et al.* (2007a) reported that the activities of MPF and MAPK (24 hpm) did not differ between ovine oocytes vitrified at GV-stage with cumulus cells and control group either denuded or cumulus-enclosed. However, the activities were decreased when the cumulus cells were removed prior to vitrification. These results suggest that, cryopreservation may induce the degradation of molecules involved in the signaling pathways known to regulate kinase activity, and this adverse effect may be more evident when oocytes are free of cumulus cells (Bogliolo *et al.* 2007a).

Efficient oocyte activation is a key step for success of nuclear transfer (Campbell 1999). Parthenogenetic activation of oocytes provides a good model for evaluation of the developmental potential of cryopreserved oocytes (Morbeck *et al.* 2009). The present study was designed to compare two protocols (1) CA+CHX/CB, (2) Sr/CB on activation of vitrified/thawed ovine GV oocytes. Interestingly, no blastocysts stage embryos developed in vitrified oocytes activated by CA+CHX/CB; however, 3.8% blastocysts were obtained following Sr/CB activation (**Table 6.2**). The results indicate that strontium is efficient in parthenogenetic activation of vitrified/thawed ovine oocytes. Parthenogenetic activation studies have shown that the most effective stimuli are those which promote multiple intracellular calcium peaks (Vitullo and Ozil 1992). In mice,

strontium has been reported to induce a series of intracellular calcium rises by displacing bound Ca^{2+} pulses, releasing it from intracellular stores and promotes Ca^{2+} pulses (Jellerette *et al.* 2000). This occurs through IP3 (Inositol-1, 4, 5-triphosphate) receptors, which is similar to that seen in normal fertilisation (Zhang *et al.* 2005). Previous studies have shown that CA plus 10 mM $SrCl_2$ could be used more effectively for activation of fresh ovine MII oocytes as compared to other concentrations of $SrCl_2$ and to CA+CHX/CB (Choi and Campbell 2008). The results in the present study suggest that, although vitrified oocytes activated by CA+CHX/CB had the ability to cleave and develop to morula stage in frequencies comparable to Sr/CB group; it failed to produce blastocyst stage embryos. However, the two activation protocols resulted in the same proportions of blastocyst development in other groups (toxicity and control). Incorporation of calcium ionophore in parthenogenetic activation of vitrified oocytes seems to exert a harmful effect on blastocyst development, an observation which requires further investigation. CB has been reported to play an important role in parthenogenetic activation, it prevents second polar body extrusion, resulting in diploid parthenotes which are less apoptotic and have higher developmental capacity as compared to haploid (Liu *et al.* 2002).

In general freshly matured oocytes from in vitro or in vivo sources are used as cytoplast recipients for NT. Development of successful cryopreservation procedures would immensely benefit nuclear transfer research and practice because it would eliminate seasonal fluctuations in oocyte quality and also the dependence on timing of slaughter and oocyte shipment. The present study evaluated the use of ovine oocytes vitrified at GV stage as cytoplast recipients for SCNT. Although, the frequencies of cleaved embryos at 24 and 48 hpa were significantly lower in vitrified group as compared to control, development to morula and blastocyst stage embryos did not differ significantly (**Table 6.4**). These results indicate that vitrified ovine oocytes can be used as cytoplast recipients for SCNT and produce high frequencies of good quality blastocyst embryos. Also, the results suggest that vitrification of immature ovine oocytes

provides an opportunity to establish oocyte banking that can be used as a tool for improvement of genetic selection, widespread applications of reproductive biotechnologies in both animal and human species, and also for preservation of endangered species. Oocyte vitrification has been reported to reduce the level of maternal mRNAs in ovine oocytes (Succu *et al.* 2008) which could be attributed to the lower cleavage in SCNT vitrified oocytes. It has been reported that, the damaging effects of freezing-thawing were apparent only up to the two-cell stage but beyond this stage no further influences were observed on the development to morula or blastocysts (Schroeder *et al.* 1990). The reduced frequencies of blastocyst development following parthenogenetic activation of vitrified oocytes as compared to SCNT indicates that the nuclear materials, surrounding microtubules and meiotic spindles were adversely affected by cryopreservation (Kubota *et al.* 1998; Dinnyes *et al.* 2000; Tominoga *et al.* 2005); however, the effects on cytoplasmic components should not be neglected (Dinnyes *et al.* 2000).

Regarding the quality of blastocysts, the results suggest that vitrification of ovine GV oocytes resulted in production of good quality blastocysts following activation and SCNT. In contrast, previous studies in cattle and pigs reported lower cell numbers in blastocysts produced by SCNT and parthenogenetic activation of vitrified/thawed MII oocytes as compared to control (Atabay *et al.* 2004; Tominaga *et al.* 2005; Somfai *et al.* 2006). Similar developmental potential reported here following SCNT of vitrified oocytes compared to control may be due to several factors include vitrification protocol, cryoprotectant combinations, and activation method. Furthermore, in these studies only morphologically high quality oocytes, i.e. uniform granular, homogeneously distributed cytoplasm surrounded by compact layers of cumulus cells were used. Given that the ability of an embryo to withstand freezing and thawing has been used in the part as a useful indicator for quality (Rizos *et al.* 2001), it is possible that the selection of best quality oocytes here may have influenced cryotolerance and hence the developmental potential of vitrified/thawed oocytes following SCNT.

In the present study, cytogenetic analysis of day 7 SCNT blastocysts revealed lower proportions of diploid embryos obtained from vitrified oocytes as compared to those produced from control, but the difference was not significant. Data regarding the cytogenetic status of NT embryos are limited and the results are confounded by differences between NT protocols, karyotyping methods, developmental stages, types of donor cells, donor cell-cytoplast cell cycle synchronization, and perhaps species (Campbell *et al.* 1999; Slimane-Bureau and King 2002; Li *et al.* 2004). The abnormal chromosome constitution reported here may also attribute to the cytoskeletal injuries resulted from vitrification and thawing (Bos-Mickich and Whittingham 1995).

In summary, the main finding in these studies is that vitrified-thawed ovine oocytes can be used successfully for the first time as recipient cytoplasts for SCNT with high frequencies of blastocyst development. This finding has important implications for nuclear transfer research. Although, lower values of MPF/MAPK activities were reported in vitrified/thawed oocytes as compared to control, the difference was not significant. Vitrified GV oocytes can develop up to morula stage following IVM and parthenogenetic activation, irrespective to activation protocol. Moreover, strontium can be used effectively for parthenogenetic activation of vitrified/thawed ovine oocytes. The results show that, parthenogenetic development forms a solid platform to test the developmental potential of vitrified immature ovine oocytes after ICSI and NT. Further studies are required to demonstrate the ability of nuclear transfer produced embryos to develop in vivo, establish pregnancy, and produce offspring.

CHAPTER 7

VITRIFICATION OF IMMATURE OVINE OOCYTES WITH CRYOLOOP: EFFECT OF CYTOCHALASIN B PRE-TREATMENT ON VIABILITY AND SUBSEQUENT DEVELOPMENT

7.1 INTRODUCTION

Cryopreserving reproductive tissues or cells has become an increasingly widespread means of preserving or salvaging genetic material for subsequent use in assisted reproduction. However, while live offspring have been produced after fertilisation of frozen-thawed oocytes in a number of species, the overall success of oocyte cryopreservation in terms of subsequent developmental competence is still very low (Tharasanit *et al.* 2006a; Ledda *et al.* 2007).

Immature oocytes differ from mature ones in that their chromatin has yet to condense and is protected within a membrane-bound vesicle (the germinal vesicle: GV), while the meiotic spindle has yet to assemble. In this latter respect, the microtubule spindle of mature (metaphase II: MII) oocytes is extremely susceptible to damage during freezing and thawing (for review, Chen *et al.* 2003). Poor oocyte survival following cryopreservation has been associated with disruption of important microstructures such as the plasma membrane (Agaca *et al.* 1998), the cytoskeleton (Saunders and Parks 1999), the chromosomes (Chen *et al.* 2003), and the cortical granules (Carroll *et al.* 1990), all of which are critical to oocyte fertilisability and subsequent developmental competence (Saunders and Parks 1999). Recently, vitrification has been used for the cryopreservation of

oocytes as an alternative to traditional slow-freezing (Vajta 2000). Many studies have been conducted to improve vitrification protocols including increases in cooling and warming rates (Vajta and Kuwayama 2006; Ledda *et al.* 2007). Moreover, stabilizing the cytoskeleton system during vitrification may also be beneficial for improvement of survival and subsequent development of vitrified/thawed oocytes and embryos (Zhang *et al.* 2009).

Cytochalasin B (CB) as a cytoskeletal relaxant was considered to make the skeletal elements less rigid (Fujihira *et al.* 2004). In mature oocytes, CB reduced damage to microtubules and may enhance stabilization of spindle microtubules during vitrification (Zhang *et al.* 2009). In the case of GV oocytes, no organised meiotic spindle is present, and this relaxant effect may preserve the functionality of the gap junctions between oocyte and granulosa cells and permit a faster and more uniform penetration of the cryoprotectants (Vieira *et al.* 2002). To date, studies on the effect of CB on viability and subsequent development of vitrified oocytes are few, whilst the results are controversial and dependent upon species. In pig and sheep Somfai *et al.* (2006) and Zhang *et al.* (2009) reported positive effects of CB pre-treatment on viability and subsequent development of vitrified/thawed MII oocytes. In contrast, other studies in cattle and sheep did not report any effects of cytochalasin pre-treatment on survival and in vitro development of vitrified/thawed GV oocytes (Vieira *et al.* 2002; Silvestre *et al.* 2006).

The aims of these studies were to investigate the effect of CB pre-treatment prior to vitrification of immature ovine oocytes (obtained from mature ewes) on viability, in vitro fertilisation status, and subsequent development following IVM /IVF/IVC.

7.2 MATERIALS AND METHODS

7.2.1 Cytochalasin B (CB) treatment

Before vitrification, selected COCs obtained at slaughter (**Chapter 2.1**) were randomly divided into two groups and incubated with or without 7.5 μg/ml CB in TCM-199 plus 10% FBS for 60 min at 39°C. Oocytes from each group were vitrified or treated as toxicity and control.

7.2.2 Vitrification and warming of COCs

After washing three times in a base medium (H-TCM 199 supplemented with 10% heat inactivated FBS), COCs were vitrified using cryoloop as described in (**Chapter 2.2.3**). Following vitrification and warming, oocytes were morphologically examined to evaluate their viability (see **Chapter 2.2.4**).

7.2.3 IVM/ IVF/ IVC

IVM/IVF/IVC and embryo evaluation were performed as previously described (**Chapter 2.3, 2.5, 2.8, 2.8.1**). Total cell numbers of day 7 blastocyst stage embryos were determined using Hoechst stain as described in (**Chapter 2.8.2**). At 18 h post-insemination (hpi), frequencies of in vitro fertilisation and polyspermy were determined as previously described (**Chapter 2.5.3**).

7.2.4 Statistical Analysis

Survival, frequencies of in vitro fertilisation, and development were analysed using Chi square test. Total cell numbers were analysed using un-paired student *t*-test. All results are considered to be statistically significant at $P< 0.05$.

7.3 RESULTS

7.3.1 Effect of CB pre-treatment on viability of vitrified COCs

The effects of CB pre-treatment on viability of vitrified/thawed GV ovine oocytes are presented in **Table 7.1** and **Fig 7.1.** No significant differences were observed in survival rates between CB treated and non treated oocytes in both vitrified and toxicity groups (values ranged from 88.4 to 93.9%).

Table 7.1 Effect of cytochalasin B (CB) pre-treatment on viability of vitrified/thawed ovine GV-oocytes.

Treatment	*No. Oocytes*	*No. Normal*	*%*
Vitrified	304	274	(90.1%)
Toxicity	216	203	(93.9%)
CB-vitrified	404	357	(88.4%)
CB-toxicity	277	250	(90.3%)

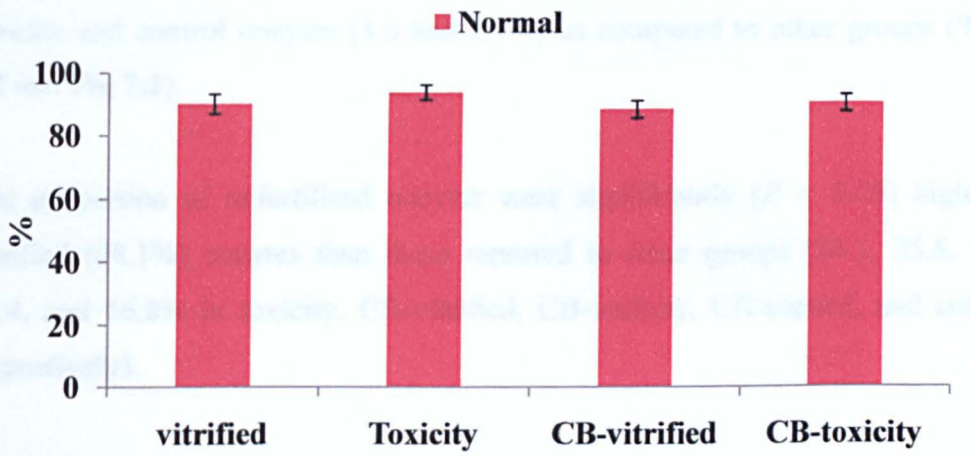


Figure 7.1 Effect of CB pre-treatment on viability of vitrified/thawed ovine GV oocytes. The graph represents the percentages (± SEM) of viable oocytes (normal) in vitrified and toxicity groups following exposure to CB prior to vitrification. Eight replicates were performed for each experimental group.

7.3.2 Effect of CB treatment on fertilisation events following IVF

The effects of CB pre-treatment on frequencies of in vitro fertilisation and polyspermy of ovine oocytes vitrified and exposed to vitrification solutions at GV-stage are presented in **Table 7.2** and **Fig 7.2**.

Although, CB treatment resulted in higher percentages of fertilisation in vitrified oocytes than those vitrified without treatment (57.0 *vs* 40.7%, respectively), the differences were not significant. No significant differences were observed in terms of fertilisation between CB-vitrified, toxicity (72.3%), CB-toxicity (75.4%) and CB-control groups (67.3%). However, significant differences ($P< 0.05$) were observed between both vitrified groups (CB treated and non treated) and control group (81.1%). CB pre-treatment did not affect the frequencies of in vitro fertilisation in toxicity and control groups (**Table 7.2**).

No significant differences in polyspermy were observed between vitrified, CB-vitrified, CB-toxicity, and CB-control groups (11.1, 17.2, 14.0, and 11.2%, respectively). However, the frequencies of polyspermy were decreased in both toxicity and control oocytes (3.6 and 2.1%) as compared to other groups (**Table 7.2** and **Fig 7.2**).

The proportion of unfertilised oocytes were significantly ($P < 0.05$) higher in vitrified (48.1%) oocytes than those reported in other groups (24.1, 25.8, 10.5, 21.4, and 16.8% in toxicity, CB-vitrified, CB-toxicity, CB-control, and control, respectively).

Table 7.2 Effect of cytochalasin B (CB) pre-treatment on in vitro fertilisation of ovine oocytes vitrified at GV-stage

Treatment	*No. Oocytes*	*Fertilisation*		
		No. Fertilised (%)	*No. Polyspermic (%)*	*No. Unfertilised (%)*
Vitrified	81	33 (40.7%)^{b}	9 (11.1%)^{ab}	39 (48.1%)^{a}
Toxicity	83	60 (72.3%)^{ac}	3 (3.6%)^{bc}	20 (24.1%)^{bc}
CB-vitrified	93	53 (57.0%)^{bc}	16 (17.2%)^{a}	24 (25.8%)^{b}
CB-toxicity	57	43 (75.4%)^{ac}	8 (14.0%)^{a}	6 (10.5%)^{c}
CB-control	98	66 (67.3%)^{ac}	11 (11.2%)^{ab}	21 (21.4%)^{bc}
Control	95	77 (81.1%)^{a}	2 (2.15%)^{c}	16 (16.8%)^{bc}

Values with different superscripts in the same column are significantly different (a, b, c $P< 0.05$). Fertilised oocytes (characterised by presence of sperm/swollen sperm head in ooplasm, or a male and/or female pronucleus). Polyspermy (oocytes with more than one sperm in ooplasm or more than two pronuclei). Unfertilised oocytes (no signs of sperm penetration). Four replicates were performed.

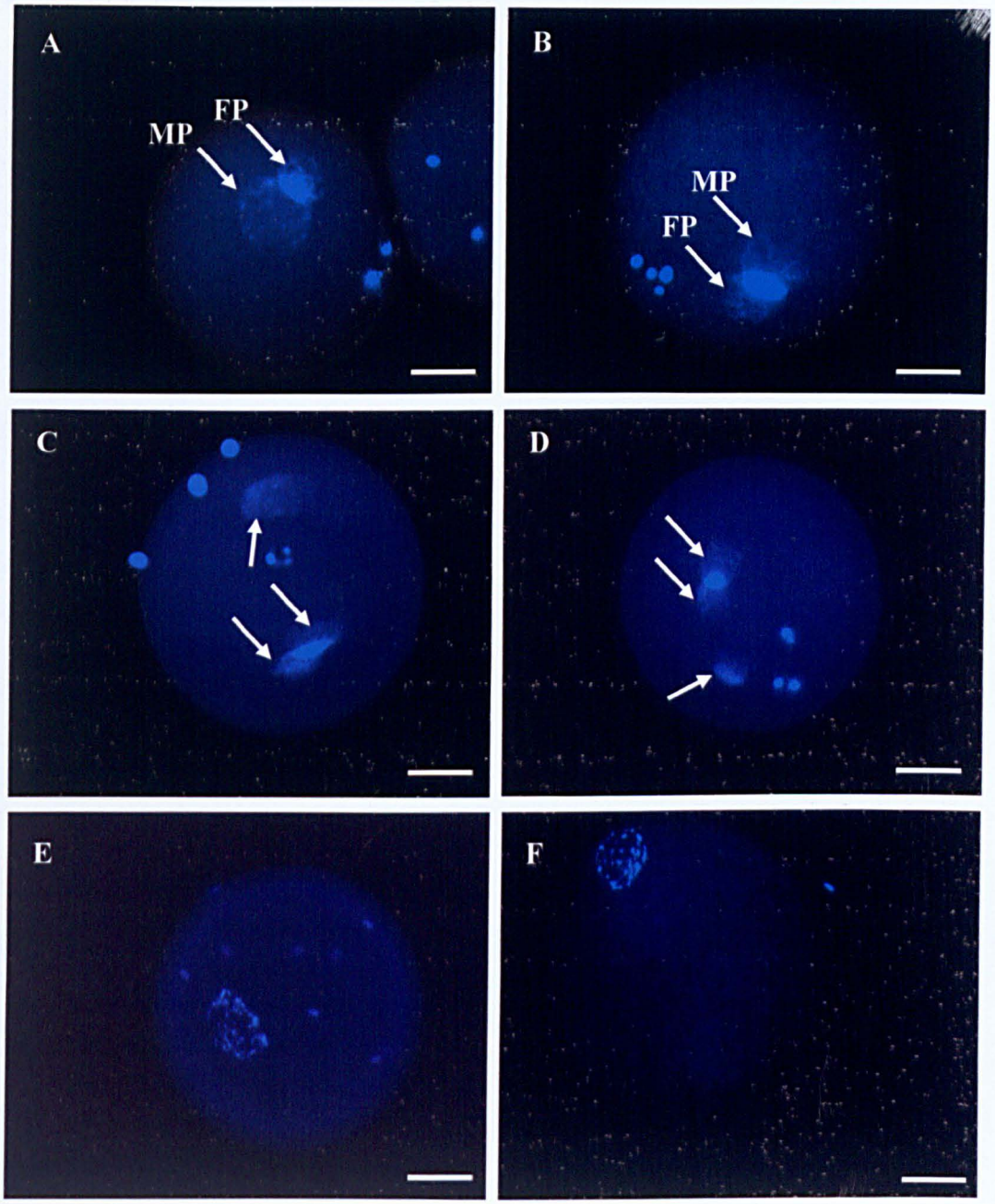


Figure 7.2 In vitro fertilisation of ovine oocytes. The figure represents fertilisation events of ovine oocytes stained with Hoechst at 18 hpi. (**A** and **B**) fertilised oocytes with male (MP) and female pronucleus (FP), (**C** and **D**), polyspermy (oocytes with ≥ 3 pronuclei), and (**E** and **F**) unfertilised oocytes (no signs of sperm penetration). Scale bar = 50 μm.

7.3.3 Effect of CB treatment on in vitro embryo development following IVF

The development of ovine oocytes vitrified at GV stage and treated with CB before vitrification is presented in **Table 7.3, Fig 7.3** and **7.4**.

Cleavage was significantly ($P < 0.05$) lower in vitrified and CB-vitrified oocytes at both 24 hpi (12.5 *vs* 9.1%) and 48 hpi (25.0 *vs* 16.2%) compared to other groups (59.5, 35.9, 77.8 and 60.4% at 24 hpi and 75.9, 67.2, 82.2, and 75.8% at 48 hpi in toxicity, CB-toxicity, CB-control, and control oocytes, respectively **Table 7.3**).

Development to morula stage was significantly lower ($P< 0.05$) in vitrified and CB-vitrified oocytes (21.4 *vs* 10.1%) than in toxicity, CB-control, and control (45.7, 50.0 and 64.8%, respectively); however, no significant difference was observed between vitrified and CB-toxicity groups.

Development to blastocysts stage (day 7 pi) was significantly decreased ($P< 0.05$) in both vitrified and CB-vitrified oocytes (4.5 and 3.0%) than other groups (31.0, 27.3, 31.1 and 48.4% in toxicity, CB-toxicity, CB-control and control oocytes, respectively). Based on the numbers of cleaved oocytes (48 hpi), 16.1 and 18.8% of the cleaved embryos were developed to blastocysts in both vitrified and CB-vitrified groups. These values did not differ significantly from CB-control (37.8%). Also, no significant difference was observed between both toxicity groups (CB treated or non-treated, ~ 40%). However, the percentage (63.8%) in control oocytes was significantly higher ($P < 0.05$) than other groups.

Expanded and hatched blastocysts stage embryos were significantly lower (**Table 7.3** and **Fig 7.3**) in both vitrified and CB-vitrified as compared to other groups. No significant differences were observed in total cell numbers between all groups (92.5 ± 25.5, 98.6 ± 8.5, 103.7 ± 19.6, 107.7 ± 8.3, 100.8 ± 7.9, and 122.6 ± 10.3 in vitrified, toxicity, CB-vitrified, CB-toxicity, CB-control, and control, respectively, **Table 7.2** and **Fig 7.4**).

Table 7.3 Effect of cytochalasin B (CB) pre-treatment on development of ovine oocytes vitrified at GV stage

Treatment	*No. Oocytes*	*No. Cleaved (%) hpi*		*No. Morula (%)*	*No. Blastocysts (% of oocytes)*				*Total No. Blastocysts (% of cleaved 48 hpi)*	*Cell No / blastocyst mean ± SEM*
		24	*48*		*Early*	*Expanded*	*Hatched*	*Total*		
Vitrified	112	14 (12.5%)^{a}	31 (25.0%)^{a}	24 (21.4%)^{a}	3 (2.7%)^{ac}	1 (0.9%)^{a}	1 (0.9%)^{a}	5 (4.5%)^{a}	16.1%^{a}	92.5 ± 25.5
Toxicity	116	69 (59.5%)^{b}	88 (75.9%)^{b}	53 (45.7%)^{bd}	10 (8.6%)^{ade}	16 (13.8%)^{b}	10 (8.6%)^{b}	36 (31.0%)^{b}	40.9%^{bc}	98.6 ± 8.5
CB-vitrified	99	9 (9.1%)^{a}	16 (16.2%)^{a}	10 (10.1%)^{c}	1 (1.0%)^{ad}	2 (2.0%)^{a}	0 (0.0%)^{a}	3 (3.0%)^{a}	18.8%^{a}	103.7± 19.6
CB-toxicity	128	46 (35.9%)^{c}	86 (67.2%)^{b}	42 (32.8%)^{ad}	7 (5.5%)^{cd}	20 (15.6%)^{b}	8 (6.2%)^{b}	35 (27.3%)^{b}	40.7%^{b}	107.7 ± 8.3
CB-control	90	70 (77.8%)^{b}	74 (82.2%)^{b}	45 (50.0%)^{b}	7 (7.8%)^{ce}	12 (13.3%)^{b}	9 (10.0%)^{b}	28 (31.1%)^{bd}	37.8%^{ac}	100.8 ± 7.9
Control	91	55 (60.4%)^{b}	69 (75.8%)^{b}	59 (64.8%)^{b}	18 (19.8%)^{b}	18 (19.8%)^{b}	8 (8.8%)^{b}	44 (48.4%)^{cd}	63.8%^{b}	122.6±10.3

Values with different superscripts in the same column are significantly different (a, b, c, d, e $P < 0.05$). Three replicates were carried out.

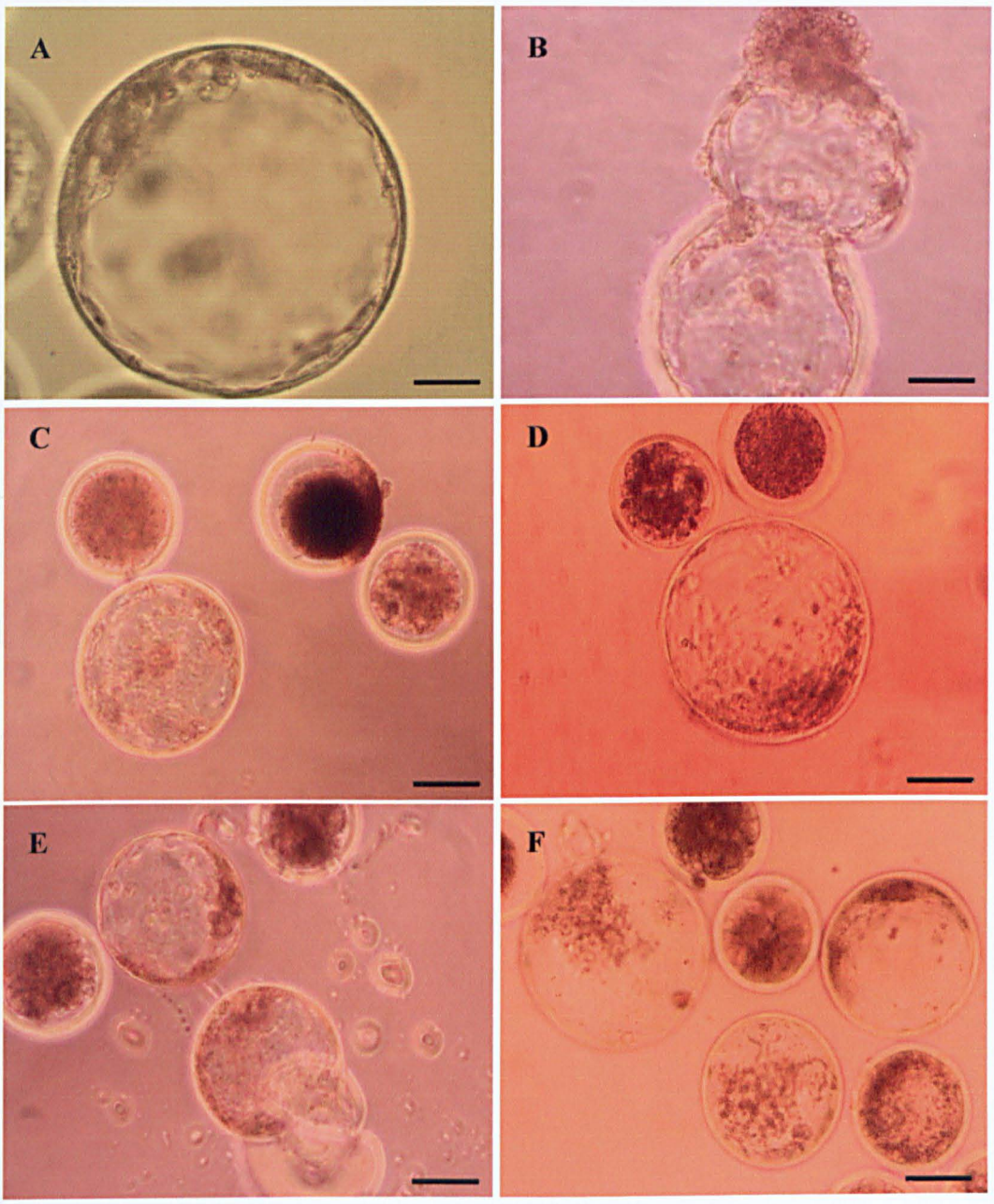


Figure 7.3 Day 7 blastocysts produced by IVF of ovine oocytes vitrified at GV stage and pre-treated with CB. The figure shows day 7 blastocysts obtained following IVM/IVF/IVC of ovine oocytes (**A** and **B**) vitrified, (**C**) CB-vitrified, (**D**) toxicity, (**E**) CB-toxicity and (**F**) control groups. Scale bar = 50 μm.

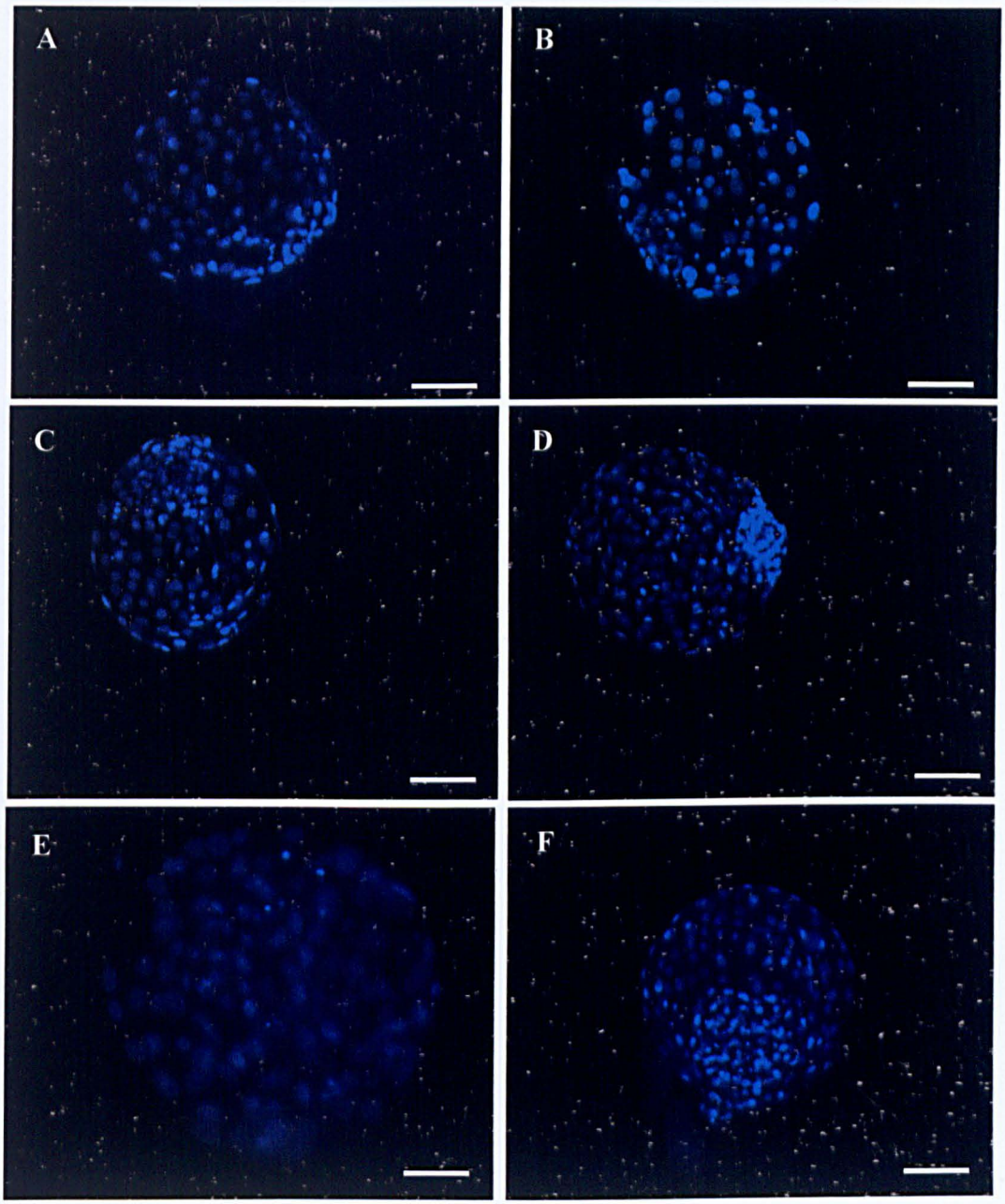


Figure 7.4 Total cell numbers in day 7 in vitro produced blastocysts. The figure shows day 7 blastocysts stained with Hoechst obtained following IVM/IVF/IVC of ovine oocytes from (**A**) vitrified, (**B**) CB-vitrified, (**C**) toxicity, (**D**) CB-toxicity, (**E**) CB-control and (**F**) control groups. Scale bar = 50μm.

7.4 DISCUSSION

Damage to the cytoskeleton is one of the major cryoinjuries in an oocyte vitrification program (Chen *et al.* 2003). In particular, the meiotic spindle of matured oocytes is vulnerable to cryoinjury with depolymerization of its microtubules occurring at lower temperatures. As the temperature drops and the ice proliferates, the concentration of electrolytes and other solutes within the oocyte can reach very high levels which may be damaging to intracellular proteins. Hence, methods for the potential protection of intracellular environment are important for successful cryopreservation (Morato *et al.* 2008a, b, c).

Stabilizing of the cytoskeleton during vitrification may be beneficial for improving the post-thaw survival and subsequent development of vitrified oocytes. Several cytoskeleton stabilizers have been used to reduce injury to oocytes and embryos during vitrification including CB (Fujihira *et al.* 2004; Silvestre *et al.* 2006; Somfai *et al.* 2006; Bogliolo *et al.* 2007a) and cytochalasin D (CD, Vieira *et al.* 2002; Magnusson *et al.* 2008). Cytochalasins inhibitors of microfilament synthesis that disrupt actin polymerization (Therodoropoulos *et al.* 1994) and prevent cytokinesis without affecting karyokinesis (Modlinski 1980). In MII oocytes, CB reduces damage to microtubules and may enhance microtubule stabilization during vitrification (Zhang *et al.* 2009). In GV oocytes, the relaxant effect of CB may preserve gap junction functionality between oocytes and cumulus cells and allow faster and uniform penetration of cryoprotectants (Vieira *et al.* 2002). The beneficial effects of CB during oocyte cryopreservation are not clear and the results are controversial, probably differ according to the species. Isachenko *et al.* (1998) reported improvement in the frequencies of in vitro maturation from 5.6 to 22.0% in vitrified/thawed porcine GV oocytes following CB treatment before vitrification. Furthermore, Fujihira *et al.* (2004) observed that treatment of porcine GV oocytes with CB at a concentration of 5.0 μg/ml for 30 min or 7.5 μg/ml for 10 or 30 min before vitrification had beneficial effects on in vitro maturation rates. Also, Somfai *et al.* (2006) suggested that, CB pre-treatment increased survival and

development of vitrified/thawed porcine MII oocytes. In ovine, Zhang *et al.* (2009) observed that pre-treatment of vitrified/thawed MII ovine oocytes with 7.5 or 10 μg/ml CB for 20–25 min had a positive effect on subsequent development to blastocyst stage following IVF. In contrast, other studies have reported no effects of CB pre-treatment on vitrified/thawed MII bovine (Vieira *et al.* 2002) and GV ovine (Silvestre *et al.* 2006) oocytes. Moreover, Bogliolo *et al.* (2007a) observed a severe reduction in viability (16%) in CB pre-treated vitrified/thawed GV ovine oocytes as compared to controls (85%). In the above mentioned ovine studies, the effect of CB pre-treatment on viability and subsequent in vitro maturation of vitrified/thawed GV oocytes were only evaluated (Silvestre *et al.* 2006; Bogliolo *et al.* 2007a). Therefore, the present study investigated the effect of CB pre-treatment prior to vitrification on viability, in vitro fertilisation and subsequent development of ovine oocytes vitrified at GV stage. The survival rates reported here in both vitrified and toxicity groups, irrespective to CB treatment (88.4 to 93.9%) were slightly higher than that obtained by Bogliolo *et al.* (2007a, 84.3%) following vitrification of GV ovine oocytes.

It has been reported that problems with cryopreserved oocytes are obvious around fertilisation (Horvath and Seidel 2008). Decreased fertilisation rates following cryopreservation may be partly attributed to alterations of the zona pellucida (Horvath and Seidel 2008). Carroll *et al.* (1990) reported lower fertilisation rates in frozen/thawed mouse oocytes as compared to control. Moreover, Tian *et al.* (2007) observed lower penetration rates following IVF of vitrified/thawed MII ovine oocytes than control, suggesting that, exposure of oocytes to vitrification solutions resulted in premature release of cortical granules and blockage of sperm entry. Earlier studies reported that, the actin cytoskeleton of the gametes during fertilisation process has been affected by cytochalasin with noticeable alterations in fertilisation (Rogers *et al.* 1989). In the present study, CB pre-treatment resulted in higher frequencies of fertilised oocytes in vitrified group (57%) than non treated (40.7%); however, the difference was not significant. The cleavage rates and development to blastocysts were significantly lower in both vitrified and CB-

vitrified oocytes as compared to other groups. However, in correlation to the numbers of cleaved embryos, no significant differences were observed in the frequencies of blastocyst development between vitrified, CB-vitrified and CB-control groups. In addition, no significant differences were noticed in terms of total cell numbers per blastocyst between treatment and control groups.

In the present study, the frequencies of in vitro fertilisation, cleavage, blastocyst formation, and the quality of blastocysts did not differ between the control and the toxicity groups. This indicates that high concentrations of cryoprotectants had no adverse effect on developmental competence of ovine oocytes. The reasons for improved results in toxicity trials may be due to the strictly controlled short exposure time to cryoprotectants during vitrification overcoming the detrimental effect of high concentrations of cryoprotectant.

These results suggest that, pre-treatment of immature ovine oocytes with7.5 μg/ml CB for 60 min has no harmful effects on viability and subsequent development following IVF. In contrast, Silvestre *et al.* (2006) and Bogliolo *et al.* (2007a) found negative effects of CB treatment on survival of vitrified/thawed GV ovine oocytes.

In conclusion, pre-treatment of immature ovine oocytes with the cytoskeleton stabilizer (CB) prior to vitrification improved the frequencies of in vitro fertilisation. In addition, the results show that cryopreservation of ovine GV ovine oocytes combined with CB pre-treatment resulted in good quality late stage pre-implantation embryo development following IVF. Further investigations are required to evaluate the effects of other chemicals which act as cytoskeleton stabilizers on the viability and developmental potential of vitrified/thawed ovine oocytes for example Taxol.

CHAPTER 8

EFFECT OF CAFFEINE TREATMENT ON SPINDLE CONFIGURATION, MPF/MAPK ACTIVITIES, AND SUBSEQUENT DEVELOPMENT OF OVINE OOCYTE-S VITRIFIED AT GERMINAL VESICLE (GV) STAGE

8.1 INTRODUCTION

Cryopreservation of immature ovine oocytes has been reported to decrease the levels of maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) after IVM (see **Chapter 6,** and Bogliolo *et al.* 2007a). MPF is a cyclin dependent serine/threonine protein kinase, its activation occurs in late G2 by de-phosphorylation of T14 and Y15 by cdc25 phosphatase (Taieb *et al.* 1997, **Fig 8.1**). Active MPF phosphorylates a range of proteins initiating entry into M-phase resulting in nuclear envelope breakdown, chromatin condensation, and microtubule organization (Verde *et al.* 1990). MAPKs are serine/threonine kinases activated by phosphorylation of the threonine and tyrosine residues (Posada and Cooper 1992). High levels of both kinases are responsible for the onset of germinal vesicle breakdown (GVBD) and required for the arrest of oocytes at MII stage (Kalous *et al.* 1993; Nurse 1990). MPF activity is regulated by a combination of cdc2 with cyclin B and phosphorylation of cdc2 at T14 and Y15.

Caffeine, a phosphodiesterase inhibitor has been reported to increase the activity of MPF by inducing the dephosphorylation of cdc2 at T14 and Y15 in pig oocytes (Kikuchi *et al.* 1999), cultured mammalian cells (Steinmann *et al.* 1991) and *Xenopus* eggs (Smythe and Newport 1992). Previous studies have been reported

that treatment of ovine oocytes with 10.0 mM caffeine from 18–24 hours post onset of maturation (hpm) increased the activities of both MPF and MAP kinases. Moreover, when used as cytoplast recipients for somatic cell nuclear transfer (SCNT), caffeine-treated oocytes increased the occurrence of NEBD and premature chromosome condensation (PCC) in the donor nucleus and improved the quality of blastocysts stage embryos obtained, in terms of total cell numbers (Lee and Campbell 2006).

Based on these observations, this study was designed to evaluate the effects of caffeine treatment during in vitro maturation on MPF/MAP kinases activities, chromatin and spindle organization, and subsequent in vitro embryo development following IVM/IVF/IVC of ovine oocytes previously vitrified at the GV stage.

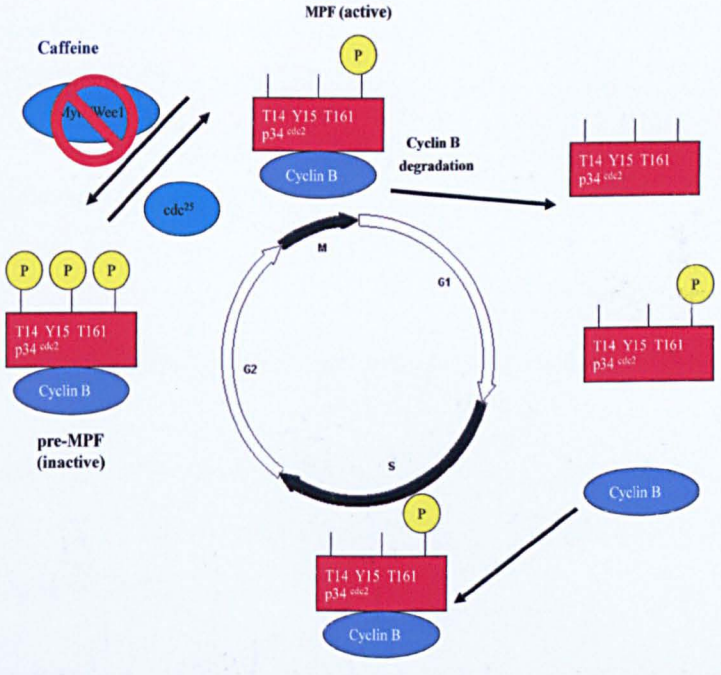


Figure 8.1 Activation of MPF. MPF activity is controlled by association of cdc2 with cyclin B and phosphorylation of cdc2 at T14 and Y15. Caffeine, a phosphodiestrase inhibitor increases the activity of MPF by inducing the dephosphorylation of cdc2 at T14 and Y15, and by inhibition of Myt1/Wee1 kinases.

8.2 MATERIALS AND METHODS

8.2.1 Oocyte vitrification and IVM

Cumulus oocyte complexes (COCs) obtained from slaughtered sheep ovaries (**Chapter 2.1**) were matured in vitro without any treatment (Control),following exposure to vitrification solutions without being plunged into liquid nitrogen (Toxicity), or vitrified using cryoloop as previously described (Vitrified, **Chapter 2.2.3**). Following vitrification and warming, viable oocytes were matured in vitro (**Chapter 2.3**).

8.2.2 Caffeine treatment

At 18 hpm, cumulus cells were removed from all groups by repeated pipetting of COCs in H–TCM 199/PVP containing 300 IU/ml hyaluronidase. After washing in maturation medium, oocytes from each group were incubated in maturation medium supplemented with (+) or without (-) caffeine (10 mM) for another 6 h till 24 hpm.

8.2.3 MPF/MAPK activities, spindle and chromosome configuration

At 24 hpm, analysis of MPF and MAPK activities were performed in all groups as described in (**Chapter 2.11**), spindle and chromatin morphology were evaluated as described in (**Chapter 2.4**).

8.2.4 In vitro fertilisation and culture (IVF/IVC)

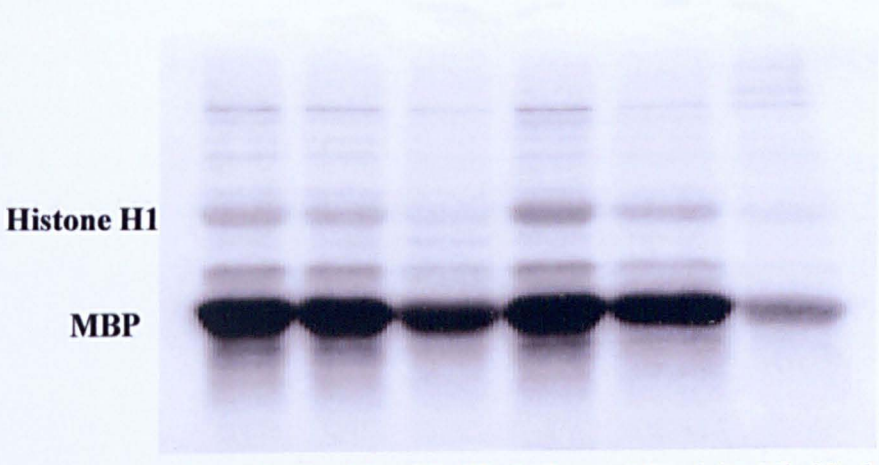
Following 24 h IVM, oocytes from all groups were fertilised and cultured as described in (**Chapter 2.5** and **2.8**).

8.3 RESULTS

8.3.1 Effect of caffeine treatment on MPF and MAP kinases activities

The effect of caffeine (10 mM, 18 to 24 hpm) treatment on MPF and MAP kinases activities of ovine oocytes vitrified and exposed to vitrification solutions at GV stage are presented in **Fig 8.1.** Supporting the previous results in **Chapter 6,** activities of both MPF and MAP kinases (24 hpm) were decreased in vitrified oocytes as compared to control; however, the activities were not affected in toxicity group (*t*-test). The activities of both kinases were decreased in vitrified oocytes treated with caffeine (vitrified +) than those non-treated (vitrified -). However, caffeine treatment increased the activities of both kinases in control oocytes as compared to untreated controls (**Fig 8.2**).

A



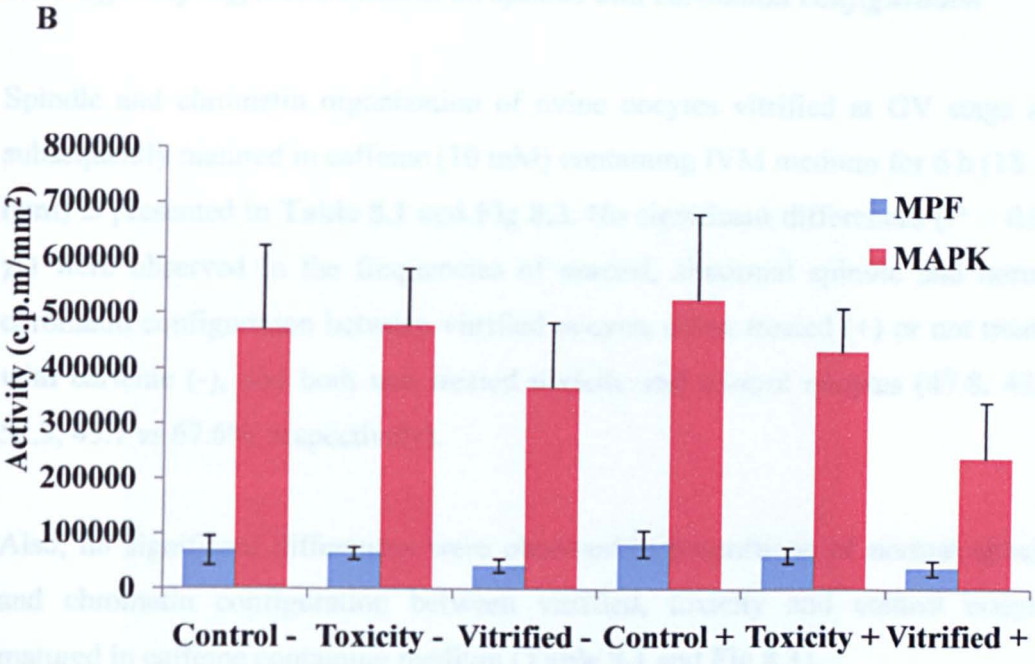


Figure 8.2 Effects of caffeine treatment on maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activities. Oocytes from different groups control, toxicity and vitrified were incubated in IVM medium supplemented with (+) or without (-) caffeine (10 mM, 18 – 24 hpm), at 24 hpm maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activities were evaluated. (**A**) Phospho-image of histone H1 (MPF) and myelin basic protein (MAPK) phosphorylation. (**B**) Relative activities (^{32}P incorporation [cpm/mm^2]) of MPF and MAPK kinases. Ten oocytes were analysed for each group, and three replicates were performed. Bars represent mean ±SEM.

8.3.2 Effect of caffeine treatment on spindle and chromatin configuration

Spindle and chromatin organization of ovine oocytes vitrified at GV stage and subsequently matured in caffeine (10 mM) containing IVM medium for 6 h (18–24 hpm) is presented in **Table 8.1** and **Fig 8.3.** No significant differences ($P > 0.05$, χ^2) were observed in the frequencies of normal, abnormal spindle and normal chromatin configuration between vitrified oocytes either treated (+) or not treated with caffeine (-), and both non treated toxicity and control oocytes (47.8, 43.8, 52.3, 45.7 *vs* 67.6%, respectively).

Also, no significant differences were observed in proportions of normal spindle and chromatin configuration between vitrified, toxicity and control oocytes matured in caffeine containing medium (**Table 8.1** and **Fig 8.3**).

The percentages of oocytes with missing spindles significantly increased in vitrified and toxicity groups and ranged from 23 to 28% as compared to control (7 to 9%). Frequencies of oocytes with abnormal chromosome significantly increased ($P < 0.05$) in both vitrified groups as compared to control oocytes (52.2 and 56.1 *vs* 22.7 and 32.4% in caffeine treated (+) and non-treated (-), respectively).

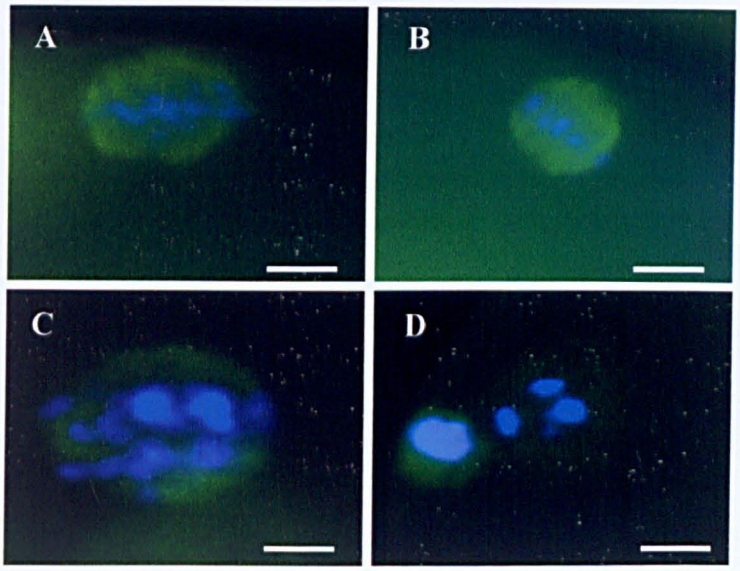


Figure 8.3 Spindle and chromatin configuration. The figure shows (**A** and **B**) normal barrel shaped metaphase II spindle (green) with compact chromosome (blue) arranged at the equator of the structure obtained following 24 h IVM of ovine oocytes vitrified at GV stage and matured either in caffeine containing medium (+) (10 mM, 18–24 hpm) or without caffeine (-) . (**C** and **D**) represent abnormal spindle configuration with dispersed chromatin. Scale bar = 50 μm.

Table 8.1 Effect of caffeine treatment on spindle and chromatin configuration of ovine oocytes vitrified at GV-stage

Treatment	*Caffeine treated*	*No. Oocytes*	*Spindle configuration*			*Chromosome configuration*	
			No. Normal (%)	*No. Abnormal (%)*	*No. Missing (%)*	*No. Normal (%)*	*No. Abnormal (%)*
Vitrified	(+)	46	22 (47.8%)^{ab}	11 (23.9%)^{a}	13 (28.3%)^{a}	22 (47.8%)^{ab}	24 (52.2%)^{a}
	(-)	57	25 (43.8%)^{a}	16 (28.1%)^{a}	16 (28.1%)^{a}	25 (43.8%)^{a}	32 (56.1%)^{a}
Toxicity	(+)	44	23 (52.3%)^{ab}	11 (25.0%)^{a}	10 (22.7%)^{ac}	23 (52.3%)^{ab}	21 (47.7%)^{ac}
	(-)	46	21 (45.7%)^{a}	13 (28.3%)^{a}	12 (26.1%)^{a}	21 (45.7%)^{a}	25 (54.3%)^{a}
Control	(+)	44	34 (77.3%)^{b}	6 (13.6%)^{a}	4 (9.1%)^{bc}	34 (77.3%)^{b}	10 (22.7%)^{b}
	(-)	102	69 (67.6%)^{ab}	26 (25.5%)^{a}	7 (6.8%)^{b}	69 (67.6%)^{ab}	33 (32.4%)^{bc}

^{a,b,c} different superscripts in the same column are significantly different ($P< 0.05$). (+) matured with caffeine (10 mM, 18–24 hpm), (-) without caffeine. Four replicates were carried out.

8.3.3 Effect of caffeine treatment on frequency of cleavage following IVF

The effect of caffeine treatment (10 mM, 18–24 hpm) on cleavage at 24 and 48 hpi of vitrified/thawed ovine immature oocytes are presented in **Table 8.2.** Cleavage was significantly lower ($P< 0.001$, χ^2) in vitrified (+ and -) at both 24 (2.8 and 4.3%) and 48 hpi (30.3 and 32.2%) as compared to other groups. At 24 hpi, frequencies of cleaved embryos were also decreased ($P< 0.05$) in toxicity (31.6 and 22.6%, + and -, respectively) and control (+, 31.5%) as compared to control (-, 45.2%). However, this trend was not observed at 48 hpi with cleavage values ranging from 58.1 to 78.6%, **Table 8.2.**

Table 8.2 Effect of caffeine treatment on cleavage rates following IVF of ovine oocytes vitrified at GV stage

Treatment	*Caffeine treated*	*No. Oocytes*	*No. Cleaved (%) hpi*	
			24	*48*
Vitrified	(+)	142	4 (2.8%)[a]	43 (30.3%)[a]
	(-)	115	5 (4.3%)[a]	37 (32.2%)[a]
Toxicity	(+)	98	31 (31.6)[b]	77 (78.6%)[b]
	(-)	93	21 (22.6%)[b]	54 (58.1%)[b]
Control	(+)	149	47 (31.5%)[b]	94 (63.1%)[b]
	(-)	96	52 (45.2%)[c]	67 (69.8%)[b]

Values with different superscripts in the same column are significantly different (a, b, c, $P< 0.05$). (+) oocytes matured in 10 mM caffeine for 6 h, (-) oocytes matured without caffeine.

8.8.4 Effect of caffeine treatment on subsequent development following IVF

Effects of caffeine treatment (10 mM, 18–24 hpm) on morula, blastocyst development and total cell numbers in blastocyst embryos after IVM/IVF/IVC of ovine vitrified/thawed GV-oocytes are presented in **Table 8.3** and **Fig 8.4.** Development to morula (day 5 pi) was significantly lower ($P< 0.05$, χ^2) in either vitrified groups (25.4 and 24% in + and -, respectively) than in toxicity (+, 45.1 %) and control (+, 41.6%) groups. However, no significant differences ($P> 0.05$) were observed between vitrified oocytes, toxicity (-, 35.5%), and control (-, 38.5%). Caffeine treatment resulted in higher frequencies of day 7 blastocyst stage embryos (4.2%) compared to non treated vitrified oocytes (1.7%); but the difference was not significant. These were significantly lower ($P< 0.001$, χ^2) than in other groups (30 – 32%).

Morphological evaluation of the blastocysts produced revealed no hatched blastocysts in either vitrified group; however, 12.2, 16.1, 16.1, and 8.3% were reported in toxicity and control oocytes (+ and -, respectively). The numbers of expanded blastocysts significantly decreased ($P< 0.05$, χ^2) in both groups of vitrified oocytes (0.7 and 1.7%) as compared to toxicity (+ and -) and control (-) groups (10.2, 12.9 and 11.5%, respectively, **Fig 8.4 A**). However, no significant differences were observed between vitrified (-) and control groups (+, 6.7%). Total cell numbers were significantly lower ($P< 0.05$, *t*-test) in vitrified (+) blastocysts (54 ± 8.5) as compared to other treatments (107.2 ± 5.9, 117.3 ± 6.2, 109.6 ± 5.4, and 100.6 ± 11.7, in toxicity and control + and -, respectively); however, this value did not differ significantly from those reported in vitrified (-, 87.0 ± 0.0) group (**Table 8.3** and **Fig 8.4 B, C** and **D**).

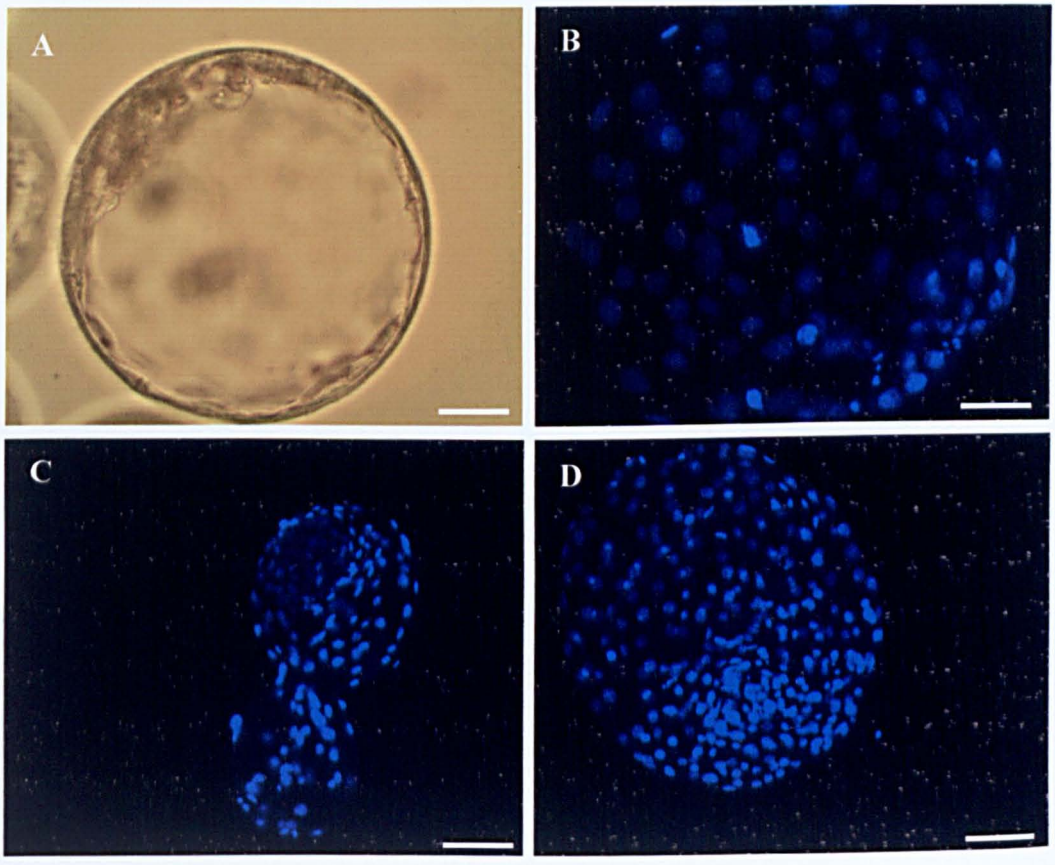


Figure 8.4 Day 7 ovine blastocysts produced by IVF. The figure shows day 7 blastocysts produced by IVF of ovine oocytes matured in IVM medium supplemented with 10 mM caffeine (18 to 24 hpm), (**A**) expanded blastocyst from vitrified oocytes, (**B, C** and **D**) blastocysts stained with Hoechst and produced from vitrified, toxicity and control oocytes, respectively. Scale bar = 50µm.

Table 8.3 Effect of caffeine treatment on subsequent development of ovine oocytes vitrified at GV stage

Treatment	*Caffeine treated*	*No. Oocytes*	*No. Morula (%)*	*No. Blastocysts (% of oocytes)*				*Total blastocysts (% of cleaved 48 hpi)*	*Total cell numbers/ blastocyst mean±SEM*
				Early	*Expanded*	*Hatched*	*Total*		
Vitrified	(+)	142	36 (25.4%)^{a}	5 (3.5%)^{ac}	1 (0.7%)^{a}	0 (0.0%)^{a}	6 (4.2%)^{a}	13.9%^{a}	54.0± 8.5^{a}
	(-)	115	28 (24.3%)^{a}	0 (0.0%)^{c}	2 (1.7%)^{ac}	0 (0.0%)^{a}	2 (1.7%)^{a}	5.4%^{a}	87.0± 0^{ac}
Toxicity	(+)	98	53 (45.1%)^{bc}	9 (9.2%)^{a}	10 (10.2%)^{b}	12 (12.2%)^{b}	31 (31.6%)^{b}	40.3%^{b}	107.2± 5.9^{bc}
	(-)	93	33 (35.5%)^{ac}	3 (3.2%)^{ab}	12 (12.9%)^{b}	15 (16.1%)^{b}	30 (32.2%)^{b}	55.5%^{b}	117.3± 6.2^{b}
Control	(+)	149	62 (41.6%)^{bc}	11 (7.4%)^{ab}	10 (6.7%)^{bc}	24 (16.1%)^{b}	45 (30.2%)^{b}	47.9%^{b}	109.6± 5.4^{bc}
	(-)	96	37 (38.5%)^{ac}	10 (10.4%)^{b}	11 (11.5%)^{b}	8 (8.3%)^{b}	29 (30.2%)^{b}	43.3%^{b}	100.6± 11.7^{bc}

Values with different superscripts in the same column are significantly different (a, b, c, $P< 0.05$). (+) oocytes matured with 10 mM caffeine for 6 h, (-) oocytes matured without caffeine. Four replicates were carried out.

8.4 DISCUSSION

Developing effective methods to cryopreserve mammalian oocytes has proven very challenging, with those from domestic species, notably cattle, pig, and sheep, being especially sensitive to cryopreservation procedures (Mullen *et al.* 2007). Many characteristics make mammalian oocytes particularly susceptible to cryoinjury, including the large amount of lipid in some taxa including ovine (McEvoy *et al.* 2000), large volume (Mullen *et al.* 2007), and the presence of MII spindle (Tharasanit *et al.* 2006a). Due to the inability of these cells to proliferate in culture and the relatively small number of oocytes obtainable from a given individual, the proportions that survive cryopreservation are important (Mullen *et al.* 2007).

As previously reported vitrification of ovine GV oocytes reduced the activities of MPF and MAP kinases as compared to fresh control oocytes (Bogliolo *et al.* 2007a). Previous studies reported that treatment of ovine oocytes with 10 mM caffeine 18–24 hpm increased the activities of both MPF and MAP kinases. Therefore, based on these observations, the present studies hypothesised that incubation of vitrified/thawed ovine GV oocytes with caffeine during IVM can restore the activities of MPF and MAPK, spindle and chromosome integrities, and subsequent development following IVF. Supporting the previous results in **Chapter 6**, vitrification of ovine GV oocytes decreased the activities of MPF and MAPK following 24 h IVM as compared to control oocytes. Caffeine treatment decreased the level of MPF and MAP kinases in vitrified oocytes than those vitrified and matured in vitro without caffeine treatment, but the difference between two groups was not significant. However, it increased the activities of both kinases in control group, confirming the previous results obtained by Lee and Campbell (2006). The reason for this is unclear, but it may be that caffeine has different action on vitrified oocytes than that on control. These observations require further investigation. One of the mechanisms that regulate MPF activity is the modification of the phosphorylation status of P^{34cdc2} kinase by Myt1/Wee1 and

cdc25. The addition of caffeine in culture medium suppresses Myt1/Wee1 activity, resulted in the inhibition of the shift from active MPF to pre-MPF, the inactive form (see **Fig 8.1**) and subsequent increase in MPF activity (Kikuchi *et al.* 1999). The mechanism of action of caffeine may differ in COCs and denuded oocytes. In COCs caffeine like other purine derivatives, possibly acts by blocking cAMP phosphodiesterase and cdc2 kinase, increasing cAMP levels and thus changing the signalling pathway (Chesnel *et al.* 1994). Regarding toxicity group (oocytes exposed to vitrification solutions without freezing), the activities of both kinases were not affected in this group as compared to control oocytes, indicating that vitrification and warming are responsible for the alterations in the activities of both kinases, not the cryoprotectants. In contrast to the observations presented here, Bogliolo *et al* (2007b) reported that caffeine treatment can increase MPF and MAP kinases activities in vitrified MII ovine oocytes and thus contributed towards maintaining a regular chromatin organization in the metaphase plate and also preventing parthenogenetic activation. Discrepancies between these results may relate to, the differences in stage at which oocytes were vitrified (MII *vs* GV), vitrification methods (cryotop *vs* cryoloop) or caffeine treatment regime. In the study by Bogliolo *et al* oocytes were treated with 20 mM caffeine for 2 h (21 to 23 hpm) and then vitrified and warmed. However, in the studies reported here, oocytes were vitrified at GV stage, incubated in maturation media until 18 hpm and then cultured in IVM medium supplemented with 10 mM caffeine for 6 h (Lee and Campbell 2006). It has been reported that, treatment of oocytes with caffeine at a concentration higher than 10 mM and for a period over 6 h decreases oocyte viability (Lee and Campbell 2006). Previous studies reported lower MPF kinase activity in ovine oocytes either obtained from mature or pre-pubertal ewes vitrified and exposed to vitrification solutions at MII stage (Succu *et al.* 2007 a, b), despite, the activity was restored after 2 h culture in oocytes obtained from mature animals but not from pre-pubertal ones. Bogliolo *et al.* (2007a) found that denudation of ovine COCs before vitrification decreases MPF and MAPK activities; however, these activities were not affected if oocytes vitrified while cumulus layer intact.

In the present study, the effects of caffeine treatment on spindle and chromosome morphology of vitrified, toxicity, and control oocytes were evaluated. The results show that, caffeine treatment did not affect the proportions of oocytes with normal spindles and chromatin organization. The effects of caffeine treatment on spindle morphology of mouse oocytes have been studied by Miao *et al.* (2007) and the results showed that oocytes have various spindle morphologies in relation to caffeine concentrations. Higher percentages of normal morphology were observed in oocytes treated with 5 or 10 mM caffeine as compared to those treated with 0 or 1 mM (Miao *et al.* 2007).

In the present study, the effect of caffeine (10 mM, 18-24 hpm) treatment on cleavage and subsequent in vitro embryo development of vitrified/thawed GV, toxicity, and control oocytes was assessed. The results showed that, caffeine treatment did not affect cleavage (24 and 48 hpi), morula development (day 5pi) in vitrified, toxicity, and control groups (**Table 8.2**). However, it increased the percentage of day 7 blastocyst stage embryos in vitrified group as compared to non treated oocytes (**Table 8.3**). Irrespective to caffeine treatment, the developmental rates in vitrified oocytes were significantly lower as compared to toxicity and control groups. A part from the various injuries that occur to the oocytes during vitrification and warming, the lower developmental potential reported here could be related to the removal of cumulus cells before fertilisation, however, these effects were not observed in toxicity and control oocytes. Thus, the results suggest that presence of cumulus cells during IVF of vitrified sheep oocytes is necessary to maintain the integrity of oocytes and subsequent development. Bogliolo *et al.* (2007a) observed that cumulus cells behave differently and display distinct morphologies, showing a lower grade of physical interaction with the oocytes, when cultured with vitrified denuded oocytes compared to the control group. Previous studies reported that denudation of ovine oocytes at 24 hpm followed by immediate fertilisation decreases the frequencies of cleaved oocytes (Maalouf *et al.* 2009). Herein, although caffeine treatment reduced MPF and MAP kinases activities and did not affect the cleavage rates in vitrified oocytes, it did increase

the frequencies of blastocyst stage embryos, suggesting that caffeine increased the developmental potential of vitrified oocytes by mechanisms other than elevation of MPF and MAPK activities. Caffeine has been reported to extend the fertilisation period of in vitro matured ovine oocytes by reducing polyspermy and retaining the ability to develop to blastocyst (Maalouf *et al.* 2009) other studies demonstrated that caffeine treatment has effects at an epigenetic level, histone acetylation in aged mouse oocytes was reduced (Huang *et al.* 2007). The percentage of blastocysts reported in these studies in vitrified oocytes matured in caffeine containing IVM medium (+, 14%) as calculated per cleaved embryo was slightly higher than that reported by Succu *et al.* (2007a) following IVF of vitrified/thawed ovine MII oocytes (12.5%). This indicates an improvement in vitrification protocol of GV oocytes. Total cell numbers were decreased in blastocysts obtained from vitrified (+) oocytes as compared to those obtained following IVM/IVF/IVC of vitrified oocytes matured in vitro without caffeine (-), but the difference was not significant. However, the value in vitrified (+) group was significantly lower than in toxicity and control groups. No significant differences were noticed between vitrified (-), toxicity (+) and control blastocysts (**Table 8.3**). Maalouf *et al.* (2009) observed no effects of caffeine treatment on the quality of in vitro produced ovine blastocysts in terms of mean cell numbers or ICM: TE ratio.

In conclusion, GV-stage ovine oocytes vitrified with cryoloop can subsequently mature in vitro without any effects on spindle configuration and subsequently following fertilisation and development to the blastocyst stage. Caffeine treatment of ovine oocytes vitrified at GV stage increased the frequencies of blastocyst development, but no effects on the quality of in vitro produced blastocysts. The results suggest that, caffeine improved blastocyst rates in vitrified oocytes by mechanisms other than an increase in MPF and MAP Kinases activities. Further studies are required to evaluate the effects of different caffeine concentrations on developmental competence of vitrified oocytes following IVF, parthenogenetic activation, and SCNT.

CHAPTER 9

EFFECT OF DEMECOLCINE PRE-TREATMENT ON VIABILITY, TIMING OF FIRST POLAR BODY EXTRUSION, SPINDLE CONFIGURATION, AND SUBSEQUENT DEVELOPMENT OF OVINE OOCYTES VITRIFIED AT GERMINAL VESICLE (GV) STAGE

9.1 INTRODUCTION

Mammalian oocytes remain one of the most difficult cell types to successfully cryopreserve (Men *et al.* 2003a). Stabilizing the cytoskeleton structure during vitrification could be beneficial for improving the post-thaw survival and subsequent development of vitrified oocytes. Several cytoskeleton stabilizers have been used to reduce injury to oocytes and embryos during vitrification including cytochalasins (**Chapter 7**) and taxol (Morato *et al.* 2008a, b; Zhang *et al.* 2009). Another chemical, demecolcine (Deme), a cytoskeletal modifier has been widely used in nuclear transfer (NT) studies to increase the efficiency of enucleation in different species including pig (Yin *et al.* 2002), cattle (Russell *et al.* 2005; Tani *et al.* 2006), mice (Ibanez *et al.* 2003), goat (Lan *et al.* 2008), and sheep (Hou *et al.* 2006). Moreover, incubation of ovine GV oocytes with demecolcine has been reported to induce germinal vesicle breakdown (GVBD) and chromosome condensation which subsequently led to meiosis resumption (Hou *et al.* 2006). Demecolcine has also shown to increase MPF and MAPK activities in cattle and goat oocytes (Tani *et al.* 2006; Lan *et al.* 2008). However, no studies have been reported the effects of demecolcine pre-treatment on viability of vitrified/thawed oocytes.

The objectives of these studies were to investigate the effects of demecolcine pre-treatment on viability, timing of first polar body (PBI) extrusion, spindle, chromatin configuration and subsequent development of vitrified/thawed ovine GV oocytes following IVM and IVF or IVM and parthenogenetic activation.

9.2 MATERIALS AND METHODS

9.2.1 Demecolcine treatment, vitrification, and in vitro maturation (IVM)

COCs obtained from mature ewes were randomly divided into three groups (1) untreated control, (2) vitrified, and (3) Deme + vitrified (oocytes were incubated with 0.1 μg/ml demecolcine in TCM-199 medium supplemented with 10% FBS for 20 min at 39°C prior to vitrification). Following vitrification and warming (**Chapter 2.2.3**), oocytes were matured in vitro for 24 h as previously described (**Chapter 2.3**). At 24 hpm (hour post onset of maturation), oocytes meiotic spindle and chromatin morphology was examined as previously described (**Chapter 2.4**).

9.2.2 In vitro fertilisation and culture (IVF/IVC)

24 hpm, oocytes from three groups were fertilised in vitro and cultured for 7 days in C.SOF medium as previously described (**Chapter 2.5** and **2.8**).

9.2.3 Parthenogenetic activation

24 hpm, in vitro matured oocytes were activated by 10 mM $SrCl_2$ (strontium chloride) and 7.5 μg/ml CB (cytochalasin B) in calcium free CZB medium for 4–5 h at 39°C and a humidified atmosphere of 5% CO_2 in air. Following activation, oocytes were cultured in vitro for 7 days as previously described (**Chapter 2.8**).

9.3 RESULTS

9.3.1 Effect of Deme pre-treatment on viability of vitrified oocytes

The effect of Deme pre-treatment on viability of vitrified/thawed GV ovine oocytes is presented in **Fig 9.1.** No significant (χ^2) differences were observed in the proportions of viable oocytes between deme+vitrified and vitrified groups (90.8%, 324/357 and 87.2%, 211/242, respectively).

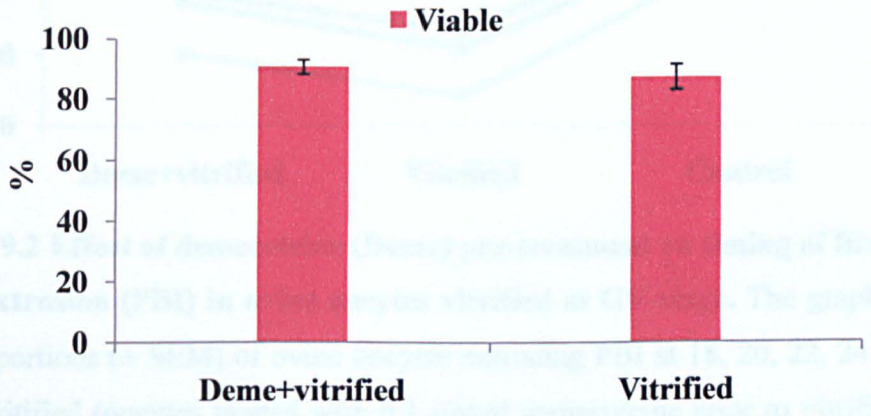


Figure 9.1 Effect of demecolcine (Deme) pre-treatment on viability of ovine oocytes vitrified at GV-stage. The graph shows the percentage (± SEM) of viable and in deme+vitrified (oocytes treated with 0.1 μg/ml demecolcine for 20 min prior to vitrification) and vitrified groups. Six replicates were carried out.

9.3.2 Timing of first polar body (PBI) extrusion

The effects of demecolcine pre-treatment on kinetics of PBI extrusion in ovine vitrified/thawed GV oocytes are presented in **Fig 9.2** and **Fig 9.3**. Numbers of oocytes showing PBI were evaluated at 18, 20, 22, and 24 hpm. At 18 hpm, the proportion of oocytes with PBI was significantly higher ($P< 0.05$, χ^2) in deme+vitrified (20.4%, 20/98) oocytes as compared to non-treated group (8.5%, 10/118); however, these percentages were significantly lower ($P< 0.01$) than

control (47.1%, 41/87). Demecolcine pre-treatment also increased the numbers of oocytes with PBI at 20, 22, and 24 hpm (34.7, 37.8, and 51.0%, respectively, **Fig 9.2** and **Fig 9.3**) as compared to the oocytes that vitrified without treatment (22.0, 27.1, and 43.2%, respectively), but, the differences were not significant. These percentages were significantly lower ($P< 0.01$) than control (55.2, 58.6, and 88.5%).

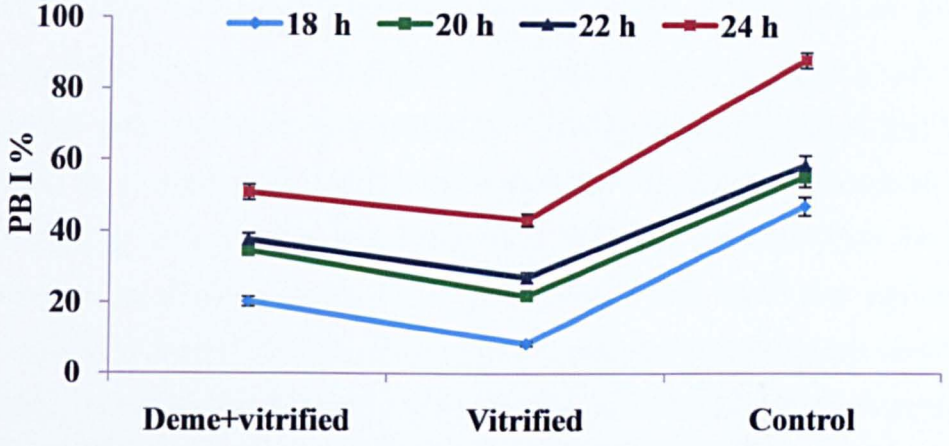


Figure 9.2 Effect of demecolcine (Deme) pre-treatment on timing of first polar body extrusion (PBI) in ovine oocytes vitrified at GV-stage. The graph shows the proportions (± SEM) of ovine oocytes extruding PBI at 18, 20, 22, 24 hpm in deme+vitified (oocytes treated with 0.1 µg/ml demecolcine prior to vitrification), vitrified, and control groups. Four replicates were performed.

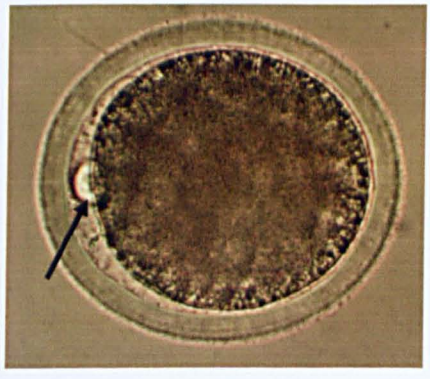


Figure 9.3 Ovine oocyte showing first polar body (PBI) following 24 h IVM. The figure shows 24 h in vitro matured oocyte with PBI (arrow) obtained from vitrified GV ovine oocytes.

9.3.3 Effect of Deme pre-treatment on spindle and chromosome organization of ovine oocytes vitrified at GV stage

Effects of demecolcine pre-treatment prior to vitrification of immature ovine oocytes on spindle and chromatin configuration 24 hpm are summarized in **Table 9.1** and **Fig 9.4.** Percentage of oocytes with normal spindle and chromatin configuration was significantly decreased ($P< 0.05$, χ^2) in vitrified groups as compared to control (42.5 and 41.8 *vs* 76.5%). Numbers of oocytes with missing spindles were significantly increased ($P< 0.001$) in deme+vitrified and vitrified groups as compared to control (47.5 and 32.7 *vs* 3.9%). Abnormal spindle morphology was significantly lower ($P< 0.05$) in deme+vitrified oocytes as compared to vitrified group (10.0 *vs* 25.5%). These were not significant as compared to control (19.6%). Proportions of oocytes with abnormal chromosome organization were significantly higher ($P< 0.05$) in vitrified groups as compared to control (57.5 and 58.2 *vs* 32.5% **Table 9.1** and **Fig 9.4**).

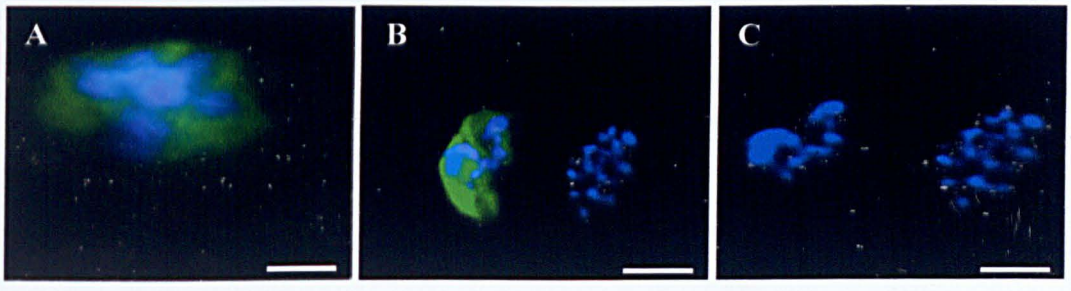


Figure 9.4 Spindle and chromatin configuration of ovine oocytes following 24 IVM. The figure shows (**A**) normal barrel shape metaphase II spindle (green) with compact chromosome (blue) arranged at the equator of the structure obtained 24 hpm of ovine oocytes vitrified at GV stage and treated with 0.1 µg/ml demecolcine prior to vitrification. (**B** and **C**) show abnormal spindle with dispersed chromatin. Scale bare = 50 µm.

Table 9.1 Effect of demecolcine (Deme) pre-treatment on spindle and chromatin configuration of ovine oocytes vitrified at GV-stage.

Treatment	*No. Oocytes*	*Spindle configuration*			*Chromosome configuration*	
		No. Normal (%)	*No. Abnormal (%)*	*No. Missing (%)*	*No. Normal (%)*	*No. Abnormal (%)*
Deme +Vitrified	40	17 (42.5%)[a]	4 (10.0%)[ac]	19 (47.5%)[a]	17 (42.5%)[a]	23 (57.5%)[a]
Vitrified	55	23 (41.8%)[a]	14 (25.5%)[b]	18 (32.7%)[a]	23 (41.8%)[a]	32 (58.2%)[a]
Control	51	39 (76.5%)[b]	10 (19.6%)[bc]	2 (3.9%)[b]	39 (76.5%)[b]	12 (23.5%)[b]

[a,b,c] different superscripts in the same column are significantly different ($P< 0.05$). Four replicates were performed.

9.3.4 Effect of Deme pre-treatment on in vitro embryo development following IVM/IVF/IVC of ovine oocytes vitrified at GV stage

Effects of demecolcine pre-treatment (0.1 μg/ml for 20 min), prior to vitrification of ovine GV oocytes on cleavage (24 and 48 hpi), morula (day 5 pi), blastocyst development (day 7 pi) and total cell numbers in blastocyst stage embryos are presented in **Table 9.2** and **Fig 9.5**.Cleavage at 24 and 48 hpi and morula development were significantly lower ($P< 0.01$, χ^2) in deme+vitrified (6.1, 43.1, and 28.5%) and vitrified groups (3.3, 30.1, and 22.9 %) as compared to control (50.4, 82.4, and 46.4%). Blastocyst development in deme+vitrified (9.8%) and control (33.6%) was significantly higher ($P< 0.01$) than in vitrified group (1.3%). The same trend was observed, when the numbers of blastocyst calculated as a percentage from cleaved embryos (22.6 and 40.8 *vs* 4.3%, in deme+vitrified, control, and vitrified groups, respectively, **Table 9.2**).

Hatched blastocysts were significantly lower ($P< 0.05$) in deme+vitrified oocytes as compared to control group (4.9 *vs* 12.8%, **Fig 9.5 A**), however, no hatched blastocysts developed in vitrified oocytes that not treated with deme prior to vitrification. Expanded blastocysts were significantly lower ($P< 0.05$) in treated groups as compared to control (1.6 and 1.3 *vs* 13.6%). No significant differences (*t*-test) were observed in total cell numbers between groups (97.0 ± 13.3, 91.0 ± 4.0, and 106.0 ± 5.1 in deme+vitrified, vitrified, and control, respectively, **Table 9.2** and **Fig 9.5 B**).

Table 9.2 Effect of demecolcine (Deme) pre-treatment on in vitro embryo development following IVM/IVF/IVC of ovine oocytes vitrified at GV-stage.

Treatment	*No. Oocytes*	*No. Cleaved (%) hpi*		*No. Morula (%)*	*No. Blastocysts (% of oocytes)*				*Blastocysts (% of cleaved 48 hpi)*	*Total cell numbers/ blastocyst mean ± SEM*
		24	*48*		*Early*	*Expanded*	*Hatched*	*Total*		
Deme +Vitrified	123	10 (6.1%)[a]	53 (43.1%)[a]	35 (28.5%)[a]	3 (2.4%)[ab]	2 (1.6%)[a]	6 (4.9%)[a]	12 (9.8%)[a]	22.6%[a]	97.0 ±13.3
Vitrified	153	5 (3.3%)[a]	46 (30.1%)[a]	35 (22.9%)[a]	0 (0.0%)[b]	2 (1.3%)[a]	0 (0.0%)[b]	2 (1.3%)[b]	4.3%[b]	91.0 ± 4.0
Control	125	63 (50.4%)[b]	103 (82.4%)[b]	58 (46.4%)[b]	9 (7.2%)[a]	17 (13.6%)[b]	16 (12.8%)[c]	42 (33.6%)[c]	40.8%[c]	106 ± 5.1

Values with different superscripts in the same column are significantly different at $P< 0.05$. Four replicates were performed.

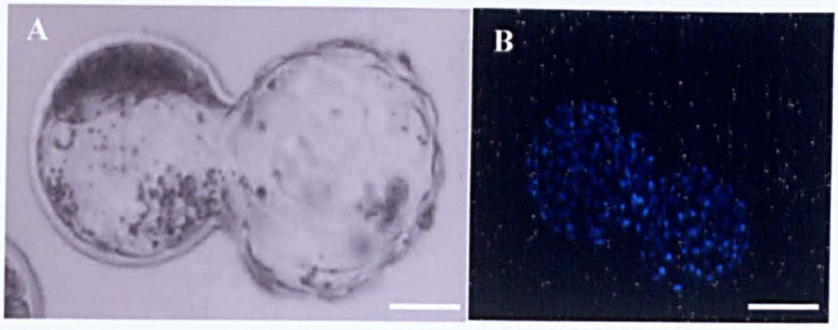


Figure 9.5 Day 7 in vitro produced blastocysts. The figure shows (**A**) day 7 hatched blastocyst (**B**) stained with Hoechst and produced by IVM/IVF/IVC of ovine oocytes vitrified at GV stage and treated with 0.1 µg/ml demecolcine for 20 min before vitrification. Scale bar = 50 µm.

9.3.5 Effect of Deme pre-treatment on parthenogenetic development of ovine oocytes vitrified at GV stage

Cleavage at 24 and 48 hpa in deme+vitrified (10.3 and 40.7%) and control groups (52.5 and 76.7%) were significantly higher ($P< 0.05$, χ^2) than in vitrified oocytes (3.3 and 30.1%). The same trend was observed for morula development (26.2 and 48.4 *vs* 9.9% in deme+vitrified, control, and vitrified groups, respectively).

Development to Blastocyst stage embryos (day 7 pa) was higher in deme+vitrified oocytes (4.8%) as compared to vitrified group (1.8%); but the difference was not significant ($P> 0.05$). These values were significantly lower ($P< 0.001$) as compared to control group (24.2%). Lower proportions of hatched blastocysts developed in vitrified groups as compared to control (**Table 9.3**). However, total cell numbers per blastocyst were not significant (84.0 ± 7.0, 86.0 ± 4.0, and 95.1 ± 5.6 in deme+vitrified, vitrified, and control groups, respectively).

Table 9.3 Effect of demecolcine (Deme) pre-treatment on parthenogenetic development of vitrified/thawed ovine immature oocytes.

Treatment	*No. Oocytes*	*No. Cleaved (%) hpa*		*No. Morula (%)*	*No. Blastocysts (% of oocytes)*				*Blastocysts (% of cleaved 48 hpa)*	*Total cell numbers/ blastocyst mean ± SEM*
		24	*48*		*Early*	*Expanded*	*Hatched*	*Total*		
Deme +Vitrified	145	15 (10.3%)[a]	59 (40.7%)[a]	38 (26.2%)[a]	3 (2.1%)[a]	3 (2.1%)[a]	1 (0.7%)[a]	7 (4.8%)[a]	11.9%[a]	84 ± 7.0
Vitrified	111	4 (3.6%)[b]	26 (23.4%)[b]	11 (9.9%)[b]	1 (0.9%)[a]	0 (0.0%)[a]	1 (0.9%)[a]	2 (1.8%)[a]	7.7%[a]	86 ± 4.0
Control	219	115 (52.5%)[c]	168 (76.7%)[c]	106 (48.4%)[c]	21 (9.6%)[b]	21 (9.6%)[b]	11 (5.0%)[b]	53 (24.2%)[b]	31.5%[b]	95.1 ± 5.6

Values with different superscripts in the same column are significantly different at $P< 0.05$. Four replicates were performed.

9.4 DISCUSSION

Demecolcine, a cytoskeletal modifier, has been reported to improve the efficiency of enucleation in NT (Yin *et al.* 2002; Russell *et al.* 2005; Tani *et al.* 2006) and induce GVBD and chromosome condensation in GV oocytes (Hou *et al.* 2006). In these studies, the effect of Deme pre-treatment on timing of PBI extrusion in vitrified/thawed ovine GV oocytes was evaluated. The results demonstrate that lower percentage (8.5%) of vitrified oocytes displayed PBI extrusion at 18 hpm; however, this value significantly increased to 20.4% when oocytes were treated with demecolcine prior to vitrification. The results suggest that vitrification of immature oocytes results in delay of PBI extrusion and demecolcine pre-treatment improve this delay. Also, Deme pre-treatment increased the numbers of oocytes with PBI at 20, 22 and 24 hpm. Cooling of GV oocytes to 0 or 4°C has been reported to affect meiotic spindle formation and polar body extrusion although the telophase I spindle divided normally into two chromosomes sets (Wu *et al.* 1999). Previous studies reported that PBI is not normally extruded following IVM of vitrified GV oocytes even after separation of chromosomes (Abe *et al.* 2005), indicating that vitrified oocytes have the ability to mature normally to telophase I stage; however, damage to some regulatory factors can be occurred which subsequently influence the extrusion of PBI (Wu *et al.* 1999). High numbers of oocytes with no visible PBI was also reported following vitrification of IVM buffalo oocytes (Boonkusol *et al.* 2007); this was due to alterations in spindle fibre formation. A delay in maturation was also reported following vitrification of GV equine oocytes (Hochi *et al.* 1994). The role of demecolcine for the increase in the rates of PBI extrusion in vitrified oocytes is unclear; and requires further investigation. Previous studies reported that incubation of ovine GV ovine oocytes with demecolcine induced GVBD, chromosome condensation and subsequent resumption of meiosis (Hou *et al.* 2006). Earlier studies also showed that demecolcine can induce germinal vesicle migration in amphibians and gold fish oocytes (Habibi and Lessman 1986). Alterations in spindle and chromosome configuration following oocyte cryopreservation have been extensively studied in

different species (Chen *et al.* 2003; Ledda *et al.* 2007; Morato *et al.* 2008 a, b, c, d). In these studies, Numbers of oocytes with normal spindles were significantly decreased in vitrified groups as compared to control. Although, Demecolcine pre-treatment decreased the numbers of oocytes with abnormal spindle morphology in vitrified group, it increased the numbers of oocytes with missing spindle. The exact explanation to this observation is unclear; but, demecolcine as a microtubule inhibitor, can bind to tubulin dimmers and prevent microtubule polymerization, thus, resulting in the loss of dynamic spindle microtubules (Ibanez *et al.* 2003; Russell *et al.* 2005). In the present study, the effect of demecolcine pre-treatment on subsequent development following IVF and parthenogenetic activation of vitrified oocytes was also investigated. Demecolcine pre-treatment increased the frequencies of cleavage, morula and blastocyst development in vitrified oocytes as compared to non treated vitrified group. The higher developmental frequencies in demecolcine treated oocytes may be due to the higher frequencies of PBI extrusion in treated oocytes which subsequently improve maturation, fertilisation and subsequent development. In addition, demecolcine has been reported to increase MPF and MAPK activities in cattle and goat oocytes, respectively (Tani *et al.* 2006; Lan *et al.* 2008), but this was not studied in sheep, which requires further investigations.

In conclusion, vitrification of GV-stage oocytes delays the kinetics of in vitro maturation as determined by extrusion of PBI; pre-treatment of oocytes with demecolcine prior to vitrification enhances this delay. Moreover, pre-treatment of vitrified oocytes with demecolcine could improve the developmental potential of ovine vitrified/thawed GV-stage oocytes. Further studies are required to evaluate the effects of different concentrations of demecolcine on viability of vitrified oocytes and to examine the incidence of chromosomal abnormalities in the blastocysts produced from oocytes pre-treated with demecolcine.

CHAPTER 10
GENERAL DISCUSSION

Oocyte cryopreservation has the potential to be an important adjunct to assisted reproductive technologies (ART) in human and domestic animals. Long-term storage of oocytes from patients in danger of losing ovarian function is one of the most important reasons for cryopreservation of human oocytes, and it extends to highly valuable females of any species. In animals, oocyte preservation permits genetic material from a female to be stored unfertilised until she expresses her potential and the appropriate mate can be selected. However, despite significant recent progress, the efficiency of oocyte cryopreservation is still very low. In contrast to cryopreservation of sperm and embryos, with oocytes the entity is a single, large, highly specialized cell that has to survive the procedure to be able to undergo fertilisation and cleavage. Oocytes are susceptible to a range of damages due to exposure to low temperature including rupture of intercellular communication between the cumulus cells and oocyte, premature release of cortical granules, hardening of the zona pellucida, change in lipid bilayers or lysis of oocyte membranes, damage to the structure of cytoskeleton or mitochondria, damage to cytoplasmic organelles such as smooth endoplasmic reticulum, decreasing the activities of maturation promoting factor (MPF) and mitogen activated protein kinase (MAPK), disorganization of meiotic spindle, loss or clumping of microtubules, scattered chromosomes, and subsequent decrease in fertilisation and developmental rates (Hochi *et al.* 1998; Wu *et al.* 1999, Men *et al.* 2003a, b; Abe *et al.* 2005; Albarracin *et al.* 2005a, b; Bogliolo *et al.* 2007a; Succu *et al.* 2007a, b). The success of oocyte cryopreservation depends on several factors include size, stage of maturity, membrane permeability, oocyte quality, presences or absences of cumulus cells, species, and other factors related to the cryopreservation procedures (Fabbri 2006). The most encouraging results in domestic animals have been reported in cattle, where cryopreservation of immature and mature oocytes has been resulted in healthy offspring following in

vitro fertilisation (IVF) and culture (Vieira *et al.* 2002; Papis *et al.* 2000). However, few experiments have been conducted on small ruminants, especially with sheep (Bogliolo *et al.* 2007a). These studies were designed to establish a technique for oocyte cryopreservation which would support subsequent development following different in vitro techniques. Sheep were chosen as an experimental model.

Currently, there are two techniques used for oocyte cryopreservation slow-freezing and vitrification. Vitrification is less harmful to the cells, such as oocytes and embryos whose chilling sensitivity is high, than conventional methods of slow-freezing (Martino *et al.* 1996b, Vajta *et al.* 1998). The key aim of any vitrification protocol is to achieve high cooling rates in combination with high concentrations of cryoprotectants (Liebermann and Tucker 2002). A balance between maximizing cooling rate and minimizing the cryoprotectant concentrations is important to minimize the osmotic or chemical toxicity that occur by using high concentrations of cryoprotectants.

The effects of exposure of immature ovine oocytes to different combinations of cryoprotectant on viability and subsequent in vitro maturation (IVM) were evaluated (**Chapter 3**). The viability of oocytes was not affected by the types and concentrations of cryoprotectants. However, following IVM, significantly lower maturation rates (16.1%) were observed in oocytes exposed to VSII (25% ethylene glycol (EG) + 25% DMSO) as compared to those exposed to VSI (20% EG + 20% DMSO, 50.0%) or to VSIII (25% EG + 25% glycerol, 45.1%). The results indicate that a combination of 20% EG and 20% DMSO can be used effectively for the vitrification of ovine oocytes.

As discussed earlier, high cooling rate remains the key factor for successful oocyte vitrification, various devises have been used to decrease the volume of vitrification solutions and subsequently increase the cooling rates (**Chapter 1.6.1.3**). Solid surface vitrification (SSV) and cryoloop share the advantages of using a

containerless system and small volumes of solution (less than 1 µl) which favours rapid heat exchange during cooling, therefore, it prevents chilling injury and cracking of zona pellucida (Dinnyes *et al.* 2000; Lane *et al.* 1999a,b). In the present study, recovery, viability, frequencies of maturation, fertilisation, cleavage and subsequent development following IVM/IVF/IVC of ovine oocytes vitrified at GV stage using SSV or cryoloop were determined (**Chapter 4** and **Chapter 5**). Following SSV, 75.7% of vitrified oocytes were recovered; of those oocytes recovered 74.8% were viable. Maturation, fertilisation, cleavage and blastocyst development were significantly decreased in SSV vitrified oocytes as compared to fresh control (**Chapter 4**). Following cryoloop vitrification, 82.6% of vitrified/thawed oocytes were recovered and 72.6% were viable. Frequencies of maturation (oocytes at MII stage) and fertilisation following IVM/IVF did not differ significantly between vitrified and control groups. However, polyspermic fertilisation significantly ($P< 0.001$) increased in vitrified oocytes as compared to control (17.0 *vs* 3.5%, **Chapter 5**). Previous studies reported that exposure of oocytes to cryoprotectants and cryopreservation resulted in clustering, degeneration, loss, and premature release of cortical granules and subsequent zona hardening which can lead to a defective blocking of polyspermy (LeGal and Massip 1999; Tian *et al.* 2007). Cleavage (24 and 48 hpi, hours post insemination), morula (day 5pi), and blastocyst (day 7pi) development were significantly decreased in cryoloop vitrified oocytes as compared to non vitrified control groups. High frequencies of cleaved embryos developed to blastocyst stage (29.4%) in vitrified group (**Chapter 5**) indicating that even though some polyspermy almost certainly occurred, this may not have been a major problem. Previous studies reported that mouse oocytes frozen and thawed in medium containing foetal bovine serum (FBS) could be fertilised and develop at frequencies similar to control (Carroll *et al.* 1993). In the present study, the oocytes were handled in vitrification and thawing media contain FBS that might be responsible for similar developmental potential of vitrified oocytes as compared to control. The results presented here suggest that exposure of oocytes to SSV (**Chapter 4**) and cryoloop (**Chapter 5**) vitrification solutions (Toxicity control)

did not affect viability, maturation, fertilisation and subsequent development as similar frequencies as compared to control were achieved. The reasons for improved the results in toxicity trials may be due to the strictly controlled short exposure time to cryoprotectants during vitrification overcoming the detrimental effect of high concentrations of cryoprotectant. Technical difficulties associated with SSV (controlling the size of the droplets) may explain the lower developmental frequencies in SSV vitrified oocytes as compared to those vitrified using cryoloop.

Parthenogenetic activation and nuclear transfer (NT) could be an alternative approaches to evaluate the developmental potential of vitrified oocytes. Here, the effectiveness of two protocols (1) a combination of calcium ionophore (A 23187), cycloheximide and cytochalasin B (CA+CHX/CB), (2) strontium and CB (Sr/CB) on parthenogenetic activation of vitrified/thawed ovine GV oocytes was evaluated. Development of somatic cell nuclear transfer (SCNT) embryos using ovine oocytes vitrified at GV stage as cytoplast recipients was also evaluated (**Chapter 6**). No blastocyst stage embryos developed in vitrified oocytes activated by CA+CHX/CB; however, 3.8% were obtained following Sr/CB activation. The results indicate that strontium is efficient in parthenogenetic activation of vitrified/thawed ovine oocytes. Following SCNT, although the frequencies of cleaved embryos were significantly decreased in vitrified oocytes as compared to control group, development to morula and blastocyst stage embryos was not significantly different. These results indicate that ovine oocytes vitrified at GV stage can be used as cytoplast recipients for SCNT and produce high frequencies of good quality blastocyst embryos. This may be due to various factors including vitrification protocol, cryoprotectant combinations, activation method, oocyte quality, and the stage of oocyte enucleation anaphase-I/telophase-I (AI/TI). Previous studies reported that enucleation at AI/TI removes significantly less cytoplasm than enucleation at MII which is beneficial for the developmental competence of the recipient cytoplast (Lee and Campbell 2006).

Cytoskeleton stabilization using cytochalasin B (CB) can be a possible approach to improve the cryotolerance of vitrified oocytes (Vieira *et al.* 2002). The effects of CB pre-treatment prior to vitrification (7.5 μg/ml for 60 min) on viability, fertilisation and subsequent development following IVM/IVF/IVC of vitrified/thawed ovine GV oocytes were evaluated (**Chapter 7**). No significant differences were observed in survival rates between CB treated and non treated oocytes in both vitrified and toxicity groups. CB treatment resulted in higher fertilisation rates in vitrified oocyte as compared to non treated group (57.0 *vs* 40.7%), but the difference was not significant. No significant differences were observed in the frequencies of blastocyst development between CB-vitrified oocytes and vitrified group. These results suggest that pre-treatment of immature ovine oocytes prior to vitrification with 7.5 μg/ml CB for 60 min has no adverse effects on viability and subsequent development. In contrast, Silvestre et al (2006) and Bogliolo *et al.* (2007a) reported negative effects of CB treatment on survival of vitrified/thawed ovine GV oocytes.

The effects of caffeine treatment during in vitro maturation on MPF/MAPK kinases activities, spindle, chromatin configuration, and subsequent in vitro embryo development following IVM/IVF/IVC of ovine oocytes previously vitrified at the GV stage were evaluated (**Chapter 8**). Caffeine treatment (10.0 mM, 18–24 hpm) decreased the levels of MPF/MAPK kinases in vitrified oocytes as compared to non treated group, but the difference was not significant. No significant differences were observed in the proportion of oocytes with normal spindle and chromatin configuration between vitrified and control groups (**Chapter 5**). Caffeine treatment did not affect these proportions in vitrified oocytes (**Chapter 8**). Caffeine treatment increased the frequencies of blastocysts development in vitrified oocytes as compared to non treated vitrified group. These results suggest that caffeine treatment improved the blastocyst development in vitrified oocytes by mechanism other than an increase in MPF/MAPK kinases activities as it does in fresh control oocytes (Lee and Campbell 2006).

The effects of demecolcine (deme) pre-treatment on viability, timing of first polar body (PBI) extrusion, spindle, chromatin organization, and subsequent development of vitrified/thawed GV oocytes following IVM and IVF or IVM and parthenogenetic activation were evaluated (**Chapter 9**). The results show that the frequencies of PBI at 18 hpm were significantly decreased in vitrified oocytes as compared to deme + vitrified (oocytes treated with 0.1 μg/ml demecolcine for 20 min prior to vitrification) and control groups (8.5 *vs* 20.4 and 47.1%, respectively). Also, demecolcine pre-treatment increased the frequencies of PBI in vitrified oocytes at 20, 22, and 24 hpm as compared to non-treated. The results indicate that vitrification of immature oocytes resulted in a delay in the kinetics of PBI extrusion and demecolcine pre-treatment improve this delay. Demecolcine pre-treatment did not affect the proportions of oocytes with normal spindle and chromatin configuration; however, it increased the numbers of oocytes with missing spindle. As previously discussed in (**Chapter 9**) demecolcine can bind to tubulin dimmers and prevent microtubule polymerization, thus, resulting in loss of dynamic spindle microtubules (Ibanez *et al.* 2003; Russell *et al.* 2005). Following IVF and parthenogenetic activation, the frequencies of cleaved embryos, morula and blastocysts development were increased in vitrified oocytes pre-treated with demecolcine prior to vitrification as compared to non treated vitrified oocytes.

In conclusion, these results demonstrate that ovine oocytes vitrified at GV stage can be matured, fertilised, and develop up to blastocyst stage embryos. Cryoloop may be superior to SSV for vitrification of ovine oocytes as this technique resulted in an increased developmental potential of vitrified/thawed oocytes after IVM/IVF/IVC. Exposure of immature oocytes to SSV and cryoloop vitrification solutions did not affect in vitro embryo production in sheep. Vitrification of immature oocytes did not significantly affect the proportions of oocytes with normal spindle, chromatin configuration and MPF/MAPK kinase activities following IVM as compared to control. Viability, maturation, fertilisation, and subsequent development of vitrified/thawed ovine GV oocyte can be improved by the treatments of oocytes with different chemicals either prior to vitrification such

as CB and demecolcine or during IVM such as caffeine. Vitrified GV oocytes can develop up to morula stage following parthenogenetic activation, irrespective to activation protocol. Strontium can be used effectively for parthenogenetic activation of vitrified immature ovine oocytes. Vitrified-thawed ovine oocytes can be used successfully for the first time as cytoplast recipients for SCNT with production of high frequencies of blastocyst development. The results demonstrate that good quality blastocysts could be produced from vitrified immature oocytes following IVF, parthenogenetic activation or SCNT. The results presented here are encouraging and vitrification protocols (**Fig 10.1**) applied in the present studies could be a promising tool for the establishment of oocyte banking (Fabbri *et al.* 2001; Fabbri 2006) for widespread application of ART in both humans and animals.

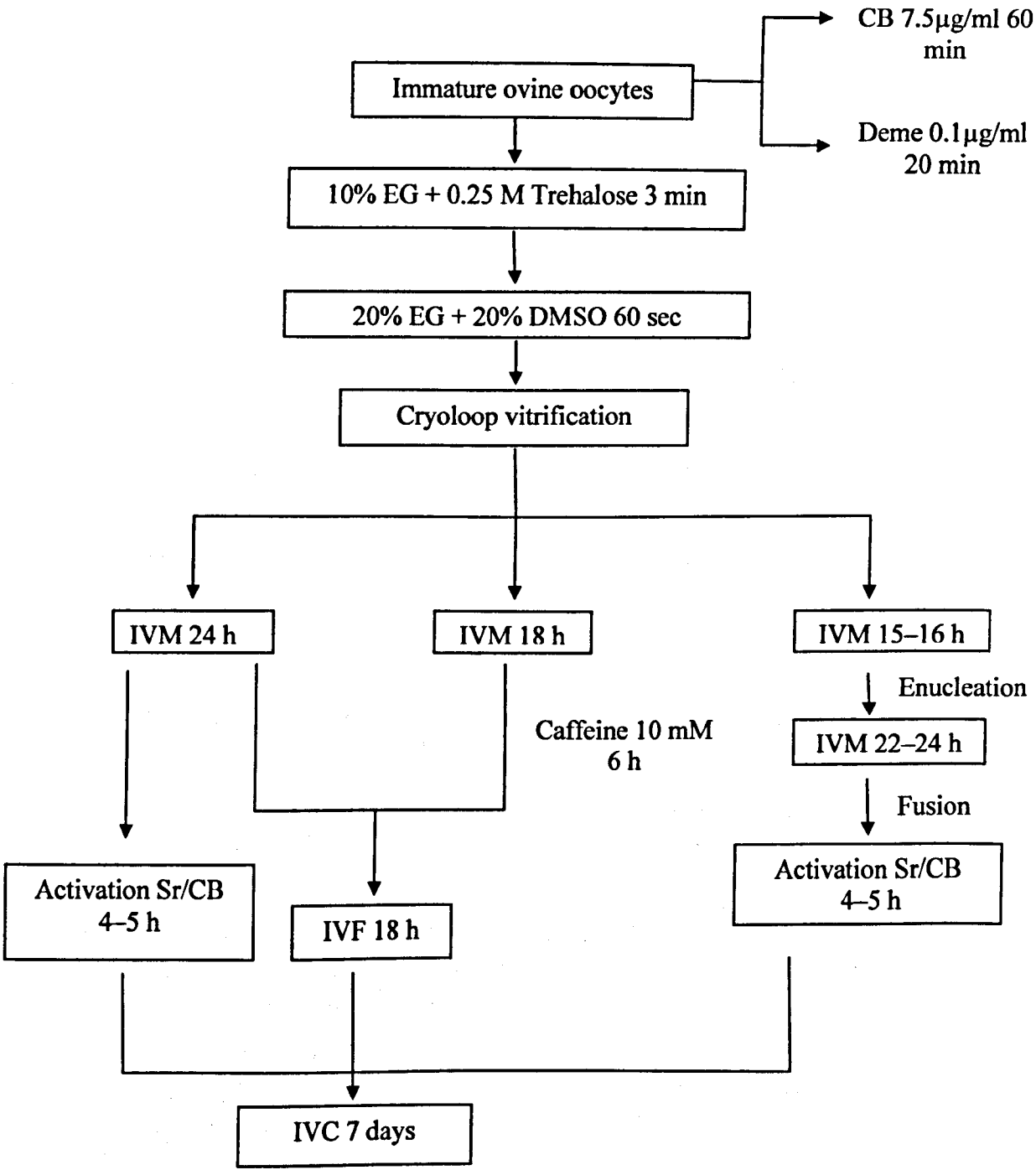


Figure 10.1The best conditions used for vitrification of immature ovine oocytes applied in this thesis and resulted in high developmental competence following IVF, parthenogenetic activation or SCNT.

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APPENDIX A

MEDIA AND STOCK SOLUTIONS

I. Media and Stock Solutions for Oocyte Culture

Ovary Collection Medium (PBS):

8	g	NaCl
0.2	g	KCl
1.44	g	Na_2HPO_4
0.24	g	KH_2PO_4

Adjust pH to 7.4 with 5 N NaOH and make up to 1 L final volume with dH_2O. Sterilise by autoclaving for 20 minutes at 15 lb/in^2.

Oocyte Dissection Medium:

18	ml	Tissue Culture Medium 199 with Earle's salts containing 25 mM HEPES, 25 mM sodium bicarbonate, 3 mM L-glutamine, L-Amino Acids (H-TCM 199)
2	ml	Heat-inactivated Foetal Bovine Serum

Sterilise by filtration through a 0.22 μm Millipore filter and equilibrate in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 39°C for at least 2 hours prior to use.

Sodium pyruvate stock:

11	mg	Sodium pyruvate (Sigma P-4506)
1	ml	TCM 199

Stock solution was stored at 4°C. Sodium pyruvate stock was diluted in maturation medium to a working concentration of 0.2 mM.

17β-estradiol stock:

1	mg	17β-estradiol (Sigma E-8875)

1	ml	Ethyl alcohol

Stock solution was stored at -20°C for a maximum of one month. 17β-estradiol stock was diluted in maturation medium to a working concentration of 1 μg/ml.

Gentamycin stock:

50	mg	Gentamycin (Sigma G-3632)
1	ml	Normal saline

Stock solution was stored at 4°C. Gentamycin stock was diluted in maturation medium to a working concentration of 50 μg/ml.

Cysteamine (2- Mercaptoethylamine) stock:

7.714	mg	Cysteamine (Sigma M-9768)
1	ml	TCM 199

Stock solution was dispensed in 20 μl aliquots, and stored at -20°C. Cysteamine stock was diluted in maturation medium to a working concentration of 100 μM.

FSH (Follicular stimulating hormone) stock:

400	mg	FSH (Folltropin®-V; Vetrepharm)
80	ml	Normal saline

Stock solution was dispensed in 20 μl aliquots, and stored at -20°C. FSH stock was diluted in maturation medium to a working concentration of 5 μg/ml.

LH (Luteinizing hormone) stock:

25	mg	LH (Lutropin -V; Vetrepharm)
5	ml	Normal saline

Stock solution was dispensed in 20 μl aliquots, and stored at -20°C. LH stock was diluted in maturation medium to a working concentration of 5 μg/ml.

Oocyte Maturation Medium:

9	ml	Tissue Culture Medium 199 with Earle's salts containing 25 mM sodium bicarbonate, 3 mM L-glutamine
1	ml	Heat-inactivated Foetal Bovine Serum
5	µg/ml	FSH (Folltropin®-V; Vetrepharm)
5	µg/ml	LH (Lutropin-V; Vetrepharm)
1	µg/ml	17β-estradiol
0.2	mM	Sodium pyruvate
100	µM	Cysteamine
50	µl/ml	Gentamycin

Sterilised by filtration through a 0.22 µm Millipore filter and equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 39°C for at least 2 hours prior to use.

II. Media and Stock Solutions for Vitrification of Ovine Oocytes

Cryoprotectants Used in Vitrification Solutions:

Ethylene Glycol	(EG, Sigma E-9129)
Dimethylsulfoxide	(DMSO, Sigma D 4540)
Glycerol	(GLY, Sigma G-6279)
Trehalose	(T, Sigma T-0167)
Polyvinylpyrrolidone	(PVP, Sigma, PVP 40)

Different Vitrification Solutions (VS):

Base Medium

18	ml	PBS
2	ml	FBS

Cryoprotectants:

Media	Base Medium	EG	DMSO	GLY	T
Equilibration	1.8 ml	0.2 ml	-	-	0.189 gm
VSI	1.2 ml	0.4 ml	0.4 ml	-	-
VSII	1 ml	0.5 ml	0.5 ml	-	-
VSII	1 ml	0.5 ml	-	0.5 ml	-
Warming I	1.8 ml	0.2 ml	-	-	0.756 gm
Warming II	2 ml	-	-	-	0.378 gm

SSV Vitrification Solutions:

Base Medium (20% FBS)

24 ml H-TCM 199

6 ml FBS

Equilibration Solution (4% EG)

9.6 ml Base medium

0.4 ml EG

Vitrification Solution (35% EG)

5 ml Base medium

1.5132 g Trehalose

0.5 g PVP

3.5 ml EG

Fill up to 10 ml

Warming Solution (0.3 M Trehalsoe)

9 ml Base medium

1.134 g Trehalose

Fill up to 10 ml

Cryoloop Vitrification Solutions:

Base Medium

18	ml	H-TCM 199
2	ml	FBS

Equilibration Solution

1.8	ml	Base medium
0.2	ml	EG
0.189	g	Trehalsoe

Vitrification Solution

1.2	ml	Base medium
0.4	ml	EG
0.4	ml	DMSO

Warming Solutions

Warming Solution I

1.8	ml	Base medium
0.2	ml	EG
0.756	g	Trehalose

Warming Solution II

2	ml	Base medium
0.378	g	Trehalsoe

III. Media and Stock Solutions for Ovine IVF

Stock C (33mM Na Pyruvate)

Na Pyruvate 36mg

Dissolve in 10mls MQ water. Filter and store at 4°C for up to 1 week.

Stock GLN (10mM glutamine)

Glutamine 73mg

Dissolve in 50mls MQ water. Filter and store at 4°C for up to 1 week or aliquot and keep at –20°C.

Stock B (250mM $NaHCO_3$)

$NaHCO_3$	1.0505g
Phenol red	5mg (or 3 drops)

Dissolve $NaHCO_3$ in 50mls MQ water and add phenol red. Filter and store at 4°C for up to 2 weeks.

Stock H (250mM Hepes)

Hepes	5.9575g

Dissolve in 100mls MQ water. Filter and store at 4°C for up to 1 month.

Stock L (330mM Na Lactate)

Na lactate (60% syrup)	1.41mls

Dissolve in 30mls MQ water. Filter and store at 4°C for up to 1 month.

Stock S3

	g/100ml	g/200ml
NaCl	6.294	12.588
KCl	0.534	1.068
KH_2PO_4	0.162	0.324

Filter and store at 4°C for 3 months.

Stock D (171mM $CaCl_2.2H_2O$)

$CaCl_2.2H_2O$	1.260g

Dissolve in 50mls MQ water. Filter and store at 4°C for up to 3 months.

Stock M (49mM $MgCl_2.6H_2O$)

$MgCl_2.6H_2O$	500mg

Dissolve in 50ml MQ water. Filter and store at 4°C for up to 3 months.

Stock G (60mM glucose)

Glucose	540mg

Dissolve in 50mls MQ water. Filter and store at 4°C for up to 3 months.

Sperm Washing Medium

To 50ml TCG water, add:

Stock S2 (S3)	10.0ml
Stock H	8.4ml
Stock B	1.6ml
Stock C	1.0ml
Stock GLN	1.0ml
Stock M	1.0ml
Stock L	1.0ml
Stock G	4.0ml
Stock D	1.0ml
BSA Fraction V	300mg
Stock Pen/Strep	0.5ml
water	up to 100ml

pH 7.2-7.4
mOsm 283 +/- 10
Filter and keep in the fridge for up to 2 months.

Fertilisation Medium

To 25ml TCG H_2O, add:

Stock S2	5.0ml
Stock B	5.0ml
Stock C	1.5ml
Stock L	1.5ml
Stock M	0.5ml
Stock GLN	5.0ml
Stock D	1.0ml
Pen/strep	0.25ml
H_2O	Up to 50ml

pH 7.9
mOsm 283 ± 10
Filter and fridge for up to 2 weeks.

IVF – add 2% sheep serum to fertilisation medium and equilibrate before use.

IV. Media and Stock Solutions for Nuclear Transfer

Composition of modified Synthetic Oviduct Fluid (mSOF) medium:

Component	MW	Concentration	g/L
NaCl	58.44	107.63 mM	6.2899
KCl	74.55	7.16 mM	0.5338
KH_2PO_4	136.10	1.19 mM	0.1620
$MgSO_4$	120.40	1.51 mM	0.1818
$CaCl_2 \cdot 2H_2O$	147.00	1.78 mM	0.2617
Sodium lactate (60% syrup)	112.10	5.35 mM	757 µl
$NaHCO_3$	84.01	25.00 mM	2.1002
Na-pyruvate	110.00	7.27 mM	0.7997
L-Glutamine	146.10	0.20 mM	0.0292
Tri-Sodium-citrate	294.10	0.34 mM	0.1000
Myo-inositol	180.20	2.77 mM	0.4992
Phenol-red		10.0 µg/ml	10 mg
ddH_2O			Up to 1L

The osmolarity of SOF was adjusted to 270 to 280 mOsm and to pH 7.4 with 1 N sodium hydroxide and stored at 4°C for a maximum of one week. For use 45.0 µl/ml BME amino acids, 5.0 µl/ml MEM amino acids and 5% heat inactivated FBS were added whenever needed. The medium was sterilised by filtration through a 0.22 µm Millipore filter and equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 39°C for at least 2 hours prior to use.

Composition of HEPES - buffered Synthetic Oviduct Fluid (H-SOF) medium:

Component	MW	Concentration	g/L
NaCl	58.44	107.63 mM	6.2899
KCl	74.55	7.16 mM	0.5338
KH_2PO_4	136.10	1.19 mM	0.1620
$MgSO_4$	120.40	1.51 mM	0.1818
$CaCl_2 \cdot 2H_2O$	147.00	1.78 mM	0.2617
Sodium lactate (60% syrup)	112.10	5.35 mM	757 µl
$NaHCO_3$	84.01	5.00 mM	0.4200
Na-pyruvate	110.00	7.27 mM	0.7997
L-Glutamine	146.10	0.20 mM	0.0292
Tri-Sodium-citrate	294.10	0.34 mM	0.1000
HEPES	238.30	20.00 mM	4.7660
Phenol-red		10.0 µg/ml	10 mg
ddH_2O			Up to 1L

Chatot Ziomek Barister (CZB) medium:

Stock media

Component	amount	Component	amount
Ultra pure water	990 ml	Na-Lactate	5.3 ml
NaCl	4760 mg	D-Glucose	1000 mg
KCl	360 mg	KH_2PO_4	160 mg
$MgSO_47H_2O$	290 mg	EDTA2Na	40 mg

Mix well, it is 1000 ml. Then make the CZBas following: $CaCl_2$ stock for CZB is 0.189 g in 10 ml water

CZB:

Component	amount	Component	amount
Stock media	99 ml	Pyruvate	3 mg
$NaHCO_3$	211 mg	Glutamine	15 mg
$CaCl_2 \cdot 2H_2O$ stk	1 ml	BSA	500 mg

Adjust pH to 7.4., For Calcium free CZB, do not add calcium chloride stock solution.

Hyaluronidase stock (300 IU/mg):

100	mg	Hyaluronidase (Sigma H-3506)
1.1	g	PVP
110	ml	Ca^{2+}-, Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS-)

Stock solution was dispensed in 1 ml aliquots and stored at -20°C.

Cytochalasin B stock:

10	mg	Cytochalasin B (Sigma C-6762)
1	ml	DMSO

Stock solution was dispensed in 10 µl aliquots, and stored at -20°C. Cytochalasin B stock was diluted in H-SOF at a working dilution of 5 µg/ml.

Hoechst 33342 stock:

25	mg	Hoechst 33342 (Sigma B-2261)
5	ml	ddH_2O

Stock solution was dispensed in 10 µl aliquots, and stored at -20°C. Cytochalasin B stock was diluted in H-SOF at a working dilution of 5 µg/ml.

Ca^{2+} Ionophore (A23187) stock:

5	mg	Ca^{2+} Ionophore (A23187) (Sigma C-7522)
1.91	ml	DMSO

Stock solution (5 mM) was dispensed in 10 µl aliquots, and stored at -20°C. Ca^{2+} Ionophore (A23187) stock was diluted in H-SOF at a working dilution of 5 µM.

Cycloheximide (CHX) stock:

5	mg	Cycloheximide (Sigma C-6255)
1	ml	Ca^{2+}-, Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS-)

Stock solution was dispensed in 20 µl aliquots, and stored at -20°C. Cycloheximide (CHXM) stock was diluted in mSOFaaci at a working dilution of 10 µg/ml.

Caffeine solution:

9.6	mg	Caffeine (Sigma C-8960)
5	ml	Maturation medium

Caffeine was dissolved for 2-3 min and sterilised by filtration through a 0.22 µm Millipore filter. Prior to use the medium was equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 39°C for at least 2 hours.

Fusion medium (0.3 mM Mannitol):

2.73	g	Mannitol (Sigma M-4125)
50	µl	100 mM $MgSO_4$ (246.5 mg/10ml)
5.96	mg	HEPES
25	mg	BSA
50	ml	ddH_2O

Stock solution was dispensed in 3 ml aliquots and stored at -20°C

Strontium stock solution:

226.62	mg	Strontium chloride hexahydrate (Sigma 255521)
or 158.53	mg	Strontium chloride anhydrous (Sigma 439665)
1	ml	ddH_2O

Stock solution was dispensed in 10 µl aliquots and stored at room temperature.

V. Media and Stock Solutions for In vitro Double Kinase Assay

Oocyte collection buffer:

6.4	mM	EDTA (pH 7.4)
10	mM	NaF
100	mM	Na_3VO_4 in DPBS

Ice cold lysis buffer:

45	mM	β-glycerophosphate (pH 7.3)
12	mM	ρ-nitrophenylphosphate
20	mM	3-(*N*-morpholino)-propanesulfonic acid (MOPS)
12	mM	$MgCl_2$
12	mM	ethyleneglycol bis (2-amino-ether) tetra acetic acid (EGTA)
1.4	mM	EDTA
20	mM	Na_3VO_4
10	mM	NaF
2	mM	dithiothreitol (DTT)
2	mM	phenylmethylsulphonyl fluoride
2	mM	benzamidine
20	µg/ml	leupeptin
20	µg/ml	pepstatin
20	µg/ml	aprotinin

VI. Reagents and Gel Preparation for SDS-PAGE Slab Gels

Bio-Rad's Preweighed Acrylamide:

37.5:1 mixture (Catalog Number 161-0106, 200 g)

90 g acrylamide/bis (30 g/ 100 ml) to 300 ml with dH_2O

1.5 M Tris-HCL, pH 8.8:

27.23 g Tris base

~80 ml ddH_2O

Adjust to pH 8.8 with 1N HCL, and make up to 150 ml with ddH_2O and store at 4°C.

10% SDS:

Dissolve 10 g SDS in water with gentle stirring and bring to 100 ml with ddH_2O.

2X SDS sample buffer

5.0 ml	0.5 M Tris-HCL, pH 6.8
4.0 ml	glycerol
0.8 g	SDS
617.0 mg	DTT
2 mg	1% (w/v) bromophenol blue
8.0 ml	to 20 ml with ddH_2O

Store at –80°C

5X electrode (Running) buffer (pH 8.3):

9 g	Tris base
43.2 g	glycine
3 g	SDS
	To 600 ml with ddH_2O

Store at 4°C. Warm to 37°C before use if precipitation occurs. Dilute 60 ml 5X stock with 240 ml ddH_2O for one electrophoretic run

Separating gel (15%) preparation – 0.375 M Tris, pH 8.8:

4.7 ml	ddH_2O
5.0 ml	4x Tris-HCl (1.5 M, pH 8.8)
10 ml	30% stock acrylamide/bis
200 µl	10% SDS
100 µl	10% (w/v) ammonium persulfate (APS)
14 µl	TEMED

A total of 10 ml is sufficient for 2 mini gels

Staking gel (4%) preparation – 0.125 M Tris, pH 6.8:

6.0 ml	ddH_2O
2.5 ml	4x Tris-HCl (0.5 M, pH 6.8)
1.4 ml	30% stock acrylamide/bis
100 µl	10% SDS
50 µl	10% (w/v) ammonium persulfate (APS)
10 µl	TEMED

A total of 5 ml is sufficient for 2 mini gels

Composition of Separating gel mixtures

Stocks solution (ml)	Final acrylamide concentration				
	6%	10%	12%	14%	15%
30% acrylamide/0.8% biacrylamide	2.000	3.330	4.000	4.700	5.000
4x Tris.Cl (pH 8.8)	2.500	2.500	2.500	2.500	2.500
10% (w/v) SDS	0.100	0.100	0.100	0.100	0.100
10%(w/v) ammonium persulfate	0.050	0.050	0.050	0.050	0.050
TEMED	0.007	0.007	0.007	0.007	0.007
ddH_2O	5.350	4.020	3.350	2.650	2.350
Total volume*	10	10	10	10	10

*A total volume of 10 ml gel mixture is sufficient for two mini gels

APPENDIX B
CRYOPRESERVATION OF OOCYTES IN DIFFERENT SPECIES

Cryopreservation of oocytes from different mammalian species.

Species	*Stage*	*CPAs*	*Freezing method*	*Viability assay*	*Results (%)*	*Reference*
Bovine	MII	EG	Vitr	IVF	15 BR	Martino *et al.* (1996a)
Bovine	MII	EG+F+S	Vitr	IVF	72.4 FR	Hochi *et al.* (1998)
Bovine	MII	EG+DM	Vitr	IVF	25* BR	Vajta *et al.* (1998)
Bovine	MII	EG+S	Vitr	IVF	10 BR	Otoi *et al.* (1998)
Bovine	GV	EG+PR+S	Slow	IVF/NT	7*/5 BR	Kubota *et al.* (1998)
Bovine	MII	EG+PR+S	Slow	IVF/NT	12*/16 BR	Kubota *et al.* (1998)
Bovine	MII	EG+DM+F+S	Vitr	IVF	33 BR	Lane *et al.* (1999a)
Bovine	MII	EG+S	Vitr	IVF	14.8* BR	Papis *et al.* (2000)
Bovine	MII	EG+PVP+T	Vitr	IVF/NT	15/17 BR	Dinnyes *et al.* (2000)
Bovine	MII	EG+DM+F+S	Vitr	IVF/ICSI	25.8/16 BR	Mavrides and Morroll (2002)
Bovine	MII	EG+DM+F+S	Slow	IVF/ICSI	15.4/9.4 BR	Mavrides and Morroll (2002)
Bovine	GV	EG+DM+ S	Vitr	IVF	6.1*BR	Vieira *et al.* (2002)

Bovine	MII	EG+DM+ S	Vitr	Comet	26.1 Degen.	Men *et al.* (2003b)
Bovine	MII	EG+DM+ S	Slow	Comet	46 Degen.	Men *et al.* (2003b)
Bovine	MII	EG+T+PVP	Vitr	NT	24 BR	Atabay *et al.* (2004)
Bovine	MII	EG+PR	Vitr	IVF	7.4 BR	Chian *et al.* (2004)
Bovine	GV	EG+S	Vitr	IVM	44.5 MR	Martins *et al.* (2005)
Bovine	MII	EG+DM+S	Vitr	IVF	2.5 BR	Albarracin *et al.* (2005a)
Bovine	MII	EG+DM+S+F	Vitr	NT	16.3* BR	Hou *et al.* (2005)
Bovine	GV	EG+S+F	Vitr	IVF	8 BR	Abe *et al.* (2005)
Bovine	MII	EG+S	Vitr	IVF/NT	12/33 BR	Tominaga *et al.* (2005)
Bovine	GV	EG+DM+S	Vitr	IVM	20 MR	Cetin and Bastan (2006)
Bovine	GV	EG+DM+S	Vitr	IVF	2.3 BR	Kim *et al.* (2007)
Bovine	GV	EG+DM	Vitr	IVM	29.2 MR	Yamada *et al.* (2007)
Bovine	MII	EG+DM+PVP	Vitr	IVF	2.6 BR	Horvath and Seidel (2008)
Bovine	GV	EG+DM+S	Vitr	IVF	5.8* BR	Vieira *et al.* (2008)
Bovine	MII	EG+DM+S	Vitr	IVF	3.2 BR	Morato *et al.* (2008b)
Bovine	MII	EG+S	Vitr	IVF/NT	0.9/7.4* BR	Yang *et al.* (2008)

Bovine	MII	EG+DM+S+F	Vitr	PA	23.2 BR	Hou *et al.* (2009)
Bovine	MII	EG+T+PVP	Vitr	IVF	8 BR	Sripunya *et al.* (2010)
Ovine	GV	EG+S	Vitr	IVM	32.6 MR	Isachenko *et al.* (2001)
Ovine	MII	EG+DM+S	Vitr	IVF	9.4 BR	Kelly *et al.* (2005)
Ovine	GV	EG+DM+S	Vitr	IVM	12.7 MR	Silvestre *et al.* (2006)
Ovine	MII	EG+DM+S+F	Vitr	IVF	28.2 FR	Tian *et al.* (2007)
Ovine	MII	EG+DM+S	Vitr	IVF	12.5 BR	Succu *et al.* (2007a)
Ovine	MII	EG+DM+S	Vitr	IVF	0	Succu *et al.* (2007b)
Ovine	MII	EG+DM+S	Vitr	IVF	34.3 CR	Berlinguer *et al.* (2007)
Ovine	GV	EG+DM	Vitr	IVF	31.2 MR	Bogliolo *et al.* (2007a)
Ovine	MII	EG+DM	Vitr	IVF	17 BR	Succu *et al.* (2008)
Ovine	MII	EG+DM+S	Vitr	IVF	2.6 BR	Zhang *et al.* (2009)
Porcine	GV	EG	Vitr	IVM	5.6 MR	Isachenko *et al.* (1998)
Porcine	GV	EG	Vitr	IVF	0 CR	Rojas *et al.* (2004)
Porcine	MII	EG	Vitr	IVF	10.4 CR	Rojas *et al.* (2004)
Porcine	GV	EG	Vitr	ICSI	13.5 BR	Fujihira *et al.* (2004)

Porcine	MII	EG+T+PVP	Vitr	PA	2.6 BR	Somfai *et al.* (2006)
Porcine	MII	EG+DM+S+F	Vitr	PA	0 BR	Shi *et al.* (2006)
Porcine	GV	EG+S+PVP	Vitr	IVF	9.5 BR	Gupta *et al.* (2007)
Porcine	MII	EG+S+PVP	Vitr	IVF	3.4 BR	Gupta *et al.* (2007)
Porcine	MII	EG+T+PVP	Vitr	IVF	9 BR	Somfai *et al.* (2007)
Porcine	MII	EG+DM+S	Vitr	PA	1.3 BR	Du *et al.* (2008)
Porcine	MII	EG+DM+S	Vitr	PA	3 BR	Lin *et al.* (2009)
Porcine	GV	EG+T+PVP	Vitr	IVF	5.1 BR	Somfai *et al.* (2010)
Equine	GV	EG+S+F	Vitr	IVM	30 MR	Hurtt *et al.* (2000)
Equine	MII	EG+DM+S+F	Vitr	Morphology	73* Viable	Maclellan *et al.* (2002)
Equine	GV	EG	Slow	IVM	1.2 MR	Tharasanit *et al.* (2006a)
Equine	GV	EG+DM+S	Vitr	IVM	27.6 MR	Tharasanit *et al.* (2006a)
Equine	MII	EG	Slow	ICSI	7.5 CR	Tharasanit *et al.* (2006a)
Equine	GV	EG+DM+S	Vitr	ICSI	1 BR	Tharasanit *et al.* (2006b)
Equine	GV	EG+DM+S	Vitr	IVM	32.6 MR	Tharasanit *et al.* (2009)

Goat	MII	EG+DM+S+F	Vitr	PA	0 BR	Begin *et al.* (2003)
Goat	GV	PR+T	Vitr	IVF	17.8 FR	Kharche *et al.* (2005)
Goat	MII	PR+T	Vitr	IVF	8 FR	Sharma *et al.* (2006)
Goat	GV	EG	Vitr	IVF	31.4 FR	Garg and Purohit (2007)
Buffalo	GV	EG	Vitr	IVF	13.5 BR	Wani *et al.* (2004b)
Buffalo	MII	EG+T+PVP	Vitr	PA	13.6 BR	Bookusol *et al.* (2007)
Buffalo	MII	EG+T+PVP	Vitr	IVF	1.5 BR	Gasparrini *et al.* (2007)
Buffalo	MII	EG+DM	Vitr	IVF	5.5 BR	Gautam *et al.* (2008)
Buffalo	MII	DM	Slow	IVF	0.6 BR	Gautam *et al.* (2008)
Buffalo	GV	EG+DM	Vitr	IVM	41.5 MR	Mahmoud *et al.* (2009)
Buffalo	MII	EG+DM	Vitr	IVF	7.8 BR	Attanasio *et al.* (2009)
Cat	GV	EG+DM	Vitr	IVF	4.3 BR	Cocchia *et al.* (2010)
Mouse	MII	PR+S	Slow	IVF	43.7* BR	Stacheki *et al.* (2002)
Mouse	GV	DM+S	Slow	IVF	18.2 BR	Ruppert-Lingham *et al.* (2003)
Mouse	MII	EG+DM+S+F	Vitr	ICSI	57* CR	Endoh *et al.* (2007)
Mouse	MII	EG+DM+S	Vitr	Morphology	98 Viable	Gomes *et al.* (2008)

Mouse	MII	EG+DM+S+F	Vitr	IVF	76.1 BR	Wang *et al.* (2009)
Human	MII	EG+S	Vitr	ICSI	65* Viable	Kuleshova *et al.* (1999)
Human	GV	EG+S	Vitr	IVF	23 BR	Hong *et al.* (1999)
Human	MII	PR+S	Slow	ICSI	58 FR	Fabbri *et al.* (2001)
Human	MII	EG+DM+S+F	Vitr	Morphology	83 Viable	Liebermann and Tucker (2002)
Human	MII	PR+S	Slow	ICSI	59* FR	Boldt *et al.* (2003)
Human	MII	EG+S+F	Vitr	ICSI	73.7* FR	Saki and Dezfuly (2005)
Human	MII	EG+DM+S	Vitr	ICSI	94.3* CR	Lucena *et al.* (2006)
Human	GV	EG+DM+S	Vitr	IVM	71 MR	Isachenko *et al.* (2006)
Human	MII	PR+S	Slow	PA	100 CR	Morbeck *et al.* (2009)
Human	MII	EG+DM+S	Vitr	PA	40 CR	Morbeck *et al.* (2009)

GV: germinal vesicle; MII: metaphase II; CPAs: cryoprotectants; EG: ethylene glycol; DM: DMSO; S: sucrose; T: trehalose; F: Ficoll; PR: 1-2 propanediol; PVP: polyvinylpyrrolidone; Vitr: vitrification; Slow: slow freezing; IVM: in vitro maturation IVF: in vitro fertilisation; ICSI: intracytoplasmic sperm injection; PA: parthenogenetic activation; NT: nuclear transfer; Comet: comet assay, BR: blastocyst rate; CR: cleavage rate; FR; fertilisation rate; MR: maturation rate; Degen: degeneration to DNA.* pregnancy and live births reported in these studies.

APPENDIX C

VITRIFICATION OF BOVINE BLASTOCYSTS AND ESTABLISHEMENT OF PREGNANCY

Achievement of pregnancy from vitrified blastocysts using Cryoloop

Day 7 bovine blastocysts produced by in vitro maturation and in vitro fertilisation of Ovum Pick Up (OPU) oocytes were vitrified using the Cryoloop method applied in the present study. After storage in liquid nitrogen for one month, blastocysts (6) were thawed and then transferred to the synchronised recipient heifers. Pregnancy diagnosis was performed 45 days after transfer. One animal was diagnosed pregnant. This indicates that the Cryoloop vitrification technique applied in this study can use effectively for preservation of bovine blastocyst stage embryos.