CRYOPRESERVATION AND *IN VITRO* MATURATION OF MURINE GERMINALVESICLE STAGE OOCYTES

Thesis presented for the

Degree of Philosophiae Doctor

by

C. J. Ruppert-Lingham, B.Sc.

Department of Obstetrics & Gynaecology

University of Wales College of Medicine

15th June 2005

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Acknowledgements

I would like to express my gratitude to Dr Sharon Paynter for her constant advice and encouragement at every stage of this study. I would also like to thank Dr Barry Fuller for his expert evaluation of the design and scope of the work. I am indebted to Dr Frank Dunstan for his guidance in the statistical analysis of the study. I would like to thank Prof. Howard Evans for his enthusiastic explanations of the importance of gap junctions and Prof. Maurice Hallett for his help with the confocal microscope. I would like to thank Prof. Robert Shaw for his advice and suggestions. I would also like to thank Jo Godfrey for her jovial support during the laboratory work. Finally I would like to thank my husband Paul, without whose patience and understanding none of this would be possible.

Contents

Tables

Figures

Abbreviations

AC	adenyl cyclase
AGA	18α-glycyrrhetinic acid
ATP	adenosine tri phosphate
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine mono phosphate
cAPK	cAMP dependent protein kinase
CEO	cumulus enclosed oocyte
CF	carboxy fluorescene
CO_2	carbon dioxide
COC	cumulus oocyte complex
CPA	cryopreservation agent
dcAMP	dibutryl cyclic adenosine mono phosphate
DO	denuded oocyte
E_2	estradiol
EGF	epidermal growth factor
ET	embryo transfer
FCS	fetal calf serum
FRAP	fluorescence return after photobleaching
FSH	follicle stimulating hormone

g	grammes
GA	18α-glycyrrhetinic acid
GDP	guanine di phosphate
GJ	gap junction
GT	gonadotrophin
GTP	guanine tri phosphate
GV	germinal vesicle
GVBD	germinal vesicle break down
H	hours
hCG	human chorionic gonadotrophin
hr	hours
ICSI	intra cytoplasmic sperm injection
IGF-1	insulin-like growth factor
ip	intra peritoneal
IVF	*in vitro* fertilisation
IVM	*in vitro* maturation
l	litres
LH	luteinising hormone
LN_2	liquid nitrogen
M	molarity
Me_2SO	dimethylsulphoxide
MEM	modified earles medium
mg	miligrammes
MII	metaphase II
min	minutes

ml	millilitres
MPF	meiosis promoting factor
NaCl	sodium chloride
PB1	phosphate buffered medium
PBS	phosphate buffered saline
PCOD	poly cystic ovarian disorder
PDE	phosphodiesterase
PEG	polyethylene glycol
PI	propidium iodide
PKC	protein kinase C
PMS	pregnant mares serum
PrOH	propanediol
rFSH	recombinant follicle stimulating hormone
RNA	ribonucleic acid
SDN-POA	sexually dimorphic nucleus – pre optic area
SMM	standard maturation medium
$T6_{16}$	tyrodes medium 16mg/ml BSA
$T6_4$	tyrodes medium 4mg/ml BSA
VSD	vitrification solution dimethylsulphoxide

Publications arising from work contained in this thesis

Full Paper

Ruppert-Lingham C.J., Paynter S.J., Godfrey J., Fuller B.J. and Shaw R.W. (2003) Developmental potential of murine germinal vesicle stage cumulus-oocyte complexes following exposure to dimethylsulphoxide or cryopreservation: loss of membrane integrity of cumulus cells after thawing. *Hum. Reprod.* **18**, 392-398.

Abstracts

Ruppert-Lingham C.J., Paynter S.J., Fuller B.J. and Shaw R.W. (2000) Differences in the developmental potential of murine germinal vesicle stage oocytes matured either as cumulus-oocyte complexes (COCs) or as COCs in the presence of follicle cells from throughout the ovary. *J. Reprod. Fertil. Abstract Series* **26**, 30.

Ruppert-Lingham C.J., Paynter S.J., Fuller B.J. and Shaw R.W. (2000) Differences in the developmental potential of murine germinal vesicle stage oocytes matured either as cumulus-oocyte complexes (COCs) or as COCs in the presence of follicle cells from throughout the ovary. *Proceedings of the 15th Annual Postgraduate Research Day*, **8**, 70.

Ruppert-Lingham C.J., Paynter S.J., Godfrey J., Fuller B.J. and Shaw R.W. (2001) Effects of Cryoprotectant Addition/Removal and Slow Cooling on the Developmental Potential of Immature Murine Cumulus-Oocyte Complexes. *Cryobiology*, **43**, 330.

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Lecture

Ruppert-Lingham C.J. (2002) Cryopreservation of Immature Germinal Vesicle Stage Oocytes. *Postgraduate Lecture Series Department of Obsetrics and Gynaecology University Hospital of Wales*

SUMMARY

Cryopreservation of unfertilised oocytes for banking or oocyte donation would be a valuable adjunct to reproductive technology. As the mature oocyte contains a temperature-sensitive meiotic spindle, cryopreservation of immature germinal vesicle (GV) stage oocytes, which do not contain the spindle, may be a practical alternative. However, one of the major obstacles to the application of immature oocyte cryopreservation is the difficulty associated with *in vitro* maturation (IVM) of the thawed oocytes prior to *in vitro* fertilisation. The cumulus cells surrounding the oocyte are essential to oocyte maturation. Thus the aim was to assess survival and function of both oocyte and cumulus cells post-cryopreservation.

Initially, culture conditions during IVM of murine GV stage cumulus-oocyte complexes (COCs) were modified. In the second part of the study, survival (morphological appearance and membrane integrity) and function (ability, *in vitro*, to mature, be fertilised and develop into blastocysts) of the oocytes and their associated cumulus cells was assessed following cryopreservation. An attempt was made to determine the stage of the protocol at which damage was incurred.

Alterations to culture conditions had little impact on the ability of fresh GV stage oocytes to develop to blastocysts, although IVM in the presence of mixed ovarian cells was found to be detrimental. Treatment with 1.5M dimethyl sulphoxide (Me_2SO) without freezing had little effect on the parameters investigated, unlike exposure to a 6M Me_2SO solution. Slow-cooled/thawed or cumulus-denuded oocytes had decreased developmental potential when compared with control oocytes. Development was not improved by co-culture with fresh cumulus cells. Much of the damage caused to the cumulus cells occurred during plunging from -60°C to -196°C. Damage was reduced by cooling at 10°C/min from -60°C to -150°C prior to plunging to -196°C. However, embryo development was not improved. Vitrification of COCs led to substantial cumulus cell damage and very poor embryo development.

Chapter 1

INTRODUCTION

1.1 PRODUCTION OF THE MATURE OOCYTE *IN VIVO*

In mammals, mature fertilisable oocytes are produced by the ovary. The ovary contains a large number of follicles that have the potential to grow and produce mature oocytes. At birth the human ovary contains 1-2 million follicles. Until recently the number of follicles in the mammalian ovary was thought to be finite. However, there is now contradictory evidence in mice (Johnson *et al*, 2004). Throughout life follicles will be lost through ovulation and atresia until, at menopause, no follicles remain. The primordial oocytes, which are present in the ovary from birth, must grow and mature in order to attain the potential to be ovulated, fertilised and undergo embryogenesis. This section details the stages of follicle formation, growth and subsequent ovulation *in vivo*. The human system will be used as a basis for the account; examples from studies of other species will also be included where relevant.

1.1.1 Follicle Formation and Growth

The reproductive organs of the early foetus consist of the Wolffian duct, the Mullerian duct and one pair of bipotential gonads. If induced by the presence of testosterone the Wolffian duct will develop into male reproductive organs and the bipotential gonads will go on to form testes. These are organisational events; they put in place the systems that will produce gametes in the future. In the absence of testosterone the Mullerian duct will go on to develop into female reproductive organs, and the bipotential gonads will become ovaries (Jost, 1971).

The ovarian follicle provides a micro-environment for oocyte growth and maturation. Follicles can be divided into categories according to their stage of development. Primordial follicles, intermediary follicles, primary follicles, secondary follicles, preantral and antral follicles (see figure1.1), with primordial follicles being the most numerous (Gougeon and Chainy, 1987). The oocytes within early stage

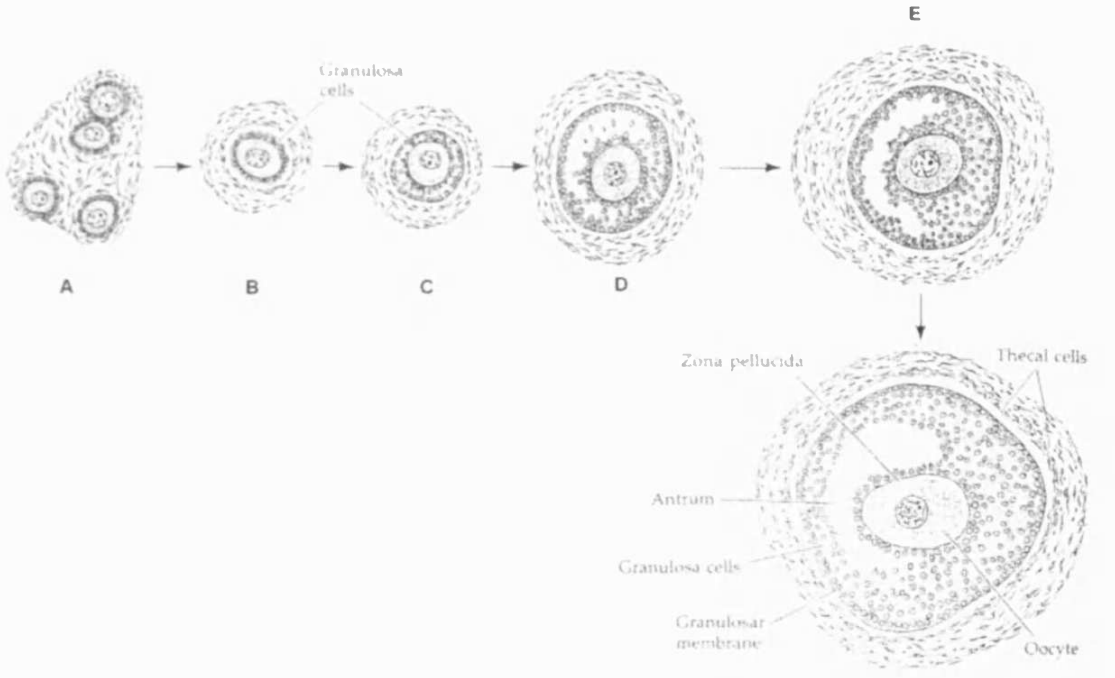


Figure 1.1 Stages of follicle growth and maturation. (**A**) Primordial follicles. (**B**) Primary follicle. (**C**) Secondary follicle. (**D**) Pre-antral follicle. (**E**) Antral /Pre-ovulatory follicle.

follicles are of a similar size and thus the progression from primordial to primary follicle does not involve growth of the oocyte, it is a slow and laborious process of maturation (Gougeon and Chainy, 1987).

Primordial follicles (figure 1.1 **A**) contain an oocyte that is arrested in the prophase stage of the first meiotic division. The oocyte is associated with a small number of flattened somatic cells or granulosa cells (Pedersen and Peters, 1968). The recruitment of primordial follicles is marked by the initiation of follicle growth and is partially mediated by gonodotrophins (GT) (Halpin *et al*, 1986). Recently it has been suggested that although gonadotrophins are required to achieve granulosa cell

differentiation, the oocyte is probably the dominant factor in determining the direction of differentiation (Eppig, 2001). As the follicle grows the number of somatic cells surrounding the oocyte increases and they undergo substantial morphological and functional transformations (Pedersen and Peters, 1968). In humans, follicular growth starts when the diameter of the germinal vesicle (GV), (the nucleus of the immature oocyte, which contains decondensed chromosomes surrounded by the nuclear envelope) reaches 19μm (Gougeon and Chainy, 1987). In mice the first event in the initiation of follicular growth is the synthesis of RNA within the nucleus of the oocyte. This occurs in response to an inductive signal provided by the somatic granulosa cells (Lintern-Moore and Moore, 1979).

As the number of granulosa cells increases and the follicle becomes multi-layered, the follicles are termed secondary or early growing follicles (figure 1.1 **C**). The oocyte increases in size and secretes glycoprotein that condenses around it to form a translucent-acellular layer called the zona pellucida. Despite the glycoprotein coat, contact is maintained between the oocyte and surrounding somatic cells via cellular projections. These traverse the zona and form direct junctions with the oolemma (Albertini and Anderson, 1974). Simultaneously the homogeneous population of somatic cells begin to differentiate. The follicle cells become cuboidal and begin to proliferate in response to the circulating GTs (Gougeon, 1986; Lintern-Moore, 1977). This is accompanied by the acquisition of high affinity steroid hormone receptors (Richards *et al*, 1976). More specifically, the development of follicles and granulosa cell differentiation is associated with changes in receptor content. These changes are subject to heteroregulation, i.e. they are hormonally regulated by specific interactions between estradiol (E_2), follicle stimulating hormone (FSH) and luteinising hormone (LH) (Richards *et al*, 1976). The somatic cells on the

periphery of the follicle continue to differentiate and develop into theca cells. The follicle acquires full responsiveness to GTs as LH and FSH receptors are expressed in both the theca and the granulosa cells (Kobayashi *et al*, 1990). From this time the secondary follicle is defined as preantral, a growing follicle (figure 1.1 **D**).

During follicle growth, just prior to antrum formation, the granulosa cells within preantral follicles adopt a cuboidal conformation, their nuclei become irregularly shaped and their cytoplasmic volume increases. Cytoplasmic projections develop on the surface of the somatic cells and these intermingle with microvilli on the surface of the oocyte. Some of these projections invade the oolemma and form regions of cell contact. These regions of intercellular contact were identified as gap junctions (Anderson and Albertini, 1976), and are found in large numbers in both antral and preovulatory follicles (Albertini and Anderson, 1974). They conjoin the oocyte to its companion granulosa cells and mediate metabolic co-operation (Anderson and Albertini, 1976).

Basal follicular growth now commences. At this stage the oocyte requires only tonic levels of GTs, it increases in volume and the granulosa cells continue to proliferate until the oocyte becomes recruitable. The volumetric growth of the oocyte is partially achieved by the accumulation of water, ions and lipids. Much of the increase is due to the rate of protein synthesis that occurs, coupled with the lack of cytokinesis. The human oocyte begins this growth stage at a size of around 35μm and takes months to reach its final size of 120μm. However, the mouse oocyte only takes 2-3 weeks to reach its final size of 80μm, from an initial size of 15μm (Salha *et al*, 1998). As the pre-antral stage concludes, the oocyte stops growing and just prior to antrum formation it becomes competent to resume meiosis (Sorensen and Wassarman, 1976).

1.1.2 Hormonal Control of Follicular Growth and Ovulation

As sexual maturity approaches, the hypothalamus begins the pulsatile release of hypothalamic release factors. These travel through the portal system to the pituitary gland. When combined with the correct levels of circulating sex steroids, the pituitary begins to secrete GTs. The area of the hypothalamus that controls the release of GTs by the pituitary is called the sexually dimorphic nucleus of the pre-optic area (SDN-POA). The SDN-POA of the female is smaller than that of the male, it facilitates both cyclic and tonic release of GTs (Arnold and Gorski, 1984). It is the cyclic nature of GT release in females that allows the mid-cycle surge of LH to occur that ultimately leads to oocyte maturation.

Late in the luteal phase, the largest healthy follicles have a diameter of 2-5mm (Gougeon, 1986). In response to increasing FSH levels their numbers and quality increase, from this group the follicle destined to ovulate will be selected (Gougeon and Lefévre, 1983). Unlike early follicles, recruitable follicles are highly receptive to cyclic levels of GTs. The rate of mitotic division of the granulosa cells (mitotic index) of these follicles increases (Gougeon and Lefévre, 1983), and thus so may the number of FSH receptors present within the follicle.

In molecular terms LH consists of a ten amino acid polypeptide and a β chain of one hundred and fifteen amino acids. In addition to its effects on oocyte maturation, LH has a direct effect on the ovary by stimulating the secretion of estrogen and progesterone by ovarian follicles. FSH is also morphogenetic in character; some of its main effects include maturation of the gonads, growth of the oocyte, growth of ovarian follicles and synthesis of estradiol by follicles. The FSH molecule consists of a ninety-two amino acid α chain and a one hundred and eighteen amino acid β chain.

FSH and LH mediate oocyte maturation via their actions on the granulosa cells that surround the oocyte. LH has been shown to cause alterations in the composition of granulosa cell membranes (Parr, 1974). GTs also cause changes in the conformation of granulosa cells (Lintern-Moore, 1977). The differentiation of granulosa cell phenotypes requires the mediation of GTs (Eppig, 2001). During the follicular phase GTs can be localised to the granulosa cell layer and the internal thecal layer (Kobayashi *et al*, 1990). Differentiation has been shown to be associated with specific changes in the expression of FSH and LH receptors. These changes are subject to heteroregulation by FSH, LH and E_2 (Richards *et al*, 1976). GTs influence the maturation of oocytes from the outset of follicular development. GT receptors have been identified in the granulosa cells of dominant follicles (Horie *et al*, 1992). FSH secretion by the anterior pituitary initiates follicular antrum formation (Greep *et al*, 1942).

The largest healthy follicle, with a diameter of 5.5-8.2mm, is selected at the beginning of the follicular phase (Gougeon and Lefévre, 1983). Apart from size and granulosa cell mitotic index there is no detectable difference between the selected follicle and the other remaining healthy follicles (Gougeon and Lefévre, 1983). It is thought that the follicle to be selected is the one whose granulosa cells have most rapidly acquired a higher receptivity to FSH. This could lead to an increase in aromatase activity following the rise in FSH levels. Recent studies have implicated FSH regulated paracrine factors in early ovarian development, these may be important for activation and selection of follicles (Albertini *et al*, 2001)

The antral phase precedes ovulation and is initiated by secretion of FSH by the anterior pituitary. In response, fluid begins to accumulate between the granulosa cells (Greep *et al*, 1942). This forms a central cavity termed the follicular antrum. The

antrum separates the differentiating somatic cell populations. The follicle cells that line the periphery of the follicle are known as granulosa cells. The somatic cells that remain closely associated with the oocyte are termed cumulus cells, and together with the oocyte they form the compact COC. These differing cell populations are morphologically and functionally different. Granulosa cells adjacent to the basement membrane elongate and cease proliferation. They become targets for luteinisation as they develop LH receptors and begin the synthesis of enzymes involved in steroid metabolism. Meanwhile, antral granulosa cells, cumulus cells, and cells of the 'stalk' region, retain their shape and continue to proliferate (figure 1.1). Few LH receptors are found in these cells and stereogenic activity is limited. However, contact is maintained between the granulosa cells via homogeneous gap junctional linkages (Gilula *et al*, 1978). Following the LH surge the granulosa cells begin to produce an extracellular matrix. The secreted matrix forces the cells surrounding the oocyte apart, causing the complex to increase in volume, to become more elastic and in the process, reducing the gap junctional associations before ovulation, after which no coupling is detected (Gilula *et al*, 1978). Eventually the complex detaches from the follicle wall. The mature oocyte is expelled from the ovary when the follicle wall ruptures.

1.1.3 Oocyte Maturation

The oocyte remains in meiotic arrest by means of inhibitory factors provided by the follicle and does not resume maturation until stimulated by the preovulatory GT surge. It has been shown that spontaneous germinal vesicle breakdown (GVBD) can be instigated by the removal of the GV stage oocyte from the follicular environment (Pincus and Enzmann, 1935; Edwards, 1965). Spontaneous maturation does not take place in oocytes originating from pre-antral follicles, as competence to undergo GVBD is associated with follicular morphology and oocyte size. Maturation prepares

the oocyte for the demands that will be placed upon it as it develops. The mRNA content of the cell reflects this. A fully grown mouse oocyte contains 0.4-0.6ng of RNA, this is around 200 times the amount found in a typical somatic cell (Olds *et al*, 1973; Wassarman and Kinloch, 1992). The RNA accumulates during maturation, when meiosis resumes the levels of RNA fall (Piko and Clegg, 1982). The high levels of mRNA are caused by increased rates of protein synthesis that are associated with the conclusion of maturation. At the GV stage, the chromosomes are not visible as the chromosomal axes are unravelled and extensive lateral loops form. It has been suggested that when in this state the chromosomes are similar to lampbrush chromosomes that are found in amphibian oocytes and are a site of intense transcriptional activity (Davidson, 1986). Initiation of the process of maturation and ovulation occur almost simultaneously. However, the two events are not causally linked, as each can occur in the absence of the other (Eppig, 1977).

1.1.3.1 Meiotic and Cytoplasmic Maturation

Oocyte maturation can be considered to occur as two distinct events. Meiotic or nuclear maturation involves the breakdown of the GV to allow the oocyte to conclude the arrest of meiosis at prophase I and to reach metaphase of the second meiotic division (MII). This division is signified by the extrusion of a polar body. Cytoplasmic maturation is achieved by an increase in the synthesis of proteins and nucleic acids in order to build up the stores of these substances for subsequent development. As GVBD approaches, the nuclear membrane becomes undulated and the number of nuclear pores increases. This alteration in the structure of the membrane is presumably caused by an increase in the nuclear-cytoplasmic traffic of molecules. The undulation of the membrane continues to become more pronounced until the membrane begins to breakdown altogether, resulting ultimately in GVBD.

Breakdown of the GV is followed by the formation of the first meiotic spindle during metaphase I and the extrusion of the first polar body. When the oocyte reaches the diplotene stage of the first meiotic division, the nucleus has developed the capability to undergo meiotic maturation. This ability seems to come about independently of its cytoplasmic state (Bao *et al*, 2002). It is likely that acquisition of competence to undergo GVBD is a multistep process. However, it has been found that spontaneous meiotic maturation does not require gene transcription (Rodriguez *et al*, 2002).

Cytoplasmic maturation is the process whereby the cytoplasm of the oocyte is prepared for fertilisation and development. Oocytes that matured spontaneously were found to have low rates of fertilisation and development. Therefore, cytoplasmic maturation was thought to not occur during spontaneous maturation (Thibault, 1977). The developmental capacity of murine oocytes derived from small antral follicles is poor compared with the development of oocytes collected from large antral follicles (Eppig *et al*, 1992). Many of the oocytes collected from small antral follicles do not develop past the two-cell stage. This is evidence of a qualitative difference between the oocytes from large and small antral follicles (Eppig *et al*, 1992). MII oocytes taken from 18 or 26 day old mice also had significantly different developmental capabilities. Eighty two percent of the oocytes from the 26 day old mice developed to blastocyst, whereas, only 27% of the oocytes from 18 day old mice reached this stage. Both groups of oocytes had undergone meiotic maturation, there was speculation that the oocytes from the younger mice were lacking certain maternal factors (Eppig, 1994). A failure in the completion of cytoplasmic maturation could lead to an inability to translate species of mRNA that were accumulated during oocyte growth. This could result in a shortage of the proteins required for fertilisation and development. For example, the cytoplasmic changes necessary for the processing of

the male pronucleus were thought to not occur after spontaneous maturation (Thibault, 1977). A study that examined the protein content of oocytes arrested at MI i.e. before meiotic maturation, showed that the MI arrested oocytes had different profiles of protein synthesis than non-arrested MI oocytes. The proteins synthesised by the arrested oocytes were similar to those synthesised by MII stage oocytes. This demonstrates that certain critical aspects of cytoplasmic maturation can occur in oocytes without the completion of meiotic/nuclear maturation (Eppig, 1994; Bao *et al*, 2002). It has been suggested that completion of meiotic/nuclear maturation is insufficient for the acquisition of developmental competence. Cytoplasmic maturation involves the synthesis, storage, reprogramming and utilisation of molecules. The regulatory signals required for this may be produced by granulosa/cumulus cells when combined with GTs (Moor *et al*, 1998).

1.1.3.2 Mediation of Maturation by Somatic Cells

Oocyte growth has been shown to be dependent on the maintenance of communication mediated by gap junctions between the oocyte and the surrounding somatic cells (Eppig, 1979). It has been hypothesised that gap junctions mediate the transfer of inhibitory molecules such as cyclic adenosine mono-phosphate (cAMP) from somatic cumulus cells to the oocyte, thereby facilitating meiotic arrest (Eppig *et al*, 1983). Intercellular communication within the COC is governed via gap junctions. However, no intercellular coupling has been detected in post ovulatory oocytes (Gilula *et al*, 1978). GT administration has been shown to reduce the extent of interaction between cumulus cells and the oocyte (Moor *et al*, 1980; Racowsky and Satterlie, 1985). The reduction in cumulus-oocyte coupling seemed to temporally correlate with meiotic maturation and mucification of the cumulus cell mass. It was hypothesised that initiation of oocyte maturation was a result of the decrease in

oocyte-cumulus coupling and the subsequent interruption in the flow of meiosis inhibiting factor to the oocyte (Dekel and Beers, 1978). However, measurement of intercellular coupling, using [^{3}H]uridine and [^{3}H]choline, before and after maturation revealed that meiotic maturation occurred in advance of any reduction in cumulus cell-oocyte coupling (Eppig, 1982). This supported the hypothesis that oocyte maturation was initiated by a change in the signal transmitted to the oocyte by the cumulus cells as a result of GT stimulation, and not a reduction in intercellular coupling.

Maintenance of threshold cAMP concentrations within the ovarian follicle inhibits oocyte maturation. Studies have indicated that meiotic arrest is maintained by the transfer of cAMP via gap junctions from somatic granulosa/cumulus cells to the oocyte since the termination of cell-cell coupling led to the disappearance of the GV and resumption of meiosis (Dekel and Beers, 1978). However, when cAMP concentration was increased within the cumulus cell compartment by treatment with FSH or cholera toxin no increase in cAMP concentration was detected within murine oocytes. Similar treatment of denuded oocytes (immature oocytes stripped of their associated cumulus cells) caused no inhibition of meiosis. Therefore, the inhibitory effect of FSH and cholera toxin was attributed to an agent other than cAMP (although cAMP may be required for its action) that is mediated by cumulus cells (Schultz *et al*, 1983). Another study, demonstrated that sub optimal concentrations of the cAMP analogue, dibutryl cyclic adenosine monophosphate (dbcAMP) had a greater inhibitory effect on cumulus-enclosed oocytes (CEO), than on denuded oocytes (DO). Since dbcAMP can readily permeate plasma membranes it can be assumed that the dbcAMP equilibration was the same in CEO and DO. This supports the hypothesis

that a cAMP dependent factor is transmitted from cumulus cells to the oocyte (Eppig *et al*. 1983).

Priming of COCs with forskolin, a stimulator of the catalytic subunit of adenyl cyclase (AC), which in turn converts adenosine tri-phosphate (ATP) to cAMP, lead to significantly higher rates of resumption of meiosis than without priming. AC is a multi-functional membrane protein. The binding of a messenger molecule such as a hormone leads to the activation of AC. The hormone binding receptor interacts with a specific G protein. The previously inactive G_s subunit becomes active by binding and converting guanine di-phosphate (GDP) to guanine tri-phosphate (GTP), (Gilman, 1984). Activation of AC is proportional to the amount of hormone present. Therefore the amount of cAMP produced is proportional to the systemic levels of hormone. Phosphodiesterase (PDE) performs a virtually opposite function. PDE is largely activated by a rise in the cytosolic concentration of calcium. However, the rate of cAMP hydrolysis is also increased by the formation of calcium/calmodulin complexes. These complexes are able to activate inactive cAMP-PDE, resulting in an overall decrease in cAMP concentration.

The two molecules are thought to operate in a reciprocal way, with each contributing to the regulation of intracellular levels of cAMP. An increase in cAMP levels causes an increase in cAMP dependent protein kinase activity, which in turn leads to an increase in the phosphorylation of proteins, ensuring the maintenance of meiotic arrest. Conversely, a decrease in cytosolic cAMP concentration leads to a fall in cAMP dependent protein kinase activity. In this situation proteins may not be phosphorylated in large enough numbers, GVBD could occur and meiosis would resume.

The diverse effects of cAMP are mediated by the action of cAMP dependent protein kinase (cAPK) and/or protein kinase A. cAPK functions to modify the activities of specific enzymes by the transfer of the terminal phosphate group in ATP to the hydroxyl group in serine, threonine and tyrosine residues of proteins. In this way the activity of an enzyme can be controlled through minor modifications. Maturation is initiated by an interruption in protein phosphorylation, which is normally catalysed by cAPK. Levels of cAMP decrease and this leads to an alteration in the pattern of protein phosphorylation within the oocyte. There are two different variations of cAPK, type I and type II. They each perform different functions at different stages of the cell cycle in both the meiotic divisions of an oocyte and in the mitotic divisions of somatic cells. A model was proposed in which, forskolin treatment caused an increase in cAMP concentration, this in turn was said to stimulate the cumulus cells to produce a secreted meiosis-inducing factor, this overcame the inhibition of resumption of meiosis and induced oocyte maturation. In the proposed model the oocyte-cumulus connections were a requirement in the initiation of the production of the secreted factor. However, they were not necessary for transferring this substance to the oocyte (Guoliang *et al*, 1994). A putative maturation factor was investigated in a number of studies by the co-culture of intact COCs and DOs with fresh cumulus cells. It was found that disassociated cumulus cells were capable of releasing a factor that acted in a positive capacity on denuded oocytes matured *in vitro* (Byskov *et al*, 1997). Isobe and Terada (2001) reported similar findings. It was demonstrated that secreted factors governed the down regulation of gap junctions, cumulus expansion and GVBD in porcine oocytes. The existence of a paracrine maturation factor was also investigated by Downs (2001). Maturation was induced in COCs treated with the gap junction inhibitor 18α-glycyrrhetinic acid (GA) but not in

denuded oocytes treated in the same way. This suggested that the cumulus cells provided a positive factor that aided maturation without the requirement for patent gap junctions. In addition to this it was discovered that no reduction in oocyte-cumulus cell coupling occurred during the period of induction of maturation, it was concluded that the positive paracrine signal is usually overridden by inhibitory signals conveyed via gap junctions (Downs, 2001).

1.1.3.3 Regulatory Molecules

The cytoplasm of cells in metaphase contain a factor that can cause meiotic maturation of oocytes arrested at the first meiotic prophase. This factor is known as Maturation or M-phase Promoting Factor. MPF consists of two subunits, cyclin B which acts as a regulatory subunit, and $p34^{cdc2}$, a serine/threonine kinase which acts as a catalytic subunit. The MPF molecule is maintained in the inactive state by phosphorylation of specific amino acid residues on $p34^{cdc2}$. This phosphorylation is principally controlled by two molecules Wee 1 and Mik kinases (Lundgren *et al*, 1991). Cyclin B builds up during maturation and is degraded after cell division (Pines and Hunter, 1989). The dominant cyclin isoform in the mouse oocyte is cyclin B1 (Chapman and Wolegemuth, 1992). This is the same variety that is found in human oocytes (Heikinheimo *et al*, 1995).

Oocyte maturation is characterised by two distinct peaks of high MPF activity. The first peak occurs at the time that meiotic activity is resumed. The second peak is higher and more sustained than the first and occurs during meiotic arrest at MII stage (Mattioli *et al*, 1991). MPF is thought to have a variety of target molecules (Heikinheimo and Gibbons, 1998). It is sometimes known as histone kinase, as the histone H1 is a classic substrate of MPF (Langan *et al*, 1989). H1 is important in DNA packaging. MPF phosphorylates H1, and in this way is thought to play a part in

chromosome condensation during maturation. Other molecules that are also thought to be influenced by MPF include; nuclear lamins, which are phosphorylated by MPF, and could be important in the disassembly of the nuclear envelope during GVBD. MPF is also responsible for the phosphorylation of pp60c-src kinase, which is responsible for cytoskeletal rearrangements during cell division. Interestingly, MPF seems to have an inhibitory effect on RNA polymerase II (Cisek and Gorden, 1989). In this context the function of MPF could be the inhibition of transcription.

MPF activity is likely to be upregulated by the protein kinase c-mos. C-mos is the cellular version of the viral oncogene, mos (Sagata *et al*, 1989). C-mos enhances MPF activity in the mouse oocyte, partially by inhibiting the proteolytic degradation of cyclin B that prompts the accumulation of cyclin B between meiosis I and II (O'Keefe *et al*, 1991). Studies have shown that if c-mos is blocked, meiosis II is prevented (Zhao *et al*, 1990). However, the capacity of c-mos to maintain the arrest of meiosis II was fully demonstrated by the production of knock out mice for the c-mos gene. Oocytes originating from these mice failed to arrest at meiosis II (Colledge *et al*, 1994).

A tyrosine kinase receptor expressed on oocyte cell membranes has been identified. The receptor, c-kit, binds to a stem cell factor that is expressed on granulosa cell membranes (Morto and Berstein, 1993). Studies involving knock-out mice demonstrated that oocytes taken from mice lacking the c-kit gene fail to mature past the primordial stage this suggests that the interaction between the tyrosine kinase receptor and its ligand is a requirement for maturation (Kuroda *et al*, 1988).

In conclusion, the production of mature, fertilisable oocytes is a complex and lengthy process. Following fertilisation, growth and development of the resultant

embryos must occur in order to produce viable offspring. Failure at any one of these stages can lead to sub fertility or infertility.

1.2 ASSISTED REPRODUCTION

In vitro fertilisation (IVF) has been successful in few species, in that satisfactory numbers of oocytes can be routinely harvested; these are capable of fertilisation, embryogenesis, implantation and development into viable offspring. IVF technology would be useful in the propagation of rare species, in animal husbandry and, in the case of humans, it is used to treat infertility. The systems used to achieve IVF in humans and in the mouse will be outlined.

1.2.1 Infertility

Infertility is defined as a failure to conceive after 1 year of unprotected intercourse. Within the United Kingdom it is estimated that 16.8% of the population will experience fertility problems (Hull *et al*, 1985). Investigations into the cause of infertility typically begin with examination of the female partner. Female fertility begins an initial decline at around the age of 30. This is caused by a depletion in the number of follicles within the ovary due to follicle recruitment and atresia (Gougeon and Chainy, 1987). Fewer viable oocytes are available for recruitment and ovulation, therefore this decline accelerates after the age of 35.

Early onset infertility can be caused by disorders of the ovary; for example, primary ovarian failure is a failure in the initial formation of ovarian follicles. Ovulatory failure can be caused by a lack of follicle response to circulating GTs or inadequate levels of GTs in the bloodstream. Ovulation can also be affected by hyperandrogenism, which is caused by disorders in steroid synthesis. Another cause of female infertility is obstruction or developmental defects in the Mullerian system

such disorders may prevent spermatozoa from reaching the oocyte or disrupt the establishment of a pregnancy. Disorders of the reproductive tract include; hostility of cervical mucus caused by abnormal secretion or due to the presence of antibodies, also tubal and peritoneal abnormalities that can be caused by disease and inflammation. By far the most prevalent tubal disorder is damage caused by *Chlamydia trachomatis*, which can lead to pelvic inflammatory disease (PID). Endometriosis affects 5-10% of pre-menopausal women, it is the growth of uterine tissue on non-uterine sites within the peritoneal cavity this can lead to adhesions and tubal blockage. Although its pathology is unclear it is thought to be caused by retrograde menstruation. Uterine problems such as uterine myomas, uterine leiomyomas (fibroids) or inappropriate responses of the uterine lining, can prevent a pregnancy from becoming established. In some cases pregnancy can occur, but not continue. This is referred to as Recurrent pregnancy loss (RPL) and effects between 0.5-1% of pregnant women.

Male infertility is assessed via a semen sample provided by the patient. Abnormal semen volume, low sperm count and poor sperm motility and morphology are early indicators of male sub-fertility. Disorders can occur in the formation of spermatozoa. Asthenozoospermia is inadequate formation of sperm and teratozoospermia is the formation of abnormal sperm. Sperm production is totally prevented by primary testicular failure, which is caused by a lack of stem cells. Antibodies may be produced by the patient's own immune system that destroy spermatozoa within the body. Low numbers of spermatozoa can also be explained by disorders of the male reproductive tract. Oligozoospermia is caused by blockage of the ducts, which leads to sperm cell death. Blockages can also be inherited or caused by infections.

In 10-15% of infertile couples investigated all test results are normal. There appears to be no physiological explanation for their failure to conceive. This group are said to have 'unexplained infertility'.

1.2.2 Treatment Procedures

1.2.2.1 Ovarian stimulation

Ovarian stimulation is required for the treatment of anovulatory patients. It is also used in the treatment of ovulatory patients to produce multiple oocytes in order to increase the probability of fertilisation *in vitro*. Controlled ovarian hyperstimulation is achieved by the administration of follicle stimulating drugs that increase the number of maturing follicles within the ovary. Urinary purified or more recently recombinant hormone preparations containing FSH are administered to recruit follicles. Follicular growth is measured either by ultrasound scanning or by levels of estrogen in the blood. When ovulation is imminent, as judged by large follicle size or high estrogen levels, human chorionic gonadotrophin (hCG) which has an effect similar to that of LH, is administered to promote oocyte maturation and ovulation. Typically around 10 oocytes will be collected. However, this amount can vary substantially depending on the drug regimen used, the response of the patient and the skill of the operator.

Ovarian stimulation of the mouse can be tailored to produce either GV stage oocytes by administration of FSH alone or MII stage mature oocytes by the additional administration of LH. The LH must be administered prior to the endogenous LH surge, which is controlled by the light/dark cycle. LH is therefore given 53hrs after FSH administration at the correct time in the light dark cycle. Approximately 20 oocytes can be collected from each mouse.

1.2.2.2 Oocyte collection

In the past human oocytes were collected from ovarian follicles via abdominal laparoscopy. Currently pre-ovulatory oocytes are aspirated from follicles on the surface of the ovary using transvaginal ultrasound-guided methods. Each follicle is aspirated beginning with the largest visualised follicle; the follicular fluid is collected and checked for the presence of oocytes. Once collected the oocytes are examined for the presence of a GV or a polar body and the quality of the oocytes is assessed by the condition of the cumulus cell mass. Oocytes are then typically cultured for 4-6hrs to allow them to reach the stage of maturity found in ovulated oocytes.

The mouse is a polyovular species. GV stage mouse oocytes can be collected, 46hrs after intraperitoneal administration of FSH, by puncturing of follicles within the excised ovary. MII stage mature oocytes can be collected 13hrs following administration of LH when ovulation of the oocyte-cumulus mass into the ampulla has occurred.

1.2.2.3 Sperm collection

Human semen is collected as a whole ejaculatory sample. Motility of the sperm is defined as the proportion of motile sperm in the ejaculate; forward progression can be assessed using light microscopy, or computational methods. The spermatozoa that are capable of fertilisation cannot be identified directly through observation, therefore the capable sperm must be separated from the sample. There are a number of methods that can be used to achieve this. Layering of medium over the freshly collected sample enables the motile sperm to enter it, after which it is centrifuged. A similar method, the swim-up method, involves centrifuging the sample and then placing the pellet into viscous media into which the spermatozoa swim. A density gradient can

also be used in conjunction with centrifugation in order to trap cell debris and abnormal sperm, while allowing normal motile sperm to be collected as a pellet.

Mouse spermatozoa is collected from the excised epididymis and incubated in a protein rich medium at 37°C to allow capacitation to occur (the process by which the sperm acquire the ability to fertilise an oocyte), (Austin, 1951).

1.2.2.4 *In vitro* fertilisation

In human IVF, insemination usually takes place 3-6hrs after oocyte aspiration typically in a commercially prepared media. Oocytes can be transferred to droplets of suspended sperm or alternatively 10X concentrate sperm preparations can be added to single oocytes in a droplet at a 1:10 ratio. Penetration of a sperm initiates the second meiotic division and the second polar body is extruded. Fertilisation is confirmed by the appearance of two pronuclei (the male and female haploid sets of chromosomes) 15-18hrs post insemination, at this stage the cumulus cells may be removed in order to visualise and score the pronuclei. The resulting embryos are cultured and transferred to the uterus at the 2-cell, 4-8-cell or blastocyst stage. The first human birth to result from this process occurred in 1978 (Steptoe and Edwards, 1978). To date in excess of 1 million IVF babies have been born worldwide (Bavister, 2002).

In a protocol similar to that used in human IVF, mouse oocytes are incubated with sperm in a media containing a high concentration of macromolecule, usually bovine serum albumin (BSA) for 6 hrs. Fertilisation is assessed as the presence of 2-cell embryos at 24hrs post insemination. Development to hatching blastocyst can be achieved *in vitro* in certain strains of mice and live young can be produced following transfer of embryos at all preimplantation stages to pseudopregnant females.

Recent Developments

Reproductive technology is a fast moving field. In recent years a number of new techniques have been developed many of which are now used routinely. Intracytoplasmic sperm injection (ICSI) is a technique used to treat male sub-fertility. The first report of successful human ICSI was made by Palermo *et al* (1992), many healthy live births have been reported since using this technique. During routine IVF the transfer of a large number of embryos increases the likelihood of a pregnancy occurring. However, the risks involved in a multiple pregnancy far outweigh the advantage afforded. For this reason only 2-3 embryos are replaced per cycle and there has been a recent trend towards replacement of a single embryo. This approach has been particularly successful when used in conjunction with extended *in vitro* culture and single blastocyst transfer.

Embryo cryopreservation can be used to store surplus embryos for use at a later date. However, the storage and manipulation of human embryos has complex moral and ethical repercussions. Oocyte cryopreservation has been cited as a possible alternative to embryo cryopreservation. In addition to the avoidance of many of the moral objections levelled at embryo cryopreservation, the cryopreservation of oocytes can be utilised in cases where embryo cryopreservation is not an option. For example, in the treatment of young female cancer patients prior to their receiving high dose chemotherapy that may leave them infertile.

1.3 CRYOPRESERVATION

Certain cell types and simple tissues e.g. skin, can be cryopreserved and are capable of normal function following recovery after thawing. However, this is not true of more complex tissues and organs. Cells can be stored at temperatures that are sufficiently low so as to lead to the cessation of biochemical reactions. During cooling

to such temperatures a number of physical and biological stresses are imposed. A primary cause of cellular damage that occurs during cooling is the formation of ice crystals. Damage can also be caused by exposure to low temperatures without ice (Farrant and Morris, 1973). Ice crystal formation is accompanied by an increase in the solute concentration of the remaining liquid phase in which the cells are suspended. In order to combat these problems, cryoprotective agents (CPAs) have been employed which, through a variety of mechanisms, reduce the incidence of intracellular ice formation. However, the use of these agents introduces a new set of problems, including cytotoxicity and osmotic stress. For the purpose of this discussion, the cryopreservation of single cells will be focused on. However, cryopreservation of multicellular systems will also be briefly discussed.

1.3.1 Cooling Rates

Long term storage of cells can be achieved if they are stored below -130°C, this is usually achieved by immersion in liquid nitrogen (-196°C). Below -130°C biological reactions cease as there is no liquid water and insufficient thermal energy, cells can therefore, be stored in this way indefinitely as, at this temperature only oxidative damage caused by free radicals can affect the sample (Ashwood-Smith and Friedman, 1979). However, it is during the process of reducing the temperature of the sample for storage, and increasing the temperature during thawing, that the opportunity for damage arises. For this reason different cooling and thawing regimes have been investigated.

1.3.1.1 Slow-Cooling

Cooling of aqueous solutions at slow rates, i.e. <1°C/sec., leads to the formation of a small number of large ice crystals. Nucleation of ice crystals can occur

spontaneously as water molecules arrange themselves into suitable configurations, this is termed homogeneous nucleation. However, in practice heterogenous nucleation normally occurs where foreign particles act as nucleation centres for the formation of crystalline lattices (Franks, 1982). In controlled-rate cooling of biological samples, ice formation is initiated (seeding) in the supercooled liquid (liquid cooled beyond the temperature required to form ice) by touching the exterior of the cryopreservation vessel with a cold metal object, such as forceps. The triggering of ice formation while the sample is held at the seeding temperature, allows time for the release and dispersal of the latent heat of crystallisation so as not to disrupt the progress of the subsequent controlled rate cooling profile. Ice formation tends to occur in the extracellular space not in the small volume that constitutes the intracellular space (Mazur, 1961). This is largely due to the high concentration of proteins and other molecules within the cells that discourage ice formation. The removal of liquid water from the extracellular environment increases the external osmotic pressure causing an efflux of water from the cellular compartment to the extracellular space. The rapid removal of water from the cell causes shrinkage that can be damaging per se as membrane proteins and intracellular enzymes are brought together that would not normally be in contact with each other and that may react in unpredictable ways (Meryman, 1970). Rapid cell shrinkage due to water efflux may result in the rupture of the cell membrane allowing entry of extracellular ice (Muldrew and McGann, 1994). The intracellular concentration of electrolytes also rises as water is removed, potentially causing damage to organelles and denaturing proteins. Extracellular ice formation can also cause damage by physically crushing and deforming the shape of the cell, which at low temperatures can cause additional damage (Mazur, 1984).

In a dilute aqueous solution, i.e. culture media, there is an increase in ionic composition following ice formation. For example, on cooling to −10°C the ionic concentration can reach approximately 3 molar, which would be lethal to cells. Cytotoxic substances that are normally present in the solution in tolerable amounts may reach toxic levels. The changes in the composition of the solution may also cause an alteration in pH (Lovelock, 1953a). Such effects are known as solution effects. It has been demonstrated that the damage inflicted on erythrocytes that were cryopreserved and thawed in an isotonic saline solution was similar to that caused by increasing the salt concentration of the solution without cooling (Lovelock, 1953a). Such increases in solute concentration have also been shown to cause damage to the lipid bilayer of cells (Lovelock, 1955). Recently these changes have been discussed comprehensively in a review by Pegg (2002).

1.3.1.2 Rapid-Cooling

Rapid cooling rates (>1°C/sec.) cause the formation of many ice nucleation centres. These crystals have less time in which to increase in size, therefore, rapidly cooled samples tend to contain many small ice crystals. In rapidly cooled biological samples, ice formation occurs before intracellular water has the opportunity to diffuse out of the cell (Mazur, 1963). The large number of nucleation centres increases the likelihood of intracellular ice formation. As water within the cell freezes extensive intracellular damage can occur (Mazur, 1970; Mazur, 1990). One advantage of rapid cooling is that it minimises the exposure of the cells to high solute concentration.

1.3.2 Cryoprotective Agents (CPA)

The optimum cooling rate of a cell is dependent on the surface area to volume ratio of the cell and the permeability of the plasma membrane. CPAs can be used to

reduce the total amount of water available for the formation of ice and reduce solution effects. This decreases the dependence of cell survival on the rate of cooling. CPAs can be either permeating (i.e. the molecules are able to pass freely across the plasma membrane) or non-permeating.

Permeating CPAs reduce ice formation via a colligative action, i.e. they provide a concentration of dissolved particles, molecules and ions, that serve to volumetrically replace water. That is, the relative number of particles reduces the relative concentration of water, hence less water is available to form ice and the relative concentration of salts is also reduced. Permeating CPAs include glycerol (Polge *et al*, 1949), and dimethylsulphoxide (Me_2SO), (Lovelock and Bishop, 1959). CPA molecules are generally inert with little evidence to suggest that they react with any of the other constituents of the solution. The exception being Me_2SO which in cardiac muscle cells reduces the requirement for ATP. Theoretically stores will build up and provide a glut of ATP following thawing (Shlafer, 1981). Permeating CPAs tend to be low in molecular weight, highly soluble in water at room temperature and have a low level of cytotoxicity. CPAs increase the unfrozen fraction at a given temperature, thereby reducing the ionic composition of the liquid phase and protecting the cells from the potentially damaging solution effects. The addition of permeating CPA can also reduce the shrinkage that occurs as intracellular water exits the cell during the early stages of cooling. CPA can enter the cell as the water is drawn out by the extracellular osmotic pressure, thus limiting the extent of cellular shrinkage (Meryman, 1970).

Non-permeating CPA reduce extracellular ice formation. This can help to reduce the deformation of cells caused by the physical pressure exerted on them by bulky ice crystals. They tend to be high in molecular weight and this reduces the

colligative effect observed with permeating CPAs. Non-permeating CPAs have been used in conjunction with permeating CPAs in an effort to reduce intracellular exposure to high concentrations of CPA (Rall, 1987). Non-permeating CPAs include dextran, sucrose and polyethylene glycol (PEG).

When CPAs are compared at the same molar concentration all have a similar protective effect. The suitability of a CPA for the cryopreservation of a given cell type will depend on the relative cellular toxicity, membrane permeability, cell size and cell type. CPA toxicity is related to the concentration at which it is used and the duration and temperature of exposure. It has been demonstrated that a decrease in duration and temperature of exposure improved cell survival (Fahy, 1984).

Other additives have also been shown to have a positive effect during cryopreservation. These include; egg yolk, milk powder and serum proteins. It is thought that these substances may work by adding bulk to the frozen matrix. More recently alternative cryoprotective agents have been proposed, in particular ethylene glycol (EG) the use of which has yielded promising results (Bafrani *et al*, 2003)

1.3.2.1 Osmotic Effects

In the case of permeating CPAs, cells are usually incubated in a solution containing the CPA prior to cryopreservation, so that a transmembrane equilibrium can be reached before cooling commences. However, as cells tend to be more permeable to water than to CPA, an osmotic disequilibrium is established. Initially the equilibrium is restored by the efflux of intracellular water, this can lead to extreme cell shrinkage that may be damaging (section 1.3.1.1). Conversely, when a cell loaded with CPA is placed into an aqueous solution it will swell and could burst as water diffuses back across the membrane into the cell in an effort to restore equilibrium. Shrink/swell injury can be caused if the difference between intracellular and

extracellular CPA concentration is too great. Shrink/swell damage can be reduced by the stepwise addition and removal of CPA so as to ensure that cell volume remains within tolerated parameters (Mazur, 1981). This is particulary important when high concentrations of CPA are used. Shrink/swell damage can also be reduced during removal of permeating CPA if dilution is carried out in the presence of a non-permeating solute, e.g. sucrose. The employment of sucrose as a diluent provides a high extracellular concentration of solute. As the CPA moves out of the cell, water is prevented from moving into the cell in excessive quantities, due to the high extracellular solute concentration.

1.3.3 Vitrification

It had been demonstrated that during rapid cooling, the cytoplasm of cells which had become dehydrated, were partially vitrified. This observation lead to a move towards vitrification rather than slow controlled rate cooling of cells. Ice formation occurs during cooling as the molecules of a liquid arrange themselves into the crystalline lattice conformation found in ice crystals. Ice formation can be avoided if a solution is cooled at a sufficiently rapid rate. This is due to the theory of vitreous state, which dictates that at sufficiently low temperatures crystallisation does not occur (Luyet, 1937). When a solution is cooled very rapidly the viscosity of the solution increases, this reduces molecular mobility to such an extent that the molecules are unable to arrange themselves into a crystalline configuration. The molecules move closer together until the temperature is too low for the formation of crystals to take place an amorphous solid is formed that has a random arrangement of molecules similar to that found in liquids (MacFarlane, 1987). Pure water can be vitrified by cooling 60μl droplets at 10°C/sec. (Macfarlane, 1986). In order to reduce the cooling rate required, solute can be added. Solute addition decreases the mobility

of water molecules and further inhibits ice formation. In order to achieve vitrification at practicable cooling rates a high concentration of CPA can be used to increase the solute concentration of the solution. The concentration required depends on the cooling rate employed. The formation of the glassy solid occurs at the glass transition temperature (T_g) for water this temperature is -140°C. However, the solute concentration at which glass transition occurs is different depending on the solute (Boutron, 1990). At solute concentrations of around 60%, the temperature of homogeneous nucleation is reduced and the glass transition temperature is increased. If solute concentration is sufficiently high it is possible to cool past the nucleation temperature without the solution freezing. True vitrification is signified by an absence of ice nucleation. In practice, true vitrification is difficult to achieve. Vitrification of biological samples, which often contain multiple nucleation sites, leads to the formation of small ice crystals. On warming, liquid is released from the amorphous matrix, these molecules can add to the small crystals formed during cooling and cause them to grow. Ice formation that occurs during warming of a previously vitrified sample is termed devitrification.

1.3.3.1 Vitrification of Cells

Vitrification of simple organisms, although theoretically possible, was found to be problematic (Luyet and Gehenio, 1939). However, in 1940 Luyet and Gehenio demonstrated low rates of survival of frog spermatozoa following vitrification. Results were shown to improve considerably following the addition of sucrose to the cooling medium. If the spermatozoa were partially dehydrated with 40-50% sucrose prior to vitrification survival rates in excess of 20% were observed. In a later study erythrocytes were cooled rapidly in different concentrations of glycerol, high rates of recovery were reported following vitrification in 8.6M glycerol compared with the

low rates of survival following vitrification at lower concentrations (Rapatz and Luyet, 1968). It was suggested that in order to achieve vitrification the interior and the exterior of the cell must be equilibrated with a high concentration of CPA. However, using high concentrations of CPA could lead to problems such as cytotoxicity and osmotic stress (Fahy, 1986). The high concentrations of CPA that are required for vitrification can be achieved by using a mixture of different CPAs without exceeding the cytotoxic concentration of any one CPA (Rall and Fahy, 1985). Osmotic stress can be limited by stepwise addition and removal of CPA (section 1.3.2.2). Vitrification has been used to successfully cryopreserve some cell types, for example monocytes (Takahashi *et al*, 1986). One major obstacle to success has been the cytotoxicity of the vitrification solution. This problem was tackled by the development of alternative, less toxic vitrification solutions (Rall, 1987).

1.3.4 Warming Rates

The rate of warming that is used to thaw a cryopreserved sample is equally important as, and dependent on, the rate at which it was cooled. Cells that have been cooled slowly will be shrunken, as much of their intracellular water will have passed out across the plasma membrane during cooling. Such cells will therefore, require a slow warming rate as they require time in which to return to their original volume. The extent of shrinkage is dependent upon the temperature at which slow-cooling ceases. This was demonstrated by Whittingham *et al* (1979) in the first report of the survival of mouse embryos following cryopreservation in Me_2SO. Mouse embryos were slow-cooled to various subzero temperatures and then cooled rapidly to the storage temperature. Cell shrinkage was more extensive when the embryos were slowly cooled to –60°C and –80°C than those cooled to –10°C or –50°C. The embryos that were slowly cooled to the lower temperatures required a slower rate of warming

in order for them to have sufficient time to requisition water and return to their original size. This observation was subsequently shown to be specific to Me_2SO in experiments using glycerol carried out by Rall and Polge (1984).

Cells that have been rapidly cooled will contain many small ice nucleation centres. These small crystals could easily be present within the intracellular space as water would have been trapped there before it had an opportunity to exit the cell when the sample became solid. Growth of existing crystals requires less energy than the formation of new crystals. On warming, as liquid water is released it could cause the growth of these nucleation centres leading to the formation of large intracellular ice crystals that would be almost certainly lethal to the cell. For this reason rapidly cooled samples should be warmed rapidly to curtail the growth of ice crystals. This was demonstrated by Rall (1987). High rates of survival and subsequent development were reported following vitrification of mouse embryos followed by rapid warming at 300°C/min. Rall *et al* (1985).

1.3.5 Multicellular Systems

When single cells are cryopreserved it is possible to optimise protocols based on cell size and membrane permeability characteristics etc. However, in multicellular systems different cell types and the complex interactions and interconnections between the cells are more difficult to accommodate. An organ or section of tissue can be considered to be a dense cell suspension, with extracellular space where nucleation and freezing could occur. However, extracellular ice formation that is harmless in cell suspensions could cause extensive damage if it were to occur within tissues (Pegg and Diaper, 1983). Unlike a suspension of single cells, a multicellular system also lacks the high surface area to volume ratio that is a requirement for the

removal of intracellular water and the diffusion into the cell of the CPA. This property means that slow cooling and warming are preferable.

In a multicellular system, cell survival can be dependent on the proportion of the sample that consists of cells (how densely packed the cells are). Packing effect, where a lack of extracellular space prevents the free exchange of CPA and water across membranes, leads to a decrease in post-thaw cell survival (Pegg and Diaper, 1983). The cells of multicellular systems are often heterogeneous and so require different cryopreservation conditions in order to ensure optimal survival. Transferral of single cell techniques to whole organs is complicated by the need for revascularisation. However, other organs such as coronary valves and skin can survive cryopreservation (Billingham and Medawar, 1952). In the case of immature oocytes, the surrounding cumulus cells must also survive the process of cryopreservation and thawing as they may be required for the post thaw maturation of the oocyte.

1.4 CRYOPRESERVATION OF MAMMALIAN OOCYTES

The development of a reliable method for cryopreservation of mammalian oocytes for the purposes of long-term storage would be an important advance in the field of reproductive biology. There have been a number of reviews that cover this topic (Parks and Ruffing, 1992; Bernard and Fuller, 1996, Paynter, 2000). In a clinical context, oocyte cryopreservation would allow the storage of oocytes from cancer patients prior to their receiving treatment with high dose chemotherapy and total body irradiation that is likely to impair ovarian function. It would also allow the optimal use of oocytes collected for the purpose of IVF by facilitating storage of oocytes where the partner is unable to produce a suitable sperm sample. Banking of oocytes that are surplus to immediate treatment needs, either for subsequent pregnancy attempts or donation, would also be facilitated. Oocyte banking would also make the

process of oocyte donation less complicated by avoiding the need to synchronise donor and recipient cycles. Long-term storage of oocytes could also lead to benefits for agriculture and for species conservation.

Unlike the cryopreservation of spermatozoa and embryos, oocyte cryopreservation has met with limited success. This is due to a number of specific features of the oocyte that make cryopreservation a more technically challenging task. The human oocyte is one of the largest mammalian cells, which means that it has a low surface area to volume ratio. This fact, together with the low permeability to water of the plasma membrane means that it is difficult to remove much of the intracellular water. Therefore, water tends to be retained on cooling and intracellular ice formation can ensue. From the onset of follicle growth the oocyte is set on a defined course of maturation, at the end of which it is receptive to fertilisation for only a short time. In order for fertilisation and development to occur the integrity of a number of specialised structures must be maintained.

1.4.1 Specific Features of Oocytes

1.4.1.1 The Zona Pellucida

The mature, ovulated oocyte is surrounded by a proteinaceous coat known as the zona pellucida. The primary function of the zona is at the time of fertilisation. Following penetration by spermatozoa, cortical granules that are produced by the golgi apparatus and positioned immediately adjacent to the plasma membrane, undergo exocytosis and release enzymes that toughen the zona. This prevents the penetration of additional spermatozoa and thus prevents polyspermy.

Mature mouse oocytes that had been exposed to 1.5M Me_2SO were found to have hardened zona pellucidas, this effect was found to coincide with the depletion of cortical granules at the oocyte cell surface (Vincent *et al*, 1990a). Similar

cryopreservation protocols that were found not to cause hardening, did not cause a significant reduction in the reservoir of cortical granules. This evidence suggests that the effect of Me_2SO on the zona is associated with cortical granule release (Vincent *et al*, 1990a). Frozen/thawed oocytes were found to have a reduced rate of fertilisation when compared with unfrozen control oocytes and control oocytes that were exposed to cryoprotectant in the absence of cryopreservation (Carroll *et al*, 1990a). Following zona drilling of thawed oocytes, the fertilisation rate was increased which strongly suggests that changes in the zona that occurred during cryopreservation were responsible for the reduced rate of fertilisation.

Experiments have shown that *in vitro* culture of mouse oocytes causes progressive hardening of the zona. Anaerobic culture and a covering of cumulus cells have both been shown to reduce this effect (DeFelici and Siracusa, 1982). It was found that the addition of serum during IVM improved penetration by spermatozoa and shortened the time required for enzymatic dissolution of the zona (Choi *et al*, 1987). Similar reports were made suggesting that the addition of serum during cryopreservation could be beneficial to the condition and function of the zona post thaw. Significantly more oocytes were fertilised following the addition of serum during cryopreservation and subsequent culture, than following the addition of poly vinyl alcohol (PVA), (Carroll *et al*, 1993). The recent adoption of ICSI (section 1.2.2.4) following cryopreservation has largely overcome the problems associated with zona hardening (Kazem *et al*, 1995), with all human live births from cryopreserved oocytes since 1997 having resulted from this treatment. Although, questions over the safety of this technique have been raised as it bypasses many of the natural checks that may prevent fertilisation of an oocyte by an abnormal sperm. Whether ICSI is required to achieve fertilisation of cryopreserved oocytes remains

uncertain. However, a cryopreservation technique that facilitates un-assisted fertilisation would allow the natural safety checks to remain in place.

1.4.1.2 The Cytoskeleton

Microfilaments

Actin polymers (microfilaments) form an integral part of the complex and dynamic cytoskeleton of the oocyte. Most of the surface of mature mouse oocytes is covered by short dense microvilli (Vincent *et al*, 1990b). In the region of cortex overlying the chromosomes and meiotic spindle, microvilli are scarce yet there is a high concentration of actin filaments (Maro *et al*, 1984). Intact microfilaments are required for a number of dynamic intracellular changes at the time of fertilisation (Maro *et al*, 1986). Under normal circumstances, following fertilisation, a meiotic furrow forms in the region of the cortex at the equator of the spindle. Two areas rich in actin form at each side of the furrow. One of these shrinks and the other expands, causing rotation of the meiotic spindle and the formation of the second polar body. The addition of an inhibitor of actin polymerisation, cytochalasin D, prevents spindle rotation, furrow constriction and pronuclear migration (Maro *et al*, 1984). It has also been demonstrated that microfilaments are involved in anchorage of the spindle to the plasma membrane. The actin-rich cortical area overlying the spindle forms a domain to which the meiotic cleavage furrow is restricted. In this way the spindle is maintained in a peripheral location, thus allowing the formation of the polar body (Webb *et al*, 1986). Although the orientation of the spindle and actin fibres in the human oocyte differs from that of the mouse, damage to the actin network could also have an effect on the ability of the cells of the embryo to divide correctly during subsequent development.

The typical cortical actin meshwork found in mouse oocytes has been shown to be disrupted by exposure to 1.5M Me_2SO at 37°C. Changes in the cell surface involving microvilli length and distribution were noted, as well as the irregular expansion of oocytes following removal of the cryoprotectant. These effects were reduced by exposure to the cryoprotectant at lower temperatures (Johnson, 1989). Exposure of mouse oocytes to 1.5M Me_2SO caused less disruption when the treatment was carried out at 4°C prior to freezing, and removed at the same temperature following thawing. Whether exposure to CPA was performed at 37°C or 4°C most of the changes were found to be reversible after removal of the Me_2SO (Vincent *et al*, 1990b).

Oocytes of other species, including rabbit and human, have a different pattern of polymerised actin distribution in their cortical region and along junctional feet. In rabbit oocytes this distribution has been shown to absent after treatment with 1,2-propanediol (PROH). However, after incubation for 30 minutes this effect was found to be reversible in some cases (Vincent *et al*, 1989).

Microtubules

Microtubules are absent from the cytoplasm of mature oocytes, as they constitute the meiotic spindle on which the maternal chromosomes are configured. The spindle is temperature sensitive and must be preserved during cryopreservation of oocytes as it is vital in the subsequent separation of chromosomal material. A process which, if compromised may lead to an increase in chromosomal abnormalities such as aneuploidy or polyploidy. Cryopreservation of mouse oocytes in the presence of Me_2SO has been shown to increase the incidence of polyploidy but not aneuploidy in first-cleavage stage embryos (Glenister *et al*, 1987). It has also been demonstrated that freezing and thawing of mouse oocytes leads to an increased incidence of

chromosomal abnormalities in one-cell zygotes (Bouquet *et al*, 1992). This subsequently leads to decreased cleavage rates and development in early embryos (Bouquet *et al*, 1993). In contrast the frequency of aneuploidy was not increased following vitrification in 6M Me_2SO, provided the exposure time did not exceed 90 seconds. Similar proportions of vitrified and control oocytes were fertilised, implanted and went on to form normal fetuses (Bos-Mikich *et al*, 1995).

Within mature mouse oocytes the meiotic spindle is held at a tangent to the plasma membrane. At fertilisation it rotates and becomes radially orientated before the second polar body is extruded (Schatten *et al*, 1985). The architecture of the spindle is maintained via a dynamic equilibrium between alpha and beta tubulin dimers or free tubulin, and polymerised microtubules. Due to this energetic relationship the microtubular spindle is both thermosensitive and chemosensitive.

Studies have shown that the spindles of oocytes that are held on ice become disorganised. Reversal of this effect was not observed at either 18°C or 37°C, the optimal temperature for tubulin re-polymerisation after cooling was found to be 25°C (Magistrini and Szollosi, 1980). Suprisingly, exposure to temperatures only slightly below physiological has also been shown to affect these sensitive structures. Cooling of oocytes to 20-25°C for 30 minutes was shown to cause progressive disassembly of the spindle, which in turn elevated the levels of free tubulin and drove the transient formation of asters (Pickering and Johnson, 1987). However, this effect was more pronounced following cooling to lower temperatures. Cooling to room temperature has been shown to have a similar effect on human oocytes. Cooling for 10-30 minutes caused a reduction in spindle size, disorganisation of the microtubules of the spindle and in some cases a complete lack of microtubules (Pickering *et al*, 1990). Ultrarapid cooling also caused loss and clumping of microtubules (Sathananthan *et al*, 1988).

More recently, cooling of human oocytes to 0°C for 10 minutes has been shown to cause the complete destruction of the spindle. However, only minor damage was found to occur if the oocytes were held at 0°C for less than one minute (Zenzes *et al*, 2001).

The exposure of oocytes to cryoprotectant can also have an influence on the structure of the spindle. Exposure to Me_2SO was shown to drive the polymerisation of microtubules that are associated with pericentriolar material, at the expense of the unravelling spindle (Johnson and Pickering, 1987). Similar effects were reported following exposure of mouse oocytes to PROH. Treatment with 1.5M Me_2SO at room temperature led to a high proportion of oocytes with abnormal spindles, while similar treatment with PROH led to absence of the spindle (Van der Elst *et al*, 1988). Equivalent observations were made in rabbit oocytes where exposure to PROH or Me_2SO led to spindle disassembly and the appearance of microtubules in the cytoplasm (Vincent *et al*, 1989). In most cases following removal of the cryoprotectant and a period in culture, spindles were found to reform. There have been reports of normal chromosomal configuration in human oocytes following exposure to and cryopreservation in PROH (Gook *et al*, 1993). In fact, in one study chromosome dispersal did not occur despite disappearance of the spindle (Zenzes *et al*, 2001). In other species disruption to the intracellular architecture has resulted in some chromosomal dispersal (Johnson and Pickering, 1987, Pickering and Johnson, 1987, Pickering *et al*, 1990, Sathananthan *et al*, 1988, Van der Elst *et al*, 1988, Vincent *et al*, 1989).

1.4.2 Cryopreservation of Mature Oocytes

One of the first reports of oocyte cooling detailed the survival and *in vivo* development of oocytes that were cooled to −10°C using glycerol as a cryoprotectant

(Sherman and Lin, 1958). Another early study looked at the cryopreservation of isolated rabbit and human oocytes again using glycerol as a cryoprotectant. High rates of morphological normality were reported in both types of oocyte (Burks *et al,* 1965).

Following the successful cryopreservation of embryos, the protocols used were adapted for oocyte cryopreservation. The first reports of live births from frozen murine embryos demonstrated that slow-cooling at a rate of 0.3-2°C/min, followed by slow warming rates of 4-25°C/min. in the presence of 2M Me_2SO, most favoured survival. There was a 50-70% rate of progression to expanded blastocyst and live births were achieved following embryo transfer (Whittingham *et al,* 1972). Similar results were obtained by Wilmut *et al* in the same year, also using Me_2SO as a cryoprotectant (Wilmut *et al*, 1972). Further studies revealed that the requirements for embryo freezing were not only species specific, but also related to the stage of development. Refinement of these protocols led to their widespread use in the establishment of embryo banks for the breeding of valuable commercial livestock. Embryo freezing was also utilised in the development and banking of mouse strains for the purpose of research. In the absence of any substantial evidence of genetic alteration or birth defects the knowledge gained from these animal studies was applied in the development of protocols for the freezing of human embryos. The first pregnancy arising from a human embryo, which was frozen at the 8-cell stage, was reported by Trounson and Mohr (1983).

1.4.2.1 Slow-Cooling of Oocytes

A number of reports of the successful preservation of rodent oocytes using the adapted slow-cooling methods outlined above quickly ensued (Tsunoda *et al,* 1976; Parkening and Chang, 1977). One of the earliest experiments using this method

involved the treatment of oocytes with 1.5M Me_2SO on ice at 4°C followed by slow cooling to –65°C or -80°C after which they were stored at –196°C. Despite poor survival this resulted in the first live births following cryopreservation of ovulated mammalian oocytes (Whittingham, 1977). In another such study, mouse oocytes were slow-cooled at 0.5°C/min to –80°C, using Me_2SO as a cryoprotectant and held at -196°C before thawing. Following culture, 94% were found to be morphologically normal. Fifty-three percent of the oocytes went on to cleave at fertilisation and when transferred to live females most of these implanted (Glenister *et al*, 1987). However, other reports of mouse oocyte cryopreservation using identical cooling profiles gave lower normality/survival rates of 69-88% (Schroeder *et al*, 1990). This could be due to the use of cryovials that may prevent sufficient cooling of the oocytes within the volume of liquid.

In a study involving cryopreservation of oocytes of different species in the presence of PROH, structural damage occurred such as cytoplasmic blebbing into the perivitelline space. This damage did not occur when a mixture of PROH and Me_2SO was used (Todorow *et al*, 1989). Cryopreservation of murine oocytes in the presence of PROH has also been shown to cause increased rates of parthenogenetic activation (Van der Elst *et al*, 1992).

1.4.2.2 Rapid-Cooling of Oocytes

If a biological sample is to be cooled rapidly a higher concentration of CPA should be used (see section 1.3.1.2). Ultra-rapid cooling in the presence of 3.5M Me_2SO led to 33-34% survival of mouse oocytes, with only 7-15% progression to blastocyst. The developmental impairment was attributed to the formation of micronuclei and a failure in the post-thaw incorporation of chromosomal material into the nuclei (Sathananthan *et al*, 1988). More favourable results were reported

following ultra-rapid cooling using a mixture of Me_2SO and sucrose. One study looked at ultrarapid cooling in the presence of 3.5M Me_2SO and a range of different sucrose concentrations. Oocytes that were rapidly cooled with 0.25 or 0.5M of sucrose and 3.5M Me_2SO developed to the blastocyst stage during post-thaw culture (Surrey and Quinn, 1990). Presumably as a non-permeating agent, the sucrose reduces the formation of extracellular ice crystals and thus decreases the damage that can be caused by these. In another study that used sucrose as a protective agent during rapid cooling, mouse oocytes survived at a rate of 80-95%, of these 56% were fertilised and 46% of the fertilised oocytes went on to form blastocysts (Van der Elst *et al*, 1991).

1.4.2.3 Vitrification of Oocytes

In order to vitrify cells at practicable cooling rates they need to be in the presence of a high concentration of CPA (section 1.3.3). Exposure to such high concentrations can result in damage caused by the cytotoxic effects of the CPA and/or by the osmotic stress imposed during equilibration (Fahy, 1986). Vitrification in the presence of high solute concentrations was used by Rall and Fahy (1985) to successfully preserve mouse embryos. They used a vitrification solution named VS1 that was a mixture of two permeating (Me_2SO and PROH) and one non-permeating polyethylene glycol (PEG) cryoprotectant, together with acetamide. Such a technique has since been applied to murine oocytes. While this was successful (Kono *et al*, 1991; Nakagata, 1990; Nakagata, 1993), exposure to VS1 was found to lead to fetal deformity. The acetamide added to the solution to reduce the cytotoxic effects of Me_2SO was itself thought to be a toxic agent (Kola *et al*, 1988). Vitrification solutions with fewer constituents have been developed since. VS2 contains PROH and PEG, VS3 contains glycerol and PEG. Improved rates of embryo survival have been reported following the use of VS2 and VS3 compared with VS1 (Rall, 1987). Good

rates of survival have also been achieved following vitrification using a single CPA Rall and Wood (1994). In addition, murine oocytes that were vitrified in 6M Me_2SO survived at a rate of over 70% (Wood *et al*, 1993). One of the most important factors in the success of vitrification is a rapid rate of warming (section 1.3.4). The poor survival rates reported by some investigators have been attributed to devitrification or ice crystal growth that occurs on warming (Shaw *et al*, 1991; Shaw *et al*, 1992).

1.4.2.4 Cryopreservation of Mature Human Oocytes

The first human live births to result from cryopreserved oocytes were achieved by the adoption of a standard slow-cool cryopreservation protocol. Oocytes were slow-cooled to –36°C in the presence of 1.5M Me_2SO and then plunged to –196°C. Seventy six percent of the oocytes survived post-thaw, of which 75% fertilised and 60% of the embryos cleaved to a multicellular stage. Seven embryo transfers were carried out, these resulted in the birth of fraternal twins (Chen, 1986), and a singleton birth (Chen, 1988). Another study used a lengthy period in which to allow equilibration of the oocytes with 1.5M Me_2SO. The oocytes were then slow-cooled to –70°C and stored in liquid nitrogen. Post-thaw only seven out of 28 oocytes survived. However, two embryo transfers were carried out resulting in one pregnancy (Van Uem *et al*, 1987).

Investigators began to perform experiments designed to compare the cryoprotective qualities of Me_2SO and PROH with respect to human oocytes. Higher rates of survival were reported following cryopreservation in PROH compared with Me_2SO (Al-Hasani, 1987; Todorow *et al*, 1989). The karyotypes of 4 oocytes that had been cryopreserved with PROH were examined and all were found to be normal (Gook, 1994). A number of live births have been reported following slow cooling in the presence of PROH and sucrose followed by rapid thawing. One study reported

that four out of 12 oocytes survived slow cooling to –30°C and then rapid cooling to –150°C, two of these were fertilised following ICSI. Although only one embryo cleaved, this was transferred and a healthy female was born (Porcu *et al*, 1997). In another study 10 oocytes were cryopreserved using a very similar method. Three of the oocytes survived and following ICSI two embryos were produced. Embryo transfer was carried out and a healthy male was born (Polak de Fried *et al*, 1998). Better survival rates (~88.9%) were reported in a similar study where ICSI produced a number of healthy embryos. Five were transferred and a triple pregnancy was established (Young *et al*, 1998).

In addition to the success reported following slow cooling of oocytes, recently, reports have been made of successful vitrification using EG and sucrose. In one study 17 mature oocytes were vitrified in 40% EG and 20.54% sucrose. Eleven oocytes survived and underwent ICSI. Five pronuclear zygotes were formed and three embryos developed from these. Each embryo was transferred to a separate patient and one pregnancy developed that resulted in the birth of a healthy female at 37 weeks (Kuleshova *et al*, 1999).

1.4.3 Cryopreservation of Immature Oocytes

One possible explanation for the poor developmental potential of cryopreserved and thawed mature oocytes is that the temperature-sensitive microtubular spindle, which is constructed during oocyte maturation and is vital to the development of the oocyte following fertilisation, is damaged during freezing. Immature oocytes are yet to form the spindle and so may be more resilient to the cooling process. The central location of cortical granules at the GV stage may also be advantageous during freezing. Additionally, during the early stages of oocyte maturation, the small size of the oocytes coupled with differences in membrane permeability, could lead to

dissimilarity in their response to cryoprotectant exposure and cooling when compared to that of mature oocytes.

1.4.3.1 Cryopreservation of Ovarian Tissue and Isolated Follicles

Early oocytes within primordial follicles are approximately 1% of the volume of fully grown mature MII stage oocytes. The oocytes and somatic cells are less differentiated; the oocyte has fewer organelles and is lacking both a zona pellucida and cortical granules (Oktay *et al*, 1998a). Also primordial follicles are extremely numerous within the ovarian cortex. These characteristics make early oocytes good candidates for cryopreservation.

The earliest studies of oocyte cryopreservation involved freezing of ovarian pieces in the 1950s. Oocyte survival was achieved following cryopreservation and thawing of immature rat ovaries in a solution containing glycerol and rat serum (Deanesly, 1957). The survival of primordial follicles from slices of rat ovary was demonstrated following cooling to –79°C. However, after grafting of the tissue the follicles degenerated (Parkes, 1958). Subsequently the births of live mice were reported following transplantation of frozen ovary to sterilised recipients (Parrott, 1960).

More recently, it has been demonstrated that fertility can be restored to sterilized mice by the transfer of ovarian tissue containing primordial follicles (Gosden, 1990). Fetal mouse ovaries were cryopreserved in Me_2SO and stored at –196°C were capable of restoring cyclicity to oophorectomised mice following transplant, at a frequency similar to that achieved following transplant of fresh untreated ovaries (Harp *et al*, 1994). Primate ovaries have a lower density of follicles within the cortex than are present within the mouse ovary. Sheep ovaries are similar to primate ovaries in size and follicle density. It has been reported that fertility can be restored to sterilised

sheep following transplant of ovarian tissue that had been slow-cooled in Me_2SO and stored in liquid nitrogen. This was confirmed by a live birth (Gosden *et al*, 1994). It has also been reported that pieces of marmoset ovary slow-cooled in 1.5M Me_2SO were capable of supporting development of large antral follicles following transplantation into immunodeficient mice (Candy *et al*, 1995).

Studies looking at the morphological normality of cryopreserved human ovarian tissue have indicated that follicles can survive freeze/thawing within the ovarian cortex. In one study human ovarian tissue was controlled-rate cooled using either Me_2SO or PROH and sucrose as cryoprotectants (Hovatta *et al*, 1996). After thawing there were no signs of tissue necrosis in either group. In addition, the proportion of atretic follicles was not significantly increased compared with fresh samples. In a subsequent study, primordial and primary follicles in cryopreserved and thawed human ovarian tissue were cultured within an extracellular matrix. Two thirds of the follicles were found to be viable after 10-15 days in culture (Hovatta *et al*, 1997). In another study, ovarian slices were slow-cooled in EG. Viable primordial follicles were isolated from cryopreserved samples at a similar frequency to those isolated from fresh ovarian slices. Electron microscopy revealed that the majority of cells lacked ultrastrucutral damage after cryopreservation (Oktay *et al*, 1997). In another study slices of human ovarian cortex were cryopreserved in PROH. The number of normal oocytes and pre-granulosa cells were significantly reduced by cooling. The highest proportion of intact primordial and primary follicles was achieved after a slow-cool/rapid thaw cryopreservation protocol (Gook *et al*, 1999).

Following cryopreservation and thawing, oocytes within the early follicles must be allowed to grow and mature before they can be fertilised. Growth and maturation of fresh follicles was attempted by the grafting of follicles within human ovarian

tissue into immunodeficient mice (Oktay *et al*, 1998b). The follicles survived for 17 weeks, grafts that were supplemented with FSH matured to the antral stage. In a later study cryopreserved cortical strips of human ovary were transplanted into a pocket of the pelvic wall of a patient. A dominant follicle was identified following hormonal stimulation (Oktay *et al*, 1999). Unfortunately, many of the studies that involve transplant of ovarian tissue to oophorectomised recipients are conducted soon after the oophorectomy. If this treatment were to be applied to human patients there could be years between the removal and the transplant of the ovaries. After this time period the site may have insufficient vascularisation, in which case a new site would be required (Oktay *et al*, 1998b). The re-establishment of cyclical activity could also be problematic. In addition it has been shown in the mouse that cryopreserved ovarian tissue containing cancer cells can cause cancer if transferred to a healthy recipient (Shaw *et al*, 1996). This was supported by a more recent study which was unable to exclude the possibility of transmission of cancer following transplantation of affected tissues (Kim *et al*, 2001).

Oocytes within cryopreserved follicles could also be matured *in vitro*. It has been demonstrated in bovine follicles that a short period of culture can cause a significant increase in the proportion of normal follicles following cryopreservation in Me_2SO (Paynter *et al*, 1999). Another study looked at the IVM of cryopreserved follicles either within the ovarian cortex or following partial isolation. Thawed ovarian cortical tissue containing primordial, primary and secondary follicles was cultured as slices. Following mechanical or enzymatic partial isolation of half of the follicles, they were all cultured in an extracellular matrix for 1-3 weeks (Hovatta *et al*, 1999). It was found that the proportion of atretic follicles was increased by partial isolation and that post-thaw culture was best carried out within the intact ovarian

cortex. In a similar study human follicles were isolated from cryopreserved ovarian slices and cultured within a collagen gel support matrix. Follicles showed an increase in the number of granulosa cell layers and an increase in oocyte diameter, similar to that observed in follicles isolated from fresh ovarian slices (Abir *et al*, 1999).

In contrast to ovarian tissue, isolated follicles are delicate and rapidly disintegrate into single cells (Oktay *et al*, 1998a). However, as demonstrated in some of the studies discussed, their structure can be supported and protected by integration within a matrix like substance (Abir *et al*, 1999; Hovatta *et al*, 1997; Hovatta *et al*, 1999). It has also been demonstrated that primary follicles can be isolated from mouse ovaries and cryopreserved in the presence of Me_2SO. Follicle growth and maturation were found to occur in the absence of surrounding ovarian tissue, to a similar degree in both the fresh and cryopreserved follicles (Carroll *et al*, 1990b). Eventually some of the follicles produced mature oocytes, these were fertilised and transferred to recipients at the 2-cell stage leading to a single live birth. In a subsequent experiment, primordial follicles were isolated from mouse ovaries and cryopreserved using Me_2SO as a cryoprotectant. The thawed follicles were suspended within plasma clots and transplanted to recipient animals. The grafts reportedly reorganised into morphologically identifiable ovaries. Normal offspring were produced following natural mating (Carroll and Gosden, 1993).

1.4.3.2 Cryopreservation of Isolated Immature Oocytes

Immature oocytes are often produced together with mature oocytes following the hormonal stimulation regimes used in conventional IVF treatment. Regimes for the collection of immature oocytes alone have subsequently been devised, involving modification of oocyte collection techniques and reduction of hormonal stimulation. Recovery of immature oocytes from unstimulated ovaries has also been reported

Therefore, the use of immature oocytes for the purpose of routine IVF is not only feasible, but could actually be preferable to traditional IVF treatment, reducing the risk of ovarian hyperstimulation syndrome (OHSS), lowering treatment costs and reducing treatment time, a factor which is particularly relevant to cancer patients. In addition, patients who only produce immature oocytes could be offered IVF treatment.

At GV stage the oocyte has reached full size and is surrounded by layers of much smaller somatic cells, called cumulus cells. At this point the chromosomes are decondensed and enclosed within the nuclear envelope, as is the case in embryos. The lack of evidence of chromosomal damage during embryo cryopreservation suggests that when in this state, chromosomes are less prone to cryoinjury (Bernard, 1991). Also at this stage the oocyte is meiotically competent. The temperature sensitive meiotic spindle is yet to form, and so theoretically, may escape damage during cryopreservation.

GV stage oocytes may be more sensitive to exposure to cryoprotectant than mature oocytes. Cryopreservation of GV stage hamster oocytes led to 63% survival, compared with 81% observed in mature oocytes (Mandelbaum *et al,* 1988). This was attributed to differences in sensitivity to the freezing and thawing protocols, due largely to changes in the permeability of the oocyte membrane during the progression of maturation. In another study matured mouse oocytes treated with Me_2SO had a survival rate of 99%, while only 88% of GV stage oocytes treated in this way survived. Mouse oocytes frozen at GV stage survived at a rate of 69%, of these 95% underwent germinal vesicle breakdown, and 90% formed a polar body. Subsequently, only 9% underwent the first cleavage to form a two cell embryo, and none of these went on to form blastocysts (Schroeder *et al,* 1990). However, in a study by Van

Blerkom and Davis (1994) GV stage oocytes had a higher rate of survival (79%) following cryopreservation than metaphase II/mature oocytes (60%). GV stage oocytes were found to mature and fertilise at a high frequency, with no significant increase in the incidence of aneuploidy. This suggested that the GV stage oocytes were more resistant to the process of freezing than mature oocytes. The increased levels of survival observed in some studies indicate that the GV stage oocyte may show an increased resilience to cryopreservation. This could be due to some of the attributes, listed earlier such as the lack of meiotic spindle or decondensed state of the chromosomes. Alternatively, it could be the due to the presence of the cumulus cells that surround the oocyte at GV stage.

Despite high rates of survival, maturation of thawed oocytes remains problematic. Cryopreservation of GV stage mouse oocytes in the presence of 1.5M Me_2SO led to high rates of survival (93%), and the rates of maturation to metaphase II and fertilisation was the same as for fresh control oocytes (Candy *et al,* 1994). Following uterine transfer, implantation rates were similar across the groups. However, the rates of embryo loss were found to be higher in the groups subjected to *in vitro* maturation. While only 24% loss was observed in mature oocytes, fresh GV stage oocytes showed a 46% loss, and frozen/thawed GV stage oocytes a 40% loss. The poor developmental potential of the GV stage oocytes was attributed to sub optimal *in vitro* maturation rather than damage inflicted during cryopreservation as similar rates of loss were recorded, both following cryopreservation and in the absence of it.

1.4.3.3 Cryopreservation of Immature Human Oocytes

A number of studies have looked at the cryopreservation of immature human oocytes. Survival post thaw is generally poor. GV stage oocytes survived at a rate of

37%, of which 20% were matured *in vitro* to the MII stage (Mandelbaum *et al*, 1988). Slow cooling and thawing of immature prophase I oocytes in the presence of PROH led to 15.6% survival and 58.3% maturation to metaphase II (Toth *et al*, 1994). Whereas, stepwise addition of PROH, followed by rapid cooling and thawing led to 43.3% survival and 27.3% maturation to metaphase II. Rapid cooling was found to result in the survival of significantly more oocytes, however, maturation frequency was not significantly altered by different cooling rates. In a study by Van Blerkom and Davis (1994), immature human oocytes were vitrified using a range of different concentrations of Me_2SO and sucrose. Although premature and apparently partial condensation of chromosomes was observed in half of the oocytes, they underwent nuclear maturation at a high frequency. It was commented that the susceptibility to cryoinjury at chromosomal and cytoplasmic level might be more related to oocyte quality than the specific freezing protocol.

One study compared cryopreservation of mature and immature oocytes (Baka *et al*, 1995). Following stepwise addition of PROH, oocytes were slow cooled to –30°C before being plunged into liquid nitrogen. Spindle evaluation revealed that 43.5% of mature MII stage oocytes that were cryopreserved had normal spindles. Whereas a significantly higher proportion, 81% of cryopreserved GV stage oocytes had normal spindles. This is similar to the percentage normality observed in untreated-control oocytes (83% and 88.9%).

Cryopreservation of GV stage oocytes from unstimulated ovaries resulted in significantly lower maturation to MII stage (59.3%) than observed in untreated control oocytes (76.8%). The oocytes that were cryopreserved at GV stage fertilised at a rate of 42.9%, of which 16.7% cleaved. This was significantly lower than the rates of fertilisation (90.5% and 81%) and cleavage (94.7% and 88.2%) associated with the

fresh control oocytes and oocytes exposed to CPA without freezing respectively (Son *et al*, 1996). In a further study using the same method GV stage oocytes were found to have a substantial amount of chromosomal and spindle abnormalities, 77.8% were aneuploidy or polyploidy. This effect may account for the frequency of poor development observed. It was also suggested that the developmental impairment was caused by a failure in the completion of cytoplasmic maturation, leading to aberrations in cell physiology at a molecular level (Park *et al*, 1997).

In the first report of human live births from cryopreserved immature human oocytes, only three of 13 oocytes survived slow-cooling and thawing in PROH and sucrose. Of these only 2 reached maturity after 30 hrs of culture. Both of the matured oocytes underwent intracytoplasmic sperm injection and were fertilised, they were transferred to a recipient and a healthy infant was born at 40 weeks gestation (Tucker *et al*, 1998). In another study, ultra-rapid cooling was used to cryopreserve GV stage oocytes that originated from the antral follicles of unstimulated patients with chocolate ovarian cysts. Oocytes were cooled in the presence of ethylene glycol and sucrose, 59% of the oocytes survived and were judged to be morphologically normal, and after *in vitro* maturation 64% were mature. Following insemination 70% of the oocytes were fertilised and 71% of these underwent the first cleavage to form two cell embryos. The transfer of two of these embryos to a recipient uterus led to a pregnancy (Wu *et al*, 2001).

The results of these studies seem to suggest that the severe impairment in development observed following cryopreservation of GV stage oocytes of other species is reflected in the case of human oocytes cryopreserved at the same stage. Embryo development is one of the most important parameters by which the success of

oocyte cryopreservation is assessed. Unfortunately, in the case of human oocytes, for ethical reasons this can be problematic.

1.5 MATURATION OF OOCYTES *IN VITRO*

The ovary of the young, mature adult contains many follicles at different stages of growth and maturation. The follicles that contain the earliest form of oocyte, primordial follicles, are also the most numerous. As follicular growth progresses there is a gradual apoptotic 'thinning out' of follicles. This facilitates the spatial distribution of follicles to allow subsequent follicle growth to progress unhindered (Nayudu and Osborn, 1992). Although primordial follicles are numerous and, therefore, represent a large pool of oocytes for use in IVF (Salha *et al,* 1998), the immature oocyte contained within would require a lengthy period of *in vitro* culture which should include the correct conditions to trigger and complete maturation. Presumably during *in vitro* culture some of these ovarian factors are absent. Also, the apoptotic 'thinning out' process may continue during *in vitro* culture and this could reduce the original number of primordial follicles within a given culture group. IVM of oocytes within primordial follicles has only been achieved in the mouse (Eppig and O'Brien, 1996). Follicles at later stages of growth and maturation are less numerous. However, IVM is more easily achieved due to the reduced period of *in vitro* culture required. IVM of oocytes within antral follicles, for example, has been achieved in a number of species including humans (Abir *et al*, 1999).

1.5.1 *In Vitro* Follicle Growth

1.5.1.1 Primordial and primary follicles

Within the ovary, primordial follicles are present in large numbers, the younger the ovary the more primordial follicles are present. Primordial follicles contain an oocyte surrounded by a layer of flattened somatic cumulus cells (as described in section 1.1.1). Primordial oocytes have not yet reached the growth phase and they are arrested at prophase of the first meiotic division (Pederson and Peters, 1968). From this reservoir of follicles only a relatively small number will go on to develop to the Graafian stage. The only report of *in vitro* growth and maturation of primordial follicles was achieved using a two-step system. During preantral development, the follicles were cultured within entire ovaries excised from newborn mice. After 8 days in organ culture the volume of the oocytes had increased 13.8 fold. COCs were then isolated from the cultured ovaries and were cultured further in fresh media in the presence or absence of FSH and epidermal growth factor (EGF). The matured oocytes showed competence to undergo GVBD and polar body extrusion. Forty two percent of the oocytes matured with FSH and EGF went on to the two-cell stage. The rate of blastocyst formation was reported to be ~2% in all groups. A single live birth was achieved (Eppig and O'Brien, 1996). In a system that aimed to mimic physiological conditions, it was demonstrated that primary murine ovarian follicles could also be grown *in vitro*. Intact follicles with theca and stroma attached were grown to the Graafian stage and then stimulated to ovulate in response to LH. The resulting oocytes were reported to be capable of fertilisation, embryogenesis and development to viable offspring (Spears *et al*, 1994).

1.5.1.2 Preantral and antral follicles

In vitro maturation of pre-antral follicles has been reported in a number of species. There are two different approaches that have been taken in their culture. One approach is the culture of whole follicles (Nayudu and Osborn, 1992). Intact follicles were manually isolated and cultured with a full complement of theca cells and intact basal lamina. Antrum formation occured and the oocytes were capable of growth and subsequent embryo development.

The second approach, that of Eppig and Schroeder (1989), and Eppig *et al* (1992), was the culture of cumulus-oocyte complexes enzymatically isolated from pre-antral follicles. Murine complexes were cultured on collagen impregnated membranes or serum coated plastic dishes. Although antrum formation did not occur, the oocytes were capable of growth and development. Live young were produced by embryo transfer at the 2-4 cell stage (Eppig and Schroeder, 1989). Other studies also reported follicle development to multilaminar stages. However, often antrum formation did not occur and isolation of the oocytes from within the collagen gel proved to be difficult (Torrance *et al*, 1989; Carroll *et al,* 1991). In an extention of the method of Eppig and Schroeder (1989), that allowed microscopic evaluation of the growing follicles and sampling of the surrounding media, granulosa cell proliferation and the formation of antral-like cavities occurred. Cortvrindt *et al* found that following culture in FSH supplemented media ~40% of the oocytes progressed to MII. The oocytes were capable of fertilisation and development to expanded blastocysts (Cortvrindt *et al*, 1996). In a similar method, Roy and Treacy (1993) cultured enzymatically isolated COCs within layers of agar. In this case, antrum formation did occur; however, oocyte growth and development were not assessed.

Antral follicles have also been cultured *in vitro*. In a morphological study of human follicles, the follicles were embedded within a collagen gel impregnated with FSH. It was hypothesised that the gel would help to conserve the three-dimensional structure of the follicle. In earlier studies the integrity of murine follicle structure had been affected by culture on a flat surface (Cortvrindt *et al*, 1996). Forty percent of the cultured follicles showed an increase in granulosa cell number and an increase in oocyte size during 24hrs of culture. However, ovulation was not achieved (Abir *et al*, 1999).

1.5.2 *In Vitro* Maturation of Oocytes

As previously stated (section 1.1.3), pre-ovulatory oocytes are capable of spontaneous maturation when cultured *in vitro* after removal from the follicular environment (Pincus and Enzmann, 1935; Edwards, 1965). However, oocytes so treated have shown reduced rates of fertilisation and development (Thibault, 1977). The addition of serum, hormones, growth factors and meiosis inhibitors to the culture media of isolated oocytes has been shown to improve rates of maturation and development in a manner similar to that demonstrated during *in vitro* maturation of follicles (section 1.5.1).

One problem encountered following *in vitro* culture of mouse oocytes was that of hardening of the zona pellucida (De Felici and Siracusa, 1982). During normal fertilisation the contents of cortical granules, that lie around the periphery of the oocyte, are released following penetration by a spermatazoon. This is thought to cause the proteolytic conversion of glycoproteins that constitute the zona pellucida, leading to hardening of the zona and prevention of further sperm penetration. *In vitro* matured oocytes were not penetrated by spermatozoa and did not become fertilised (Choi *et al*, 1987). In addition, enzymatic digestion of the zona pellucida by α-chymotrypsin was

lengthier following IVM than following ovulation. This effect was reduced by the addition of fetal calf serum (FCS) to the culture media, leading to improved sperm penetration and shorter duration of enzymatic dissolution (Choi *et al*, 1987). Calf serum contains a glycoprotein called fetuin that acts as a protease inhibitor. Incubation of murine oocytes in serum-free media with added fetuin did not cause zona hardening and the oocytes were capable of fertilisation (Schroeder *et al*, 1990b). A human homologue of this molecule, alpha 2-HS-glycoprotein, is found in human follicular fluid (Kalab *et al*, 1993).

Growth factors perform a number of important functions in FSH-dependent folliculogenesis. They are regulatory proteins produced by the oocyte and the granulosa cells within the ovary, for this reason they may be beneficial during maturation *in vitro* (Erickson and Shimasaki, 2001). When combined with FSH, the addition of EGF during IVM of mouse oocytes improved the rate of fertilisation (Merriman *et al*, 1998). Addition of EGF and insulin-like growth factor I (IGF-I) to bovine COCs from small pre-antral follicles, during IVM lead to a significant increase in the frequency of first polar body extrusion (Sakaguchi *et al*, 2002). Other molecules that have important regulatory functions *in vivo* include; inhibin, activin and follistatin, which control the synthesis and secretion of pituitary FSH. These proteins also have important roles in folliculogenesis, oocyte maturation and corpus luteum function. The intraovarian functions of these regulatory molecules include; granulosa cell proliferation and regulation of FSH and LH receptor expression by granulosa cells (Knight and Glister, 2001). Activin A and inhibin A were reported to stimulate GVBD, progression to MII stage and improve subsequent rates of fertilisation in GV stage primate oocytes that were matured *in vitro* (Alak *et al.*, 1996). The maturation regulatory molecule MPF (see section 1.1.3.3) which is

activated *in vivo* by the endogenous LH surge, has been shown to be activated in a similar way during *in vitro* maturation (Trounson *et al*, 2001).

1.5.2.1 *In vitro* maturation of isolated murine COCs

Considerable effort has been focused on the elucidation of IVM conditions that will lead to optimum levels of maturation, fertilisation and embryo development. Due to the scarcity of human oocytes, a large majority of these studies have been carried out on mouse oocytes. Numerous studies concerned with *in vitro* maturation of isolated COCs have examined the roles of GTs. Addition of GTs to culture media during IVM of mouse COCs caused an initial inhibition of GVBD (Eppig *et al*, 1983). However, GT addition was shown to cause only transient inhibition of GVBD. After which GVBD occurred at an accelerated rate compared with that of oocytes that were induced to mature spontaneously (Downs, 1990) It was suggested that the initial inhibition of GVBD resulted from a rise in intracellular cAMP that occurs in response to hormone binding (Eppig and Downs, 1987). Addition of FSH during oocyte maturation has also been shown to increase the rate of transition from the 2-cell stage to blastocyst stage (Downs *et al*, 1986). Maturation was promoted in GV stage mouse COCs by sequential exposure to FSH and then LH. This led to a significant increase in the rate of blastocyst development. FSH contamination of the LH preparation was ruled out with the use of FSH antisera, it therefore was concluded that the acquisition of developmental competence, and possibly the completion of cytoplasmic maturation, was due to sequential GT treatment (Jinno *et al*, 1990). However, the completion of meiotic maturation is not always sufficient for the progression of embryo development.

Studies have indicated that an important factor in the acquisition of developmental competence is the signals originating from somatic cells, which govern

cytoplasmic maturation and subsequent development (Moor *et al*, 1998). It was demonstrated that denuding of oocytes before maturation *in vitro* lead to fewer embryos progressing to the expanded blastocyst stage (Schroeder and Eppig, 1984). In another study evidence was presented that ^{3}H-uridine and ^{3}H-leucine were incorporated into oocytes that were surrounded by cumulus cells. However, incorporation occurred to a lesser extent when the oocytes were denuded and co-cultured with the dissociated cells. This demonstrated that molecules of low molecular weight could pass between cumulus cells and the oocyte. It was also concluded that the junctional associations between cumulus cells and oocyte, and the signals passed across them, were required for maturation *in vitro* to be completed (Eppig, 1979). Although previously the oocyte was thought to be a passive recipient of these signals, it now seems that the intercellular communication is bi-directional. It has been suggested that the signals form part of an oocyte-granulosa cell regulatory loop, within which the oocyte is probably the dominant factor (Eppig, 2001).

1.5.2.2 *In vitro* maturation of isolated human COCs

Human and primate oocytes spontaneously resume meiosis at a low rate *in vitro,* compared with other species (Edwards, 1965). The human oocyte has a size dependent ability to resume meiosis and complete maturation *in vitro*. Resumption of meiosis increases significantly as the diameter of the oocyte increases from 90 to 120μm (Durinizi *et al*, 1995). Following the hormonal stimulation used to promote superovulation of mature metaphase II oocytes, around 5.7% of the oocytes recovered are described as immature or GV stage. These oocytes require *in vitro* culture in order to reach a stage of maturity at which they can be utilised for conventional IVF treatment. It has been demonstrated that immature oocytes from GT stimulated ovaries can be matured *in vitro* (Veeck *et al*, 1983). However, as an alternative to the

administration of high doses of the GTs that are required to stimulate superovulation, techniques have been developed that allow the retrieval of immature oocytes from unstimulated ovaries via ultrasound guided methods (Trounson *et al*, 1994). Oocytes collected from unstimulated ovaries matured *in vitro,* are capable of being fertilised and the embryos able to cleave. The transfer of 6 embryos at various stages of development into the uterus of one patient resulted in a pregnancy that was delivered at 36 weeks (Russell *et al*, 1997).

Under *in vitro* conditions the human oocyte is capable of the resumption of meiosis completion of the first meiotic division; progression to MII and fertilisation. However, subsequent development is retarded and this is thought to be due to a failure in the completion of cytoplasmic maturation (Moor *et al*, 1998). Very few studies involving *in vitro* maturation of human oocytes have investigated development to the blastocyst stage. Due to the clinical context of these studies, many early embryos are transferred to recipients and the stage of developmental failure is difficult to deduce. An important aspect of cytoplasmic maturation is the accumulation of proteins and RNA in preparation for subsequent development and embryogenesis. Protein profiling was carried out on mature MII stage oocytes that had been matured *in vivo* following GT administration, and on MII stage oocytes that had been matured *in vitro*. The *in vitro* matured oocytes had a reduced protein content compared with the *in vivo* matured oocytes. Iso-electric focusing and SDS-page indicated that nine specific human oocyte proteins were expressed by the *in vivo* group but not by the *in vitro* group. These proteins could be necessary for cell cycle events and embryo development (Trounson *et al*, 2001). The protein content of the oocyte is partially a result of the protein content of the culture media. It has been demonstrated that immature oocytes from unstimulated ovaries can be matured to MII by culture in

media supplemented with fetal cord serum, and that such oocytes are capable of being fertilised. However, improved rates of progression to MII and fertilisation were achieved when the culture media was supplemented with follicular fluid taken from follicles containing mature MII stage oocytes. Five of the embryos produced were transferred to a recipient uterus and a triplet pregnancy was established (Cha *et al*, 1991). In a separate study IVM of immature human oocytes was carried out sequentially in two separate culture media both of which were designed to support the completion of maturation (including the sequestering of RNA and proteins) and increase development to blastocyst. The oocytes were cultured in the first media for 24hrs, it contained elevated levels of sodium lactate and sodium pyruvate, but a low concentration of glucose. The second media in which the oocytes were also cultured for 24hrs, contained lower levels of sodium lactate and sodium pyruvate and a high concentration of glucose. Six embryos were produced by intracytoplasmic sperm injection, only one developed to the blastocyst stage. The blastocyst was transferred to a recipient patient and a healthy pregnancy and birth resulted (Barnes *et al*, 1995).

The somatic/follicular cells that surround the oocyte could also contribute to the protein content of the oocyte via gap-junction-mediated transfer of molecules. These molecules could potentially activate intracellular cascades of phosphorylation and/or dephosphorylation of regulatory proteins. Although some studies have claimed that cumulus cell cover and condition have little bearing on the maturational or developmental potential of immature GV stage oocytes (Barnes *et al*, 1996), other studies have highlighted the importance of the maintenance of these associations. It has been demonstrated that intact cumulus-enclosed GV stage human oocytes originating from ovarian follicles progress to MII stage at a rate significantly higher than that reported in the denuded GV stage oocyte group (Kennedy and Donahue,

1969). The role of a putative paracrine factor was investigated by a study that examined the effect of co-culture of oocytes with follicular cells during maturation. Immature GV stage oocytes that were matured with additional cumulus cells fertilised at a rate of 54%. COCs matured under normal culture conditions fertilised at a rate of 20%, subsequent development was not assessed in either case (Dandekar *et al*, 1991). Benefits were reported following co-culture of cumulus free immature oocytes with vero kidney cells. However, the investigators did not look at the subsequent development of the oocytes, therefore its relevance to cytoplasmic maturation is unclear (Janssenswillen *et al*, 1995). Improved development to the blastocyst stage has been demonstrated following co-culture of immature oocytes with human ampullary cells (Hwu *et al*, 1998). Similarly retention of the cumulus during maturation *in vitro* improves rates of nuclear and cytoplasmic maturation. This was evidenced by the elevated rates of embryo cleavage, which did not occur in the denuded group (Goud *et al*, 1998). These studies fully illustrate the importance of the conservation of the associations between the oocyte and the cumulus cells during the final stages of oocyte maturation in order to optimise subsequent embryo development. However, there is also evidence that a maturational benefit can be gleaned by the co-culture of oocytes with other cell types, this could indicate a deficiency in the culture media that is overcome by the presence of the additional cells. This could also be indicative of a paracrine aspect to the completion of cytoplasmic maturation.

1.6 HYPOTHESIS

Cryopreservation negatively affects both the immature oocyte and the cumulus cells of the COC. The aim of this work was to identify the conditions which would maximise viability of both of these components.

Aims of this study;

- To establish methods for the recovery and culture of immature murine COCs
- To evaluate the success of cryopreservation and *in vitro* maturation by performing murine IVF, with culture of resultant embryos to the blastocyst stage.
- To carry out membrane integrity assessment of the cumulus cells of the COCs.
- To compare slow-cooling and vitrification of COCs for damage to both oocytes and cumulus cells.
- To define points of damage to the COC and attempt to select protocols that would minimise damage.

Chapter 2

MATERIALS AND METHODS

The chemicals used, and their supplier are listed in appendix I. All dry chemicals were weighed using Thomas Scientific 6"x 6" weighing sheets (cut in to 3"x 3" pieces) with a Mettler-Toledo, MonoBloc (B204-S) balance accurate to four decimal places, with a maximum capacity of 220g. Liquids whose volume was in excess of 1ml were measured using Falcon serological pipettes and a Bibbyjet pump. Liquids required in volumes of less than 1ml were measured using a Gilson pipette. Solutions were made up in a Microflow biological safety cabinet (class 2) and filter sterilised using a Ministart filter with a pore size of 0.2μm or a pressure filtration unit (Sartolab P) again, with a 0.2μm pore size. Where stated, media was placed in a Heraeus 6000 humidified incubator at 37°C in an atmosphere of 5% CO_2 in air until the media had reached the desired temperature.

2.1 SUPEROVULATION

In order to collect large numbers of oocytes, mice were induced to superovulate by exogenous administration of gonadotrophins. The prepared hormone was stored at –20°C for a period not exceeding one month. The hormone preparation was warmed to 37°C prior to administration.

2.1.1 Pregnant Mares Serum (PMS) Gonadotrophin

PMS is used to mimic FSH, which initiates recruitment of follicles into the growing pool. PMS was prepared by the dissolution of a vial of Folligon in 0.9% saline. A sterile saline solution was made up by adding 0.45g NaCl to 50ml of sterile distilled H_2O. The saline solution was filter sterilised. Twenty ml of saline solution

was placed in a 25cm^3 culture flask. Two 21g needles (Kendall, tyco/Healthcare) were inserted through the membrane at the top of a vial of Folligon and a few mls of the 20ml of saline solution were added to the vial using one of the needles and a 1ml syringe. The vial was agitated to aid the dissolution of the hormone powder. When all of the powder had dissolved, the solution in the vial was added to the saline contained within the culture flask. This process was repeated three times. The final concentration of hormone was 50IU/ml. The hormone preparation was divided into 1ml aliquots and placed into 1.5ml eppendorf tubes.

2.1.2 Human Chorionic Gonadotrophin (hCG)

hCG is used to mimic the action of LH, which stimulates ovulation of recruited follicles. A similar procedure to that described in section 2.1.1 was used to prepare hCG (Chorulon) the only difference being the vial of Chorulon was dissolved in 15ml of saline to give a final concentration of 100IU/ml. The preparation was again divided into 1ml aliquots and stored in 1.5ml amber eppendorf tubes (due to the sensitivity of the preparation to UV light).

2.1.3 Administration of Hormones

For each experiment a minimum of 12 mice were used and each experiment was repeated a minimum of 3 times. CBA/CaxC57BL/6 mice bred from stock (obtained from Harlan, Bicester, UK) were kept under controlled conditions in a room with lights set to turn on at 8am and turn off at 10pm (giving 14h of light and 10h of dark) and fed water and pellets *ad libitum*.

To retrieve immature GV stage oocytes, virgin female mice (6-8 weeks old) were administered 0.1ml PMS, 50IU/ml at 12 noon or 1pm via intraperitoneal (i.p.) injection. After 46 hours, these animals were euthanised by cervical dislocation. To

retrieve *in vivo* matured oocytes, virgin female mice (6-8 week old) were injected with 0.1ml PMS by i.p. on day 1 at 4pm, followed by 0.1ml of hCG 100IU/ml administered by i.p. 53 hours later (day 3 at 9pm). The timing of administration of hormones relative to the light/dark cycle is important. The endogenous LH surge occurs in response to PMS approximately 20hrs post mid-point of the second dark period following PMS injection. It is therefore, important to administer hCG prior to the endogenous LH surge in order to obtain high numbers of oocytes of similar maturity (Gates, 1971). After a further 13 hours, these animals were euthanised by cervical dislocation.

2.2 COLLECTION AND CULTURE OF OOCYTES

2.2.1 Phosphate Buffered Medium (PBS)

PBS media was used for handling tissues/cells during manipulations in air. When supplemented with BSA, PBS was designated PB1 and used as a holding medium for oviducts, ovaries and epididimi. When supplemented with FBS, PBS was designated PBF and used for all 'open-air' manipulations of oocytes including loading of CPA and cryopreservation. To prepare PBS, one vial of Dulbecco's PBS powder (detailed in table 2.1) was emptied into a 1L volumetric flask. Sterile distilled H_2O was used to flush out the vial and was placed in the flask. The volume within the flask was then made up to around 500ml. The following additions were then made; 60mg of penicillin; 36mg of sodium pyruvate; 1g of glucose and 12mg of phenol red and the contents were mixed. To prevent precipitation, the calcium chloride (0.159g) that was supplied with the PBS powder was dissolved separately in approximately 100ml of sterile distilled H_2O and then added to the volumetric flask, after which the volume was made up to 1L with more sterile distilled H_2O. To ensure that all the contents were mixed, a 2cm magnetic flea was added to the flask and the opening of the flask

was sealed with Nesco film. The flask was then placed on a magnetic stirrer for approximately 10 minutes or until the medium was clear. The medium was filter-sterilised and stored in 75cm^3 culture flasks (Falcon) at 4°C for a period not exceeding one month. For PB1, BSA was added to the medium at a concentration of 4mg/ml, twenty-four hours before it was required. The medium was then filter sterilised and stored in a tissue culture flask on a heated block at 37°C. To make up PBF, 5% foetal bovine serum (FBS) was added to PBS. It was filter sterilised and stored in a tissue culture flask on a heated block at 37°C. All PBS media used in manipulation of GV stage oocytes was also supplemented with 0.1mg/ml dcAMP immediately prior to use.

	g/l
KCl	0.20
KH_2PO_4	0.20
$MgCl_2$ (anhydrous)	0.049
NaCl	8.00
Na_2HPO_4	1.15

Table 2.1 Constituents of Dulbecco's PBS powder

2.2.2 Standard Maturation Medium (SMM)

Standard maturation medium was used for holding immature GV stage COCs in an incubator at 37°C in an atmosphere of 5% CO_2 in air prior to maturation or cryopreservation. SMM was supplemented with 0.1mg/ml dcAMP to ensure that the GV stage oocytes within the COCs remained at the GV stage throughout the manipulations (Carroll *et al*, 1991). Alternatively it was supplemented with gonadotrophins (FSH or FSH/LH) and used for maturation culture of COCs prior to IVF. The gonadotrophin concentration and type was arrived at after a number of alterations that are detailed in a later section. SMM constituted 1 litre of MEM Earles supplemented with 10% FBS; 27.5mg sodium pyruvate; 50mg streptomycin; 60mg

penicillin; 1μg epidermal growth factor and 2.5ml L-glutamine. The medium was filter sterilised and supplemented with either 0.1mg/ml dcAMP, or 0.75IU/ml Gonal-F (rFSH), or 7.5IU/ml Humegon (FSH/LH). Ten ml aliquots of SMM supplemented with dcAMP, and two ml aliquots of SMM supplemented with gonadotrophin were stored at –20°C until required.

	g/l
$CaCl_2$ (anhydrous)	0.20
KCl	0.40
$MgSO_4$	0.098
NaCl	6.8
$NaHCO_3$	2.2
NaH_2PO_4-H_2O	0.14

Table 2.2 Constituents of MEM Earles

2.2.3 Mineral Oil

Oocytes and preimplantation embryos were cultured in small (30μl) droplets of medium. In order to prevent evaporation of the droplets and to buffer against changes in pH and temperature during handling outside the controlled atmosphere of the incubator, the droplets were covered with mineral oil. Two hundred ml of mineral oil was mixed with 50ml of either SMM (for the culture of oocytes) or T6 (for the culture of embryos), (without the addition of BSA or serum) in a $75cm^3$ culture flask (Falcon) by vigorously shaking the flask for one minute. The flask was held at 4°C for 24h after which time the mineral oil was poured off into a fresh $75cm^3$ culture flask (Falcon) and stored in the dark (by wrapping the flask in aluminium foil) at 4°C for a period not exceeding one month.

2.2.4 Dissection and IVM Culture Dishes

Organ culture dishes in which the dissection of isolated ovaries was to take place were prepared in a laminar flow cabinet. The inner well of Falcon centre-well

organ culture dishes was filled with 0.9ml of SMM supplemented with dcAMP while 1ml of the same medium was placed in the outer reservoir (figure 2.1A).

Dishes in which the COCs were to be held after dissection from the ovary (Falcon easy grip tissue culture dishes) were prepared by placing six 30µl-droplets of SMM + dcAMP as shown in figure 2.1B. The dishes were flooded with six ml of mineral oil (prepared as in section 2.2.3).

Dishes in which the initial phase of *in vitro* maturation of oocytes was to take place were prepared by arranging droplets of medium as described above. The only difference being that the medium in this case was supplemented with 0.75IU/ml FSH. These were used for the initial four hour incubation of the maturation protocol, prior to exchange of the media for SMM supplemented with 7.5IU/ml FSH/LH. All dishes were incubated in a humidified atmosphere of 5% CO_2 in air for between 18 and 24h, before use.

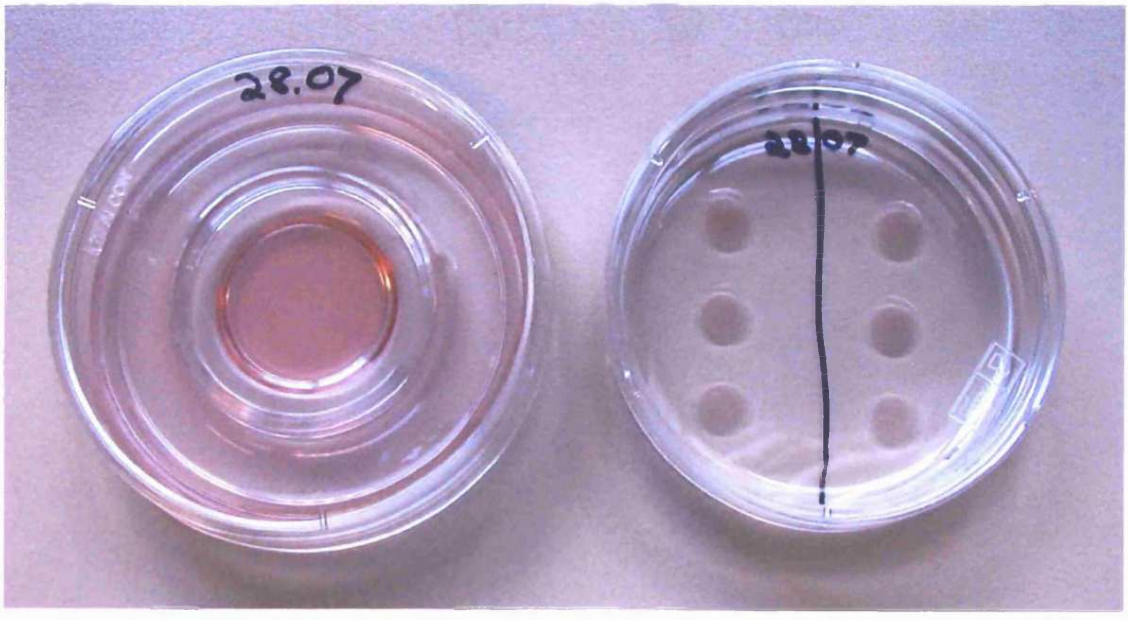


Figure 2.1 (A) Centre well organ culture dish. Filled with SMM for dissection of ovaries (section 2.2.4) or $T6_{16}$ for IVF (see section 2.5.2) (B) Droplet culture dish. Filled with SMM for culture of oocytes (section 2.2.4) or $T6_4$ for culture of embryos (see section 2.5.2).

2.2.5 Isolation of Ovaries and Oviducts

Following hormonal stimulation by intraperitoneal injection and euthanasia female mice were laid on their backs and sprayed with Hydrex. Hard Surface spray (Adams Health Care) to dampen the fur and reduce the risk of contamination. The skin of the abdomen was cut horizontally and pulled back, the peritoneum was cut and pulled back to expose the abdominal cavity. The loops of intestine were lifted out of the way and the two horns of the uterus were identified (figure 2.2). At the top of these, the ovaries and the oviducts were located and removed using forceps and scissors (figure 2.3). Ovaries are easily damaged; therefore, they were retrieved whilst still attached to the oviduct and a section of uterus, for ease of handling. Oviducts, on the other hand were removed after severing connections with the ovary and cutting half way along the uterus. Ovaries were collected where retrieval of immature oocytes was required. Whereas, if *in vivo* matured oocytes were needed, oviducts were collected, following the appropriate hormonal stimulation (see section 2.1.3). The ovaries or oviducts were placed in PB1 that had been held overnight at 37°C on a heated block.

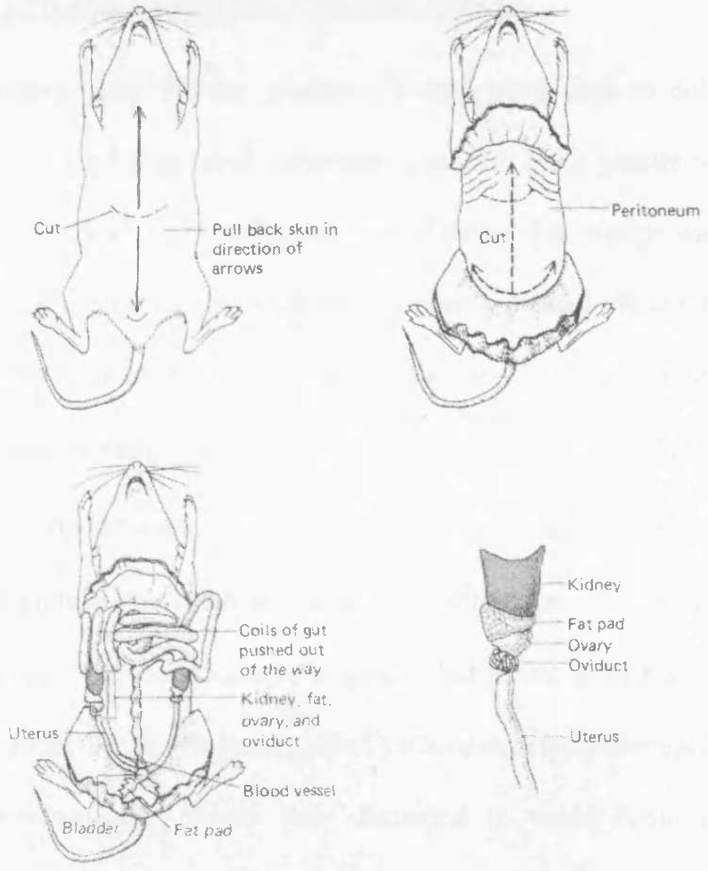


Figure 2.2 Dissection of female mouse showing orientation of ovaries and oviducts. Hogan *et al* (1986).

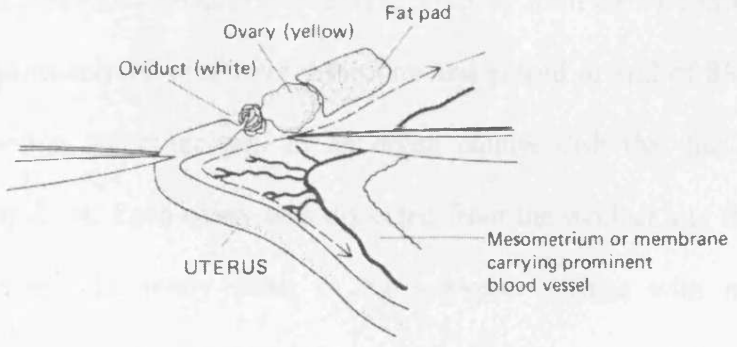


Figure 2.3 Orientation of ovary, oviduct and uterus *in vivo*. Hogan *et al* (1986).

2.2.6 Pulling Pipettes for Handling COCs and Oocytes

Disposable glass Pasteur pipettes (Volac) were used to collect and transfer COCs and oocytes using hand generated pressure. Each pipette was held with its narrowing point above a gentle Bunsen burner flame. The pipette was rolled to ensure that the circumference of the section was evenly heated. When the glass became molten the two ends of the pipette were pulled apart to create a mid-section with a diameter of approximately 80-200μm. The pipette was then broken at an appropriate point so as to provide a diameter slightly larger than the COC (150μm) or the oocyte (100μm) depending on which was required. Before use the tip of the pipette was examined under a stereomicroscope to ensure that it was smooth and free from cracks or shards of glass that might damage the COCs or oocytes. After each manipulation of COCs or oocytes, the pipette was discarded to avoid cross contamination of experimental groups or culture groups.

2.2.7 Retrieval of Immature/GV Stage COCs and *In Vivo* Matured Oocytes

The ovaries attached to a section of uterus were removed from the PB1 in which they were held approximately 10 min. after dissection and placed in 1ml of SMM + dcAMP contained within the outer well of an organ culture dish that had been prepared as in section 2.2.4. Each ovary was dissected from the oviduct and the fat pad that partially covers the ovary using a 28g monoject syringe with needle (Kendall). The ovaries were then placed into the central well of the same dish, which contained 0.9ml of SMM + dcAMP. The contents of the ovarian follicles were released by repeated puncturing with a 28g monoject syringe with needle (Kendall). Using an Olympus stereomicroscope GV stage oocytes, surrounded by at least two layers of cumulus cells (figure 2.4), were identified and placed into a 30μl droplet of

SMM + dcAMP (dish prepared as described in section 2.2.4) using a pulled Pasteur pipette (detailed in section 2.2.6). COCs were held in this medium; under mineral oil at 37°C in a humidified atmosphere of 5% CO_2 in air until all COCs had been isolated from the ovaries (a maximum of 120 minutes). All of the COCs collected were pooled in one droplet and randomly allocated to the experimental groups.

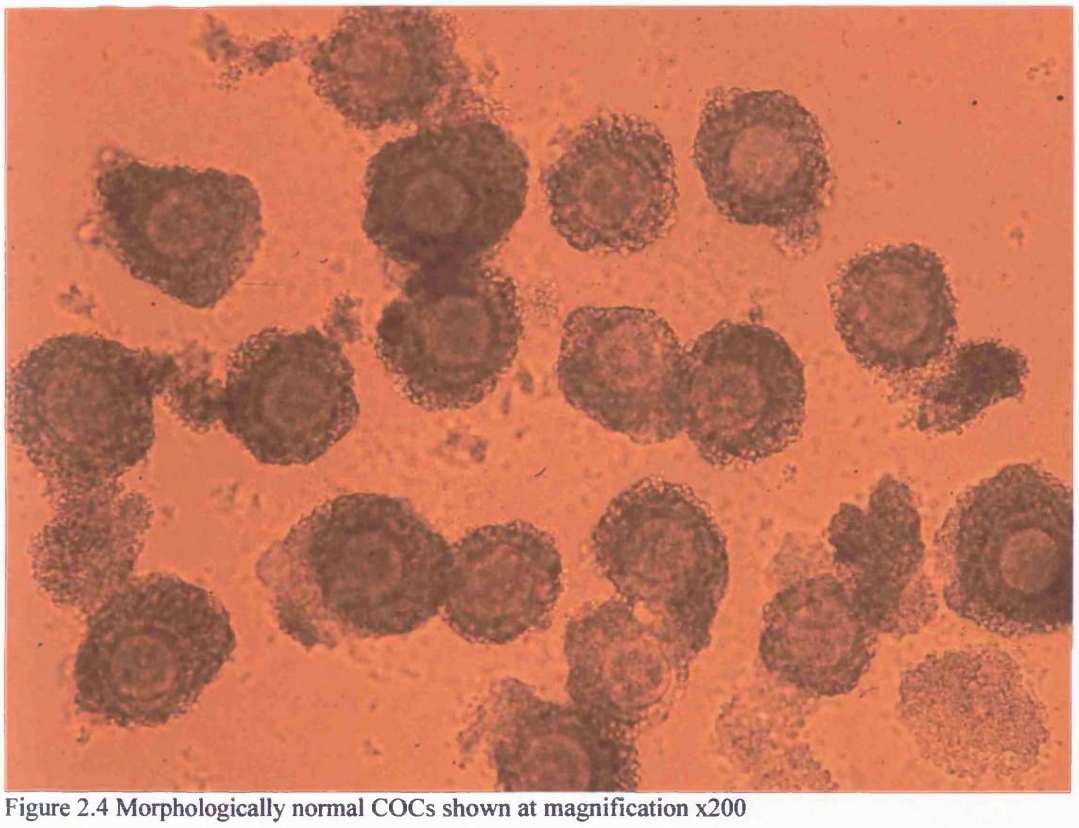


Figure 2.4 Morphologically normal COCs shown at magnification x200

Retrieval of In Vivo Matured Oocytes

After about 10 min in PB1 the oviducts were placed into the central reservoir of a fertilisation dish (as detailed in section 2.5.2). Using the stereomicroscope the oviducts were identified and where they were swollen with ovulated oocytes they were torn open to release the oocytes using monoject syringes fitted with 25g needles (Kendall). The oviducts were removed leaving the oocytes within loose cumulus masses in the central reservoir of the dish. The dish was returned to 37°C in a humidified atmosphere of 5% CO_2 in air for 10 min.

2.2.8 Removal of Cumulus Cells

Where denuded GV stage oocytes were required, freshly collected GV stage COCs, held in 30μl droplets of SMM + dcAMP, were drawn up and down a pulled Pasteur pipette (section 2.2.6) with an internal diameter of 80-90μm. Morphologically normal oocytes (i.e. spherically shaped oocytes with clear cytoplasm and no fragmentation) were selected. Oocytes and cumulus cells were transferred into separate 30μl droplets of SMM + dcAMP and held at 37°C in a humidified atmosphere of 5% CO_2 in air until required.

2.2.9 *In Vitro* Maturation (IVM)

Intact COCs i.e. at least two layers of cumulus cells present (figure 2.4); denuded oocytes (see previous section); denuded oocytes to which cumulus cells derived from twice the number of COCs were added; thawed oocytes or thawed oocytes with freshly removed cumulus cells added in excess as for denuded oocytes, were placed in 30μl droplets of SMM + dcAMP which had been kept at 37°C in an atmosphere of 5% CO_2 in air. The cells were then moved into a 30μl droplet of SMM containing 0.75IU/ml Gonal-F (FSH), under mineral oil and held for 4 hours at 37°C in an atmosphere of 5% CO_2 in air. After this time approximately 70% of the droplet was removed using a pulled Pasteur pipette. In most cases the COCs adhered to the dish and were not disturbed. The medium was replaced, using a Gilson pipette, with 30μl of SMM containing 7.5IU/ml Humegon (FSH/LH at a 1:1 ratio). Once again approximately 70% of the droplet was removed using a pulled Pasteur pipette and 30μl of SMM containing 7.5IU/ml Humegon was added using a Gilson pipette, the dish was then placed at 37°C in a humidified atmosphere of 5% CO_2 in air for a further 18 hours. Approximately 20 oocytes were cultured in each 30μl droplet.

2.3 CRYOPRESERVATION OF IMMATURE OOCYTES

2.3.1 Cryopreservation Solutions

(All cryopreservation solutions and diluents contained 0.1mg/ml dcAMP)

1.5M Me_2SO

For slow controlled-rate cooling of COCs a 1.5M solution of Me_2SO was prepared by adding 0.585ml of Me_2SO to 4.415ml of PBF (prepared as detailed in section 2.2.1) in a two position capped 13ml polystyrene tube (Falcon). The solution was held on ice at 4°C for a period not exceeding 2h.

4X PB1

In the preparation of the vitrification solution it was ensured that the molar concentration of electrolytes was equivalent to that in PB1 i.e. the salts and CPAs were added prior to the solution being made up to the final volume. To facilitate this a concentrated PB1 solution was made in which the salts were dissolved in ¼ volume of H_2O i.e. 250ml. The calcium chloride to be added to the PB1 was made up in 10ml of sterile distilled H_2O to give a 100x concentration. Both solutions were filter sterilised and stored at 4°C in culture flasks (Falcon) for a period not exceeding three months.

Vitrification Solution – VSD+PEG (6M Me_2SO + PEG)

The solution was made up in a sterile 10ml volumetric flask in a laminar flow cabinet. To prevent precipitation of the salts, the constituents were added in a specific order. Firstly, 2.5ml of 4XPB1 was mixed with 1.5ml of sterile distilled H_2O, 4.69ml of Me_2SO was then added. PEG (0.01g) was added to the solution and if necessary, the flask was sealed with Nesco film and placed on a heated stirrer set to a moderate heat to aid dissolution of the PEG. If the solution remained clear then 0.5ml of FBS was added, sterile distilled H_2O was added to the flask until the level was just below

the 10ml mark. The 100X$CaCl_2$ (100µl) was then added to the solution that was then made up to 10ml with sterile distilled H_2O. If any of the PEG remained out of solution the flask was placed on a heated stirrer set to a moderate heat until the solution was clear. The vitrification solution was stored in a two position capped 13ml polystyrene tube (Falcon) marked 100% VSD+PEG. The solution was held at room temperature for a period not exceeding 7 days. Before an experiment 25% and 65% dilutions of VSD+PEG were made up. The 25% dilution was made up by mixing 0.5ml of 100% VSD+PEG with 1.5ml of PBF supplemented with 5% FBS. Similarly, the 65% solution was made up with 1.3ml of 100% VSD+PEG and 0.7ml of PBF (5% FBS). Both dilutions were stored in two position capped 5ml polystyrene tubes (Falcon) at room temperature for a minimum of 1h.

Sucrose

For dilution of the CPA following slow controlled-rate cooling a 0.1M sucrose solution was prepared by adding 0.171g of sucrose to 5ml of PBF in a two position capped 13ml polystyrene tube (Falcon). The solution was held at room temperature for a period not exceeding 5h. For dilution of CPA following vitrification a 1M sucrose solution was used. It was prepared in an identical manner to the 0.1M sucrose solution using 1.71g of sucrose.

2.3.2 Slow Controlled-Rate Cooling

All solutions and media were equilibrated to the required temperatures prior to collection of the COCs and oocytes. In all cases the COCs or oocytes were transferred between the solutions using pulled glass pipettes in the minimum volume possible.

Exposure of COCs to cryoprotectant without freezing

COCs were transferred, from SMM supplemented with dcAMP (as detailed in section 2.2.7) into 1ml of freezing solution i.e. 1.5M Me_2SO + 0.1mg/ml dcAMP (detailed in section 2.3.1). The COCs were held in the cryoprotectant solution at 4°C on ice for 15 minutes. After this time the COCs were transferred into an organ culture dish containing 1ml of 0.1M sucrose (detailed in section 2.3.1). After being held in 0.1M sucrose at room temperature for 5 minutes, the COCs were placed into 1ml of PBF + 0.1mg/ml dcAMP at room temperature for 5 minutes. They were finally placed into a fresh dish containing 1ml of the same solution on a hot plate at 37°C for 5 minutes. Following exposure to and dilution of the cryoprotectant, the COCs were either stained for membrane integrity or were placed into the *in vitro* maturation protocol.

Slow-Cooling Protocol

Freshly collected COCs were transferred, using a pulled Pasteur pipette, from 30µl droplets of SMM supplemented with dcAMP into 1ml of cryoprotectant solution containing 1.5M Me_2SO, (as detailed above). After 5 minutes of exposure to the solution at 4°C, the oocytes were pipetted into a small column (15-20µl) of the same cryoprotectant solution within freezing straws (IMV, Cassou straws, France), (figure 2.5). The freezing straws were prepared before collection of the COCs. They contained a cotton plug, which was pushed half-way down the straw with a plastic straw holder. The straws were then partially filled with 0.1M sucrose (detailed above) using a monoject syringe fitted with 21g needle (Kendall, tyco/Healthcare) to replace some of the air within the straw, to wet the cotton plug and hence form an airtight seal and to act as an instant diluent to the CPA solution following freezing and thawing.

The straws were then stored on ice. Approximately 20 COCs were placed into each straw. The straws were sealed with plastic plugs that had been slightly wetted with 1.5M Me_2SO, and then stored on ice. When the COCs had been in the presence of Me_2SO at 4°C for a total of 15 minutes, the straws were further cooled at -2 ° C/min to -6 ° C in a controlled rate freezer (Planer Kryo10, Series III) that had been precooled

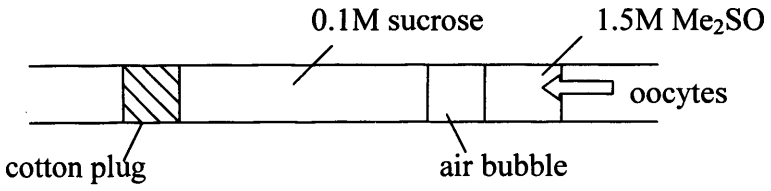


Fig.2.5 Schematic diagram of freezing straw.

to 4 ° C. The straws were held at this temperature (–6 ° C) for 10 minutes to allow the liquid to be supercooled (i.e. cooled below freezing point without ice formation) after which ice nucleation was instigated by quickly removing the straws from the chamber and briefly touching the straws simultaneously at the top of the column of Me_2SO with forceps cooled in LN_2 until ice formation was observed. The straws were held at -6 ° C for a further 10 minutes to allow the release of latent heat of crystallisation. Cooling then continued at a rate of 0.3 ° C/min to -60 ° C. When the samples reached a temperature of -60°C the straws were either held at that temperature for 10 minutes or were removed from the controlled rate freezing machine and plunged into LN_2; or remained in the freezing machine and were cooled at a faster rate of 10°C/min to -150°C before being plunged into LN_2. Straws held in LN_2 (–196°C) were stored for 1-12 weeks.

Thawing

The straws were removed from liquid nitrogen storage and placed in a controlled rate freezing machine precooled to -70°C. Samples that had been cooled to

-60°C and exposed to liquid nitrogen and those that had been cooled to -60°C and not been exposed to liquid nitrogen were placed in a precooled freezing machine at -60°C. The samples were then warmed at a rate of 8°C/min to 4°C. The straws were removed from the machine and placed on a paper towel. Each straw was held at the opposite end to the COCs and using a scalpel were cut just above the cotton plug. A 1ml syringe (Kendall, tyco/Healthcare) containing 1ml of 0.1M sucrose was attached to the cut end of the straw. The plastic plug was then removed by gentle twisting and the contents of the straw were flushed into an organ culture dish. After being held in this solution at room temperature for 5 minutes, the COCs were placed into 1ml of PBF + 0.1mg/ml dcAMP at room temperature for 5 minutes. They were finally placed into a fresh dish containing 1ml of the same solution on a hot plate at 37°C for 5 minutes. Following thawing, the oocytes were either stained for membrane integrity or were placed into the *in vitro* maturation protocol.

2.3.3 Vitrification

Again, all solutions and media were equilibrated to the required temperatures prior to collection of the COCs and oocytes. In all cases the COCs or oocytes were transferred between the solutions using pulled glass pipettes in the minimum volume possible. COCs were exposed to stepwise addition and dilution of CPA by moving through droplets of CPA solution arranged on Falcon easy grip tissue culture dishes. The lids of the dishes were replaced after each manipulation to prevent evaporation of the droplets. The original vitrification protocol used 50μl droplets for the exposure of mature oocytes to increasing CPA concentrations. However, it was found that due to the larger size of the COCs, more medium was transferred between the droplets during each transfer. To counteract any dilution of the droplets, a larger volume of 0.5ml was used for each droplet.

Exposure to the vitrification solution without cryopreservation

For each experiment approximately 20 freshly collected COCs were transferred into a 0.5ml droplet of the 25% dilution of the vitrification solution (VSD+PEG detailed in section 2.3.1) and held at room temperature for 3-5 min. The COCs were then moved to a 0.5ml droplet of the 65% dilution of the vitrification solution. The COCs were then immediately moved to a 0.5ml droplet of 100% vitrification solution. The COCs were moved through the two latter droplets as quickly as possible, thus exposure time was no longer than 10 seconds. The vitrification solution was diluted by placing the COCs in 1ml of 1M sucrose contained in an organ culture dish (as detailed previously) at room temperature for ~2 min. The COCs were then moved into a 0.5ml droplet of 1M sucrose at room temperature for ~2 min. The COCs were moved through 2x 0.5ml droplets of PBF also at room temperature for ~5 min/drop. Finally the COCs were moved into 1ml of PBF at 37°C and held for 5 min. After this time the COCs were either stained for membrane integrity assessment or subjected to IVM, IVF and embryo culture in order to assess developmental potential.

Vitrification

COCs to be vitrified were exposed to 25% and 65% vitrification solutions as described above. After being placed into the 100% vitrification solution, the COCs were loaded into straws that had been pre-prepared in an identical manner to those used for slow cooling but 1M sucrose solution replaced the 0.1M sucrose and 100% vitrification solution replaced the 1.5M Me_2SO. The straws were held at room temperature until required. After loading of the COCs into the vitrification solution the straws were plugged (as described previously) and held in LN_2 vapour at –140°C, the glass transition temperature, as measured using a microprocessor thermometer

(Comark). The straws were held in the vapour for 3 min. before being plunged into liquid nitrogen for 5-10mins. Although direct immersion of the straws in liquid nitrogen gives a very fast cooling rate of approximately 2500°C/min. (Rall, 1987), cooling at this rate can cause the plastic straws to form cracks, which become favourable sites for ice nucleation during warming. By first holding the straws at –140°C the formation of these cracks can be avoided and a cooling rate of ~200°C/min can be achieved (Rall, 1987).

Warming

Straws were warmed by being held at room temperature for 10 seconds and then being plunged into water at 20°C for 10 seconds. This gives a warming rate of >250°C/min (Rall, 1987). Each straw was cut (as detailed in section 2.3.2), the plug removed and the straw flushed with 1ml of 1M sucrose. Dilution continued in an identical manner to that used for COCs that were exposed to the vitrification solution without cooling. Similarly the COCs were either stained for membrane integrity assessment or subjected to IVM, IVF and embryo culture in order to assess developmental potential.

2.4 ASSESMENT OF MEMBRANE INTEGRITY

2.4.1 CF/PI Solution

Carboxy fluorescein (CF) is an indicator of membrane integrity (Rotman and Papermaster, 1966). Cells are permeable to the non-fluorescent CF, which is hydrolysed by intracellular enzymes to the fluorescent product fluorescein. If the plasma membrane is intact, the fluorescein is retained and the cell fluoresces green when exited with light at a suitable wavelength. Propidium iodide can be used as a

counter stain with CF it is a DNA stain that can only enter cells with compromised membranes and fluoresces red.

The stain was prepared by adding 50μg of propidium iodide and 50μg of carboxy fluorescein diacetate to 4.9ml of PBF in a test tube (Falcon) to make a final concentration of 0.1mg/ml. To prevent photo bleaching of the light sensitive dye the test tube was wrapped in aluminium foil and placed on a heated block at 37°C for a period not exceeding 1h.

2.4.2 Membrane Integrity Staining

During the staining procedure all incubations were carried out in dishes (Falcon centre-well organ culture dishes) that were covered in aluminium foil to prevent exposure of the photosensitive stains to light. Freshly collected GV stage COCs and COCs that had been subjected to CPA exposure or cryopreservation, were incubated in the dark at 37°C in one ml of PBF containing 0.1mg/ml carboxy fluorescein diacetate and 0.1mg/ml propidium iodide contained within the central well of the culture dish, for 10 minutes. The COCs were then washed twice in one ml of fresh PBF at 37°C. They were then placed into a droplet of PBF on a 76x26mm cavity slide (Superior, Germany) and viewed using an Optiphot-2 microscope (Nikon, Tokyo, Japan) fitted with filters capable of detecting fluorescence in the range 450-490nm. Cells with an intact cell membrane fluoresced green whereas those with a damaged cell membrane fluoresced red. COCs were photographed in experimental groups within 2 min of dilution of the stain (using Kodak Professional 35mm, E200 Ektachrome colour reversal film) and later they were blind scored from mounted slides for membrane integrity of the cumulus cells using the scoring system detailed in figure 2.6.

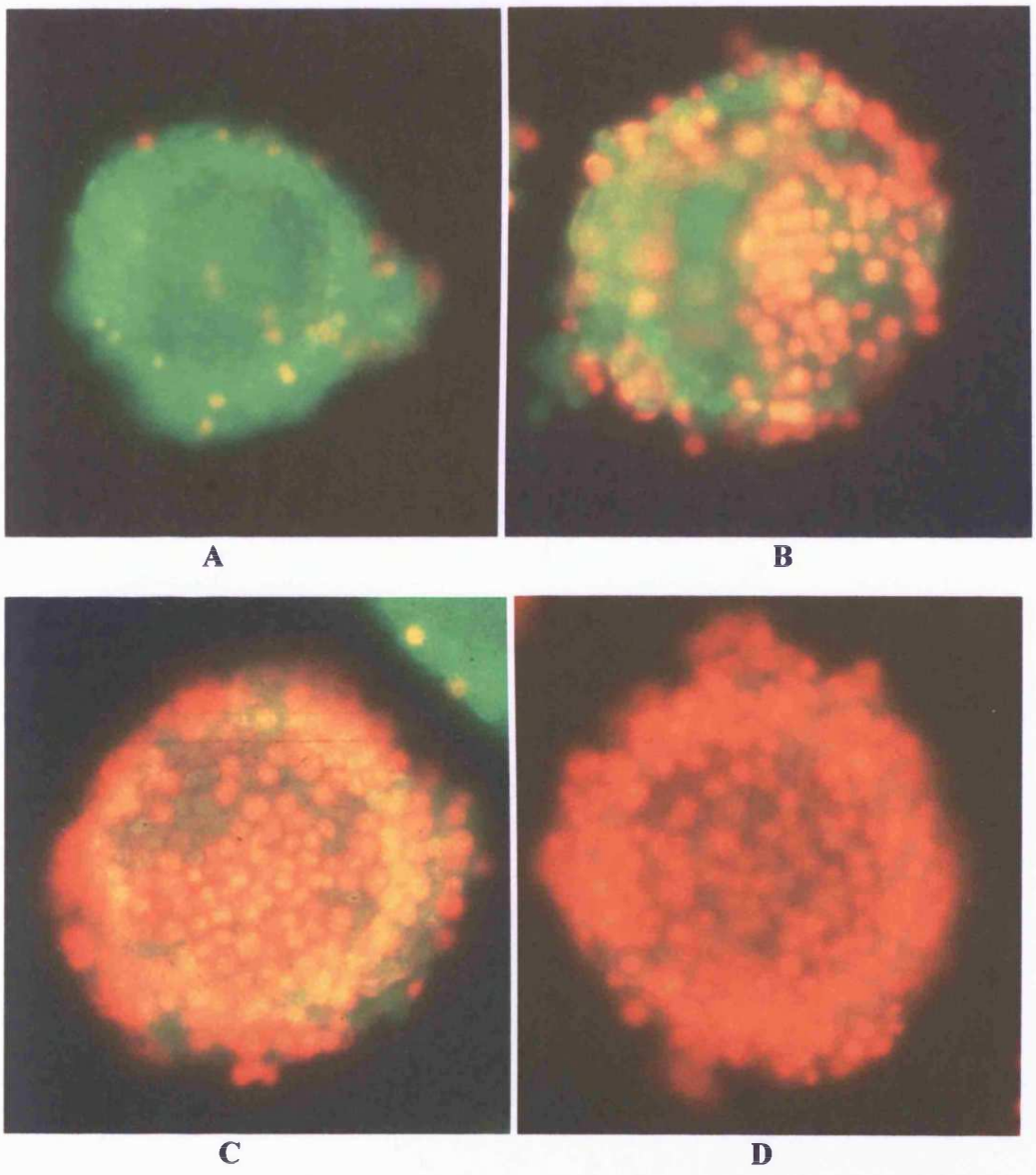


Figure 2.6 (A) A COC with between 76-100% of the surface of the oocyte covered by cumulus cells with intact membranes, scored as 1. (B) A COC with 51-75% of the oocyte covered by cumulus cells with intact membranes, scored as 2. (C) A COC where the oocyte is covered by between 26-50% intact cumulus cells, scored as 3. (D) A COC where 0-25% of the oocyte was surrounded by intact cumulus cells, scored as 4.

2.5 *IN VITRO* FERTILISATION AND ASSESSMENT OF EMBRYO DEVELOPMENT

2.5.1 Tyrode's Medium (T6)

Tyrode's medium was used for capacitation of spermatozoa, IVF and subsequent blastocyst culture. The contents of one vial of Tyrode's salts (detailed in table 2.3) were placed in a 1L volumetric flask. The vial was then rinsed with sterile

distilled H_2O and the water was placed in the flask. The following substances were then added to the flask, 60mg of penicillin, 50mg of streptomycin, 3.5ml of lactic acid, 2.106g of sodium bicarbonate, 55mg of sodium pyruvate, and 5mg of phenol red. The contents were mixed and then the volume was made up to 1L with sterile distilled H_2O. Once the contents were dissolved the medium was filter-sterilised and stored in $75cm^3$ culture flasks (Falcon) at 4°C. Twenty-four hours before the medium was required, BSA was added as a source of protein. The addition of BSA is useful as it reduces cohesion of the embryos and may also absorb trace contaminants and/or supply trace requirements. The BSA was added at either a concentration of 16mg/ml ($T6_{16}$), which was used for sperm capacitation and oocyte culture during fertilisation or 4mg/ml ($T6_4$) to be used in the culture of resultant embryos.

	g/l
$CaCl_2$	0.20
KCl	0.20
$MgCl_2$	0.0469
NaCl	8.00
$NaH_2PO_4.H_2O$	0.05

Table 2.3 Constituents of Tyrode's Salts

2.5.2 IVF and Embryo Culture Dishes

Dishes (Falcon centre well organ culture) in which IVF was to take place were prepared by placing 0.9ml of $T6_{16}$ in the central well and one ml of $T6_{16}$ in the outer reservoir (figure 2.1A). Dishes (Falcon easy grip tissue culture) in which resultant embryos were to be cultured were prepared by placing six 30µl-droplets of $T6_4$ in two parallel rows (figure 2.1B). The embryo culture dishes were then flooded with six ml of mineral oil (prepared as in section 2.2.3). Both types of dish were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for a minimum of 16h, before use.

2.5.3 Collection of Spermatozoa

CBA/CaxC57BL/6 mice bred from stock (obtained from Harlan, Bicester, UK) were kept under controlled conditions and fed water and pellets *ad libitum*. Male mice (>13 weeks old) were euthanised, laid on their backs and sprayed with Hydrex hard surface spray to help prevent fur from entering the body cavity and contaminating the dissection. The skin of the abdomen was cut horizontally and pulled back. The peritoneum was cut horizontally at the base of the abdomen. Below the bladder, one of the two fat pads attached to the testicles was identified and pulled with forceps out

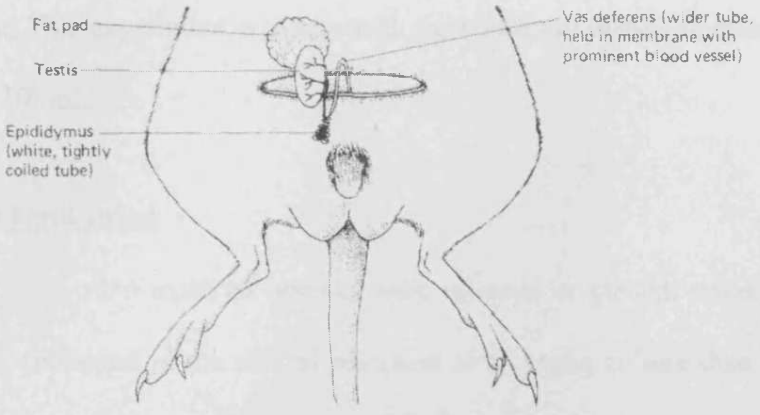


Figure 2.7 Dissection of male for collection of spermatozoa. Hogan *et al* (1986).

of its original position in order to visualise the testicle (figure 2.7). The epididymus was cut just below the testicle; the vas deferens was cut and the majority of the vessel was placed in PB1 that had been warmed overnight to 37°C on a heated block. After about 10 min the sections were placed into 1ml of $T6_{16}$ contained in the outer reservoir of a fertilisation dish (as detailed in section 2.5.2). Using the stereomicroscope, the dark mass of spermatozoa was identified within the vessels, which were torn at the testicular end using monoject syringes fitted with 25g needles (Kendall, tyco/Healthcare). The spermatozoa were released into the medium by pressing on the vessel with the needles and pushing them out of the opening. The dish

was incubated for 1-1.5 hours at 37°C in a humidified atmosphere of 5% CO_2 in air. This allowed the spermatozoa to become capacitated i.e. become capable of penetrating and fertilising an oocyte.

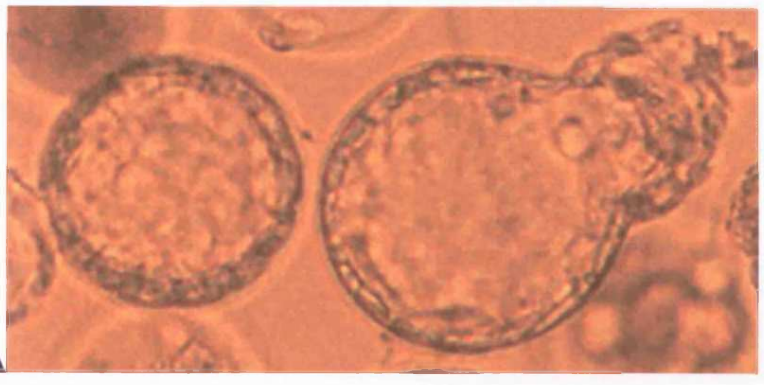
The approximate concentration of motile sperm was assessed by pipetting a 10µl droplet of capacitated sperm suspension onto the grid of a Makler Counting Chamber (Sefi-Medical Instruments). The number of motile spermatozoa in each square on the grid was counted; this amount was divided by 10 to give a motile sperm count equal to the number of motile spermatozoa multiplied by one million per ml. This was repeated three times for the same sperm suspension in order to obtain an average count. This experiment was repeated eight times to obtain an overall average count of $4.7x10^6$/ml.

2.5.4 *In Vitro* Fertilisation

In vivo or *in vitro* matured oocytes were released or placed, respectively, into 0.9ml of $T6_{16}$ contained in the central reservoir of an organ culture dish (detailed in section 2.5.2) and incubated for 10 minutes at 37°C, in an atmosphere of 5% CO_2 in air. After this time 0.1ml of capacitated sperm was added to the oocytes and the mixture was incubated at 37°C, in an atmosphere of 5% CO_2 in air for 5 hours. Oocytes were then transferred, using a pulled glass pipette, through 3 droplets of $T6_4$ under mineral oil (detailed in section 2.5.2) to remove excess spermatozoa. The oocytes were incubated in the final droplet at 37°C, in a humidified atmosphere of 5% CO_2 in air for five days during which time, normality, fertilising capacity and embryo development were assessed.

2.5.5 Assessment of Fertilisation and Development of Blastocyts

After incubation for 24h post insemination the oocytes were assessed for normality. Abnormal oocytes were defined as having dark or fragmented cytoplasm, or being irregularly shaped or shrunken. Normality was calculated as a percentage of the total group of oocytes. At the same time progression to the 2-cell stage (fertilisation) was assessed and calculated as a percentage of normal oocytes. The number of normal blastocysts and normal hatching blastocysts was counted following a further four days incubation at 37°C, in a humidified atmosphere of 5% CO_2 in air (figure 2.8). Blastocyst formation was calculated both as a percentage of the number of fertilised oocytes and of the total number of oocytes in the cohort. Hatched blastocysts were calculated as a percentage of the number of blastocysts.



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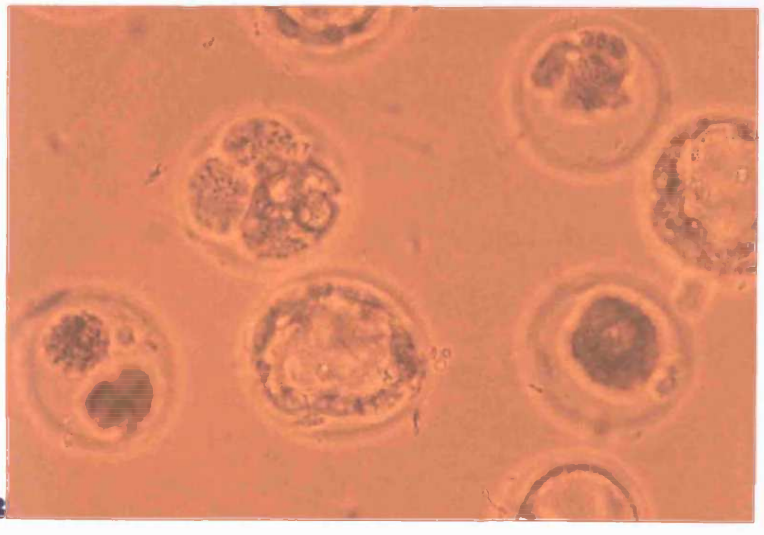

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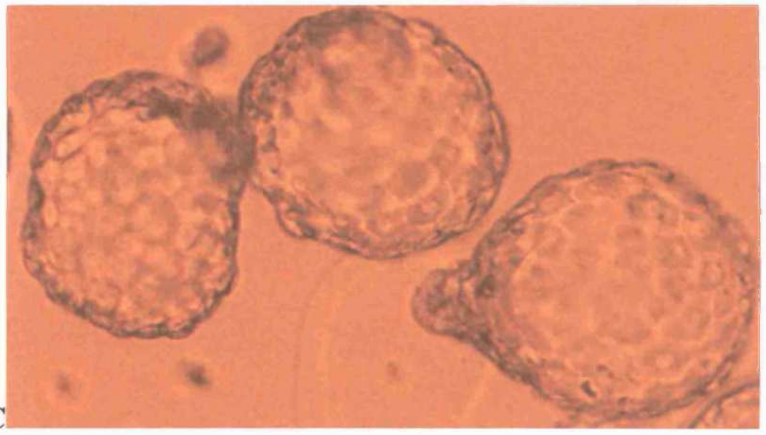
C 

Figure 2.8 Scoring of Blastocysts (A) Normal blastocyst and normal hatching blastocyst. Photos were taken 5 days post-insemination. (B) Abnormal embryos which have arrested before reaching the blastocyst stage. Photos were taken 5 days post-insemination. (C) Normal hatched blastocysts. Photos were taken 6 days post-insemination.

2.6 STATISTICAL ANALYSIS

For membrane integrity data, comparisons were made between freshly collected COCs, COCs that had been exposed to cryoprotectant without freezing and cryopreserved COCs using the Chi-squared test (95% confidence interval, two degrees of freedom). With data obtained following *in vitro* fertilisation, three data sets were compared using the Kruskal Wallis test (95% confidence interval): either one treatment group and *in vivo* and *in vitro* matured controls for maturation experiments; or two treatment groups (either exposure to cryoprotectant or cryopreservation) and an *in vitro* matured control group. Where a significant difference was found using the Kruskal Wallis test, pair wise comparisons were made for this data set using the Mann Whitney U test (95% confidence interval). These non-parametric tests were selected in order to analyse data, which was not normally distributed.

Chapter 3

IN VITRO MATURATION OF GV STAGE MURINE COCS

3.1 INTRODUCTION

GV stage oocytes must be matured to MII in order to be capable of being fertilised. As stated previously, when released from the follicular environment, spontaneous maturation occurs, i.e. the oocyte undergoes GVBD, divides by extrusion of the first polar body and can be fertilised (Edwards, 1965). However, the developmental capability of such oocytes is poor. Studies have shown that oocytes matured *in vitro* in the presence of FSH and LH have improved developmental potential compared with oocytes that were matured without hormone (Jinno *et al*, 1990; Schramm and Paprocki, 2000). Somatic follicle cells are required *in vitro* for the maturational response of immature human oocytes to GTs to occur (Trounson *et al*, 2001). Indeed, it has been reported that the presence of cumulus cells during IVM of oocytes is the single most important factor in the completion of maturation (Fukui and Sakuma, 1980; Häberle *et al*, 1999). For hormonal stimulation of maturation to occur, both cumulus cells and functioning gap junction associations between the cumulus cells and the oocyte are required (Eppig *et al*, 1983; Fagbohun and Downs, 1991). It has been demonstrated that disruption of oocyte-cumulus cell associations can have a detrimental effect on development of the resulting murine oocytes (Eppig, 1979).

Since human oocytes are not readily available for research studies, murine oocytes are often used as a model system for refining techniques of *in vitro* maturation. Numerous protocols for the *in vitro* maturation of immature murine oocytes that develop to the blastocyst stage, following fertilisation, have been

reported. One such maturation protocol involves the co-culture of immature oocytes with follicle cells from throughout the ovary, with sequential exposure to FSH and FSH+LH (Cooper *et al,* 1998). GV stage oocytes, surrounded by cumulus cells, were collected by repeated puncturing of ovarian follicles of female mice that had been stimulated with PMS (active ingredient FSH). Ovaries were collected 48 hours after stimulation, a time point at which numerous follicles would have been recruited and contain fully grown, but immature GV stage oocytes. Initially, the COCs were placed in media supplemented with dibutyryl cyclic adenosine monophosphate (dcAMP) to prevent spontaneous oocyte maturation. This molecule is a membrane permeable analogue of cAMP and like cAMP, it can maintain meiotic arrest (Carroll *et al*, 1991). The COCs were then transferred to the maturation protocol, which involved incubation in medium containing FSH for 6 hours. This was followed by incubation for 13 hours in medium supplemented with FSH/LH (in a 1:1 ratio) on a follicle cell monolayer. This combination of gonadotrophins and co-culture was intended to simulate the *in vivo* environment. The work reported in this chapter involved modification of this maturation protocol in an attempt to improve the yield of mature, fertilisable and developmentally competent murine oocytes.

3.1.1 Modification of Hormonal Milieu During *In Vitro* Maturation

3.1.1.1 Concentration of FSH

In vivo the LH surge, which stimulates oocyte maturation and ovulation, is approximately ten times higher in concentration than the FSH surge that precedes it. The original maturation protocol described above (Cooper *et al,* 1998) involved sequential exposure to media supplemented with FSH then FSH/LH. Hormone preparations were each added to the maturation media at a concentration of 7.5IU/ml. It is hypothesised that a tenfold decrease in the FSH concentration of the first medium

to 0.75IU/ml would simulate the *in vivo* ratio of hormones involved in the LH surge and could possibly lead to an improvement in blastocyst development.

3.1.1.2 Purified-Urinary versus recombinant FSH

Studies have reported that recombinant FSH is more successful than purified hormone at stimulating multifollicular maturation *in vivo* (Schats *et al,* 2000; Anderiesz *et al,* 2000). It is therefore hypothesised that substitution of the purified FSH with a recombinant FSH preparation in the first maturation medium may lead to a greater number of mature oocytes being produced at the conclusion of the maturation protocol.

3.1.2 Modification of The Culture Technique Used For *In Vitro* Maturation

3.1.2.1 COCs matured either in isolation or in the presence of additional follicle cells from throughout the ovary.

The established maturation protocol (Cooper *et al,* 1998) involves the incubation of COCs with additional follicle cells collected from throughout the ovary. It is possible that, within the ovary, follicle cells that were in close proximity to immature oocytes, which had not been recruited for maturation, could exert a negative influence on the maturing oocytes when cultured with them *in vitro*. This could lead to the inhibition of the maturation process and subsequently, poor blastocyst formation. To test this hypothesis, COCs were matured either in isolation or in the presence of additional follicle cells.

3.1.2.2 Conservation of the interactions between cumulus cells of the COC

The original maturation protocol involves the transfer, by pulled Pasteur pipette, of COCs from a droplet of the first maturation medium to a droplet of the medium containing both FSH and LH. It is proposed to exchange the medium within the

droplet rather than moving the COCs. It is hypothesised that reducing manipulation of the COCs, and the disruption this may cause, will ultimately be beneficial to oocyte maturation and to the developmental potential of the oocyte.

3.2 MATERIALS AND METHODS

3.2.1 *In Vitro* Maturation (IVM)

3.2.1.1 Collection of COCs

COCs were collected as described in section 2.2.7 and transferred into droplets of SMM supplemented with 7.5IU/ml FSH (Metrodin HP) and incubated at 37°C, in an atmosphere of 5% CO_2 in air for 4 hours.

3.2.1.2 Culture of monolayer of follicular cells

Follicle cells that were released from whole ovaries during collection of COCs, described above, were transferred by pulled pasteur pipette into a 30μl droplet of SMM supplemented with 7.5IU/ml FSH covered by mineral oil in a droplet dish as described previously and incubated at 37°C, in an atmosphere of 5% CO_2 in air for 4 hours. The medium covering the monolayer of follicle cells was then drawn off using a pulled Pasteur pipette and replaced with 30μl of SMM supplemented with 7.5IU/ml FSH/LH (Humegon) and incubated for 10 minutes at 37°C, in an atmosphere of 5% CO_2 in air.

3.2.1.3 Co-culture of GV stage oocytes and follicular cells

The COCs that had been incubated with SMM + FSH for 4 hours as described above were washed with SMM + FSH/LH before being placed on the follicle cell monolayer, prepared as described above. The COCs and follicle cells were incubated at 37°C, in an atmosphere of 5% CO_2 in air for a further 18 hours.

3.2.2 Modification of Hormonal Milieu During *In Vitro* Maturation

i) The method described in section 3.2.1 was compared with one which was identical except that the FSH preparation was diluted to 0.75IU/ml by dissolving the 75IU vial of hormone in 100ml of SMM media instead of dissolving it in 10ml of SMM media (to produce the 7.5IU/ml preparation).

ii) COCs matured using the method described in section 3.2.2 i) with the lower concentration of FSH, were compared with those matured using the same protocol except that the 0.75IU/ml Urinary purified FSH (Metrodin-HP) was replaced with 0.75IU/ml recombinant FSH preparation (Gonal-F).

3.2.3 Modification of The Culture Technique Used For *In Vitro* Maturation

i) COCs were cultured either as described in section 2.2.9 i.e. 0.75IU/ml Gonal F or in the presence of identical GTs and the presence of a follicle cell monolayer (described in section 3.2.1) during incubation in the FSH/LH preparation.

ii) COCs were either cultured as described in section 2.2.9, i.e. the medium containing FSH and the FSH/LH preparation were removed and added respectively, using a pipette with the COCs remaining in situ; or COCs themselves were transferred from droplets containing FSH into the pre-prepared droplets of medium containing FSH/LH.

For each set of experiments the two groups underwent IVF and developmental assessment as described in section 2.5. *In vivo* matured, ovulated oocytes simultaneously underwent IVF, using the same pool of spermatozoa, and were subjected to the same assessment of development in order to act as controls.

3.3 RESULTS

3.3.1 Modification of Hormonal Milieu During *In Vitro* Maturation

3.3.1.1 FSH concentration of 0.75 or 7.5IU/ml

The percentage of normal embryos that were fertilised was generally lower for oocytes matured in SMM containing 0.75IU/ml FSH than those matured in 7.5IU/ml, although the difference was not statistically significant (Table 3.1). The proportion of

Table 3.1 Fertilisation and development of *in vitro* matured GV stage murine COCs initially matured in 7.5IU/ml FSH compared with COCs initially matured in 0.75IU/ml and *in vivo* matured controls.

Protocol	No. of oocytes	Median % (range)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
In vivo matured control (n=4)	462	95.8 (94.4-98)	79.3 (31.7-92.3)	77.1[a] (61.5-91.7)	58.7 (30.5-80.7)
In vitro matured 7.5IU/ml (n=4)	187	95.1 (95-100)	77.7 (56.4-94.1)	31.9[a] (12.5-36.4)	18.9 (11.8-30.7)
In vitro matured 0.75IU/ml (n=4)	203	97.8 (94.8-100)	57.4 (24.4-84.1)	11.9 (5.7-73.9)	5.8 (2.2-47.2)

a - values within columns with the same superscript are significantly different $p<0.05$.
n= number of repeats of experiment
Normality was assessed 24h after insemination and calculated as a percentage of the total number of oocytes. Fertilisation was assessed 24h after insemination and calculated as the number of 2-cell embryos formed from normal oocytes. Blastocyst development was assessed 120h after insemination and calculated as the number of blastocysts formed from 2-cell embryos. Total blastocyst development was calculated as the number of blastocysts formed as a percentage of the total number of oocytes.

fertilised oocytes that went on to develop into blastocysts was significantly lower for oocytes matured in 7.5IU/ml FSH than for *in vivo* matured oocytes. No significant difference was found in blastocyst formation between the oocytes matured in 0.75IU/ml FSH and *in vivo* matured oocytes, however there was a broad range of results obtained from the oocytes matured in 0.75IU/ml. Comparison of total blastocyst development between the groups showed no significant differences. There

were no significant differences in the proportion of blastocysts that went on to hatch between the three groups; *in vivo* control = 72.2% (range 56.9-91.9%), 7.5IU/ml = 47.8% (range 25-50%), 0.75IU/ml = 33.3% (range 0-75%).

3.3.1.2 Purified-Urinary versus recombinant FSH

There were no significant differences between the treatment groups and the control group for any of the parameters investigated (Table 3.2).

Table 3.2 Fertilisation and development of *in vitro* matured GV stage murine COCs initially matured in 0.75IU/ml of purified urinary FSH compared with COCs initially matured in 0.75IU/ml recombinant FSH and *in vivo* matured controls.

Protocol	No. of oocytes	Median % (range)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
In vivo matured control (n=3)	111	91.9 (88.5-94.9)	83.9 (76.5-93.5)	59.6 (53.8-67.4)	47.5 (37.8-55.8)
In vitro matured purified-urinary FSH (n=3)	61	91.4 (73.1-91.7)	46.9 (36.8-63.6)	42.9 (28.6-66.6)	16.7 (11.5-28.6)
In vitro matured recombinant FSH (n=3)	61	90.6 (34.5-100)	55.2 (20-100)	51.3 (50-52.6)	25 (3.4-52.6)

a - values within columns with the same superscript are significantly different $p<0.05$.
n= number of repeats of experiment
Normality was assessed 24h after insemination and calculated as a percentage of the total number of oocytes. Fertilisation was assessed 24h after insemination and calculated as the number of 2-cell embryos formed from normal oocytes. Blastocyst development was assessed 120h after insemination and calculated as the number of blastocysts formed from 2-cell embryos. Total blastocyst development was calculated as the number of blastocysts formed as a percentage of the total number of oocytes.

3.3.2 Modification of Culture Techniques Used for *In Vitro* Maturation.

3.3.2.1 COCs matured either in isolation or in the presence of additional follicle cells from throughout the ovary.

The proportion of normal embryos that went on to develop to the 2-cell stage was lower in the groups that had been matured *in vitro* than the group matured *in vivo*

(Table 3.3). However, this difference was not found to be significant. Formation of blastocysts was significantly lower when COCs were matured in the presence of the follicle cell monolayer, when calculated as a proportion of the original number of oocytes matured. There were no significant differences in the proportion of blastocysts that went on to hatch between the three groups; *in vivo* control = 61.36% (range 50-77.8%), *In vitro* matured on follicle cell monolayer = 66.7% (range 0-100%), *In vitro* matured in isolation = 62.5% (range 0-100%).

Table 3.3 Fertilisation and development of *in vitro* matured GV stage murine COCs, matured in isolation compared with COCs matured on follicle cell monolayers and *in vivo* matured controls.

Protocol	No. of oocytes	Median % (range)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
In vivo matured control (n=5)	395	93.1 (81.5-96.5)	92.6 (81.3-96.2)	59.2 (3.2-81.9)	39.7 (2.8-48.7)
In vitro matured on follicle cell monolayer (n=5)	137	94.4 (82.6-100)	58.8 (10.5-91.7)	9.6 (5.9-33.3)	4.7[a] (0-5.6)
In vitro matured in isolation (n=5)	96	88.9 (80-95.8)	62.5 (56.3-95.7)	20 (18.2-33.3)	15.5[a] (10.0-17.6)

a - values within columns with the same superscript are significantly different $p<0.05$.
n= number of repeats of experiment
Normality was assessed 24h after insemination and calculated as a percentage of the total number of oocytes. Fertilisation was assessed 24h after insemination and calculated as the number of 2-cell embryos formed from normal oocytes. Blastocyst development was assessed 120h after insemination and calculated as the number of blastocysts formed from 2-cell embryos. Total blastocyst development was calculated as the number of blastocysts formed as a percentage of the total number of oocytes.

3.3.2.2 Conservation of the interactions of the cumulus cells of the COC

There were no significant differences between the treatment groups, nor between the treatment groups and the controls with respect to the proportion of normal oocytes that were fertilised (Table 3.4). The proportion of fertilised oocytes that went on to develop to blastocysts was slightly lower than the control for both

treatment groups; however, this was not found to be significant. Statistical analysis of the proportion of original oocytes that went on to develop into blastocysts showed that the difference in developmental potential was not significant. No significant differences were found in the proportion of blastocysts that went on to hatch between the three groups (*in vivo* control = 79.6% (range 67.5-90.9%), media moved = 33.3% (range 0-75%), COCs moved = 63.1% (range 33.3-63.6%).

Table 3.4 Fertilisation and development of *in vitro* matured GV stage murine COCs, matured in isolation without disruption, compared with COCs that were disrupted by transfer during *in vitro* maturation and with *in vivo* matured controls.

Protocol	No. of oocytes	Median % (range)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
In vivo matured control (n=4)	196	90.9 (88.8-93.9)	79.9 (75.8-89.5)	89.4 (64.7-93.8)	60.6 (53.7-67.4)
In vitro matured COCs moved (n=4)	137	97.5 (97.2-100)	60.2 (44-80)	28,9 (15.0-42.1)	23.9 (12.0-26.7)
In vitro matured media moved (n=4)	203	97.8 (94.8-100)	57.4 (24.4-84.1)	11.9 (5.7-73.9)	5.8 (2.2-47.2)

n= number of repeats of experiment
Normality was assessed 24h after insemination and calculated as a percentage of the total number of oocytes. Fertilisation was assessed 24h after insemination and calculated as the number of 2-cell embryos formed from normal oocytes. Blastocyst development was assessed 120h after insemination and calculated as the number of blastocysts formed from 2-cell embryos. Total blastocyst development was calculated as the number of blastocysts formed as a percentage of the total number of oocytes.

3.4 DISCUSSION

3.4.1 Modification of Hormonal Milieu During *In Vitro* Maturation

3.4.1.1 Concentration of FSH

The tenfold decrease in FSH concentration of the initial maturation medium did not reduce the normality of oocytes, when assessed 24 hours post insemination, in comparison with those matured in the presence of the higher FSH concentration, nor

with *in vivo* matured control oocytes. For all of the other parameters investigated there were no significant differences between the group matured in the presence of the lower concentration of FSH and the other *in vitro* and *in vivo* matured groups. However, there was great variability in these parameters for the oocytes matured in the presence of 0.75IU/ml FSH. COCs matured in the presence of 7.5IU/ml FSH had a significantly lower percentage of development to blastocyst (from fertilised oocytes) than oocytes that were matured *in vivo*.

The tenfold reduction in FSH concentration had a variable effect on the subsequent developmental potential of the oocytes. It is possible that the variability seen in the experiments using lower concentrations of FSH resulted from varying degrees of dilution of FSH when the COCs were added. However, the gonadotrophins that are released prior to ovulation, during oocyte maturation *in vivo,* are present in the blood stream and reproductive tissues in minuscule amounts. In contrast to this, the concentrations of gonadotrophins used in this study are relatively high. Despite the seemingly drastic tenfold decrease in concentration of FSH in the initial maturation media, the concentrations of FSH and FSH/LH used in the maturation protocol are considerably greater than the amounts that would be found *in vivo*. It is possible that the changes made to the hormonal concentration of the IVM medium would have a negligible effect, as they are present in excessive amounts.

3.4.1.2 Purified-Urinary versus recombinant FSH

No significant differences in normality, fertilisability or developmental potential were found between COCs matured in the presence of purified-urinary FSH or recombinant FSH. Although a study with a larger sample size may illustrate any differences more fully, reports of stimulation of multifollicular recruitment via administration of recombinant FSH (Anderiesz *et al*, 2000; Goud *et al*, 2000) may

depend on the presence of ovarian or follicular factors that are not present during *in vitro* maturation. However, recombinant FSH is subject to less variation in quality and reactivity between batches than purified-urinary FSH. Recombinant FSH may, therefore, be a more reliable and consistent source of gonadotrophin.

3.4.2 Modification of The Culture Technique Used For *In Vitro* Maturation

3.4.2.1 COCs matured either in isolation or in the presence of additional follicle cells from throughout the ovary.

No significant difference was detected between the proportion of normal oocytes and the proportion of fertilised oocytes across the three groups. However, the proportion of the total number of oocytes that went on to form blastocysts was significantly higher in the group of COCs matured without additional follicle cells compared with those matured in the presence of additional follicle cells.

The poor development of blastocysts that arose after IVF of COCs that were matured in the presence of the follicle cell monolayer suggests that the completion of cytoplasmic maturation had been affected by the presence of the additional follicle cells during maturation. The follicle cells appeared to have an inhibitory effect. It is possible that, within the ovary, follicle cells that were in close proximity to immature oocytes, which had not been recruited for maturation, could exert a negative influence on the maturing oocytes when cultured with them *in vitro*. This could lead to the inhibition of the maturation process and developmental impairment. However, this finding is in direct contrast to that reported by Cooper *et al* (1998) where maturation in the presence of a follicle cell monolayer was found to significantly improve hatched blastocyst development compared to maturation in isolation. This discrepancy could be explained by the fact that in the former study the follicle cell monolayer

comprised cells that were released by puncturing of ovarian follicles. While in the current study the monolayer may have contained damaged follicular cells, as the cells were collected following near total maceration of the ovary which was carried out in order to release the COCs. The inconsistency of the results could also be a result of the source of the FSH used in the initial IVM culture. Although Metrodin-HP was used in both studies, the current study used Metrodin that had been returned by patients following the completion of their treatment. Therefore there may have been variation in the suitability of storage conditions and the period by which the hormone preparation was out of date. The ability of the blastocysts to hatch was not affected by the presence or absence of additional follicle cells during oocyte maturation.

3.4.2.2 Conservation of the interactions between cells of the COC

Transferral of oocytes, by means of a pipette, from one medium to another was not found to have an impact on the normality, fertilisability or developmental potential of oocytes matured using the current system. The disruption caused to the COCs by pipetting may have been insignificant. Alternatively, the disruption caused by the transfer may have been temporary, the cells being capable of re-establishing any interactions. It is also possible that the cells which become detached during the transfer were able to fulfil their role in maturation via paracrine interactions that did not require physical contact.

3.4.3 Summary

Maturation experiments that involved changes in the constituents of the maturation medium i.e. changing the concentration and source of FSH, did not show any clear effect on the fertilisation and developmental potential of oocytes. These results are in agreement with those reported in other studies (Trounson *et al*, 2001).

On the other hand, modification of the culture technique was found to be important. It has been established that the maturation of COCs in the presence of follicle cells from throughout the ovary can have an inhibitory effect on the maturation process. Differences in the developmental potential of the oocytes suggested that the completion of cytoplasmic maturation, in particular, had been impeded by co-culture with additional ovarian follicle cells. Therefore in future experiments, the co-culture of the COCs with the additional follicle cell monolayer will be omitted and COCs will be cultured in isolation during the *in vitro* maturation protocol. It has been established in previous studies that it is possible to mature oocytes *in vitro* when they remain within the follicle (Spears *et al*, 1994). However, the cells within a follicle are associated with the oocyte that is being matured, which is different from this situation where follicle cells from throughout the ovary were collected and cultured for the purpose of co-culture. The inhibitory effect that was observed could be due to the fact that these follicle cells were not specifically associated with the maturing oocytes with which they were cultured.

Avoiding the transfer, by pipette, of fresh COCs from one maturation medium to the next did not have any significant effect on their fertilisation and development. However, the adoption of this technique may be beneficial when maturing cryopreserved COCs since cryopreservation is likely to undermine cumulus cell attachment.

For subsequent experiments COCs will be subjected to the following maturation protocol. COCs will be held in SMM + 0.1mg/ml dcAMP at 37°C in an atmosphere of 5% CO_2 in air. COCs will then be placed into a droplet of SMM containing 0.75IU/ml recombinant FSH, under oil for 4 hours at 37°C in an atmosphere of 5% CO_2 in air.

After this time, the medium will be replaced by SMM containing 7.5IU/ml FSH/LH at 37°C in an atmosphere of 5% CO_2 in air for 18 hours.

Chapter 4

SLOW-COOLING OF GV STAGE MURINE COCS

4.1 INTRODUCTION

The proportion of immature human oocytes that reach maturity following cryopreservation and maturation *in vitro* is low. Cryopreserved GV stage human oocytes have been shown to be capable of completing nuclear maturation and becoming fertilised (Toth *et al*, 1994, Wu *et al*, 2001). However, development of embryos resulting from cryopreserved immature human oocytes is often impaired (Son *et al*, 1996, Toth *et al*, 1994, Wu *et al*, 2001).

Refinement of IVM protocols for fully-grown non-cryopreserved murine GV stage oocytes has resulted in oocytes with a similar level of developmental competence to that observed among *in vivo* matured oocytes when the donors were pre-treated with gonadotrophins (Schroeder and Eppig, 1984, 1989). High rates of nuclear maturation have been reported following cryopreservation and IVM of murine GV stage oocytes using a variety of cryopreservation techniques (Candy *et al*, 1994, Schroeder *et al*, 1990, Van Blerkom and Davis, 1994, Van der Elst *et al*, 1993). In some cases the rate of fertilisation was similar for thawed and fresh control oocytes (Candy *et al*, 1994). However, there have also been reports of decreased rates of fertilisation following cryopreservation (Schroeder *et al*, 1990, Van Blerkom and Davis, 1994, Van der Elst *et al*, 1993). In general the developmental capacity of cryopreserved GV stage murine oocytes is poor (Candy *et al*, 1994, Schroeder *et al*, 1990, Van Blerkom and Davis, 1994, Van der Elst *et al*, 1992, Van der Elst *et al*, 1993).

It has been established that coupling of somatic cumulus granulosa cells with the GV stage oocyte is vital to the progression of oocyte maturation and subsequent embryo development (Fagbohun and Downs, 1991). Studies have shown that GV stage oocytes that are stripped of cumulus cells have a reduced developmental capacity compared with that of cumulus enclosed GV stage oocytes (Schroeder and Eppig, 1984). Thus, in attempting to cryopreserve immature oocytes it would seem prudent to preserve the cumulus cells surrounding the oocyte and to maintain connections between the cells.

The three dimensional COC is likely to be particularly prone to physical disruption caused by ice crystal formation. Even if ice crystal formation is avoided, the vast difference in size between the oocyte and it's associated cumulus cells means that they are likely to react very differently to the stresses applied during cryopreservation. The low levels of developmental competence observed in freeze/thawed GV stage oocytes combined with the apparently high levels of intracellular normality of the oocyte could be an indication of damage and/or disruption to the somatic cumulus cells and their association with the oocyte.

Previous studies of the cryopreservation of immature oocytes have concentrated on the subsequent viability of the oocyte. The initial aim of this study was to investigate the effects of cryopreservation on the oocyte and the cumulus cells surrounding it. The COCs were either exposed to CPA (in order to determine whether the osmotic fluxes that occur during cryoprotectant addition/dilution were sufficient to cause the damage that is apparent following cryopreservation), or cryopreserved using a standard slow cool/slow warm protocol (as detailed in section 2.3.2).

One mechanism by which cumulus cells may act in the hormonally directed maturation of the oocyte is via the paracrine release of secretory maturation factors

(Byskov *et al*, 1995; Downs, 2001). It has been established that cryopreservation causes damage and disassociation of the cumulus cells of thawed COCs. It is therefore likely that the functionality of the cumulus cells is compromised by this treatment. Thus secretory maturation factors may be absent or at insufficient concentrations to promote maturation in thawed COCs. Therefore, in the second set of experiments cryopreserved and fresh COCs were matured in the presence of fresh cumulus cells.

A third set of experiments was conducted to investigate the damage caused by the two main cooling steps of the cryopreservation protocol, i.e. slow cooling to –60°C and rapid cooling from –60°C to –196°C by plunging into LN_2. Previously, it has been reported that cooling to lower sub zero temperatures prior to exposure to –196°C led to higher rates of survival, fertilisation and development (Trounson and Kirby, 1989). Therefore in the fourth set of experiments the cooling profile was modified to include an additional cooling ramp to –150°C.

Following IVM, IVF was used to assess the developmental potential of the oocytes. In separate experimental groups membrane integrity staining of the treated COCs was carried out in order to assess the extent of damage caused to the cumulus cells immediately following thawing. Untreated GV stage COCs were simultaneously assessed as controls.

4.2 MATERIALS AND METHODS

4.2.1 Exposure of GV Stage COCs to1.5M Me_2SO without freezing

COCs were equilibrated with cryoprotectant and then the cryoprotectant was removed from the cells without freezing as detailed in section 2. COCs were assessed for membrane integrity immediately after removal of the CPA. In a separate set of experiments COCs were assessed for normality, fertilisability and developmental

potential following IVM and IVF. Non-treated *in vivo* matured and *in vitro* matured COCs were simultaneously assessed as controls.

4.2.2 Slow Cooling of GV Stage COCs

The normality, fertilisability and developmental potential of immature murine oocytes were determined following cryopreservation using a standard slow cooling method (detailed in section 2.3.2) and IVM. Non-cryopreserved *in vivo* matured and *in vitro* matured COCs were simultaneously assessed as controls. The viability of the cumulus cells of separate batches of thawed COCs were assessed immediately following thawing using stains to determine membrane integrity.

4.2.3 IVM of Denuded GV Stage Oocytes

Fresh COCs were denuded of their associated cumulus cells (as described in section 2.2.8) divided into two groups, one group was matured in co-culture with freshly disassociated cumulus cells. The other group was matured without the addition of cumulus cells. Fresh intact COCs were matured *in vitro* simultaneously. Following *in vitro* fertilisation the three groups were assessed for normality, fertilisability and developmental potential.

4.2.4 IVM of Freeze/Thawed GV Stage COCs in the Presence of Fresh Disassociated Cumulus Cells

COCs were cryopreserved as outlined above. Following thawing they were divided into two groups, one group of COCs were matured *in vitro* in the presence of freshly disassociated cumulus cells. The other group was matured without the addition of fresh cumulus cells. Fresh COCs were *in vitro* matured simultaneously. Following *in vitro* fertilisation the three groups were assessed for normality, fertilisability and developmental potential.

4.2.5 Comparison of GV Stage COCs Slow Cooled to –60°C and those Slow Cooled to –60°C and Plunged into LN_2

GV stage COCs were slow cooled (as described in section 2.3.2) to –60°C, some straws were then plunged into LN_2 while the remainder were held at –60°C before both groups of straws were warmed. After removal of the CPA, both groups were either assessed for membrane integrity (as described previously) or normality, fertilisability and developmental potential were assessed following IVM and IVF. Non-treated *in vitro* matured COCs were assessed simultaneously as controls.

4.2.6 Comparison of Slow Cooling of GV Stage COCs to –60°C or to –150°C prior to plunging into LN_2.

GV stage COCs were slow cooled (as described in section 2.3.2) to –60°C and then either plunged into LN_2 or cooled at a rate of –10°C/min to –150°C before being plunged into LN_2. Both groups were removed from LN_2 and warmed from –70°C to 4°C at 10°C/min (as described in section 2.3.2). After removal of the CPA the COCs were either assessed for membrane integrity or normality, fertilisability and developmental potential were assessed following IVM and IVF. Non-treated *in vitro* matured COCs were assessed simultaneously as controls.

4.3 RESULTS

4.3.1 Cryopreservation of GV Stage COCs

4.3.1.1 Assessment of development of GV stage COCs exposed to 1.5M Me_2SO without freezing

Normality was assessed following IVM and IVF. Exposure to 1.5M Me_2SO did not cause a decrease in normality of the oocytes when compared with *in vitro* and *in*

vivo matured control oocytes (Table 4.1). Rates of fertilisation were similar for COCs exposed to cryoprotectant and the *in vitro* matured control group. Both groups had lower fertilisation rates, though not significantly lower, than those in the *in vivo* matured control group. Exposure to 1.5M Me_2SO did not cause a decrease in developmental potential when compared with *in vitro* matured control oocytes. No differences in proportions of blastocyst hatching were detected between the three groups (*in vivo* control = 63.4%, range 60.6-79.1%, *in vitro* control = 65.2%, range 0.0-100%, Me_2SO treated = 74.3%, range 33.3-93.3%).

Table 4.1 Fertilisation and development of GV stage murine COCs exposed to 1.5M Me_2SO compared with *in vivo* and *in vitro* matured controls.

Protocol	No. of oocytes	Median % (range)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
In vivo matured (n=4)	283	94.0 (91.5-98.7)	92.4 (89.7-94.4)	69.4 (47.1-84.3)	60.0 (41.8-72.9)
In vitro matured (n=4)	124	87.6 (75.0-100)	66.1 (33.3-81.1)	46.9 (20.0-57.1)	16.7 (14.8-39.5)
1.5M Me_2SO + *in vitro* matured (n=4)	150	91.3 (80.6-100)	64.6 (28.0-78.9)	46.8 (42.9-58.3)	29.5 (9.7-40.0)

n= number of repeats of experiment
Normality was assessed 24h after insemination and calculated as a percentage of the total number of oocytes. Fertilisation was assessed 24h after insemination and calculated as the number of 2-cell embryos formed from normal oocytes. Blastocyst development was assessed 120h after insemination and is expressed both per fertilised 2-cell embryo and per oocyte (total blastocyst).

4.3.1.2 Assessment of development of slow cooled and thawed GV stage COCs

The normality of GV stage COCs that were slow-cooled in the presence of 1.5M Me_2SO was significantly ($p<0.05$) reduced compared with *in vitro* and *in vivo* matured controls (Table 4.2). Oocytes that were assessed as normal became fertilised in similar proportions for all groups. Thawed COCs were found to have low developmental potential compared with controls when development was calculated

from the number of fertilised oocytes or from the total number of oocytes in that group. The proportion of the blastocysts that hatched was found to be significantly reduced ($p<0.05$) after cryopreservation (median = 0%, range 0.0-33.3%) compared with *in vivo* matured controls (median = 80.7%, range 58.6-95.2%). However, neither of these values was found to be significantly different from that obtained for *in vitro* matured oocytes (median = 42.9%, range 0.0-62.5%).

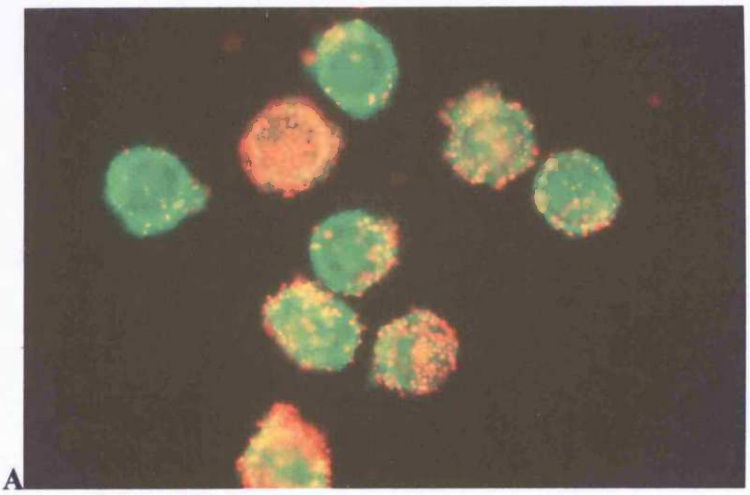
Table 4.2 Fertilisation and development of freeze/thawed GV stage murine COCs compared with *in vivo* and *in vitro* matured controls.

Protocol	No. of oocytes	Median % (range)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
In vivo matured (n=5)	325	95.8[a] (93.9-100)	86.4 (80.4-96.9)	57.9[c] (52.3-83.8)	51.5[e] (42.0-63.3)
In vitro matured (n=5)	64	96.0[b] (88.2-100)	95.9 (80.0-100)	65.4[d] (64.0-66.7)	59.3[f] (47.1-58.3)
Freeze/Thawed + *in vitro* matured (n=5)	126	50.0[ab] (0.0-71.4)	91.6 (61.1-100)	18.2[cd] (0.0-22.7)	8.3[ef] (0.0-11.9)

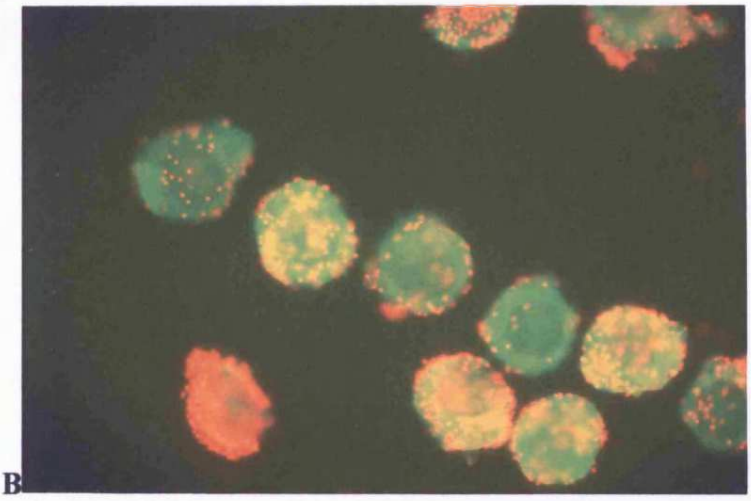
a, b, c, d, e and f - values within columns with the same superscript are significantly different $p<0.05$.
n= number of repeats of experiment
Normality was assessed 24h after insemination and calculated as a percentage of the total number of oocytes. Fertilisation was assessed 24h after insemination and calculated as the number of 2-cell embryos formed from normal oocytes. Blastocyst development was assessed 120h after insemination and is expressed both per fertilised 2-cell embryo and per oocyte (total blastocyst).

4.3.1.3 Cumulus cell membrane integrity staining

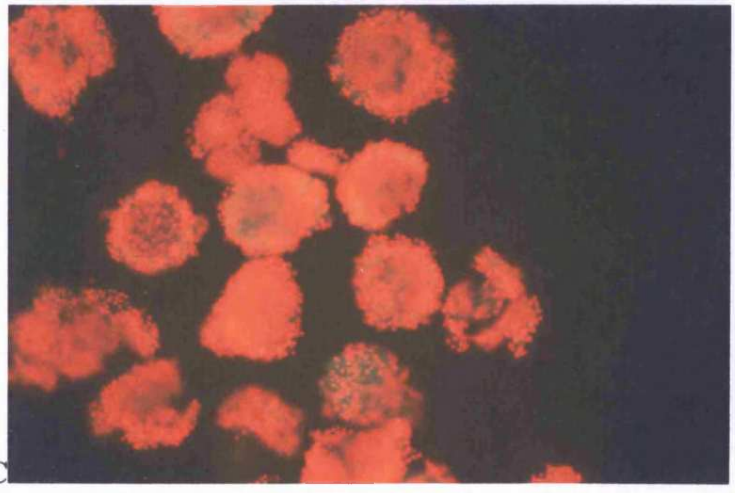
Staining of fresh COCs, COCs exposed to1.5M Me_2SO and slow-cooled COCs is shown in figure 4.1. The majority of the fresh COCs (65 COCs assessed) had a high proportion of cumulus cell membrane integrity (figure 4.1A) and a median score of 1. Following exposure to 1.5M Me_2SO (89 COCs assessed), the percentage of the surface of the oocyte covered with cumulus cells that had intact membranes was found to be similar to that observed in fresh intact COCs (figure 4.1B) these COCs also had a median score of 1. However, few of the cumulus cells of the frozen/thawed



A



B



C

Figure 4.1 Fluorescent micrographs depicting carboxy fluorescein/propidium iodide membrane integrity staining of: (**A**) Freshly-collected intact untreated GV stage COCs: (**B**) GV stage COCs exposed to 1.5M Me_2SO at 4°C for 15 min; and (**C**) COCs slow-cooled and thawed in 1.5M Me_2SO. Magnification 200x.

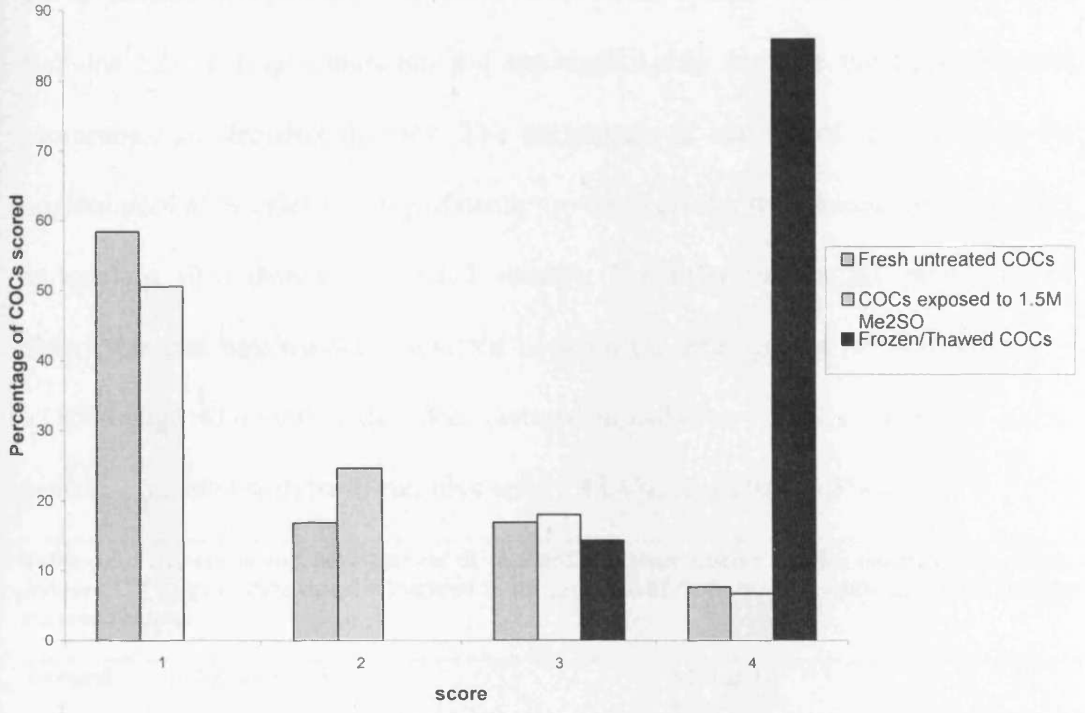


Figure 4.2 Distribution of scores assigned following membrane integrity staining of freshly collected COCs, cryoprotectant treated COCs and frozen/thawed COCs.
Scoring system - Percentage of the surface of the oocyte in contact with cumulus cells with intact membranes. 100-76% =1, 75-51% =2, 50-26% =3, 25-0% =4.

COCs (49 COCs assessed) retained an intact plasma membrane (figure 4.1C) these COCs had a median score of 4. There was found to be significantly less direct contact between the surface of the oocyte and cumulus cells with intact membranes in cryopreserved COCs compared with the fresh and Me_2SO-treated COCs (figure 4.2).

4.3.2 Effect of Cumulus Cell Removal and Co-Culture on Maturation of Immature Oocytes

4.3.2.1 Assessment of the development of denuded *in vitro* matured GV stage oocytes either in the presence of disassociated cumulus cells or in isolation.

Normality and fertilisation were similar in these groups and in the intact *in vitro* matured group (Table 4.3). When development was calculated from the total number

of oocytes both groups of denuded oocytes were found to have a low level of developmental competence, compared with intact controls. Co-culture with fresh cumulus cells during maturation did not significantly improve the developmental competence of denuded oocytes. The percentage of blastocysts formed from the original pool of oocytes was significantly ($p<0.05$) greater for cumulus intact oocytes matured *in vitro* than the denuded oocytes. No differences in the proportion of blastocysts that hatched were detected between the three groups (*in vitro* control = 67.0%, range 40.0-90.0%, denuded, matured in isolation = 43.8%, range 0.0-100%, denuded, matured with fresh cumulus cells = 43.4%, range 0.0-83.3%).

Table 4.3 Fertilisation and development of denuded GV stage murine oocytes matured in isolation, denuded GV stage murine oocytes matured in the presence of fresh cumulus cells, and intact *in vitro* matured controls.

Protocol	No. of oocytes	Median % (range)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
Control					
Intact *In vitro* matured (n=6)	135	100 (90.0-100)	92.6 (77.3-100)	53.6 (35.7-77.8)	47.6[ab] (33.3-70.0)
Denuded oocytes					
In vitro matured (n=6)	106	92.5 (81.8-100)	78.9 (35.3-95.2)	40.7 (14.3-60.0)	26.2[b] (5.3-54.5)
In vitro matured + fresh cumulus cells (n=6)	116	100 (93.3-100)	82.2 (40.1-100)	29.4 (10.0-66.7)	19.7[a] (9.1-60.9)

n= number of repeats of experiment
a, b - values within columns with the same superscript are significantly different $p<0.05$
Normality was assessed 24h after insemination and calculated as a percentage of the total number of oocytes. Fertilisation was assessed 24h after insemination and calculated as the number of 2-cell embryos formed from normal oocytes. Blastocyst development was assessed 120h after insemination and is expressed both per fertilised 2-cell embryo and per oocyte (total blastocyst).

4.3.2.2 Assessment of development of cryopreserved GV stage COCs matured *in vitro* in the presence of fresh disassociated cumulus cells

GV stage COCs were slow-cooled and thawed in the presence of 1.5M Me_2SO then matured *in vitro*, either in the presence of fresh disassociated cumulus cells or in isolation. Normality was found to be similar in all groups (Table 4.4). Cryopreserved oocytes were found to have a significantly ($p<0.05$) lower rate of fertilisation and poorer development than *in vitro* matured oocytes. Co-culture of cryopreserved COCs with fresh cumulus cells during *in vitro* maturation did not improve rates of fertilisation or development of blastocysts compared with similarly treated non co-cultured COCs. Cryopreservation caused a decrease in proportion of blastocysts that hatched (i.e. *in vitro* control = 76.9%, range 30.8-100%, cryopreserved and matured in isolation = 0.0%, range 0.0-6.0%). However, maturation of cryopreserved COCs in the presence of fresh cumulus cells led to a significant increase ($p<0.05$) in the proportion of hatched blastocysts (= 40.0%, range 0.0-66.7%).

Table 4.4 Fertilisation and development of freeze/thawed GV stage murine COCs matured in isolation, freeze/thawed GV stage murine COCs matured in the presence of fresh cumulus cells, and intact *in vitro* matured controls.

Protocol	No. of oocytes	Median % (range)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
Control					
Intact *In vitro* matured (n=7)	144	100 (100)	91.3[ab] (83.3-100)	52.9[c] (35.7-88.9)	53.3[de] (47.4-88.9)
Freeze/thawed oocytes					
In vitro matured (n=7)	109	80.0 (40-91.7)	77.8[a] (61.5-88.9)	14.3[c] (11.1-33.3)	8.3[d] (4-20)
In vitro matured + fresh cumulus cells (n=7)	115	73.3 (52.4-100)	72.7[b] (35-81.8)	40.0 (0.0-57.1)	20[e] (0-44.4)

n= number of repeats of experiment
a, b, c, d, and e - values within columns with the same superscript are significantly different $p<0.05$
Normality was assessed 24h after insemination and calculated as a percentage of the total number of oocytes. Fertilisation was assessed 24h after insemination and calculated as the number of 2-cell embryos formed from normal oocytes. Blastocyst development was assessed 120h after insemination and is expressed both per fertilised 2-cell embryo and per oocyte (total blastocyst).

4.3.3 Comparison of GV Stage COCs Slow Cooled to –60°C and Those Slow Cooled to –60°C and Plunged into LN_2.

4.3.3.1 Assessment of development

GV stage murine COCs were slow cooled to –60°C and were then either held at this temperature or plunged into LN_2 before being warmed from –60°C. The COCs were matured *in vitro* and their development assessed following IVF. Normality was found to be significantly ($p<0.05$) reduced by cooling to –60°C when compared with controls (Table 4.5). However, no significant differences in normality were detected between the two cooled groups. No differences in fertilisation were observed between the three groups.

Table 4.5 Fertilisation and development of GV stage murine COCs slow cooled to –60°C and GV stage murine COCs slow cooled to –60°C and plunged into LN_2, compared with *in vitro* matured controls.

Protocol	No. of oocytes	Median % (range)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
Control *In vitro* matured (n=5)	128	100[ab] (92.6-100)	94.4 (69.2-100)	76.9 (55.6-87.1)	72.2[c] (38.5-87.1)
Slow cooled –60°C + LN_2 (n=5)	120	45.8[a] (0.0-47.8)	64.3 (0.0-90.9)	33.3 (0.0-90.0)	12.5[c] (0.0-21.7)
Held at –60°C (n=5)	126	75.0[b] (35.0-100)	80.0 (74.1-90.5)	63.2 (55.0-100)	30.6[c] (24.0-50.0)

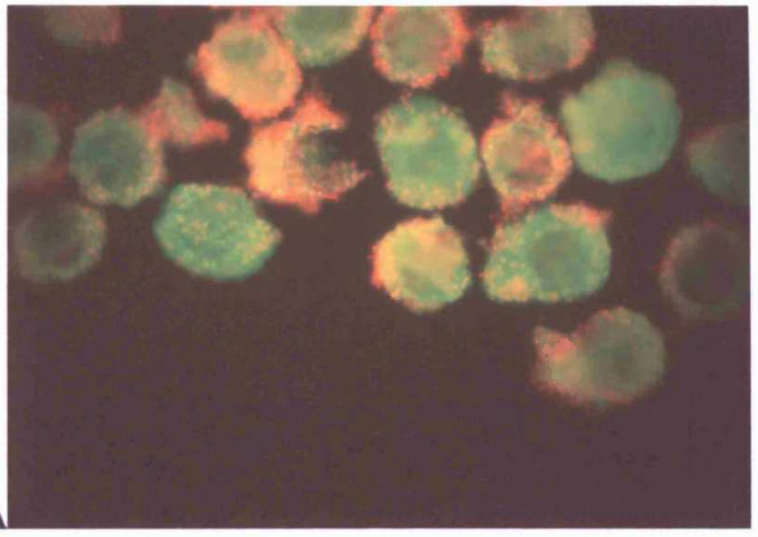
n= number of repeats of experiment
a, b, c - values within columns with the same superscript are significantly different $p<0.05$
Normality was assessed 24h after insemination and calculated as a percentage of the total number of oocytes. Fertilisation was assessed 24h after insemination and calculated as the number of 2-cell embryos formed from normal oocytes. Blastocyst development was assessed 120h after insemination and is expressed both per fertilised 2-cell embryo and per oocyte (total blastocyst).

When development of blastocysts was calculated from the total number of oocytes in each cohort, it was found to be significantly ($p<0.05$) lower following

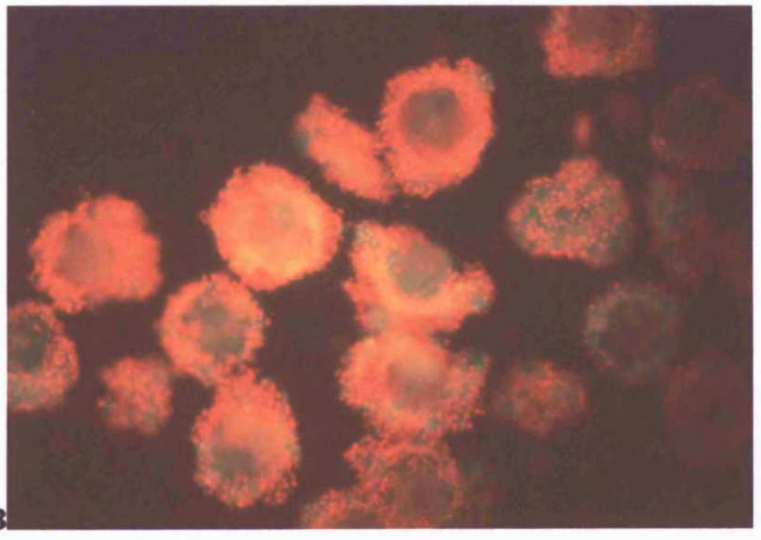
cooling to –60°C than in untreated control oocytes. In addition, cooling to, and warming from –196°C was found to cause a significant ($p<0.05$) reduction in blastocyst development compared with cooling of COCs to –60°C without plunging into LN_2. No differences in proportion of blastocysts hatched were detected between the three groups (*in vitro* matured control = 60.0%, range 53.8-66.7%, slow cooled to –60°C + plunging into LN_2 = 44.4%, range 0.0-100%, slow cooling to –60°C without plunging into LN_2 = 58.3%, range 50.0-80.0%).

4.3.3.2 Cumulus cell membrane integrity staining

Staining of fresh COCs (128 COCs assessed) revealed a low incidence of cumulus cell membrane damage and little disassociation of cumulus from the oocyte (figure 4.3A). Amongst the fresh COCs that were scored, COCs fell into all of the four scoring categories and there was a median score of 1 (figure 4.4). Staining of COCs that had been plunged into LN_2 following cooling to –60°C (86 COCs assessed) revealed extensive cumulus cell membrane damage. The majority of cumulus cells sustained damage to their plasma membranes (figure 4.3B). These COCs had a median score of 4 with over half of the COCs stained being in this category (figure 4.4). Staining of COCs that had been cooled to –60°C without exposure to LN_2 (97 COCs assessed) showed a lesser degree of cumulus cell damage than that observed in COCs that had been plunged into LN_2 (figure 4.3C). However, the loss of membrane integrity of the cumulus cells was substantial. These COCs had a median score of 3. COCs that were cooled to –60°C without exposure to LN_2 were evenly distributed between the four scoring categories. Only 33% of these COCs were scored as 4 (figure 4.4).



A



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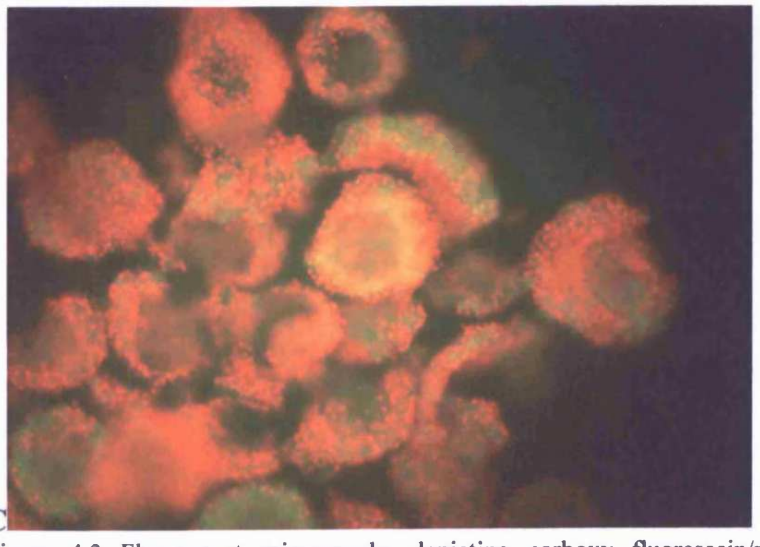


C

Figure 4.3 Fluorescent micrographs depicting carboxy fluorescein/propidium iodide membrane integrity staining of: (**A**) Freshly-collected intact untreated GV stage COCs: (**B**) GV stage COCs plunged into LN_2 following cooling to –60°C; and (**C**) GV stage COCs cooled to –60°C without exposure to LN_2. Magnification 200x.

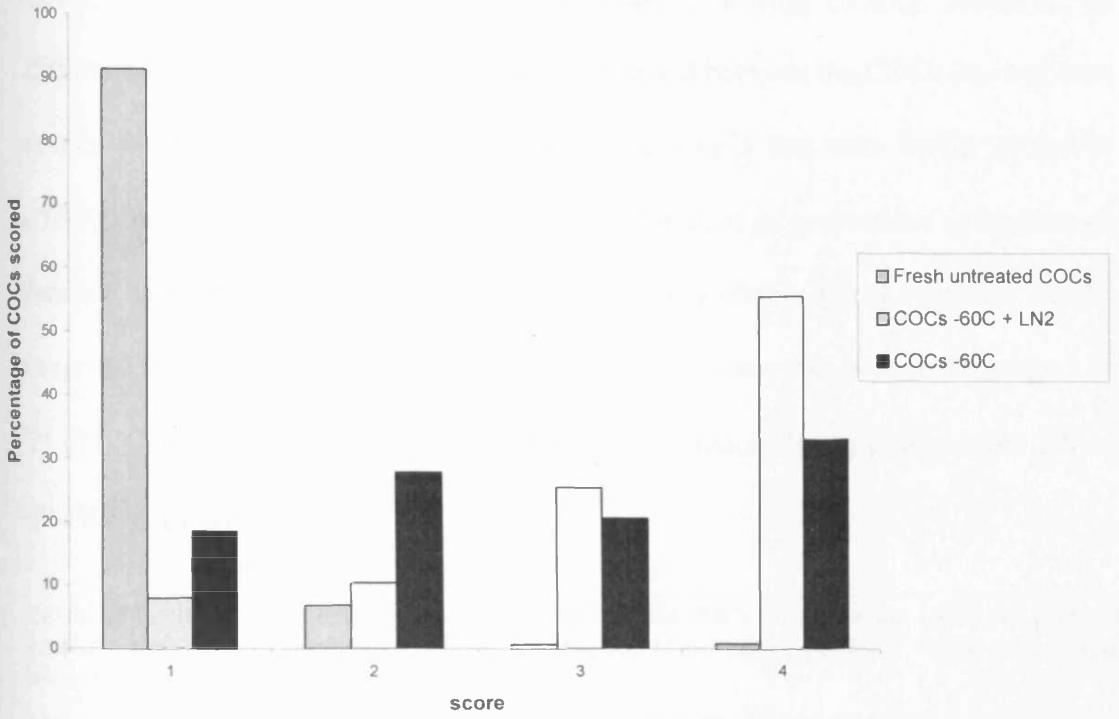


Figure 4.4 Distribution of scores assigned following membrane integrity staining of freshly collected COCs, COCs plunged into LN_2 following cooling to –60°C and COCs cooled to –60°C without exposure to LN_2.
Scoring system - Percentage of the surface of the oocyte in contact with cumulus cells with intact membranes. 100-76% =1, 75-51% =2, 50-26% =3, 25-0% =4.

4.3.4 Comparison of GV Stage COCs After Slow Cooling to –60°C or to –150°C Prior to Plunging into LN_2

4.3.4.1 Assessment of development

GV stage COCs were slow cooled to –60°C and were then either plunged into LN_2 or further cooled to –150°C at a rate of -10°C/min. before being plunged into LN_2. The COCs were then warmed slowly from –70°C, matured *in vitro* and development assessed following IVF. Normality of the oocytes was significantly reduced by cooling and exposure to LN_2 (Table 4.6). However, no significant differences in normality were detected between the two cooled groups. Also, no

differences in fertilisation were observed between the three groups. When development of blastocysts was calculated from the total number of oocytes in each cohort, it was found to be significantly lower following cooling. However, no differences in developmental capacity were detected between the COCs that had been cooled to –60°C before exposure to LN_2 or the COCs that were further cooled to –150°C before being plunged into LN_2. No differences in proportions of blastocysts hatched were detected between the three groups (*in vitro* matured control = 70.0%, range 40.0-92.3%, COCs cooled to –60°C before exposure to LN_2 = 0.0%, range 0.0-75.0%, COCs that were further cooled to –150°C before being plunged into LN_2 = 40.0%, range 0.0-50.0%).

Table 4.6 Fertilisation and development of GV stage murine COCs slow cooled to –60°C and plunged into LN_2, and GV stage murine COCs slow cooled to –60°C and further cooled to –150°C and plunged into LN_2, compared with *in vitro* matured controls.

Protocol	No. of oocytes	Median % (range)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
Control					
In vitro matured (n=5)	108	100[ab] (100)	85.7 (81.6-94.7)	72.2 (62.5-80.6)	61.9[cd] (58.8-65.8)
Slow cooled					
–60°C + LN_2 (n=5)	111	52.9[a] (9.1-75.0)	91.7 (50.0-100)	54.5 (3.3-100)	5.6[c] (4.5-47.1)
Cooled to –150°C + LN_2 (n=5)	108	50.0[b] (14.3-71.4)	90.9 (11.1-100)	41.7 (14.3-54.5)	20.8[d] (4.8-25.0)

n= number of repeats of experiment
a, b, c, d - values within columns with the same superscript are significantly different $p<0.05$
Normality was assessed 24h after insemination and calculated as a percentage of the total number of oocytes. Fertilisation was assessed 24h after insemination and calculated as the number of 2-cell embryos formed from normal oocytes. Blastocyst development was assessed 120h after insemination and is expressed both per fertilised 2-cell embryo and per oocyte (total blastocyst).

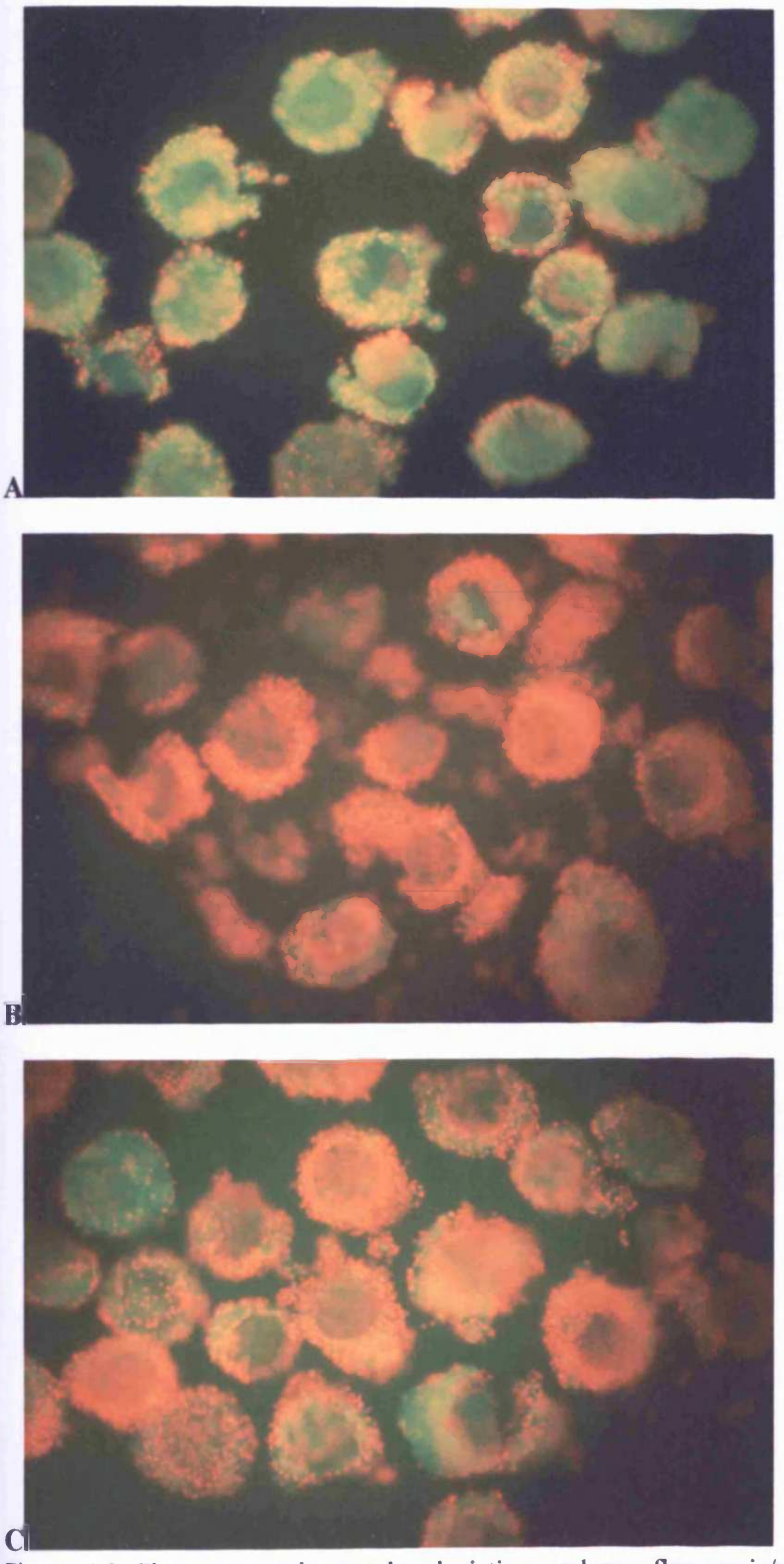


Figure 4.5 Fluorescent micrographs depicting carboxy fluorescein/propidium iodide membrane integrity staining of: (**A**) Freshly-collected intact untreated GV stage COCs: (**B**) GV stage COCs plunged into LN_2 following cooling to –60°C; and (**C**) GV stage COCs cooled to –60°C and then further cooled to –150°C before being plunged into LN_2. Magnification 200x.

4.3.4.2 Cumulus cell membrane integrity staining

Staining of fresh COCs (141 COCs assessed) once again revealed a low incidence of cumulus cell membrane damage (figure 4.5A) with a median score of 1. COCs that had been exposed to LN_2 showed high levels of cumulus cell damage (figure 4.5B and 4.5C). However, the distribution of the cooled COCs across the four scoring categories was different depending on the cooling profile employed (figure 4.6). COCs that were plunged into LN_2 following cooling to –60°C (126 COCs assessed) had a median score of 4, 68.3% of these COCs were scored as such. In contrast to this, the median score of the COCs that were cooled to –150°C before

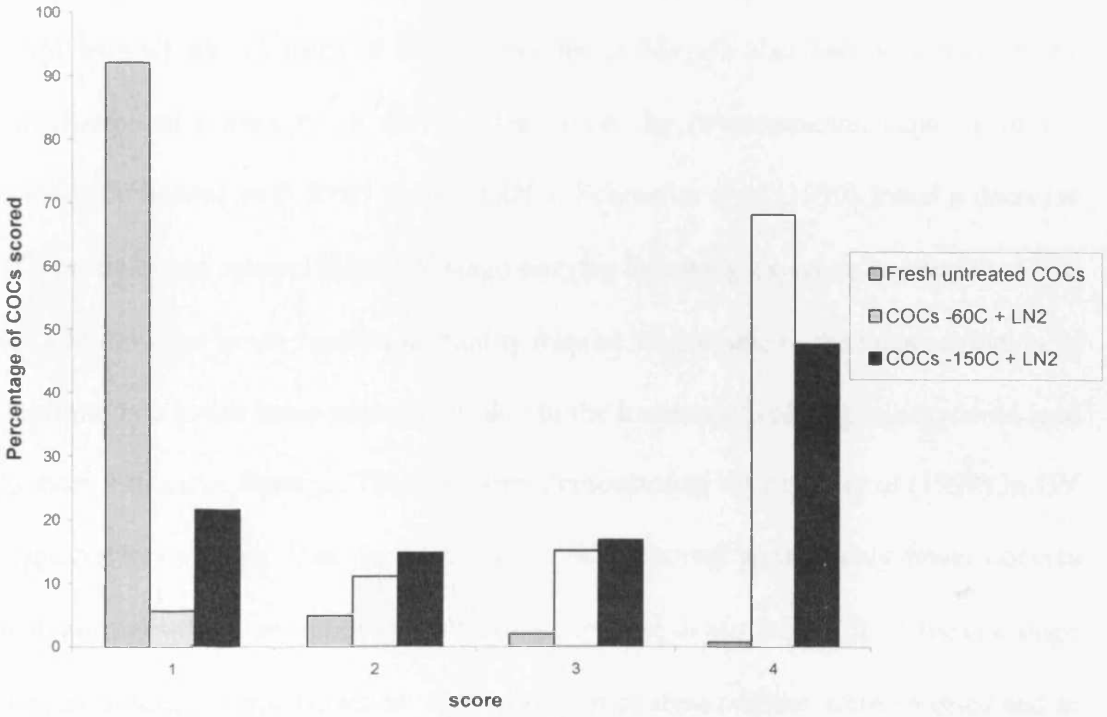


Figure 4.6 Distribution of scores assigned following membrane integrity staining of freshly collected COCs, COCs plunged into LN_2 following cooling to –60°C and COCs cooled to –60°C and then further cooled to –150°C before being plunged into LN_2.
Scoring system - Percentage of the surface of the oocyte in contact with cumulus cells with intact membranes. 100-76% =1, 75-51% =2, 50-26% =3, 25-0% =4.

exposure to LN_2 (149 COCs assessed) was 3, 47.7% were scored as 4. Also, a much larger proportion of the COCs cooled to –150°C were scored as 1 (21.5%) than COCs that were cooled to –60°C (5.6%).

4.4 DISCUSSION

4.4.1 Cryopreservation of GV Stage Murine Oocytes

This study aimed to determine at what stage of the cryopreservation process damage occurred and whether damage was inflicted on the oocyte or the cumulus cells or both. Following membrane integrity staining, the scores assigned to fresh control COCs and to COCs exposed to Me_2SO were similar. Thus no substantial disassociation of the oocyte and cumulus cells occurred as a result of exposure to 1.5M Me_2SO for 15 mins at 4°C. Exposure to Me_2SO also had no effect on the morphological normality of the oocytes or on the developmental capacity of the oocytes compared with fresh control COCs. Schroeder *et al* (1990) found a decrease in morphological normality of GV stage oocytes following exposure to Me_2SO at 0°C for 30 mins. The lower rates of normality may be attributable to the longer duration of exposure at a lower temperature that, due to the kinetics of chilling injury, could lead to more extensive damage. This has been demonstrated by Zeron *et al* (1999) in GV stage bovine oocytes. Van der Elst *et al* (1992) reported significantly fewer oocytes with normal spindle morphology following exposure to Me_2SO at 0°C at the GV stage than in control groups. However, no chromosomal abnormalities were reported and in all cases the chromosomes were located at the equatorial plane of the spindle.

Following cryopreservation, normality of oocytes was found to be highly variable. This parameter was assessed at 47hrs post-treatment and, therefore reflects the ability of the oocyte to survive the freeze/thaw process, to achieve maturation to metaphase

II and to survive in culture for this period. In contrast, high survival at 1-5hrs post-thaw has been reported (Schroeder *et al*, 1990; Van der Elst *et al,* 1992). However, fertilisation was significantly reduced compared with non-frozen controls. Candy *et al* (1994) on the other hand, reported 93% survival at 16hrs post-thaw with 83% maturation *in vitro* and 70% fertilisation. This high survival rate may be attributable to differences in the cooling profile compared with the current study. However, a more plausible explanation may be that survival was increased by the use of a rapid thawing protocol which may have been more successful than the controlled rate thawing protocol utilised in the current study. Despite the differences in survival between the two studies, in both studies oocytes that were morphologically normal following cryopreservation fertilised in similar proportions to controls.

Further culture of the embryos allowed assessment of blastocyst development, which was found to be poor. Developmental impairment of GV stage oocytes following cryopreservation has been demonstrated following *in vitro* post-fertilisation culture (Van der Elst *et al,* 1993). In this study, developmental competence was found to be low despite full nuclear maturation. This emphasises the importance of the completion of cytoplasmic maturation for the progression of embryonic development. In the study of Candy *et al* (1994) transfer of embryos derived from fresh and freeze-thawed GV stage oocytes was carried out at the two-cell stage. Similar proportions of embryos from both groups implanted, suggesting that no developmental impairment occurred as a result of freezing at the GV stage. However, all of the embryos were being re-absorbed at the time of examination, therefore none of these could have lead to the production of live offspring. The fact that the embryos were able to implant suggests that development progressed to the blastocyst stage. Although the failure to develop beyond this stage means that abnormal development cannot be ruled out.

Membrane integrity staining revealed extensive loss of plasma membrane integrity of the cumulus cells of thawed COCs. Thawed COCs, therefore, had significantly less direct contact with intact cumulus cells compared with fresh COCs and COCs exposed to Me_2SO without freezing. Previous studies have reported a loss of cumulus cells from the COC following cryopreservation and thawing (Cooper *et al*, 1998; Goud *et al*, 2000). In the present study, careful pipetting allowed retention of the majority of cumulus cells and assessment of the membrane integrity of COCs immediately post thawing. However, most of the cumulus cells of the thawed COCs became disassociated from the oocyte following a short period of culture. The reduced developmental capacity of the thawed oocytes could, therefore, be due to a loss of integrity and/or functionality of cumulus cells.

4.4.2 IVM of Cumulus Denuded and Co-Cultured Oocytes

Denuding of oocytes prior to *in vitro* maturation lead to a decrease in developmental potential. Loss of cumulus cells has previously been implicated in the poor development of embryos derived from denuded oocytes (Schroeder and Eppig, 1984). Association of the oocyte with somatic cumulus cells promotes competence to undergo fertilisation and pre-implantation development by supporting the completion of cytoplasmic maturation (Buccione *et al,* 1990). The mechanism by which this benefit is realised is unclear. It has been suggested that a paracrine factor secreted by the cumulus cells is, in part, responsible for the maturation of the oocyte (Byskov *et al,* 1995). Disassociated cumulus cells appear to produce a factor that acts in a positive capacity on denuded oocytes to stimulate meiotic resumption (Downs, 2001). Although the presence of a positive paracrine factor has been indicated, evidence fails

to support this as a primary mechanism for hormone-induced maturation in isolated mouse oocytes (Downs, 2001).

In the current study denuded oocytes that were matured *in vitro* in the presence of freshly removed cumulus cells showed no increase in fertilisation or developmental capacity compared with the denuded oocytes that were matured *in vitro* in isolation. This does not provide support for the existence of a secreted maturation factor despite the fact that the concentration of any paracrine factor would be expected to be higher in the present study, where oocytes were cultured in the presence of excess cumulus cells and where culture was performed in a lesser volume than that of Downs (2001). The failure in the completion of cytoplasmic maturation, as evidenced by a lack of improvement in fertilisation and development in the presence of disassociated cumulus cells, emphasises the importance of the conservation of the original association between the GV stage oocyte and the cumulus cells during maturation *in vitro*. A requirement for physical contact with the somatic cells would suggest that intercellular connections such as gap junctions are important at this stage. This therefore supports the view that paracrine factors are overridden by signals transmitted by gap junctions (Downs, 2001).

The addition of fresh cumulus cells to thawed COCs did not improve maturation or development of the oocytes. Therefore, as with denuded oocytes, no benefit of co-culture with fresh cumulus cells during *in vitro* maturation was established. This is evidence against the significance of a secretory maturational factor that is lacking in thawed COCs, but supports the proposal that the metabolic coupling which exists between the cumulus cells and the oocyte has a maturational role that is disrupted by the process of slow-cooling and thawing. This evidence supports the assertion that direct physical contact between the oocyte and cumulus cells, possibly via

intercellular junctions, is a requirement for the completion of cytoplasmic maturation and subsequent embryo development.

4.4.3 GV Stage COCs Slow Cooled to –60°C or Slow Cooled to –60°C and Plunged into LN_2

The stages of the cooling profile at which damage occurs were initially investigated by cooling COCs to –60°C with and without exposure to LN_2. There was not a significant decrease in normality or fertilisation of the oocytes when they were exposed to temperatures below –60°C compared with those held at –60°C. This indicates that plunging the COCs into LN_2 following slow cooling to –60°C causes no further damage with regard to ability to be fertilised. This is in agreement with previous studies that report good rates of survival and fertilisation using similar cryopreservation protocols that involved plunging in to LN_2 following slow cooling to –80°C and –40°C, respectively (Whittingham, 1977; Candy *et al*, 1994). However, in this study a significant decrease in developmental potential was detected with respect to exposure to temperatures below –60°C. This difference was mirrored in the results of the cumulus cell membrane integrity staining.

Cumulus cell damage and developmental impairment was most extensive after direct plunging into LN_2 from –60°C. The increase in damage to the cumulus cells that occurs on cooling below –60°C suggests that much of this damage is yet to occur during the controlled rate cooling to –60°C, but could occur during the plunging of the COCs into the LN_2. This damage could be a result of there being insufficient time for removal of intracellular water or loading of CPA within the cumulus cells, leading to increased lethal intracellular ice formation (Pegg and Diaper, 1983). This effect is exacerbated in clusters of cells (Levin *et al,* 1977). Rapid cooling from a relatively

high sub-zero temperature has been shown to reduce survival and fertilisation of mature murine oocytes. Following slow cooling to –36°C and plunging into LN_2 survival and fertilisation were 15% and 3% respectively, whereas following slow cooling to –80°C and plunging into LN_2 they were 72% and 42% respectively (Trounson and Kirby, 1989).

The cell-cell contacts that metabolically couple the cumulus cells and the oocyte provide numerous nucleation sites that could instigate ice formation at cellular junctions if the complex was to come into contact with ice that has formed in the extracellular space surrounding the COC. The extent to which this process occurs would be expected to increase the lower the temperature to which the cells were cooled. The formation of extracellular ice is an important consideration, particularly when attempting to cryopreserve tissues or clusters of cells such as the COC. Both the occurrence of extracellular ice and its precise location within a tissue can have a dramatic effect (Taylor and Pegg, 1983). Cooling to a lower temperature would increase the extent of ice formation thereby increasing the chances of cell deformation and damage. In addition, exposure of cells to temperatures below –60°C may increase damage per se.

Adding a second stage of cooling between –60°C and –150°C at –10°C/min. would have allowed extra time for the movement of water and CPA across cell membranes, thereby potentially reducing intracellular ice formation, and may also have altered the configuration of extracellular ice.

4.4.4 GV Stage COCs Slow Cooled to –60°C or –150°C Prior to Plunging into LN_2

Previous studies have reported good rates of survival and morphological normality of GV stage oocytes following such cooling to –150°C. In one study, GV stage

murine COCs cooled at 10°C/min to –150°C following slow cooling to at 0.3°C/min –70°C, had an overall rate of morphological normality of 81% (Cooper *et al*, 1998). In another study, GV stage human oocytes were slow cooled to –30°C before further cooling to –150°C at 50°C/min. Seventy three percent of these oocytes survived (Boiso *et al*, 2002). In the current study no significant differences in normality, fertilisation or blastocyst development were detected between COCs cooled to –60°C or –150°C before being plunged into LN_2. The proportion of blastocysts arising from the overall cohort of COCs (total blastocyst formation) was higher in the group cooled to –150°C. However, the difference between the two groups was not significant. Cumulus cell membrane damage was less extensive in the COCs that were subjected to the two-stage cooling protocol. These results suggest that there is some advantage to the inclusion of an additional cooling ramp to –150°C during cooling of GV stage murine COCs, particularly with respect to cumulus cell integrity.

4.4.5 Summary

The structure of the GV stage COC, in particular the tight packing of the cumulus cells and the intricate network of cell-cell contacts that are vital for the maturational function of the complex, make survival and development following cryopreservation by slow-cooling extremely problematic. The formation of intra-complex ice crystals may inflict damage that impedes the process of cytoplasmic maturation, which is vital for subsequent embryo development. While some progress has been made in improving the survival of cumulus cells, the modification of the protocol was insufficient to significantly impact subsequent embryo development. Vitrification and the avoidance of ice crystal formation may therefore prove to be a preferable method for the storage of GV stage COCs.

Chapter 5

VITRIFICATION OF GV STAGE MURINE COCS

5.1 INTRODUCTION

Slow-cooling of GV stage COCs has been shown to result in good levels of oocyte survival and fertilisation (section 4). However, subsequent embryo development was impeded, possibly due to a failure in the completion of maturation caused by damage inflicted on the COC by ice crystal formation. It was hypothesised that, by avoiding ice crystal formation through the use of vitrification, maturation of the oocyte would not be hindered and embryo development would be improved, compared with that observed following slow-cooling of the COCs.

In general, the vitrification of mature murine oocytes has resulted in good rates of survival and subsequent fertilisation (Nakagata, 1989; Wood *et al,* 1991; Wood *et al,* 1993). Similar results were reported following vitrification of immature murine oocytes (Van Blerkom and Davis, 1994). Evidence of morphological and developmental abnormalities in both mature (Kola *et al,* 1988; Shaw *et al,* 1990) and immature (Van Blerkom and Davis, 1994) oocytes has been reported. Despite this between 40-51% of vitrified mature murine oocytes were capable of pre-implantation development, many of these studies also reported the development of normal young and live births (Kono *et al,* 1991; Nakagata, 1989; Wood *et al,* 1991; Wood *et al,* 1993).

Vitrification has also been used to cryopreserve human oocytes. A live birth was reported following transfer of three embryos derived from mature human oocytes that were vitrified using EG and sucrose (Kuleshova *et al*, 1999). A more recent study also using EG and sucrose but with a larger sample size, reported the transfer of 125

embryos arising from vitrified oocytes to 28 patients. Six of the recipients achieved clinical pregnancies all of which resulted in the delivery of healthy offspring (Yoon *et al*, 2003). Embryo development has also been reported following vitrification, again using EG and sucrose, of immature human oocytes obtained from the antral follicles of patients with chocolate ovarian cysts. High levels of progression to the two-cell stage were reported (71%) and a pregnancy was established (Wu *et al*, 2001).

In the current study Me_2SO was used as the CPA. However, for vitrification a higher concentration is required (6M) than that required during slow-cooling (1.5M). The high concentration of CPA was used in conjunction with the macromolecule PEG. The constituents of the solution were arrived at as a result of work carried out in order to elucidate a combination of CPAs and macromolecules for the vitrification of mature murine oocytes. The solution has been used successfully in the preservation of mature murine oocytes (O'Neil *et al*, 1997). As with the original study using mature oocytes, COCs were briefly exposed to the CPA at room temperature. The solution was modified to contain dcAMP, to prevent resumption of meiosis in immature/GV stage COCs. A further modification to the protocol involved the use of droplets of a greater volume (0.5ml as opposed to 50µl) to minimise dilution of the droplets during transfer of the COCs, which are larger than denuded mature oocytes, from one droplet to another (section 2.3.3).

5.2 MATERIALS AND METHODS

GV stage COCs collected as in section 2.2.7, were treated with vitrification solution as described in section 2.3.3. Following treatment with 6M Me_2SO, half of the COCs were vitrified as described, while the CPA was diluted immediately from the remaining COCs (as described in section 2.3.3). The vitrified COCs were warmed in a 25°C water bath prior to dilution of the CPA by a method identical to that used

for the COCs exposed to CPA without cryopreservation. After removal of the CPA the COCs were either assessed for membrane integrity (as described in section 2.4) or normality, fertilisability and developmental potential were assessed following maturation and *in vitro* fertilisation (section 2.5). Freshly collected COCs were stained as a control group for membrane integrity and freshly collected *in vitro* matured COCs served as controls for IVF. I*n vitro* matured COCs that had not undergone treatment were simultaneously assessed as controls.

5.3 RESULTS

5.3.1 GV Stage COCs Treated With 6M Me_2SO Without Cryopreservation and COCs Vitrified in 6M Me_2SO

5.3.1.1 Assessment of development

Morphological normality, fertilisation and blastocyst development were significantly reduced following vitrification when compared with both untreated controls and oocytes treated with vitrification solution without cryopreservation (Table 5.1). However, normality and fertilisation were similar to controls in COCs that were treated with the vitrification solution without cryopreservation. In the latter group blastocyst development of the COCs that had been fertilised was less than that of controls and similar to the level observed in vitrified COCs (Table 5.1). However, when blastocyst development was calculated as a percentage of the original pool of COCs, the CPA treated COCs showed significant developmental impairment compared with the untreated controls. The development of the CPA treated group was significantly better than that of the vitrified COCs.

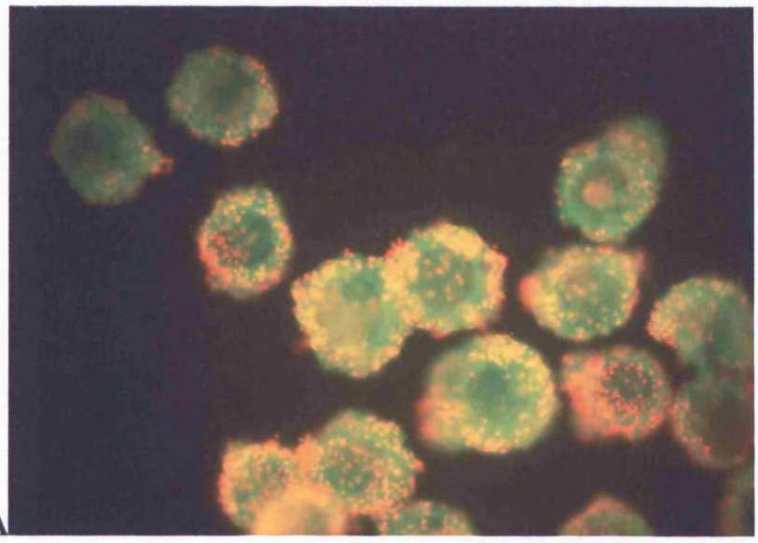
Table 5.1 Fertilisation and development of GV stage murine COCs exposed to a 6M Me_2SO vitrification solution without cooling and GV stage murine COCs exposed to a 6M Me_2SO vitrification solution and vitrified, compared with *in vitro* matured controls.

Protocol	No. of oocytes	Median % (range)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
In vitro matured (n=5)	117	95.3[a] (90.9-100)	80.0[c] (73.2-95.8)	60.9[ef] (46.7-81.0)	53.3[g] (32.6-73.9)
6M Me_2SO + *in vitro* matured (n=5)	97	94.1[b] (92.6-100)	71.4[d] (36.0-93.8)	35.8[e] (22.2-55.6)	23.5[g] (7.4-31.3)
Vitrified + *in vitro* matured (n=5)	106	71.4[ab] (47.6-90.9)	18.2[cd] (10.0-37.5)	0[f] (0.0-50.0)	0[g] (0.0-4.5)

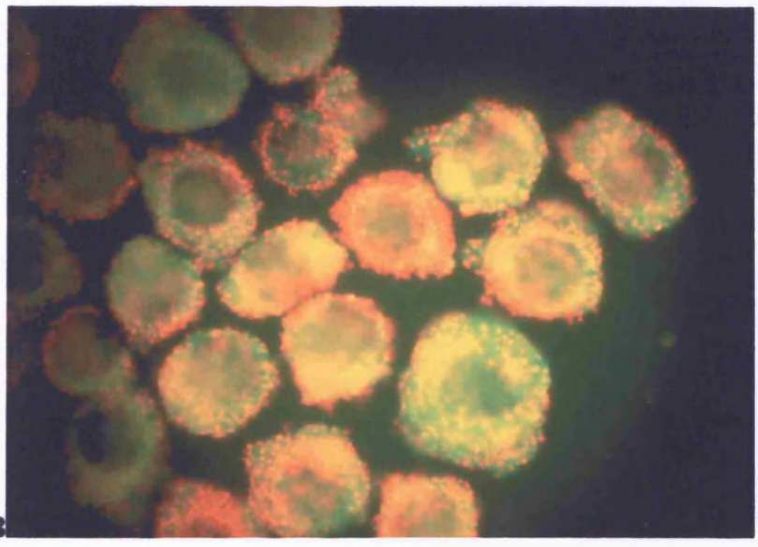
a, b, c, d, e, f, and g - values within columns with the same superscript are significantly different $p<0.05$.
n= number of repeats of experiment
Normality was assessed 24h after insemination and calculated as a percentage of the total number of oocytes. Fertilisation was assessed 24h after insemination and calculated as the number of 2-cell embryos formed from normal oocytes. Blastocyst development was assessed 120h after insemination and is expressed both per fertilised 2-cell embryo and per oocyte (total blastocyst).

5.3.1.2 Cumulus cell membrane integrity staining

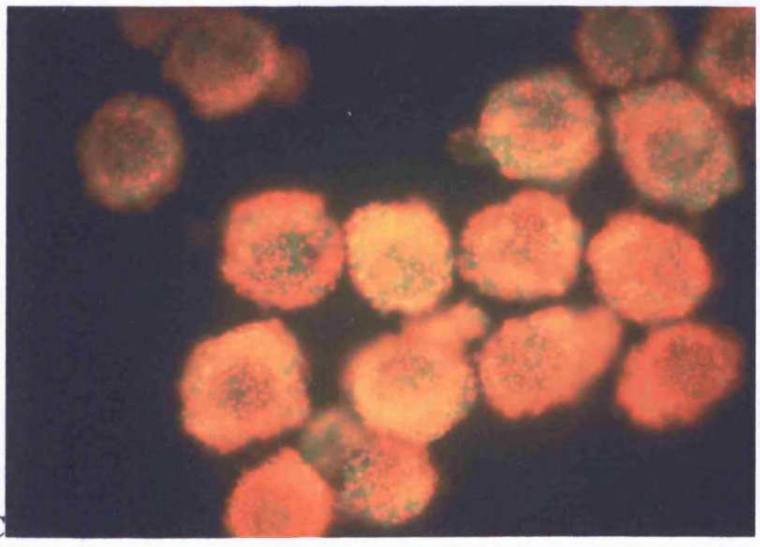
Staining of fresh COCs, COCs exposed to vitrification solution without cooling and vitrified COCs is shown in figure 5.1. The majority of the fresh COCs (96 COCs assessed) showed a high proportion of cumulus cells with intact membranes (figure 5.1A) and achieved a median score of 1 (see figure 5.2). Following exposure to vitrification solution without cooling (73 COCs assessed), the percentage of the surface of the oocyte covered with cumulus cells that had intact membranes was more variable (figure 5.1B). The scores of COCs from this group were spread across the four staining categories (figure 5.2), with a median score of 2. Few of the cumulus cells of the vitrified COCs (104 COCs assessed) retained an intact plasma membrane (figure 5.1C) these COCs had a median score of 4 (figure 5.2).



A



B



C

Figure 5.1 Fluorescent micrographs depicting carboxy fluorescein/propidium iodide membrane integrity staining of: (**A**) Freshly-collected intact untreated GV stage COCs: (**B**) GV stage COCs exposed to vitrification solution at room temperature; and (**C**) vitrified COCs. Magnification 200x.

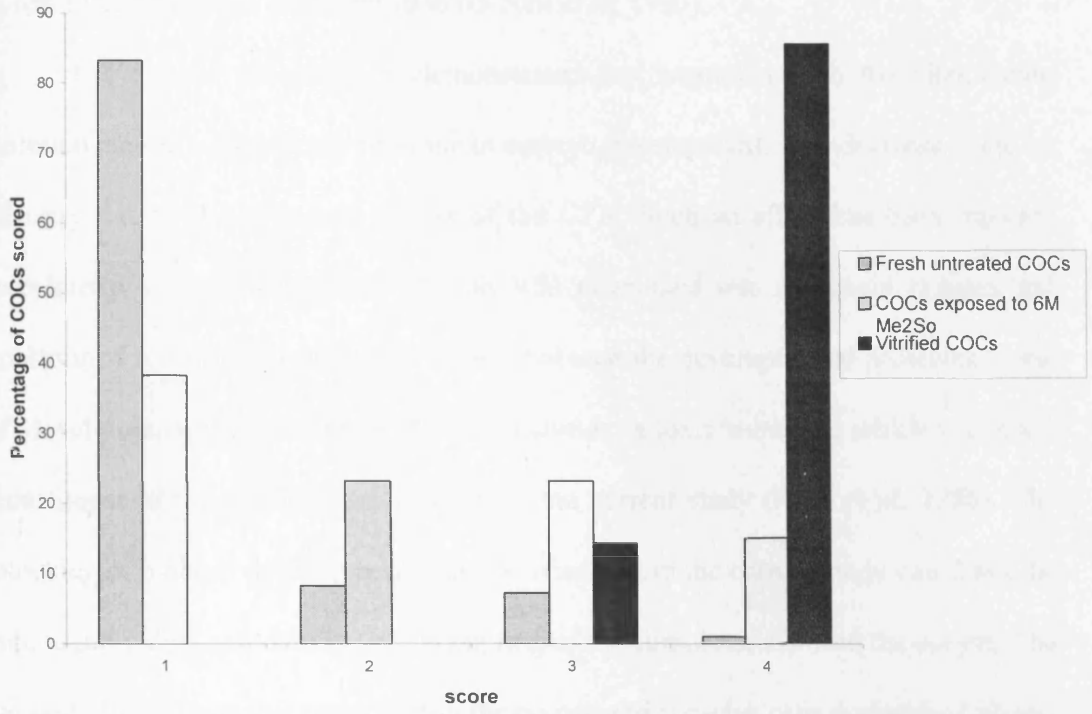


Figure 5.2 Distribution of scores assigned following membrane integrity staining of freshly collected COCs, COCs treated with 6M Me_2SO and vitrified COCs.
Scoring system - Percentage of the surface of the oocyte in contact with cumulus cells with intact membranes. 100-76% =1, 75-51% =2, 50-26% =3, 25-0% =4.

5.4 DISCUSSION

5.4.1 GV Stage COCs Treated With 6M Me_2SO Without Cryopreservation

The results indicate that treatment with the Me_2SO based vitrification solution without cooling, does not lead to significant decreases in morphological normality and fertilising capacity. Exposure to high concentrations of cryoprotectant can cause damage to cells in the absence of the additional stresses of cooling. It has previously been reported that exposure to glycerol can cause swelling and degeneration of oocytes (Wood *et al*, 1991). However, in concurrence with the current study it was found that treatment of mature murine oocytes with 6M Me_2SO led to good levels of both normality (65%) and cleavage to the two-cell stage (72%), (Wood *et al*, 1991). An ensuing study, which used an identical vitrification solution to that used in the

current study, reported improved levels of normality (94%) and fertilisation (91%) when PEG was added to the solution (O'Neil *et al*, 1997).

The current investigation demonstrated that treatment with the vitrification solution caused a significant decrease in embryo development. This decrease could be directly due to the cytotoxic effects of the CPA. Such an effect has been reported previously; where oocytes treated with VS1 developed into aneuploid zygotes and malformed fetuses. However, this study attributed the developmental problems to the VS1 solution, which contained 15.5% acetamide, a toxic substance which was not a constituent of the vitrification solution in the current study (Kola *et al*, 1988). The blocking of embryo development that was observed in the current study could also be due to individual cell damage or uncoupling of the cumulus cells from the oocyte. The volume fluctuations that occur within the oocyte and cumulus cells during loading and dilution of the CPA could cause the metabolic coupling to be compromised, potentially leading to a failure in the completion of cytoplasmic maturation and/or subsequently poor embryo development. Similar disruptions were reported following treatment with VS1, where membrane blebbing was caused by disruption of cytoskeletal microfilaments caused by volume changes during exposure to the solution (Shaw *et al*, 1990). In the current investigation this theory is supported by the cumulus cell membrane integrity staining results, where extensive cumulus cell membrane damage was not evident and the majority of the cumulus cells remained within the structure of the COC and did not become disassociated from the oocytes. Presumably while the majority of the cells remained intact, the coupling between the cells was compromised.

5.4.2 Vitrification of GV Stage COCs

Vitrification was found to cause significant decreases in all of the parameters investigated, when compared with non-treated controls. Although morphological normality was significantly reduced by vitrification, the proportion of normal oocytes was similar to that reported in other studies that employed Me_2SO as a primary constituent of the vitrification solution (Wood *et al,* 1991; Wood *et al,* 1993; Van Blerkom and Davis, 1994; O'Neil *et al*, 1997). In contrast to the findings of other studies, only a small number of the vitrified oocytes were fertilised and very few developed to the blastocyst stage. For example one study, in which denuded mature oocytes were vitrified, reported that 79% of two-cell stage embryos implanted and in 40% of cases normal fetal development occurred (Wood *et al,* 1993). This discrepancy could be due to the immaturity of the oocytes in the current study and/or the presence of cumulus cells.

An alternative explanation for the poor development of the vitrified oocytes could be devitrification of the sample. During the current investigation the small volume of vitrification solution that contained the oocytes became opaque on warming. Devitrification was reported during the vitrification and warming of mature murine oocytes and was found to lead to poor survival (Shaw *et al*, 1991; Shaw *et al*, 1992). Devitrification is characterised by the formation of small ice crystals during warming. The putative ice crystals could disrupt the structure of the COC, causing damage to the membranes and disassociation of the cumulus cells. Although cumulus cell membrane damage is indicated by the membrane integrity staining images, substantial disassociation of the cumulus cells did not occur. Thus unlike during slow-cooling, the extent of ice crystal formation during devitrification was insufficient to cause cumulus cell disassociation. However, despite this, the developmental

capability of vitrified GV stage oocytes was inferior to that of slow cooled GV stage oocytes.

Chapter 6

DISCUSSION

The current study consisted of two parts; the initial investigation was focused on the improvement of an existing protocol for *in vitro* maturation of murine GV stage COCs. While the second phase of the study involved examination of cumulus cell and oocyte survival following cryopreservation. Cell survival within the COC was determined using membrane integrity staining following cryopreservation. The IVM protocol developed in part one was applied to thawed COCs, whose overall level of functionality was tested by the assessment of embryo development *in vitro* following IVF.

6.1 *IN VITRO* MATURATION OF GV STAGE MURINE COCS

6.1.1 Importance of Culture Medium

Changes made to the hormonal content of the IVM media had a negligible effect on the normality, fertilisation and subsequent development of the oocytes. This was attributed to the high concentrations of gonadotrophin used, which in the current study were far in excess of those that would be found *in vivo*. However, these concentrations may have been reduced by leaching of gonadotrophin from the medium during culture. A recent study, which examined the effect of mineral oil overlay on the maturation and development of porcine oocytes, found that the concentration of steroid hormones in media that had been overlaid with mineral oil was lower than in identical media that was not overlaid. In addition to this, the rate of development to the blastocyst stage was reported to be higher in the oocytes that had been matured in media that was not overlaid (Shimada *et al*, 2002). It is possible that

this was due to a failure in the completion of cytoplasmic maturation due to a shortfall in the supply of hormones required for maturation. It is possible that leaching of hormone from the IVM media could have contributed to the inconsistent results in the current study as the mineral oil used was pre-equilibrated with media which did not contain hormone. In future this could be minimised by pre-equilibrating the mineral oil with the same media as that to be used for IVM.

6.1.2 Importance of Co-culture

Co-culture of COCs with additional follicle cells from throughout the ovary was found to have an inhibitory effect on the subsequent development of the oocytes. The process of spacing of dominant follicles in the ovary during oocyte growth *in vivo* could explain this phenomenon. It has been demonstrated that when follicles are in contact with each other during growth, one follicle will degenerate while the other will excel. It has been suggested that this is due to a paracrine action, possibly mediated by follicular cells (Gougeon and Chainy 1987; Nayudu *et al,* 1992). A similar paracrine interaction could have occurred in the current study leading to partial degeneration of the oocytes, or perhaps more likely, failure to achieve cytoplasmic maturation during the final stages of maturation. In a separate study where pairs of mouse follicles were cultured *in vitro,* follicle dominance only occurred when the follicles were in contact with each other. When the follicles were cultured in shared medium, dominance did not occur (Spears *et al,* 1996). While this is evidence against a paracrine factor, it could nonetheless explain the inhibition observed in the current study as during culture the COCs were in direct contact with the follicle cell monolayer.

6.1.3 Significance of Mechanical Disruption

Partial disruption of the COCs caused by transfer by pipette during IVM did not cause a significant decrease in any of the parameters investigated. While it is possible that the disruption was insufficient to impact on the maturation of the oocytes, it could also be an indicator of a paracrine factor capable of promoting maturation of the oocytes without direct contact via cellular junctions. It has been reported previously that for maturation to be completed the original cellular contacts must be maintained (Eppig, 1979). This view was supported by more recent investigations, which demonstrated that direct contact between the cells within the intact COC is required for the stimulation of cumulus cells to produce a meiosis-inducing factor, which overcomes inhibition and induces maturation of the oocyte. However, although conservation of the interaction is required for the initial production of the factor, once released it is able to act on the oocyte via a paracrine mechanism (Guoliang *et al*, 1994). In the current study the disruption caused may have had a minimal effect on maturation due to the increased dependence on the paracrine aspect of maturation induction. The alteration of the protocol from transfer of the COCs into the media to, replacement of the media without disruption of the COCs, may have not caused a significant increase in cytoplasmic maturation in this case. However, COCs that have been treated with CPA or cryopreserved and thawed may be more prone to dissociation of the cumulus cells. Therefore, during IVM of such COCs the alteration in IVM protocol may benefit maturation by preventing premature loss of cellular contact.

6.2 CRYOPRESERVATION OF GV STAGE MURINE COCS

6.2.1 Assessment of Damage to Cumulus Cells and Oocyte

In the current study slow-cooling and thawing of GV stage COCs has been shown to cause a loss of membrane integrity of cumulus cells and a corresponding decrease in the developmental potential of resultant embryos. However, the cause of this decrease is unclear. A possible explanation may be that the loss of membrane integrity of the cumulus cells (as a consequence of ice crystal formation) leads to cell necrosis and an inability to perform designated functions during maturation. Indeed it has been reported that during cryopreservation and thawing, cumulus cells sustain damage to their DNA (Lindley *et al*, 2001). The extent of such damage may be greater than that revealed by the membrane integrity assay used in the present study, as demonstrated by a study in which the effects of co-culture of bovine embryos with freeze/thawed cumulus cells were examined. Although the freeze/thawed cells were able to promote embryo development in a similar manner to fresh cumulus cells, the proportion of cells that were judged to have intact membranes, through Trypan blue dye exclusion (80-90%), was greater than the proportion that were found to be capable of attaching to the surface of culture dishes to form a confluent monolayer (60-80%), (Broussard *et al*, 1994). This indicates that damage that occurs during freezing and thawing, other than membrane damage could compromise the functionality of the cumulus cells. Alternatively, cells that sustain damage during cryopreservation (including membrane damage) may be capable of a certain degree of cellular repair following thawing and a period in culture. Even if cells regain membrane integrity their functionality could remain compromised. Despite this possibility the current investigation has shown that in the case of cryopreserved COCs, membrane integrity of cumulus cells is a good predictor of embryo

development, and indeed this implies that membrane integrity is indicative of normal cell function.

6.2.2 Importance of Retention of Intercellular Contact Within the COC

The loss of oocyte-cumulus cell association following cryopreservation has been reported in mouse (Cooper *et al*, 1998) and human oocytes (Goud *et al*, 2000). Studies have demonstrated that the completion of cytoplasmic maturation is dependent on the integrity of the gap junction associations between the oocyte and its surrounding cumulus cells (Moor *et al,* 1998). Experiments conducted in this study to examine the effects of co-culture of fresh cumulus cells with denuded oocytes or freeze-thawed COCs failed to provide any support for the existence of a secreted maturation factor. This finding is in agreement with others who have compared IVM of untreated oocytes with and without cumulus cell co-culture and found a lower rate of progression to MII stage following co-culture of the oocytes (Häberle *et al*, 1999). The importance of the conservation of the original cell-cell contacts of the COC in the metabolic coupling of the cumulus cells and oocyte was emphasised in a previous series of studies (Cross and Brinster, 1974; Wassarman and Letourneau, 1976; Eppig, 1979). It was demonstrated that fully grown oocytes incorporate more radio labelled uridine and leucine when the cumulus cells remained attached to the oocyte than when the two cell types were unattached but cultured in the same dish. It may be postulated that the culture of immature oocytes upon cumulus cell monolayers is ineffective due to the destruction of the cumulus cell complex. This point was later reiterated by Eppig (1994) who pointed out that the development of cumulus cells does not occur in isolation or in two dimensions (as in cultured monolayers) rather, the original three-dimensional structure of the complex must be maintained for maturation of the oocyte to progress. In the study by Häberle *et al*, (1999) which

found no advantage in the co-culture of cumulus cells with GV stage human oocytes, the cumulus cells were disaggregated and suspended in culture media prior to co-culture. The loss of the original cell linkages could explain the lack of maturational support provided by this system of co-culture. However, the use of co-culture during the development of bovine and human embryos has been shown to result in increased levels of development to blastocyst (Broussard *et al*, 1994; Hwu *et al*, 1998).

6.2.2.1 Importance of cumulus cell attachment during cryopreservation

It has previously been demonstrated that removal of cumulus cells prior to cryopreservation does not affect post-thaw survival rates of mature mouse or hamster oocytes (Whittingham, 1977; Mandelbaum *et al*, 1988). However, more recently a number of studies have highlighted the advantage of cryopreserving oocytes with their associated cumulus cells. A study by Pellicer *et al*, (1988) suggested that attachment of cumulus cells during cryopreservation is beneficial during freezing of immature rat COCs. The COCs were cryopreserved with different amounts of associated cumulus cells. Post-thaw survival rates increased significantly with increasing layers of cumulus cells. This finding was perplexing, as pointed out by Trounson and Kirby (1989). Fertilised oocytes and embryos have far superior survival rates following cryopreservation and thawing, when compared with that of unfertilised oocytes. Fertilised oocytes and embryos are also not surrounded by cumulus cells (as is the case with unfertilised oocytes). It was therefore proposed that the removal of the cumulus cells from MII stage mouse oocytes might increase rates of survival of the unfertilised oocytes by improving the passage of water and cryoprotectant across the oocyte membrane. However, removal of cumulus cells prior to cryopreservation caused a reduction in survival. When denuded oocytes and intact COCs were treated with Me_2SO at 0°C and cooled to –80°C prior to being plunged

into liquid nitrogen, 72% of intact COCs survived while only 5% of the denuded oocytes survived. Development beyond the two-cell stage was only assessed in the intact COC group where 55% of two-cell embryos transferred to recipients implanted and 30% went on to develop into normal fetuses (Trounson and Kirby, 1989). Similar results were reported following the removal of cumulus cells from MII stage human oocytes (Imoedemhe and Sigue, 1992; Fabbri *et al*, 1998). The benefit afforded by the presence of the cumulus cells was attributed to a putative effect whereby the cumulus cells provide protection to the oocyte by reducing the effects of sudden osmotic stresses inflicted by CPA equilibration and removal (Imoedemhe and Sigue, 1992). The reduction in cytotoxicity of Me_2SO caused by the presence of cumulus cells has previously been indicated (Johnson and Pickering, 1987). It was suggested that the cumulus cells might also provide protection during cryopreservation by a mechanism not yet understood (Imoedemhe and Sigue, 1992). The dense packing of the cumulus cells in immature COCs (which may prevent adequate dehydration) and the extensive network of cell-cell junctions could provide many possible nucleation sites for ice formation. It is possible that a greater proportion of ice would form at these sites earlier during slow-cooling than within the oocyte, allowing adequate dehydration of the oocyte and reducing the incidence of ice formation within it. The cumulus complex could therefore act as a sacrificial site within which ice may form preferentially thus protecting the oocyte from much of the damage caused by intracellular ice formation. Ice formation within the complex is of no consequence when freezing MII stage oocytes as the maturational role of the cells has already been fulfilled. However, in the case of GV stage oocytes the conservation of the original cell-cell contacts and three-dimensional architecture of the complex is of vital importance if the oocyte is to be matured *in vitro* post- thaw.

Interestingly, when coupling between the oocyte and cumulus cells was compared in fresh and frozen GV stage oocytes only a borderline significant difference in coupling index (fresh 16.7 ± 2.0 and frozen 10.6 ± 1.4) GV stage oocytes was detected. However, coupling in frozen/thawed oocytes was higher than that found in the positive control oocytes (1.3 ± 0.5) whose coupling was inhibited by the addition of retinoic acid. This suggests that although cell-cell coupling may facilitate ice formation within the cumulus complex, some coupling does remain in the rat COC following cryopreservation and thawing (Pellicer *et al,* 1988). Whether this is also true of human and mouse COCs is yet to be proven. However, similarities in the structure and cellular arrangement of the COCs suggest that this may be the case.

6.2.3 Nature of Damage Following Slow-Cooling of GV Stage Murine COCs

The poor development of embryos arising from slow cooled and thawed COCs may be attributable to a severing of cell-cell connections caused by the osmotic stress and physical disruption that occurs during the addition and dilution of CPA. However, this is unlikely to be the cause of the developmental impairment in this study, as it was not found to occur in COCs that were treated with CPA without freezing.

In an effort to determine the nature of the damage caused by slow cooling, experiments were conducted to examine the damage caused by the different stages of cooling. It was found that during the slow-cooling protocol much of the damage inflicted on the cumulus cells occurred during plunging of the COCs from –60°C to –196°C and/or warming from this temperature. The addition of a cooling ramp from –60°C to –150°C at a rate of 10°C/min, while not leading to an improvement in blastocyst development, did improve cumulus cell survival. It is possible that while the slower rate of cooling allowed increased dehydration of the cumulus cells, the

inevitable ice formation could have destroyed the cell-cell connections thus preventing the completion of maturation post-thaw. It has been ascertained that slow cooling at a rate of 0.5°C/min to –80°C (followed by slow warming), rather than cooling to –36°C (followed by rapid warming) allows sufficient dehydration prior to plunging into liquid nitrogen and rehydration on warming to significantly improve survival of MII stage mouse oocytes (Trounson and Kirby, 1989). Subsequently, controlled rate cooling to temperatures lower than –80°C was shown to be beneficial. In one study, cooling of human COCs at -0.3°C/min to -35°C then cooling at -10°C/min to -135°C resulted in levels of survival (54.5%), progression to the two-cell stage (75%) and development to blastocyst (50%), that were comparable with the current study (Imoedemhe and Sigue, 1992). However, the oocytes were cryopreserved at MII stage; therefore the maturational functionality of the associated cumulus cells was of no consequence. A subsequent study seemed to contradict these findings. Carroll *et al* (1993) carried out experiments to examine the effects of adding different macromolecules to cryopreservation solutions during slow cooling. Cooling at ~0.5°C/min to -80°C before plunging into liquid nitrogen, was compared with cooling at 0.3°C/min to -40°C and then at -10°C/min to -150°C before plunging. In the presence of FCS, the number of implantation sites created following transfer of embryos derived from frozen thawed oocytes were found to be 81% when the oocytes were cooled to -80°C before plunging, compared with 60% following the two step cooling protocol. However, these results may be explained by the different ways in which the oocytes were treated prior to cryopreservation. Intact COCs were cooled to -80°C before plunging whereas denuded oocytes were cooled using the two-step protocol. Although again in this case the oocytes were mature and therefore no maturational benefit could be gleaned from conservation of the cumulus cells, it may

be that their presence offers some physical benefit during cryopreservation. In another more recent study GV stage human COCs that were cooled at -0.3°C/min to -30°C followed by cooling at -50°C/min to –150°C were found to mature at similar rates to untreated controls. However, this only represents an ability to resume meiosis due to the completion of nuclear maturation (Boiso *et al*, 2002). Differences between these groups may have been apparent if progression to blastocyst, and thus cytoplasmic maturation, had been investigated. While increasing the extent of dehydration may allow increased survival of both the oocyte and cumulus cells, disruption to the structure of the COC remains an obstacle to improved embryo development.

The extensive network of junctions present in the COC could contribute to the damage inflicted on the cumulus cells during cryopreservation. It has previously been demonstrated that intercellular junctions within tissues are susceptible to the toxic and osmotic effects that are associated with CPA dilution (Armitage *et al*, 1995). However, this type of damage was not apparent in the current study, as developmental capability was not affected by exposure to Me_2SO without cooling. In another study, evidence has been reported for the conduction of ice formation between neighbouring cells via cell-cell contacts. Chinese hamster fibroblasts were cultured as multicellular spheroids in order to provide a 3D model for the behaviour of multicellular junction-linked tissues during cryopreservation. It was observed that often, after one cell froze intracellular ice would form in an adjacent cell. These would eventually form clusters of frozen cells dispersed throughout the colony. It was proposed that, using this mechanism, the cell-cell contacts facilitated intracellular ice nucleation (Acker *et al*, 1999). In some cases a similar pattern of cumulus cell damage was identified amongst the cryopreserved COCs in the current study, although in most cases the damage was extensive and patterns of formation of intracellular ice were impossible to postulate.

The low rates of development observed in the current study indicate that insufficient coupling remains following slow cooling and thawing for cytoplasmic maturation to be completed. The presence of two very different cell types (oocyte and cumulus cells) and the complicated nature of their vital intercellular interactions may mean that extracellular ice formation cannot be tolerated. Hence vitrification may be the best recourse.

6.2.4 Nature of Damage Following Vitrification of GV Stage Murine COCs

Unlike exposure to the cryopreservation solution used for slow cooling, exposure to the vitrification solution was found to cause a greater degree of damage to the cumulus cells and a lower rate of embryo development when compared with that observed in untreated COCs. Although devitrification was observed on warming of the vitrified samples, disruption to the structure of the COC was less extensive than that caused by slow cooling. It is therefore possible that modification of the vitrification solution, to reduce toxicity and the incidence of devitrification, may lead to an improvement in these parameters.

Despite the reduction of ice crystal formation during cryopreservation and thawing of the COCs, both survival and the ensuing development of the oocytes was inferior to that achieved following slow cooling. This finding is in contrast to the observations of other researchers who report high rates of the same following vitrification of MII stage oocytes (Nakagata, 1989; Wood *et al,* 1991; Wood *et al,* 1993; O'Neil *et al*, 1997). However, despite high rates of survival and fertilisation following vitrification, Wood *et al* (1993) reported high post-implantation loss which suggests a failure in embryo development. One reason for inferior survival post vitrification in the current study could be the immaturity of the oocytes used.

Transmission electron microscopy has revealed a number of cellular abnormalities in GV stage horse oocytes following exposure to vitrification solution and vitrification. For example, mitochondrial swelling which was associated with reduced matrix density of the mitochondria. In addition to this, numerous vacuoles were present in the periphery of the ooplasm. The formation of the vacuoles was attributed to the destruction of gap junctions caused by the drastic contraction of the ooplasm that occurs in response to osmotic pressures. The destruction of the gap junctions was found to be detrimental to the maturation process. While most of these effects were observed after exposure to the vitrification solution without cooling, in general the effects were more exaggerated after vitrification. For example, the destruction of gap junctions was three times greater following vitrification (Hochi *et al*, 1996). While the uncoupling of the cumulus cells may eventually lead to suppression of the developmental potential of the oocytes the additional effects on mitochondria that were reported may adversely influence the metabolism of the oocyte and could ultimately lead to numerous problems during maturation, fertilisation and cell division.

Severe structural disruptions to GV stage oocytes have been observed following vitrification of GV stage mouse oocytes. However, despite these, high rates of resumption of meiosis and completion of nuclear and cytoplasmic maturation (90%) were reported. Both chromosomal and cytoplasmic perturbations were observed immediately following thawing, such as premature chromosomal condensation and mixing of nuclear and cytoplasmic components prior to germinal vesicle breakdown. Externalisation of chromatin fragments into the cytoplasm was also reported; such events could lead to deletion defects that could have a deleterious impact on subsequent development. Interestingly, most of these disruptions were

found to return to normal after a period of culture (Van Blerkom, 1989). It was suggested that this short period of culture allowed the re-ordering of cellular components, and a return to a similar structure to that which existed before vitrification. In the current study the vitrified and thawed COCs were held at 37°C in culture media for a total of only 15 minutes. This period may be insufficient to allow re-organisation of the cellular components that could have been perturbed during vitrification and warming. The premature exposure of the oocytes to gonadotrophin may have been particularly futile as the gap junctions required to convey maturational stimuli might have been irreversibly compromised.

6.3 EXPERIMENTAL DESIGN

6.3.1 Variation in Outcome

In the current study the variation in outcome between replicates within each experiment and variation between control groups may be attributable to a number of different factors. Biological variation (i.e. genetic and environmental) was minimised by the use of an F1 generation of mice which were kept under controlled conditions with consistent feed, light/dark exposure and temperature. Technical and/or systemic variations were minimised in order to prevent experimental variables. However, despite these provisions variation did occur. Variation between *in vitro* matured groups may be due to the storage of the maturation medium prior to use (section 2.2.2). The medium was stored at -20°C following addition of the GTs, this form of storage may have had a detrimental effect on the bioactivity of the GTs or on the medium itself.

Slight variation was also evident in the *in vivo* matured groups. However, this was to a lesser extent and may be attributable to different batches of serum which was added as a source of protein in the medium. Serum is poorly defined and its

constituents are subject to disparity between batches. Additionally, serum has been shown to contain factors which inhibit the function of gap junctions (Salha *et al*, 1998).

In the current study paired groups were used to control for differences between different replicates. More consistent results may have been achieved if fewer replicates with more oocytes in each group were used. However, this design would have been subject to alternative experimental variables and may have been less significant.

6.3.2 Limitations of Experimental Endpoints

In the current study one of the main endpoints used to assess the success of the various cryopreservation and IVM methods was the development of blastocysts following IVF and culture of the treated oocytes. Although development to the blastocyst stage is a good indicator of the completion of cytoplasmic maturation, the failure of many of the cryopreserved oocytes to reach this stage demonstrates that a more sensitive endpoint may be required. For example assessment of the different pre-blastocyst stages reached by the embryos before development arrested. In addition to this, the blastocysts which are formed could be assessed by blastocyst spread and determination of TCN (total cell number) which is achieved by fixing and spreading of the blastocysts with cold methanoliacetic acid followed by nuclei staining (Archer *et al*, 2003). In addition to this, successful development could be confirmed by embryo transfer and examination of implantation sites, or production of live offspring.

The second endpoint used to assess the extent of cumulus cell damage was membrane integrity staining. Although there was found to be positive correlation between the extent of cumulus cell membrane damage and failure to complete cytoplasmic maturation (as demonstrated by blastocyst development), there are

certain limitations to the sole use of membrane integrity staining to assess cell viability. It has been shown that there are differences between different fluorescent probes in their ability to identify non-viable cells (Pintado *et al*, 2000). It would therefore be prudent to use more than one variety of cell staining combined with a cell viability assay such as the ability of the cells to attach to a surface or the ability to metabolise substrates. The use of more than one method may also avoid false positive results which may occur due to experimental artefacts (Ciapetti *et al*, 1998). In addition to this it has been shown that some methods are flawed in that they over estimate the numbers of non-viable cells (Tamuli and Watson, 1994). Although, in the current study the use of propidium iodide as a counter stain should have improved the accuracy of the viability assessment.

6.4 FUTURE WORK

Further investigation into IVM of GV stage COCs could include modification to the maturation medium. For example the addition of insulin or insulin-like growth factors which have been shown to enhance the response of ovarian cells to gonadotrophins (Guidice, 1992). When combined with FSH, insulin has also been shown to maintain gap junctions in porcine granulosa cells (Amsterdam *et al*, 1988). It may also be of interest to investigate the effects of the use of alternative energy substrates. A recent study reported increased rates of maturation *in vitro* to the MII stage following culture in medium containing both pyruvate and glucose, as opposed to medium containing only one of the substrates (Downs and Hudson, 2000). The addition of glucose to the medium used for *in vitro* maturation in the current study may result in a similar improvement. Modifications could also be made to the method of maturation of thawed oocytes. For example, it may be possible to place thawed oocytes that have been denuded of their cumulus cells within fresh ooectomised

COCs, thus conserving the three dimensional structure of the cumulus cells. It would be of interest to investigate the capability of the thawed oocyte and fresh cumulus cells to form novel intercellular linkages.

It may be possible to improve the method of slow cooling by using cryopreservation solutions supplemented with extracellular solutes such as 0.3M sucrose, as demonstrated recently in the controlled rate cooling of mature human oocytes (Fabbri *et al*, 2001; Fosas *et al*, 2003). The findings of the current study could also be expanded on by further investigation into the effects of different cooling regimes. Firstly, it would be of interest to ascertain the impact of plunging the COCs into liquid nitrogen following cooling to –150°C. It has already been established that cooling to –150°C before plunging to –196°C causes less damage to the cumulus cells of the COC than cooling to –60°C prior to plunging. The effect of plunging from –150°C could be investigated with a series of experiments comparing COCs cooled to –150°C and warmed with COCs cooled to –150°C, plunged into nitrogen and then warmed. However, the occurrence of additional disruption on plunging from –150°C is unlikely as at this point the glass transition temperature has already been breached. If this was found to cause additional damage and/or decrease in developmental potential then it may be worth conducting a series of experiments where COCs are cooled at a controlled rate (e.g.-10°C/min) to temperatures lower than –150°C prior to plunging to –196°C.

Another approach to improving cumulus cell survival could be to provide alternative ice nucleation sites during cooling. Extensive damage caused by ice crystal formation and growth has been identified within the cumulus cell mass of the COCs, suggesting that the cell mass provides numerous ice nucleation sites. Adding clumps of cumulus cells that have been removed from other COCs may reduce the overall

formation of ice at these sites by providing alternative sites at which formation can occur. The developmental potential of the COCs and cumulus cell membrane integrity staining of the COCs and the cumulus cell clumps could be conducted to assess the ice formation and cumulus cell damage in each. This approach may be further refined to include equilibration of the COCs with an anti-nucleating substance. Such substances have been shown to inhibit nucleation activity and are discussed in a recent review (Holt, 2003). Presumably ice would form preferentially at sites within the untreated cumulus cell clumps.

If the membrane integrity and functionality of the cumulus cells of the COC cannot be increased using slow cooling methods it may become necessary to pursue vitrification as a means of cryopreservation. Based on the vitrification experiments carried out in the current study, one possible improvement to the vitrification protocol could be the addition of an anti-nucleating substance that could reduce the incidence of devitrification during warming. Different vitrification solutions could also be used, for example ethylene glycol, which was used recently in the vitrification of human oocytes and resulted in a number of live births (Yoon *et al*, 2003). Alternatively, novel low volume techniques such as vitrification in pulled straws could be employed to increase cooling and warming rates. Vitrification has also been achieved by placing embryos into droplets immobilised on electron microscope grids, which are then submerged in liquid nitrogen, reportedly achieving cooling rates of between 11000 and 14000°C/min (Mazur *et al*, 1992). However, a major drawback associated with many low volume strategies is that they require direct contact between the cryopreservation media and the liquid nitrogen. In a clinical context the risk of viral cross contamination may be thought to be unacceptable.

Although cumulus cell survival is important, it is of equal importance to conserve the communication between the cumulus cells and the oocyte. This was demonstrated by the results of the final series of slow cooling experiments in the current study where an improvement in cumulus cell survival did not significantly improve subsequent embryo development. One explanation for this discrepancy could be disruption of the gap junctions that facilitate and regulate the bi-directional exchange of signalling molecules between cumulus cells and the oocyte. This aspect was briefly investigated in the current study. Fluorescence return after photo bleaching (FRAP) was used in order to assess the movement of molecules across gap junctions (see appendix II). However, cumulus cell survival after cooling and thawing was insufficient for these experiments to provide meaningful results. If cumulus cell survival can be improved then this technique could be used to assess the condition of the gap junctions. If they were found to be less active than those found in fresh control COCs then their activity could be increased with the use of a gap junction activator such as staurosporine, which inhibits protein kinase C (PKC) regulated phosphorylation of the connexion molecules that form gap junctions (Tenbroek *et al,* 1997).

6.5 CONCLUSIONS

- The presence of mixed ovarian cells during IVM culture of GV stage COCs was shown to cause inhibition of maturation and subsequent embryo development.
- A method of IVM of GV-stage oocytes was established that consistently produced embryos capable of development to the blastocyst stage.

- Staining for membrane integrity of the cumulus cells of the COC has been shown to be a good indicator of future embryo development.
- Slow cooled GV-stage mouse oocytes were capable of survival and, following maturation and fertilisation *in vitro*, were capable of development to the blastocyst stage again *in vitro*.
- During slow cooling the majority of damage was shown to occur during plunging to and warming from -196°C.
- COCs survived vitrification and were capable of maturation, being fertilised, and of developing to the blastocyst stage *in vitro*. However, there is scope for improvement of the vitrification protocol.

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APPENDIX I

Chemicals and Suppliers

All dry chemicals were weighed using a Mettler-Toledo, MonoBloc (B204-S) balance accurate to four decimal places, with a maximum capacity of 220g. Liquids were measured using Falcon serological pipettes or a Bibbyjet pump.

Albumin fraction V powder (BSA)	Sigma, Poole, Dorset, UK
AGA	Sigma, Poole, Dorset, UK
$CaCl_2$	Invitrogen, Paisley, UK
Carboxy fluorescein diacetate	Sigma, Poole, Dorset, UK
Chorulon	Intervet UK Ltd., Milton Keynes, UK
Crystapen benzyl penicillin sodium BP	Britannia, Redhill, Surrey, UK
dcAMP	Sigma, Poole, Dorset, UK
Dulbecco's PBS powder	Invitrogen, Paisley, UK
EGF	Sigma, Poole, Dorset, UK
Fetal Bovine Serum	Invitrogen, Paisley, UK
Folligon	Intervet UK Ltd., Milton Keynes, UK
Glucose	Sigma Poole, Dorset, UK
Gonal-F (rFSH)	Serono, London, UK
Humegon (FSH/LH)	Organon, Cambridge, UK
Lactic acid	Sigma, Poole, Dorset, UK
L-Glutamine	Invitrogen, Paisley, UK
Me_2SO	Sigma, Poole, Dorset, UK
MEM earles medium	Invitrogen, Paisley, UK
Mineral oil	Sigma, Poole, Dorset, UK
NaCl	Sigma, Poole, Dorset, UK
PEG-8000	Sigma, Poole, Dorset, UK
Phenol red	Sigma, Poole, Dorset, UK
Propidium iodide	Sigma, Poole, Dorset, UK
Sodium bicarbonate	Invitrogen, Paisley, UK
Sodium pyruvate	Invitrogen, Paisley, UK
Streptomycin-sulphate BP	Evans, UK
Sucrose	Aristar, BDH, Poole, Dorset, UK
Tyrode's salts	Gibco BRL, Life technologies, UK

APPENDIX II

Inhibition of Gap Junctions within COCs and Visualisation of Gap Junction Function Through FRAP Analysis

Previous experiments have demonstrated that slow cooling and vitrification of GV stage COCs leads to extensive cumulus cell damage, in particular a loss of membrane integrity (sections 4 and 5). However, loss of membrane integrity may not be the only factor that is limiting the ability of the GV stage oocyte to complete cytoplasmic maturation. Exposure to cryoprotective agents and cooling may also affect the operation of the gap junctions (GJ) that couple the cumulus cells and the oocyte, thus inhibiting or preventing exchange of the molecules required for the completion of cytoplasmic maturation.

The following summarises a brief set of experiments which aimed to investigate the impact of chemical inhibition of GJs on the cytoplasmic maturation and subsequent embryo development of the oocytes. Also to examine the activity of GJs in fresh, cryoprotectant treated, cryopreserved and GJ-inhibited COCs.

1 INTRODUCTION

GJs have been heavily implicated in the maturation of oocytes. They are thought to facilitate communication between the oocyte and its associated cumulus cells (Moor *et al*, 1980). To investigate this, oocytes were matured in the presence of 18α-glycyrrhetinic acid (AGA) which has been shown by others to uncouple the GJs within murine GV stage COCs leading to complete suppression of oocyte and cumulus cell coupling (Downs, 1995). Initially, the concentration of AGA required to inhibit completion of cytoplasmic maturation in the presence of serum was determined. Secondly, fluorescence return after photobleaching (FRAP) was used to

examine the communication across gap junctions in fresh and cryopreserved COCs and also in COCs matured in the presence of AGA. The COCs were stained using CF. The laser of a confocal microscope was then used to bleach a patch of cumulus cells of their fluorescence. The COCs were then photographed at one minute intervals to capture the return of fluorescence as the CF molecules diffused back through the gap junctional network.

2 MATERIALS AND METHODS

2.1 Maturation and Culture in the Presence of AGA

The methods of collection and culture of the oocytes were identical to previous experiments (section 2.2). To determine the concentration of AGA required to inhibit cytoplasmic maturation, experiments were carried out where GV stage COCs were matured in maturation medium containing different concentrations of AGA (4mM, 400μM, 1mM and 2mM). Simultaneously, control COCs were matured in identical medium which did not contain AGA (section 2.2.9).

2.2 Fluorescence Return After Photobleaching (FRAP)

The thawed COCs used for FRAP analysis were slow-cooled as detailed previously (section 2.3.2). The COCs for FRAP analysis were stained with CF as described previously (section 2.4.2). The stained COCs were subjected to FRAP analysis with the use of a Leica LSC scanning head coupled to an inverted microscope (Leica RM). The 488nm line of a Ar/Kr laser was used for excitation and diachronic filters for emission detection. The photomultiplier tube was set at 950V and the offset and laser strength were automatically adjusted to optimise the image. Five fresh COCs, and five COCs that had been cultured in the presence of 2mM AGA were

stained with CF. Five slow cooled and thawed COCs (section 2.3.2) were stained in an identical manner.

3 RESULTS

3.1 Maturation and Culture in the Presence of AGA

3.1.1 AGA (4mM)

Unlike the control COCs, the COCs matured in the presence of AGA did not adhere to the culture dish and the cumulus cells of these COCs did not expand following incubation in media containing LH. After insemination, most of these cumulus cells remained attached to the oocytes, around which many sperm were observed that had bound to the oocyte and cumulus cells. None of the oocytes matured in the presence of AGA were fertilised (Table IIi).

Table IIi Fertilisation and development of GV stage murine COCs matured in the presence of 4mM AGA compared with *in vitro* matured controls.

Protocol	No. of oocytes	% (No.)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
In vitro matured control	32	96.9 (31)	96.8 (30)	63.3 (19)	59.3
In vitro matured + 4mM AGA	34	0 (0)	0 (0)	0 (0)	0

3.1.2 AGA (400μM)

At 400μM AGA, no differences were found between the experimental and the control oocytes. The oocytes were fertilised and a similar number of the fertilised oocytes went on to develop to the blastocyst stage as those that developed in the control group (Table IIii).

Table IIii Fertilisation and development of GV stage murine COCs matured in the presence of 400μM AGA compared with *in vitro* matured controls.

Protocol	No. of oocytes	% (No.)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
In vitro matured control	38	100 (38)	92.1 (35)	42.9 (15)	39.5
In vitro matured + 400μM AGA	32	100 (32)	90.6 (29)	48.3 (14)	43.8

3.1.3 AGA (1mM)

At 1mM AGA, the oocytes were fertilised in similar numbers to the control group. However, development to blastocyst was less than that observed in the control group (Table IIiii).

Table IIiii Fertilisation and development of GV stage murine COCs matured in the presence of 1mM AGA compared with *in vitro* matured controls.

Protocol	No. of oocytes	% (No.)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
In vitro matured control	39	97.4 (38)	89.5 (34)	82.4 (28)	71.8
In vitro matured + 1mM AGA	31	100 (31)	90.3 (28)	57.1 (16)	51.6

3.1.4 AGA (2mM)

Table IIiv Fertilisation and development of GV stage murine COCs matured in the presence of 2mM AGA compared with *in vitro* matured controls.

Protocol	No. of oocytes	% (No.)			
		Normality	Fertilisation	Blastocyst	Total Blastocyst
In vitro matured control	25	100 (25)	80.0 (20)	65.0 (15)	52.0
In vitro matured + 2mM AGA	24	100 (24)	87.5 (21)	33.3 (7)	29.2

Once more, fertilisation rates were similar in both groups. However, when oocytes were matured in the presence of AGA, development to the blastocyst stage was approximately half of that observed in the control group (Table IIiv).

3.2 Fluorescence Return After Photobleaching

The cryopreserved and thawed COCs had only few cumulus cells which were intact, therefore most of the fluorescence leaked out of the cells before bleaching could commence. The technique worked well when used on fresh COCs, as fluorescence could be detected returning through the gap junctional network. COCs matured in the presence of AGA showed a delay in the return of fluorescence. However, the resolution of the images was insufficient to obtain useful data.

4 DISCUSSION

It is clear from these results that an AGA concentration of 4mM is too high to allow the progression of maturation and fertilisation, whereas a concentration of 400μM AGA was too low to produce an effect. At 1mM AGA a partial effect was evident and following maturation in the presence of 2mM AGA there was a clear effect on the development of the embryos, with only half the amount progressing to blastocyst compared with control oocytes. A similar dose dependent effect of AGA on GJs in alveolar epithelial cells has been demonstrated (Guo *et al*, 1999). However, poor development is not in itself evidence of gap junction inhibition, although this may explain the decrease. If inhibition is occurring it is not entirely effective. It may be possible to quantify the extent of inhibition through FRAP analysis.

This preliminary study using FRAP analysis has demonstrated that it is possible to observe the diffusion of the CF molecules through the GJ network. If a higher

resolution image could be obtained, this may prove to be a useful technique in examining the functionality of gap junctions. If the integrity of the membranes of the cumulus cells of cryopreserved and thawed COCs could be improved this technique could be used to examine the functionality of the GJs that couple the cumulus cells and the oocyte.