CELL MEMBRANE PERMEABILITY COEFFICIENTS OF MURINE AND HUMAN OOCYTES: FLUXES OF WATER AND CRYOPROTECTANTS DURING CRYOPRESERVATION PROCEDURES.

by

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To my mother and sister.
ABSTRACT

Cell Membrane Permeability Coefficients of Murine and Human Oocytes: Fluxes of Water and Cryoprotectants During Cryopreservation Procedures.

The aim of this thesis was to determine the cell membrane permeability characteristics of human pre-ovulatory oocytes and to apply this information to the development of a successful cryopreservation protocol. Currently, although there is no reliable method for the cryopreservation of human oocytes, there is an urgent need for such a technique in clinical I.V.F. programmes. The permeability of cells to both water and cryoprotectants is important in determining whether a cell will survive the cryopreservation procedure, and these basic parameters are unknown for human oocytes.

A microscope diffusion chamber was developed to allow direct measurement of cell membrane permeability under controlled environmental conditions of temperature and solute exposure. Volumetric responses of individual oocytes were recorded using video microscopy, and cell volume data processed using a computer algorithm based on the Kedem-Katchalsky membrane transport equations. From these values the membrane water permeability (Lp), the temperature-dependent Arrhenius activation energy (Ea) of the Lp, and the solute permeability (w) were calculated. The values of Lp and Ea
for fresh pre-ovulatory human oocytes were compared to those calculated for mouse oocytes for which values have previously been determined and failed-to-fertilise human oocyte (Ff). Values for Lp and Ea in human oocytes were of the same range as those determined for the mouse, but human pre-ovulatory oocytes were inherently more diverse than mouse oocytes. The most reliable value for Ea of the human oocytes was obtained by studying individual fresh oocytes at several temperatures, and this yielded a mean value for Ea similar to other mammalian cells.

At low temperatures, both mouse and human oocytes, responded to hypertonic perfusion with an altered morphology and non-spherical shrinkage. Although this response was morphologically reversible, it led to a reduction in the subsequent fertilisation and embryonic development. However, exposure of oocytes to cryoprotectants before cold shrinkage provided some protection to the oocytes as judged by subsequent fertilisation and reduced the damage to the developmental potential. Taking the values for Lp and Ea into account, protocols were chosen to investigate cryopreservation of human oocytes using glycerol or dimethyl sulfoxide as cryoprotectants. Fertilisation was achieved using both systems following cryopreservation, but further embryonic development was inhibited.
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ABBREVIATIONS

I.V.F.  
In vitro fertilisation

G.I.F.T.  
Gamete intrafallopian tube transfer

E.T.  
Embryo transfer

FSH  
Follicle stimulating hormone

LH  
Luteinising hormone

PMS  
Pregnant mares' serum

hCG  
Human chorionic gonadotrophin

BSA  
Bovine serum albumin

HSA  
Human serum albumin

PBS  
Phosphate buffered saline

PB1  
Modified Phosphate buffered saline (Whittingham, 1971)

EBSS  
Earles balanced salt solution

T6  
Tyrodes salt solution

c.AMP  
Cyclic adenosine monophosphate

O₂  
Oxygen

CO₂  
Carbon dioxide

N₂  
Nitrogen

HCO₃  
Bicarbonate

NaCl  
Sodium chloride solution

Na⁺  
Sodium ion

Ca²⁺  
Calcium ion

K⁺  
Potassium ion

FF  
Failed-to-fertilise human oocyte

CPA's  
Cryoprotective agents

T  
Temperature (°K)
$T_g$ Reference temperature 20°C

$Lp$ Membrane water permeability (also $Lp^g$)

$Lp_0$ Membrane water permeability at reference temperature 20°C

$P(T)$ Permeability of a species at temperature $T$

$P_g$ Permeability of a species at reference temperature $T_g$.

$V_b$ Osmotically inactive volume

$E_a$ Arrhenius activation energy (also $\mu$)

$A$ Surface area to volume ratio

$K$ Velocity of the reaction (reaction rate)

$L_f$ Latent heat of fusion of ice

$B$ Cooling rate $dT/\ dt$

$R$ Universal gas constant ($8.3 \text{ J K}^{-1} / \text{ m}^{-1}$)

$d_1$ Cell diameter in hypertonic solution

$d_0$ Cell diameter in isotonic PBS

$C_1$ Concentration of the hypertonic solution

$C_0$ Concentration of the isotonic PBS

$V$ Relative cell volume

$A_t$ Change in the cell surface area with time

$V_t$ Change in cell volume with time

$C_{s_{ext}}$ Extracellular concentration

$C_{s_{int}}$ Concentration inside the cell

$R_{m_{t}}$ Measured cell radius at time $t$

$R_{c_{t}}$ Calculated cell radius at time $t$

$J_v$ Total volume flow
J_D  differential volume flow
L_D  diffusional coefficient
L_p  hydraulic coefficient
L_{PD}  coupling coefficient between osmotic and diffusional flow
\Delta P  difference in hydrostatic pressure
\Delta \pi  difference in osmotic pressure
J_s  flux of species
D  diffusivity
L  membrane thickness
\Delta C_s  concentration gradient
w_s  solute permeability
\sigma_s  reflection coefficient (ranges between 1 and 0 with 1 corresponding to an ideal semipermeable membrane and all the solute is reflected).
N_s  number of moles of solute
v^o  molar volume of water
v_w  molar volume of solute
R  is the osmolal ratio of additive:electrolyte prior to freezing
M^e NaCl  concentration of electrolyte in the presence of an additive
M^e'NaCl  concentration of electrolyte in the absence of an additive
n  number of data points
m  slope of a straight line
x and y  coordinates (variables) of data points for a straight line
Introduction

1.1 Infertility

Infertility, usually diagnosed when a couple attempting to become pregnant have not done so within 12 months, may result from a number of causes. Often, infertility is a result of multiple factors both male and female (Figure 1). It can be divided into primary infertility - when the couple have never achieved a pregnancy, or secondary - when there has been a previous conception. In 1979 Steptoe and Edwards successfully developed the techniques of in vitro fertilisation (I.V.F.) and embryo transfer (E.T.) for clinical use with patients. This enabled treatment of a subgroup of infertility patients, those women in which the fallopian tubes were blocked.

Progress in the treatment procedures involved in I.V.F. has resulted in the techniques being applicable to a wider range of infertility problems. Since the stimulation procedures in I.V.F. may produce oocytes in excess of a patient's requirements, if consent is obtained excess gametes can be donated for transfer into the uterus of a recipient female to establish a successful pregnancy (Trounson et al., 1983). Thus it is now possible to treat women from whom oocytes cannot be collected, either due to
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Tubal Disease  Cervical Factor  Male Factor

<table>
<thead>
<tr>
<th>Reconstructive Surgery</th>
<th>Antibodies</th>
<th>Irregular Ovulation</th>
<th>Oligospermia</th>
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<td>Repair Treatment</td>
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<td>Danazol Therapy</td>
<td>Unexplained</td>
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In Vitro Fertilisation

Figure 1 Causes of infertility and Criteria for In Vitro Fertilisation
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Indications for *In Vitro Fertilisation*

i) Tubal disease: unsuitable for surgery
   unsuccessful surgery
   patent but abnormal

ii) Unexplained Infertility

iii) Endometriosis

iv) Male subfertility: oligospermia
   low motility
   abnormal morphology
   antisperm antibodies

v) Failed donor insemination

vi) Cervical hostility

vii) Failed ovulation induction

viii) Therapy for female cancer (embryo freezing prior to chemotherapy / cytotoxics)

ix) Premature menopause - Donor oocytes
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a failure to produce oocytes as a result of premature menopause or failure to stimulate the ovary, by utilising the technique of oocyte donation. In addition, donation would overcome the risk of transmission of genetic disease to an embryo from parents who are known carriers of such diseases. Although few techniques exist to improve the quality of semen such as sperm density, motility, and morphology, I.V.F is a feasible option for couples where the man presents subnormal semen characteristics. Fertilisation in vitro can occur with sperm concentrations in the range of $2 \times 10^5 - 2 \times 10^6$ / ml (Trounson et al, 1981). The low sperm density required for fertilisation allowed the treatment of male factor infertility patients by I.V.F. (de Krester, 1985). In addition I.V.F. is now used with those for couples whose infertility remains unexplained (Mahadeven et al, 1983).

1.2 The History of In Vitro Fertilisation

The culture of embryos outside of the maternal uterus was reported for rabbits in 1890 (Heape, 1890) and since this time many improvements have been made which have made culture and transfer techniques increasingly simple for embryos of many species and of various pre-implantation stages. In 1939 Pincus and Saunders collected and matured the first mammalian oocytes in vitro, as determined by chromosome studies. By 1965, this technique was applied to
human oocytes which developed to the point of extrusion of the first polar body (Edwards, 1965a, 1965b). Meanwhile investigations into the role of the pituitary hormones lead to the ability to induce superovulation. Adult mice were hormonally stimulated and pregnancies established with greater than normal numbers of offspring (Fowler and Edwards, 1957). Within a few years human menopausal gonadotrophins (HMG) and human chorionic gonadotrophins (hCG) were being used to stimulate women with abnormal ovulation cycles (Gemzel, 1967). With the advent of laparoscopy it was possible to collect oocytes immediately prior to ovulation and successfully fertilise them (Edwards et al, 1969).

The major part of the basic research for in vitro embryo culture was carried out on the mouse (Brinster, 1965, Brinster, 1970, Whittingham, 1971, Whitten, 1971). Although mouse embryos were able to grow in a variety of conditions optimal growth occurred within the range pH 7.2 - 7.3, with a medium osmolality of approximately 280 mOsm in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂. Supplements of lactate and pyruvate were found to be required for the growth of the early embryo since glucose can not be utilized until after the initiation of the first cleavage division (Brinster, 1971). Serum albumin and amino acids were also used to act as sources of nitrogen prior to the formation of the two cell embryo.
Although embryos cultured *in vitro* may lag behind those developing *in vivo* (Binkero et al, 1979) they can easily progress to the hatching blastocyst stage. Even though it was possible to fertilise and grow human oocytes on to the blastocyst stage *in vitro* (Edwards et al, 1969, 1970), replacement of embryos into the uterus of an infertile woman did not result in pregnancy (Edwards, 1973). Deficiencies in the luteal phase were indicated as the major source of the problem and various combinations of luteal support were investigated until an ectopic pregnancy was established (Steptoe and Edwards, 1976). This was followed by the first development to term and live birth of an *in vitro* cultured and fertilised oocyte following natural cycle folliculogenesis (Steptoe & Edwards, 1979). Stimulated cycles using HMG or clomiphene were reintroduced, successful implantations achieved and the advantages of replacing more than a single embryo were recognised (Jones et al, 1984, Trounson et al, 1981, Edwards and Steptoe, 1983). Endocrine stimulation techniques were altered to prevent spontaneous or unexpected ovulation, controlling the day of collection (Porter, 1984).

Although the stimulation and embryo culture techniques are continuously being modified and improved there are still problems with the treatment. Successful I.V.F. combined with cryopreservation of the embryos / gametes
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would be beneficial, in a variety of species, particularly endangered species, but application of the methods have so far been limited. Much of the detail concerning the development of embryos of particular species is unknown or not fully understood. Even in the mouse, where much of the basic research has been performed, 2-cell blocks are common in many strains, preventing on-growth of the embryos in culture. Embryogenesis is extremely complex, involving multiple cell divisions and differentiations which combine to determine the fate of individual cells or groups of cells in the embryo. Births following the transfer of human in vitro fertilised embryos are now common. However, although I.V.F. is often considered to be the solution to infertility and is used in numerous centres around the world, the success rates are not consistent. They have improved little in recent years, the established pregnancy rate being 15% compared to the initial rate of approximately 8%. The techniques involved are extremely sensitive and the success of individual I.V.F. programmes is still very variable with some units achieving success rates of approximately 40%. Each of the procedures in I.V.F. including cryopreservation, are the subject of ongoing study in an attempt to improve the overall success of the treatment.
1.3 Oogenesis and Ovulation

Although superovulatory techniques have created an abundance of material from a single stimulation cycle leading to an excess of oocytes, and thus potentially embryos, for transfer, the overall number of primary oocytes available in the ovaries are limited. There are several factors that contribute to the sparse numbers of oocytes in mammalian species the most important of which is that the number of primordial follicles is fixed from birth. Unlike the male of the species, where spermatogenesis is carried on throughout life, the female has completed the initial differentiation of the germ cells to the primordial oocyte stage prior to birth and is unable to recruit any further cells if this finite stock is depleted (Figure 2). If all of the oocytes are destroyed such as by exposure to X-irradiation or radio/chemotherapy in cancer treatment, they will not be replaced from the stem cells, resulting in infertility. The migration of primordial germ cells to the ovary is followed by a period of mitotic cell proliferation and these oogonia subsequently differentiate into primary oocytes. Many of the oogonia fail to mature and degenerate before reaching the primary oocyte stage, and the small number that do undergo a final mitotic division then replicate their DNA and enter meiosis. The completion of mitosis and switch to meiosis is a result of an initiation factor secreted from
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PRIMORDIAL GERM CELL
- migration

GONAD
- proliferation

OOGONIA

PRIMARY OOCYTE
- arrested in prophase I
- followed by a period of growth

1° OOCYTE
- maturation - formation of egg coat, cortical granules

2° OOCYTE 1st POLAR BODY

MATURE OVUM 2nd POLAR BODY
- haploid

Figure 2 Development of the oocyte from the germ cell stage

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the rete ovarii tissue. Removal of the rete tissue prevents meiosis from occurring.

In meiosis following the replication of the DNA strands to produce chromatids, prophase is initiated with the attachment of the chromosomes to the nuclear envelope via attachment plaques (leptotene). The DNA then condenses, the homologous chromosomes pair with the formation of the synaptonemal complex, a protein axis between the homologous chromosomes, ensuring they are kept closely aligned forming bivalents (zygotene) (Figure 3). Coiling causes chromosome thickening which is accompanied by the formation of recombination nodules (pachytene). Once in pachytene, the cell may remain in this stage for an extended period after which the chromatids cross over leading to the appearance of the chromosomes as meiotic figures (diplotene). During this period desynapsis occurs and the chromatin decondenses while RNA is synthesised (Figure 4). At the onset of the diplotene phase the majority of the primary oocytes are arrested in the nucleated phase with the nucleus visible (germinal vesicle). The oocyte is enclosed by a single layer of presumptive granulosa cells that are connected to the oocyte via gap junctions which allow the passage of small molecules or metabolites and are maintained even when the oocyte has synthesised the zona pellucida. Oocytes are enclosed in a follicle which severs the links between the daughter cells of a single
oogonium. These primordial follicles then enter a "rest" period lasting from just prior to birth to the onset of sexual maturation which may be many months or years depending on the species. During this period there is active transcription of the mRNA, loading the oocyte with maternal genome products for the process of maturation and development which is accompanied by growth of the oocyte. A small proportion of the follicles begin to develop, the follicle cells enlarge and increase in numbers producing a multilayered coating. These granulosa cells secrete a glycoprotein, forming an acellular layer around the oocyte, the zona pellucida. In addition the oocyte plasma membrane looses its smooth appearance, developing microvilli, and a period of oocyte growth occurs (Figure 5). For the primary follicle to continue development past the initial growth phase the pituitary gonadotrophin Follicle Stimulating Hormone (FSH) must be released, stimulating the formation of an antrum or fluid-filled cavity in response to the accumulation of oestrogen. The granulosa cell layer develops gap junctions between the cells, and membrane receptors for oestrogen and FSH while the surrounding vascularised thecal layer develops luteinizing hormone (LH) receptors. There is a continuous progression of follicles through the growth phase and they can only progress on to the antral stage if the tonic level of FSH
Chromosomal Synapsis and Desynapsis

Leptotene

Pachytene

Synaptonemal Complex

Desynapsis

Interphase

Zygotene

Diplotene

Figure 3.
Meiotic Cell Division I. The Homologous Chromosomes During Cell Division.
and LH in circulation are sufficiently high. The granulosa and thecal cells increase in numbers so that there is follicular growth, with continued synthesis of RNA and proteins although there is little accompanying increase in the size of the oocyte. The thecal cells form two distinct layers, the vascular interna and the fibrous externa. Fluid forms between the granulosa cells which, upon aggregation in a single cavity forms the follicular fluid-filled antrum. The oocyte initially remains embedded in the follicle cells (cumulus oophorus) which remain compact until maturation is nearing completion.

The outer granulosa cells develop LH receptors allowing both the thecal and granulosa cells to bind LH. If this coincides with the LH surge, the follicles can continue through to the pre-ovulatory phase (Figure 6). Cytoplasmic maturation commences with the Golgi apparatus synthesising cortical granules which migrate to a subcortical position. The chromosomes recondense and the nuclear envelope surrounding the germinal vesicle breaks down. The thecal layer becomes less prominent in response to the initial rise in LH, and immediately after the LH peak the outer granulosa cells exhibit a reduced ability to bind with FSH. The cumulus cells expand to become the corona radiata and initiate withdrawal of the cytoplasmic connections with the follicle cells. The oocyte remains attached to the peripheral granulosa cells of the follicle.
wall by a thin column of cells which suspend the oocyte in the fluid filled antrum (Figure 7). This may permit the primary oocyte to develop into a secondary oocyte as the follicular cells may release an inhibitory factor which reduces the binding of FSH to the granulosa cells or the accumulation of cAMP. The uncoupling of the oocyte from the follicle wall reduces the level of the inhibitor. The LH surge may act to prevent the production of this inhibitor, reduce cAMP or cause the production of a maturation promoter.

With the onset of maturation the oocyte resumes meiotic cell division with the chromosomes attaching to the spindle equator and the homologs separating to opposite poles. One haploid set of chromosomes, along with a small amount of cytoplasm, is extruded but retained inside the zona pellucida as the first polar body. The asymmetrical cell division results in the formation of the secondary oocyte, at which point there is a further interruption in the meiotic process and a period of arrest while the oocyte is ovulated (Figure 8). The oocyte, which now has only a cursory attachment to the outer granulosa cells, becomes increasingly prominent on the ovarian surface. This leaves only a thin layer of epithelial cells between it and the peritoneal cavity and as the wall becomes avascular the intervening cells appear to degenerate and the follicle ruptures. The follicular fluid leaves the follicle and
Preantral follicle
(a) Primordial follicle suspended in dictyate stage
(b) Primary oocyte following the growth period
Figure 6 Maturation of the oocyte, a) breakdown of the germinal vesicle, b) chromosome condensation, c) termination of cytoplasmic connections, d) migration of the cortical granules to the surface and completion of meiotic division I.
Figure 7 Development of the antral phase follicle, b) proliferation of the thecal and granulosa cells c) formation of the fluid filled antrum.
carries the cumulus mass containing the oocyte into the oviduct.

1.4 Fertilisation

Ciliary action on the fimbria of the oviduct moves the cumulus egg mass into the ostium and the oocyte moves down the oviduct to the ampulla where it may be eventually fertilised (Figure 9). The second meiotic cell division in mammalian species is usually stimulated by penetration of a spermatozoon, inducing the chromosomes to rapidly orientate on the equator and the separation of sister chromatids to opposite poles. The cell division is again asymmetrical and a second polar body is extruded which, along with the first, will eventually degenerate. The delay of the second meiotic division means the oocyte is diploid for most of the growth and development phase, thus avoiding any deleterious effects of recessive alleles which are extremely important when considering the long period of arrest.

A period of capacitation, in which the glycoprotein coatings of the spermatozoon are removed by breakdown of interactions between the charged molecules, is required before fertilisation of the oocyte can occur. Modification of the plasma membrane, accompanied by an influx of Ca$^{2+}$ through ion channels may initiate activation of the spermatozoon in which the plasma membrane of the head fuses
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Figure 8 Ovulation and Fertilisation of the Mouse Oocyte.
Summary of Preimplantation Development in the Mouse, Location and Timing, Showing Overlap in Developmental Stages

- **Ovary**
- **Bursa**
- **Infundibulum**
- **Ova**
- **Ampulla** (swollen after ovulation)
- **2-cell**
- **3-4 cell**
- **5-8 cell**
- **Compacted Morula**
- **Blastocyst**
- **Implantation in uterus**

<table>
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<tr>
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<td>Compaction</td>
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<tr>
<td>Hatching</td>
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<td>Implantation</td>
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Days post Coitum:

- Ovulation (13 hrs =)
- Fertilisation
- Early Cleavage
- Compaction
- Hatching
- Implantation

**Figure 9**
with the acrosomal membrane, exposing the contents of the acrosomal vesicle to the exterior. The tail movements of the spermatozoon become exaggerated and the membrane in the posterior section of the head develops the capacity to fuse with the oocyte membrane.

The spermatozoon and oocyte are brought into close proximity in the ampulla where the acrosome reaction occurs in response to secretions in the female genital tract. This includes release of hyaluronidase, an enzyme which digests the hyaluronic acid matrix holding the cumulus cells together. The dispersal of the cumulus mass allows the spermatozoon access to the zona pellucida, which is composed of several layers (the outer being loosely woven and filamentous, while the inner zone is compact with a number of small pores). The zona has many mucoproteins or mucopolysaccharides which can be digested by enzymes. In addition there are numerous carbohydrate receptors on the inner acrosomal membrane of the spermatozoon which attach to those on the zona. The binding formed initially at the tip of the sperm head is temporary, being succeeded by a more permanent binding in which the sperm orientates to lie horizontal to the zona. In many mammals the binding sites on the zona are highly species specific and prevent cross species fertilisation. The zona pellucida is digested by enzymes from the spermatozoon and the spermatozoon penetrates, using exaggerated oblique movements of the tail
to lie in the perivitelline space. After fusion of the post acrosomal area of the spermatozoon head plasma membrane with the vitelline membrane of the oocyte, movement of the spermatozoon ceases (Figure 10).

Although many spermatozoa may attach to the zona pellucida, normally only one fuses with the oocyte to create a diploid cell. To avoid multiple spermatozoon penetration (polyspermia) there is a rapid block to further spermatozoon fusion once a single spermatozoon has penetrated. There is a propagated wave of rapid depolarization of the oocyte membrane, the change in the electrical charge of the membrane spreads from the point of fusion and results in a transient increase in the level of cytosolic free Ca\textsuperscript{2+}. In response to the release of bound Ca\textsuperscript{2+}, the cortical granules fuse with the vitelline membrane and release their enzymes which act on the zona to block further spermatozoon penetration. The glycoproteins of the zona become crosslinked and the receptors are no longer able to bind to the spermatozoon.

The penetration and fusion of the spermatozoon causes the oocyte to initiate the second meiotic division and extrusion of a haploid set of chromosomes in the second polar body. Sperm fusion occurs at a point removed from the site of the formation of the second meiotic spindle, possibly due to an absence of microvilli in the area. This is advantageous as it reduces the risk of interference in
formation of a diploid cell containing two different sets of chromosomes. While the second polar body is in the process of extrusion, the sperm cytoplasm (including the head, midpiece, and a large portion of the tail) passes into the oocyte. The nuclear membrane surrounding the male chromosomes breaks down and the chromatin decondenses in response to factors in the ooplasm. Membranes form around the male and female haploid chromosomes creating pronuclei located subcortically. In many mammalian species the male pronucleus is larger than the female. In the mouse the two pronuclei condense asynchronously, the male to a lesser degree than the female. The pronuclei enlarge as nucleolar activity continues, mitochondria and Golgi apparatus become concentrated in the ooplasm surround the pronuclei which gradually migrate to a central position while synthesising DNA. Although they assume adjacent positions, the pronuclei of the human or mouse do not fuse. After the chromosomes have condensed and duplication is completed, the nuclear membrane breaks down and the chromosomes locate on the spindle equator where there is intermingling of the male and female homologs. This is followed by the first cleavage division and the production of a two-cell embryo.

1.5 Embryogenesis

After fertilisation of the oocyte, the single cell embryo remains in the oviduct for a period of time which is
species specific, during which the embryo undergoes numerous cell divisions. A combination of ciliary action and muscle contraction are thought to move the developing embryo through the oviduct into the uterus where implantation occurs. The rate of movement down the oviduct is constant and the embryo at particular stages of division can be located at various points in the oviduct (Fig. 9). In the human the embryo remains in the ampulla for 72 h, of which for 30 h it is at the ampulla - isthmic junction, and then moves rapidly through the isthmus to the uterus.

After the 8-cell stage the plasmalemma and cytoplasm alter, the cells become flattened and cell-cell contact increases resulting in the formation of tight junctions. Compaction of the embryo is accompanied by a rapid increase in the RNA and protein synthesis, in conjunction with a restriction in the developmental potential of the individual cells. There are rapid alterations in the morula at the 32-64 cell stage with the cells differentiating into inner and outer cell masses. When the embryo has developed to the blastocyst stage, the cells have developed an apical and basement membrane. The outer trophoderm is characteristic epithelium with apical junctional complexes and acts as a seal to the external environment. This surrounds the blastocoelic cavity and the inner cell mass. The size of the embryo does not increase during the initial cell divisions, and in the mouse and human there is only a
CHAPTER 1 - INTRODUCTION

1. Binding
   1st. Polar Body
   Acrosomal Vesicle
   Follicle Cells
   Cortical Granules
   Oolemma

2. Acrosome Reaction
   2nd. Meiotic Spindle
   Cytosol
   Perivitelline Space

3. Penetration

4. Fusion

Fertilisation of the Oocyte Following the Activation of Spermatozoa

Figure 10.
slight increase by the blastocyst stage.

The cells of the blastocyst are pulsatile and force blastocoelic fluid into the space between the zona pellucida and the embryo. The contraction of the blastocyst in conjunction with the release of a trypsin-like enzyme from the trophoblast cells causes the embryo to hatch (Wassarman et al, 1984). After the blastocyst leaves the zona, and if the uterine wall is in close presentation and has reached the appropriate stage of development, it invades the uterine epithelium, the underlying basal lamina and the endometrium, initiating implantation and establishing a pregnancy. The endometrium is extremely sensitive to implantation if it has been primed with high levels of progesterone preceded by exposure to oestrogen, as in the natural cycle luteal phase.

1.6 Cryopreservation of the Unfertilised Oocyte: The Reasons Why Cryopreservation is Required.

Although the prenatal female has large numbers of oogonia (in the human this can reach approximately 7 million), most fail to progress to the primary oocyte stage due to degeneration, possibly caused by abnormal spindle formation. This process of atresia is continued through the prolonged meiotic arrest and by birth many of the primary oocytes have themselves degenerated. In the early follicle stages, the atresia is recognisable by the
dark granular appearance of the cytoplasm while in the growing follicle it is often seen as maturation and segmentation inappropriate of follicular development. Even after birth, and prior to puberty, the number of viable oocytes continues to decrease, such that in the human only 300,000 primary oocytes remain. In the mouse more than half the primordial follicles present at birth have been lost by the time of sexual maturity. During the reproductive span of the human female only approximately 400 oocytes would be naturally ovulated due to the release of only a single oocyte per cycle.

Natural folliculogenesis involves recruitment of the small antral follicles during the first few days of the menstrual cycle controlled by gonadotrophins released from the pituitary gland. Initially, FSH and LH are produced at a basal level on day 1 to day 6 causing the growth of a cohort of pre-antral follicles. This is followed by a rise in the FSH above a threshold level, allowing oocytes at the appropriate stage of maturity to continue development to ovulation. The time the FSH stays at an elevated level is a "gate" through which follicles must pass to avoid atresia and determines the number of follicles developing to ovulation (Figure 11). From the group of synchronously developing follicles only one normally sustains the process to the pre-ovulatory Graafian follicle stage, while the remainder become atretic. The selection process by which
the dominant follicle is chosen is not understood, nor is it fully elucidated how once dominant it continues to grow in an environment inhospitable to the rest of the cohort. However the dominant follicle does secrete a regulatory protein which may decrease the sensitivity of others to stimulation by gonadotrophins. In addition there is a local increase in the oestradiol levels in the selected follicle which encourages growth.

The control over production of a single oocyte during a natural stimulation cycle can be bypassed by the administration of exogenous gonadotrophins. Follicular stimulation regimes in I.V.F. programmes act to extend the FSH "gate" through which the oocytes pass on to maturity, resulting in multiple ovulation. The patient is initially down-regulated, in which the natural hormonal secretion is prevented by administering an analog which binds with the gonadotrophin releasing hormone (GnRH) receptors. This keeps the FSH and LH at a basal level. Exogenous FSH is administered to stimulate follicular growth, and hCG to mimic the natural LH surge. The introduction of these superovulatory techniques in the treatment of infertility has resulted in the development of numerous follicles since it has been impossible to accurately control the numbers even when the follicular growth was monitored using ultrasound scanning and estimation of oestradiol 17β and the hormonal stimulation varied accordingly. If all of the
follcicles were emptied, this would result in the collection of large numbers of oocytes from a single treatment cycle. Fertilisation of all of the oocytes collected would, if successful, create numerous embryos of which only a limited number could be replaced. It has long been established (Wood et al, 1984, 1985, Steptoe, 1985) that the greater the number of embryos replaced, the higher the chance of establishing a successful pregnancy. However, it has also been shown that the greater the number of embryos transferred, the higher the risk of a multiple pregnancy (Mettler et al, 1984, Seppela, 1985) which is often detrimental to both the maternal and foetal health. A voluntary code of conduct was agreed upon by the parties interested in I.V.F. in the U. K. along the lines suggested by the Warnock Report (Warnock, Department of Health and Social Security, 1984). The Voluntary Licensing Authority was set up to monitor I.V.F. centres and regulate both the research and therapeutic aspects of the work. The recommendations of this committee were to limit the number of embryos replaced in a single cycle to a maximum of three in I.V.F. and four oocytes in gamete intrafallopian tube transfer (G.I.F.T.), which would serve to maximize the increased chances of pregnancy while minimising the risks of multiple pregnancy.

The limit on embryo replacement has meant that all of the embryos produced in a stimulation cycle can not be
Follicular Growth as a Function of Hormone Level.

Figure 11 Follicular growth as a function of hormone level.
replaced in that cycle and must be either disposed of or stored for replacement in a subsequent cycle. Both approaches to handling excess embryos have produced areas of contention and disapproval, on religious, ethical and legal grounds (Robertson, 1987, Grobstein and Flower, 1985, Scott, 1985). Problems arising from the disposal of embryos are complicated by the question of "ownership" upon the dissolution of the partnership or death of one of the members.

It is possible to maintain cells in a viable state for only a very limited time in culture even under conditions of controlled temperature, pH, osmotic pressure, and nutrient composition. Since storage time is usually only a few days, a method to prolong this period needed to be established. The processes that in a cell limit viability in culture are either biochemical (often requiring metabolic energy) or physical (for example passive diffusion) which depend solely on the rate of molecular motion. Long term preservation of tissues and cells can be achieved by a reduction in the temperature of storage.

Chemical reactions require an energy of activation and the relationship between the reaction rate and the temperature was described by Arrhenius (1889) as the log of the reaction rate (K) being proportional to the reciprocal of the absolute temperature (T). An Arrhenius plot of K against 1/T for a process containing a single
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rate limiting step is a straight line with a slope equal to the activation energy ( E_a ) / R where R is the gas constant.

\[ \ln K = \text{a constant} - \frac{E_a}{RT} \]

equation 1.1

As the temperature decreases, the reaction rate generally exhibits a decreasing linear relationship and any breaks suggest alterations in the reaction mechanisms. The affect of decreasing the temperature has a complex effect on integrated metabolic pathways which may result in the reactions becoming uncoupled. In theory lowering the temperature to -150°C reduces molecular motion to such an extent that molecular interactions are negligible and the cell is held in a state of suspended animation. Reducing the temperature further to -273.16°C ( absolute zero ) means there is insufficient thermal energy for any molecular motion to proceed on. At the temperature of liquid nitrogen ( -196°C ), the most commonly used cryogen in cryopreservation for the past 20 years, water can only exist in either a crystalline or a glassy state. Thus diffusion and other physical processes in the cell are too slow for reactions between molecules. Apart from free radical damage, most deleterious events that occur during freezing and thawing procedures are at high subzero
temperatures and, if these can be avoided, the cells can be stored for many years with no adverse affects.

Of those oocytes that are viable, there is only a small percentage that can be successfully fertilised when ovulated in a natural cycle or collected in I.V.F. Of the subsequent embryos, again only a limited number would be capable of implantation, and development to full term. Any preservation protocol developed for the unfertilised oocyte must have a high rate of successful recovery if it is going to be ethically or logistically practicable in human I.V.F.

These reasons, in addition to the scarcity of the human material, makes it ethically questionable to use human oocytes in preservation methods that are based on a purely empirical approach. The possible combinations of freezing using a particular cryoprotectant, choice of cooling and warming rate are so numerous that success is unlikely to be achieved by guesswork. Many oocytes could be wasted, with no guarantee that a successful protocol would ever be devised. Successful cryopreservation of animal oocytes of several species has now been reported, and it has been possible to determine many of the characteristics that are important for cell recovery from cryopreservation. However for the human oocyte none of these parameters are known to date, the investigation of these characteristics have formed the basis of the work in my thesis.
1.7 Cryopreservation

1.7.1 The History of Cryopreservation

Interest in the effect of freezing on biological material has been recorded over many centuries and these early studies have reported many pertinent observations. For example in 1897 Hans Molisch published a report on the response of cells to subzero temperatures and ice formation. The observations of Molisch recorded both intracellular ice formation in amoebae when cooled to \(-9^\circ C\) and cell shrinkage in *Spirogyra* as ice crystals formed in the extracellular solution at approximately \(-3^\circ C\) to \(-6^\circ C\). Molisch concluded:

"The death of cells by freezing does not always occur in the same manner, but in three distinctive ways:

a) Cells freeze and actually solidify in the protoplast (*amoeba*, *Phycomyces*, filaments of the anther of *Tradescantia*).

b) Death by freezing occurs although the cell itself does not freeze. In this case water escapes from the cell and freezes on the outer surface of the cell wall where forms a sheath of ice which closely adheres to the cell. This is usually accompanied by tremendous shrinkage of the cell, (*Spirogyra*, *Cladophora*, *Derbesia*).
c) The processes described above may occur simultaneously in the same cell, ie dehydration and ice formation may occur at different sites of the same cell (Codium).

These observations were to prove very relevant for attempts at freezing cells over the next century. Molisch had described the relationship between the cells' aqueous compartments, the tendency for water to leave cells (accompanied by cell shrinkage) or freeze inside those that did not shrink during freezing. These differing responses have been used as a basis for developing hypotheses of freezing damage or survival. Invariably, the early work concluded that cells were damaged by cooling to low temperatures and freezing for any length of time.

1.7.2 Cryoprotectants

The discovery of the protection afforded by glycerol, the first chemical additive that was reported to protect cells during freezing and thawing, by Polge et al, in 1949 was a major breakthrough in the field of cryobiology. The survival of fowl sperm frozen in Ringers solution containing 10% - 40% glycerol led to the investigation of other chemicals for cryoprotective abilities. In the original study (Polge et al, 1949), propylene glycol and ethylene glycol, both of which are now used in cryopreservation protocols, were used and found to offer
some protection, although they were more toxic than glycerol to the spermatozoon. Following the discovery of glycerol, the other most commonly used cryoprotectant today, dimethyl sulphoxide (DMSO), was found to protect red blood cells from haemolysis following freezing (Lovelock and Bishop, 1959).

Cryoprotective compounds all have in common a low toxicity to the cells to which they are added and a high solubility in aqueous solutions. Due to their high solubility in water when ice nucleation occurs in the cell suspension the cryoprotective agents (CPA's) remains in solution in preference to crystallizing out. Water molecules are highly polar structures, the oxygen atoms having two unpaired electrons orientated away from the $O-H$ bonds. This gives rise to positively and negatively charged regions, which result in a single oxygen atom being bonded tetrahedrally to four hydrogen atoms, two covalently and two by $H$-bonds so each water molecule has four neighbours. In biological samples the structure of liquid water is more complicated due to the presence of solute molecules which interact with the water molecules. Polar solutes that can act as either acceptors or donators of electrons may result in the water molecule being orientated incorrectly for ice formation and inhibit crystallization. The presence of several cryoprotectants creates mixed solvent systems with properties very different to those of
pure water.

The method of action of the CPA varies with the additive used, although the non-electrolytes like glycerol, DMSO or sucrose have a colligative effect, reducing the increased concentration of the electrolytes and increased chemical potential of water caused by ice formation at a given temperature. The concentration of extracellular solutes in the unfrozen fraction increases as the temperature decreases, but independently of the original concentration prior to freezing. The concentration of solutes continues to increase during cooling until the eutectic temperature is reached, at which the mixture solidifies. Thus the total osmolal concentration of the unfrozen fraction is not influenced by the addition of CPA's while the relative concentration of salts is reduced in accordance to:

\[
M^e \text{ NaCl} = \frac{M'^e \text{ NaCl}}{(1 + R)}
\]

equation 1.2

where

- \( M^e \text{ NaCl} \) is the concentration of electrolyte in the presence of an additive.
- \( M'^e \text{ NaCl} \) is the concentration of electrolyte in the absence of an additive (assuming that NaCl is the major electrolyte).
- \( R \) is the osmolal ratio of additive : electrolyte prior to freezing.
Addition of any water soluble non-toxic chemical in high concentrations will reduce the amount of the system that has formed ice crystals at any particular temperature, and thus reduce the concentration of solutes. CPA's greatly depress the nucleation temperature, 8-cell mouse embryos nucleate intracellular ice at -10 to -15°C whereas the addition of 1 - 2 M glycerol or DMSO decrease this value to -38 to -44°C (Rail et al, 1983). Those CPA's that penetrate the cell may reduce the osmotic imbalance between the cell and the extracellular environment. However, some cell shrinkage is advantageous during freezing since it reduces the water content of the cell and thus the probability of intracellular ice formation.

It has been suggested (Arakawa et al, 1990) that CPA's stabilize proteins at low temperature due to the hydrophilic nature of the molecules. Cryoprotectants can be preferentially excluded from the hydration shell around the surface of a protein molecule and thus the protein is hydrated relative to the bulk solution. Although this situation is unstable denaturation would increase the surface area excluding more solvent, thus the native form of the protein has a greater stability. The decrease in surface area resulting from polymerisation results in the polymer being more stable than the individual units. At higher temperatures the hydrophobic nature of the solvent molecules is predominant and thus the solvent interacts
with the protein molecule and favours the denaturation. Thus, while at lower temperatures the CPA's can protect and stabilise proteins at higher temperatures they can be toxic.

However, the ability to protect cells from freezing damage is not merely a property of additives that can permeate a cell. Sugars such as sucrose and polymers such as polyethylene glycol can not enter across the plasma membrane but have some cryoprotective effects. Bovine red blood cells can be frozen equally well whether glycerol is allowed to permeate, or insufficient time is allowed for permeation in the same concentration of glycerol, or when an equivalent osmolality of sucrose is used (Mazur et al, 1974). As the solution containing the non-penetrating solute freezes, there is a concentrating effect in the unfrozen fraction which causes a progressive depression of the freezing point of the remaining solution. In addition to this colligative mechanism which is similar to that of the penetrating CPA's, the protective action of polymers may partially be due to their ability to "bind" water and prevent it from freezing.

1.7.3 Freezing Injury

Freezing cells to very low temperatures in physiological media results in the survival of only a few of the original cells, usually those frozen at extremely
rapid rates. Only with the discovery of cryoprotectants whose colligative properties protect against intracellular ice crystal formation was the interaction of more than one factor evident since the presence of intracellular ice usually leads to lethal cell damage. The discovery of glycerol and cell survival after freezing using CPA's allowed the investigation of other factors influential in cell mortality during a freeze / thaw cycle. These highlighted the importance of the velocity of cooling and warming on the rates of cell survival.

The initial investigations were performed on a wide range of cell types including human spermatozoa (Parkes, 1945), bull spermatozoa (Smith and Polge, 1950), mammalian ova (Smith, 1952), and red blood cells (Luyet, 1949). The survival curves of numerous cell types when plotted against rate of cooling were in the form of an inverted U, indicating an optimum rate of cooling. This suggested that there was an interaction of events causing cell mortality with opposite dependence on the velocity of cooling. Lovelock (1953a) proposed that damage caused during freezing at relatively slow rates in red blood cells was due to the high concentration of electrolytes caused by ice crystallization and the reverse dilution factor shock as ice melted on thawing. It was also suggested by Lovelock (1953b) that the protective ability of glycerol was due to the colligative effects of reducing the concentration of
these electrolytes. Peter Mazur in 1965 used these facts and his own observations to describe a "Two factor hypothesis" for freezing damage in which it was proposed that cell injury from a freeze / thaw cycle may be from separate effects depending on the freezing conditions. The first resulted from intracellular ice crystal formation at fast cooling rates and the second from prolonged exposure to high concentrations of solutes in the extracellular liquid phase during slow cooling. Thus, the degree to which each of these contribute to cell damage is dependent upon the cooling rate. These were explained as follows:

As the temperature of a cell suspension decreases the media and the cells remain in an unfrozen state (supercooling to about -5°C) before ice crystals start to form in the extracellular media. The precise temperature of nucleation is determined by the composition of the extracellular media, the presence of protective solutes and their concentration, and presence of nucleating agents in the mixture. Ice formation does not occur consistently at the equilibrium freezing point temperature and liquids in biological samples will supercool since a suitable interface must be created for a nucleation event to occur. Nucleation can be considered as a statistical phenomenon in which water molecules must form a crystal-like configuration. Random molecular motions produce a cluster of water molecules in a crystal nucleus form. These nuclei
must exist for a period of time long enough to allow condensation of other water molecules onto the nucleus and thus allow crystal growth. The lifetime of such a nucleus is inversely proportional to temperature and directly proportional to nucleus size. As the temperature decreases the likelihood of clusters existing for sufficient time to permit crystal growth is increased while the critical size of the nucleus required for crystal growth is decreased. In aqueous solutions this form of homogeneous ice nucleation is unusual and heterogeneous nucleation more common. The interface required for crystal nucleation is provided by particulate matter present in the solution and crystals form above the homogeneous nucleation temperature for pure water.

When ice forms in a cell suspension, due to the fact that the probability of nucleation is related to the size of the compartment, it occurs preferentially in the extracellular medium (Hobbs, 1974). Crystals propagate throughout the solution to re-establish thermodynamic stability, excluding cells and solutes into the residual liquid channels. Water leaves the liquid phase to form crystals, excluding solutes and so causing an increase in the concentration of the extracellular environment. As a result the residual solution and the cell are no longer in osmotic equilibrium. The water within the cell remains supercooled, with a higher chemical potential than that in
the surrounding medium. The chemical potential of water is a measure of the osmotic pressure, the activity of water, the volume of water and the solute concentration. Excessive supercooling is deleterious to the survival of the cell due to the tendency for intracellular ice crystal growth (Whittingham, 1977). The presence of an osmotically active intact semi-permeable plasma membrane may protect the cell from ice crystal propagation from the external medium.

The events that follow extracellular nucleation are dependent on the rate of any further temperature decrease imposed on the system. A cell cooled slowly will dehydrate rather than nucleate intracellular ice crystals, the plasma membrane acting as a barrier to crystal propagation from the extracellular solution. If the cell suspension is cooled slowly the water is able to leave the cell and cell contents approach equilibrium with the chemical potential of the extracellular water, even though the ice phase is continuously growing. The cell avoids the probability of intracellular ice crystal formation by this efflux of water and dehydration. If the rate of cooling is rapid, some water is able to leave the intracellular environment as ice forms extracellularly. However the water is unable to diffuse quickly enough to re-establish or maintain an equilibrium with the surrounding medium. The water becomes increasingly supercooled and eventually intracellular ice
crystals nucleate which serves to reduce the difference in the chemical potential of water between intra and extracellular environments (Fig 12).

The terms "rapid" and "slow" cooling are qualitative descriptions but an approximation can be applied to individual cell types which is determined by the membrane water permeability of the cell. Rapid cooling can be said to be the rate of cooling that does not permit osmotic shrinkage, and slow cooling as that allowing equilibrium shrinkage of the cell in response to increasing external solute concentration during a reduction of temperature.

At slow cooling rates, the probability of intracellular ice nucleation is low but the length of time taken for the freezing process exposes the cell to high extracellular solute levels for long times. However if the cooling rate is fast, the damaging events from high concentrations of solutes may be avoided but insufficient time is available for the cell to dehydrate to equilibrium water content, and intracellular ice crystals form. If the two curves for cell mortality resulting from these two forms of injury overlap then cell recovery will not be possible. It has since been shown in numerous cell types, e.g. unicellular algae (Morris, 1977), yeast cells (Mazur and Schmidt, 1968), and mammalian embryos (Whittingham et al, 1972), that survival curves take the form of an inverted U shape (Figure 13). This is consistent with the two dominant
factors, i.e. the solute effects at slow cooling rates and intracellular ice formation on rapid cooling, interacting to cause mortality.

It was proposed by Mazur (1963) that three characteristics of a cell have a major role in determining the response to freezing:

i) the membrane water permeability (hydraulic conductivity, $L_p$).

ii) the temperature coefficient of the membrane water permeability (the activation energy, $E_a$).

iii) the surface area to volume ratio.

These properties are all related to the water flux across the cell, which highlights the major importance of water in determining cell survival during freezing.

Evidence supporting the assumption that intracellular ice crystal formation is one cause of lethal cell damage can be obtained through experimental observations or mathematical modelling. The probability of intracellular ice formation is a function of the water retained within the cell at temperatures below the freezing point. Mazur (1973, 1977) used the qualitative description of the events observed during freezing to develop mathematical
Behaviour of Cells at Subzero Temperatures at Various Rates of Cooling

Figure 12
Survival as a Function of Cooling Rate for Various Mammalian Cells

Figure 13
equations to predict the rate of water loss from a cell and the degree of supercooling at a given cooling rate (Mazur, 1963, 1970, 1977, 1984). Mazur predicted curves for the cell water content as a function of the rate of temperature decrease and calculated the degree of supercooling in the cell as a function of the deviation of the calculated water content from the equilibrium value. The quantitative calculations for supercooling required that the cell membrane water permeability and its temperature coefficient were either known or could be estimated.

The degree of supercooling assuming a constant rate of cooling:

\[
\frac{L_f \cdot A \cdot L_p^g}{B \cdot v^o}
\]  
equation 1.3

where:

- \(L_f\) is the latent heat of fusion of ice
- \(A\) is the surface area to volume ratio
- \(L_p^g\) is the permeability coefficient of water
- \(B\) is the cooling rate \(\frac{dT}{dt}\)
- \(v^o\) is the molar volume of water

When a cell is cooled at an infinitely slow rate it is able to loose water and maintain zero gradient in the
osmotic pressure between the extra and intracellular environment. The water content within the cell at any point during cooling at this rate is the equilibrium water content. Since cells avoid intracellular ice formation during equilibrium cooling by dehydrating before reaching the internal ice nucleation temperature, the extent of supercooking provides an indicator to the probability of freezing. A cell with near-to-equilibrium water content will have almost 0 probability of intracellular ice formation whereas extensive supercooling will produce a probability of almost 1.

Using the equations derived by Mazur to predict the cooling rate and Lp values necessary to cause intracellular freezing, in particular cells, it has been found that the predicted conditions correspond closely to those in which cell death following freezing is high (Mazur, 1972, Diller, 1975, Leibo et al, 1984).

1.7.4 Fast Cooling

Rapid rates of cooling causes the water within the cell to become progressively supercooled and this, as predicted by Mazur, leads to ice nucleation of numerous small crystals. The presence of ice crystals is detrimental to cells resulting in injury which becomes visible on thawing. Many cell types have been investigated and been found to exhibit a relationship between cell mortality and
intracellular ice formation. Red blood cells (Rapatz et al, 1968, Diller et al, 1975), yeast cells (Mazur and Schmidt, 1968), HeLa cells (McGrath et al, 1975), hamster tissue culture cells (Mazur, 1972), and mouse ova (Leibo et al, 1984) have all exhibited increasing loss of cell viability as the occurrence of observed intracellular ice increases.

1.7.5 Slow Cooling

In contrast to damage resulting from intracellular ice formation, slow cooling, although protecting against intracellular ice, may have other deleterious affects. Damage from slow cooling may be mediated in numerous ways:

i) as a physical consequence of the presence of extracellular ice crystals.

ii) by a reduction in temperature per se.

iii) through prolonged exposure to high solute concentrations.

The mechanical effects of extracellular ice are possibly damaging to cells only when the suspension is densely packed or the cells from part of the integrated structure of a tissue. Individual cells may be injured by the
increase in solute concentration resulting in compression and torsion on the cells (Lovelock, 1953, Mazur et al., 1981). It is difficult to differentiate between these factors since they occur concurrently. Extracellular ice crystal formation leads to an increase in the osmolality and a decrease in the amount of solution remaining. Due to the decreasing residual liquid volume, the cells are forced into close contact. Either this physical proximity, or the high solute concentrations, may result in cell damage. Alternatively, the mechanical stress of the ice crystals may disrupt cell interactions or individual cell plasma membranes. Increases in solute concentrations can cause denaturation of proteins (including enzymes) by disrupting the molecular interactions responsible for maintenance of protein structure (Jaenicke, 1981). Hypertonic exposure can lead to membrane protein displacement (Neidermeyer, 1976) and release (Heber, 1981) which may involve damage to membrane pumps or other regulatory proteins. The proteins associated with the membrane will be exposed to high concentrations of solutes and alteration of pH, which may cause their denaturation. In addition removal of water from the cell and the cell membrane may cause a switch between the phase of the membrane lipids.

As the temperature is decreased, and the extracellular concentration increases, the cell dehydrates changing the
cell volume which will cause an associated surface area alteration. Cell shrinkage is associated with a decrease in the surface tension of the plasma membrane. A reservoir of membrane material may exist in the interior of the cell which may accommodate excess membrane displaced from the cell surface during shrinkage. Conversely as the concentration of the extracellular environment decreases during thawing and the cell re-expands, the membrane material may be available to be re-incorporated into the plasma membrane. If the change in the extracellular environment is too rapid, as the cell tries to re-expand the increase in surface tension may not be countered by reincorporation of sufficient of the membrane components into the plasma membrane to prevent expansion-induced lysis. Although this mechanism of cell injury has been observed in plant protoplasts mammalian cells have numerous microvilli and folds on their plasma membranes, this reservoir of material may be sufficient to allow the cells to increase their volume after dehydration without membrane lesion.

1.7.6 Rewarming

The extent to which the velocity of warming influences cell survival following freezing is dependent on the rate of cooling used for cryopreservation. Following cooling, any intracellular ice is present in a cell to various
degrees according to the details of the procedure used. Ice crystals present in a cell following rapid cooling can grow during the rewarming phase of a cryopreservation protocol when the rate of warming is slow enough to allow reconfiguration of small crystals into larger crystals which is thermodynamically favourable. When rapid thawing rates are applied damage to the cell under these conditions can be reduced since insufficient time exists for ice crystal growth before the melting point is reached. However during rapid thawing, the cell is potentially in osmotic imbalance with the extracellular environment which becomes progressively hypotonic and is subjected to "dilution shock" as the extracellular ice melts. This may cause rapid water influx into the cell. If the cell is unable to survive the large, rapid volume fluctuations, lysis may occur. In practice, rapid rates of warming have been used effectively with the majority of cell types, allowing good cell survival. However some exceptions to this have been observed, including embryos frozen at slow cooling rates using DMSO (Whittingham, 1977). In this situation, slow rewarming was found to be better for embryo survival. Any one or combination of these factors may contribute to the lethal cell damage occurring from the freeze/thaw process.
1.7.3 The Importance of Individual Parameters of Cell Composition in Predicting Cell Survival.

Since Mazurs' original quantitative theory, many of the simplifying assumptions made in the calculations have been examined (Silvares et al., 1975, Pushkar et al., 1976, and Mansoori, 1975). Whereas the introduction of alterations in most of the variables created little difference, in the amount of predicted water loss, variations in the Lp and Ea were extremely influential. Mansoori in 1975 queried the assumptions that the protoplasm of a cell is an ideal solution with a uniform temperature and a constant surface area. Cell dehydration during cooling alters the volume and thus decreases the surface area which in conjunction with the existence of a radial temperature gradient across the cell, may produce erroneous predictions of water loss during freezing. However, the non-ideality of the cell protoplasm was found to be significant only at high cooling rates and the temperature gradient across the cell only influential when a cell had a low initial water content (Mansoori, 1975). Varying the membrane surface area could cause considerable deviation from the predicted water loss and thus the degree of supercooling. More importantly, variations of the Lp or its activation energy could also create major deviations in the calculated water content of the cell at any particular temperature. These studies highlighted the fundamental importance of a cell membrane
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water permeability in calculating the velocity of water loss during freezing and thus the likelihood of intracellular ice formation.

1.8 Cryopreservation of Oocytes and Embryos

The survival of single-cell fertilised rabbit ova following the stepwise addition of glycerol to a final concentration of 15% when accompanied by slow cooling to -15°C was first reported by Smith in 1952. Although the survival after supercooling the solution was high (69% of the fertilised ova continued to divide when returned to culture conditions) in the same study dramatic reductions in the survival rate (1%) were observed on slow cooling to -79°C. In contrast to the work by Smith on fertilised eggs Sherman and co-workers investigated the effects of low temperature storage on the unfertilised oocyte. Ovulated mouse oocytes were found to retain viability as determined by staining techniques on cooling to 0°C and to fertilise after permeation by 5% glycerol (Sherman and Lin, 1958a). An extremely small number of oocytes frozen in solutions of medium containing glycerol fertilised to produce normal foetuses (Sherman and Lin, 1958b). In this study, it was observed that cells which froze internally as indicated by "black flashing" were invariably damaged, with disorganized cytoplasm and vitelline membrane.

Parkes and Smith (1953) established the survival of
the endocrine function of ovarian tissue after exposure to 15 % glycerol and freezing to -79°C or -196°C. Limited survival of primordial follicles (1 - 5 %) was achieved from grafts of cryopreserved ovarian tissue (Deanesly, 1957). Parott (1960) equilibrated tissue from rabbit ovary with 12 % glycerol for 30 - 40 minutes and retrieved a small number of viable oocytes after freezing which successfully fertilised to produce live young. Although promising, the work performed on ovarian tissue grafts was not pursued, probably due to problems associated with graft rejections encountered when transplanting tissue.

Subsequent investigations moved away from the unfertilised oocyte and once again concentrated on fertilised eggs. Concurrent reports by Whittingham et al, 1972 and Wilmut, 1972 reported live births following the transfer of 8-cell cryopreserved mouse embryos. Both reports were in agreement that for optimal survival using DMSO and slow cooling rates of between 0.22°C - 0.3°C / min were required to preserve the mouse embryo. It was assumed that with minor technical changes the protocol developed for the mouse would be transferable to other species. Indeed this was the case for rat (Whittingham, 1975) and rabbit embryos (Whittingham and Adams, 1976) which were successfully frozen using similar techniques. However it was discovered that for many species including sheep (Willadsen et al, 1976), cows (Wilmut and Rowson, 1973,
CHAPTER 1 - INTRODUCTION

Trounson et al, 1976), and goats (Bilton and Moore, 1976), fast thawing rates were necessary to achieve survival of cryopreserved embryos.

The difficulty experienced in transferring techniques for preservation of embryos of one species to others was highlighted by the work on animals. However, differences between species were complicated further by differences between the various stages in embryonic development used in cryopreservation. It became apparent that recovery was stage dependent from research into survival following freezing cow and pig embryos at various developmental stages (Polge et al, 1974). Prior to the 8-cell stage, the cow embryo was discovered to be extremely susceptible to freeze damage, whilst at the later developmental stages (the expanded blastocyst) it was relatively resistant to damage (Wilmut, 1973, Trounson et al, 1974, Wilmut et al, 1975).

The storage of human pre-implantation embryos at -196°C was recognized by Edwards and Steptoe (1977) as being extremely important in clinical I.V.F. programmes. The development of cryopreservation procedures for animal embryos had shown the possibility of freezing as a useful means of preserving genetic material without any harmful side effects from the storage period. It had been possible to successfully freeze and store the embryos of other mammalian species since 1972. However it was not until 1983
that the first pregnancy following the transfer of an 8-cell human embryo was reported (Trounson and Mohr, 1983).

Following research into and development of a successful cryopreservation protocol for embryos, research into low temperature storage of the unfertilised oocyte resumed. Techniques for the cryopreservation of species of rodent were studied in a series of investigations by Tsounda and co-workers (Tsounda et al, 1976, Parkening et al, 1976, Parkening and Chang, 1977). Using a slow cooling rate to various end point temperatures, a slow thaw rate and a variety of concentrations of DMSO, a small number of morphologically normal oocytes were recovered. In this study and the investigation by Whittingham (1977), fertilisation and subsequent development to the blastocyst level was achieved, although in both the numbers developing were expressed for a selected group of morphologically normal oocytes, thus overestimating the overall recovery of the initial population of oocytes.

Until Schroeder and Epigg (1990) reported successful preservation of immature oocytes at the germinal vesicle stage all research had concluded that immature oocytes were even more susceptible to damage on freezing. Using the protocol of slow freeze / slow thaw designed by Whittingham and previous workers for ovulated oocytes, in conjunction with techniques developed for in vitro maturation, high percentages of fertilisation and development of immature
oocytes were achieved. However, once again this was from a selected number of recovered oocytes.

Whilst much of the work on cryopreservation has focused on rodent ova, oocytes of other species including rabbit (Burks et al, 1965), and primates (De Mayo et al, 1985) have been fertilised following freezing. It has become increasingly attractive to be able to preserve the unfertilised human oocyte, a satisfactory and reproducible technique remains elusive. Although animal oocytes have now been frozen transferal of the technique has had limited success with human oocytes. Trounson, 1984 obtained 20% morphological survival of 80 oocytes released from an ovarian wedge, cultured for 48 h and subsequently frozen with fertilisation was the first published report. Even though three successful pregnancies and live births from oocytes frozen with 1.5 M DMSO have been recorded worldwide (Chen, 1986, 1988, Van Uem et al, 1987, Al Hasani, 1988) there is a great deal of room for improvement. The number of pre-ovulatory human oocytes surviving equilibration with cryoprotectants, freezing to subzero temperatures, storage and thawing is still too small to be a viable clinical option. The studies in my thesis aim to investigate the parameters of water movement across the cell membrane (Lp and Ea) which will have a major effect on designing a cryopreservation protocol.
2.1 Materials

All powdered media for culture and handling of oocytes, both mouse and human, were from Gibco Ltd, (Scotland) and any additional components were analytical grade obtained from either Sigma Chemical Company Ltd or BDH Chemicals Ltd. All water used in the preparation of media was Kendall Sterile Distilled Water (Water for injection), Kendall Laboratories Ltd, England.

2.2 Preparation of Media:

2.2.1 Stock Solutions

The methodology for preparation of Phosphate Buffered Saline Solution (PBS), Earles Balanced Salt Solution (EBSS), and Tyrodes' Salt Solution (T6) (Table 1, 2, 3) was similar in all cases. One litre of Sterile Distilled Water was used to dissolve contents of a single powder container, the contents were emptied into a sterile glass flask and a small quantity of water used to rinse out the container to ensure all traces of the powder were removed. The solution was gently agitated until the powder had dissolved and then made up to 500 mls, and the additional components for each medium added to this, taking
# CHAPTER 2 - MATERIAL AND METHODS

Table 1: The Composition of Dulbecco's Phosphate Buffered Saline Solution (PBS)

<table>
<thead>
<tr>
<th>POWDER</th>
<th>g / L</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$ (anhyd.)</td>
<td>0.159</td>
</tr>
<tr>
<td>KCl</td>
<td>0.200</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.200</td>
</tr>
<tr>
<td>MgCl$_2$ (anhyd.)</td>
<td>0.047</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.000</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>1.150</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OTHER COMPONENTS (MODIFIED)</th>
<th>g / L</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>1.000</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.012</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>0.036</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.600</td>
</tr>
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</table>
# Table 2: The Composition of Earle's Balanced Salt Solution (EBSS) Basic:

<table>
<thead>
<tr>
<th>Powder (Inorganic Salts)</th>
<th>g / L</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$ (anhyd.)</td>
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</tr>
<tr>
<td>KCl</td>
<td>0.4000</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.0977</td>
</tr>
<tr>
<td>NaCl</td>
<td>6.8000</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>2.2000</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$.H$_2$O</td>
<td>0.1400</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other Components (Modified)</th>
<th>g / L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Bicarbonate</td>
<td>1.000</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>0.011</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.600</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.500</td>
</tr>
</tbody>
</table>

**Culture Media:**

Remove 200 ml from Basic Media and add:

| Sodium Bicarbonate | 0.220 |

**Flushing Media:**

To the remaining 800 ml add:

| Hepes Solution (1M) | 16 ml |
| Heparin Injection B.P. (5000 iu/ml) | 1.6 ml |
Table 3: The Composition of Tyrode's Salt Solution (T6)

<table>
<thead>
<tr>
<th>POWDER (INORGANIC SALTS)</th>
<th>g / L</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ (anhyd.)</td>
<td>0.2000</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2000</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.0470</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.0000</td>
</tr>
<tr>
<td>Na₂HPO₄·H₂O</td>
<td>0.0500</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OTHER COMPONENTS (MODIFIED)</th>
<th>g / L</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>1.000</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.005</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>2.106</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>0.055</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.600</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.500</td>
</tr>
<tr>
<td>Sodium Lactate (70 % w/w)</td>
<td>3 ml</td>
</tr>
</tbody>
</table>
CHAPTER 2 - MATERIAL AND METHODS

care that each had totally dissolved. Those components that may have caused precipitation of salts, such as calcium chloride, were added after the solution had been diluted further almost to the litre mark.

The final few mls of water were added using a Pasteur pipette, the solution was then shaken thoroughly to ensure complete mixing of the components and bottled in sterile 250 ml flasks (Falcon, Becton Dickinson and Co, U.K.) for storage at 4 - 5°C for later use. Fresh media were made every 2 weeks. For individual components of each medium refer to Tables 1, 2, and 3.

A slight alteration from the general technique should be noted for preparation of the media for human I.V.F. once made upto the litre (See page 95).

2.2.2 Hormones For Superovulation

The administration of exogenous gonadotrophins to mimic FSH and LH is common practise for studies requiring mouse oocytes. The hormonal stimulation produces large numbers of oocytes from a small number of animals. Both pregnant mares' serum gonadotrophins (PMS) used in animal stimulation to mimic FSH, and hCG used to simulate the natural LH surge, are water soluble and were made - up into working concentrations of 50 I.U. / ml by dilution with 0.9 % saline solution (0.9 % NaCl, Baxter Healthcare Ltd, U.K.). The stock solutions were frozen in 1ml aliquots for
CHAPTER 2 - MATERIAL AND METHODS

use later when animals were injected with 0.1 ml.

i) 1000 I.U. stock PMS (Folligen, Intervet, UK.)
diluted with 20 mls 0.9 % saline solution to a
final concentration of 50 I.U. / ml.

ii) 5000 I.U. stock hCG (Gonadotrophin L.H., Pabryn
Ltd, U.K.) diluted with 100 mls of 0.9 % saline
solution to a final concentration of 50 I.U. / ml.

2.2.3 Hyaluronidase Medium

Removal of the cumulus cells was achieved using 150 iu
/ ml bovine testicular hyaluronidase (Sigma, U.K.) 10 mg
of 295 units / mg solid hyaluronidase were dissolved in
19.67 ml of PBS and 1 ml aliquots dispensed for freezing
and storage for later use.

2.3 Glassware
2.3.1 Preparation of Micropipettes

Preparation of micropipettes was simple and rapid, and
to avoid contamination they were made as required. However,
this meant the technique must consistently produce
micropipettes with a small diameter capillary bore to avoid
delays in handling the oocytes. The thin section of a
sterile Pasteur pipette was rotated in the flame of a
bunsen burner until the glass was soft, the pipette was then removed from the flame and pulled rapidly to a fine bore capillary. The pipette was snapped and the bore size compared microscopically to that of the oocytes for transfer. If the pulled pipette was too small for the oocytes, it was broken further up the barrel. Trying to aspirate an oocyte in a mismatched pipette could result in severe damage such as zona breakage, ooplasm rupture, or, if the pipette had a wide diameter, loss of the oocyte. In addition, the capillary was not used if the end was jagged since this could result in puncturing the oocyte.

2.3.2 Sterilisation of Glassware

All glassware used in both mouse and human I.V.F. and media preparation was soaked in 7X-PF Phosphate Free Laboratory Detergent (Flow Laboratories, UK.) overnight and washed in sterile water to remove all traces of detergent. The glassware was then placed in an oven at 250°C for 24 h, allowed to cool and stored with silver foil caps in sterile conditions until required.
2.4 Mouse Oocyte Collection and Culture

2.4.1 Media for In Vitro Fertilisation in Mouse

Mouse oocytes were fertilised and cultured in Tyrodes' Balanced Salt Solution (T6, Table 3). This was prepared by gassing 10 ml of T6 stock solution with a compressed gas mixture of 5% O₂, 5% CO₂, and 90% N₂ using a sterile pipette (Serological pipet, Falcon, Becton Dickinson & Co, U.K.) until there was a distinct colour change from pink to orange as a result of stabilisation of the pH brought about by the CO₂ / HCO₃⁻ buffer system at pH 7.3. The alteration in colour was a result of the presence of phenol red indicator in the medium which was insoluble in liquid paraffin oil and non toxic to mouse ova and embryos. When the pH was stable 40 mg of Bovine serum albumin, (Catalogue No. 7888, Fraction V, 96.99% Albumin, BSA, Sigma, U.K.) were added to the 10 mls of T6 stock solution (T6, 4 mg / ml BSA) and dissolved by gentle agitation of the flask to avoid frothing of the protein. 5 ml of the T6 4 were removed, while to the remainder a further 60 mg of BSA were added and allowed to dissolve (T6, 16 mg / ml BSA). Using a 5 ml sterile syringe (Plastipak, Becton Dickinson & Co, U.K.) and filling tube (Universal Hospital Supplies Ltd, U.K.) the medium was filtered (20 μm pore size, Millipore, Molsheim, France) to remove impurities.

Microdroplets of approximately 40 μl in volume T6-4 to
allow observation of embryo development were pipetted using a sterile Pasteur pipette onto culture dishes (Falcon, Becton Dickinson & Co, U.K.). The droplets were covered with liquid paraffin oil (at 37.8°C Kinematic viscosity 30 centistoke, BDH Ltd, U.K.), to prevent changes in composition and concentration due to evaporation from the small volume microdroplets. In addition, the coating of oil prevented pH changes and rapid alterations in temperature when the dishes were removed from the incubator for observation.

T6-16 was used to prepare fertilisation dishes for mouse oocytes. An organ culture flask (Falcon, Becton Dickinson & Co, UK.) was used for fertilisation, and 1 ml of medium was placed in the outer chamber with 0.9 ml in the centre well. The dishes were placed in a CO₂ incubator (5% CO₂ in air, LEEC, UK) and stored in an inner chamber (Modular Incubator Chamber, Flow U.K.) which was gassed with 5% CO₂, 5% O₂, and 90% N₂ for a 5 - 10 minute period before the valves leading into and out of the chamber were sealed and the dishes allowed to equilibrate overnight at 37°C.

The liquid paraffin oil used to coat the culture droplets was sterilised prior to use by heating in an oven at 160°C for 1 h, after which it was removed, cooled and 30 ml of sterile T6 added. The oil was equilibrated with the T6 medium and vigorously gassed with 5% CO₂, 5% O₂, and 90%
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N₂ to ensure the oil did not absorb the gases or any of the constituents from the T6 medium which may be slightly soluble in oil. Any such oil solubility could have resulted in alterations in the composition of the microdroplet. The oil was allowed to settle from the aqueous phase and decanted into tissue culture flasks (250 ml, Falcon, Becton Dickinson & Co, UK), which were stored at 4 - 5°C and re-gassed prior to use.

2.5 Isolation of Mouse Oocytes
2.5.1 Collection of Ovulated Oocytes

Virgin C57Bl x CBA F₁ hybrid females aged between 8-10 wks were superovulated by intraperitoneal injections of 5iu of PMS followed 48 - 52 h later by 5 iu of hCG. The oviducts were isolated 13 h post hCG injection after ovulation when the oocytes were located in a single large mass in the ampullary section of the oviduct. The stimulated females were sacrificed by cervical dislocation, placed on their backs on absorbent paper. The abdomen was soaked with 70% alcohol to reduce the risk of contamination from the hair as the skin was removed. Forceps were used to grasp the skin and a small cut made at the midline. The skin was pulled sharply backwards towards the head while holding the skin below the cut firmly to expose the body wall.

A fine pair of forceps was used to hold the peritoneum
and fine scissors used to make two lateral cuts to reveal the organs in the body cavity. The coils of the intestine were placed to one side to expose the two horns of the uterus, the oviducts and the ovaries. The mesenteric attachments to the ovary and oviducts and the surrounding fat were cut away to avoid interference with oocyte identification on liberation. A cut was made between the oviduct and approximately 1 mm below the utero-tubal junction, and then by holding the severed end of the oviduct, a second cut was made between the ovary and the ampulla (Fig 14). To prevent drying, the ovaries were transferred immediately into Modified Dulbecco's Phosphate Buffer (PBS, Gibco, Whittingham, 1974) supplemented with 4 mg/ml BSA.

A dissection microscope was used to locate the ampulla (Fig 14, 15a) which was identifiable by its expanded appearance and prominent longitudinal epithelial folds. The caudal tube of the uterus was grasped using a pair of watchmaker forceps and the oocytes were released into PB1 by tearing the membrane with the needle of a fine insulin syringe, using ciliary action or gentle stroking of the ampulla to aid the release (Fig 15b). A pipette with an aperture wide enough to allow the cumulus mass to pass easily through was filled with medium to prevent the large, ovarian "sticky" cell mass adhering to the glass pipette, and the mass was removed.
Dissection of Reproductive Organs of a Female Mouse
A) Alimentary Tract displaced to reveal reproductive organs
B) Reproductive organs

Figure 14. Dissection of female mouse.
Figures 15a and 15b: Magnification Factor x 20.

Figure 15c: Magnification Factor x 75.

Figure 15d: Magnification Factor x 900.

Figure 15e: Magnification Factor x 2100.

Figure 15f: Magnification Factor x 600.
Figure 15. a) Swollen ampulla isolated 13h post hCG injection. b) Cumulus mass released from the ampulla. c) Cumulus free mouse oocytes. d) Ovary isolated 8h post hCG injection. e) Pre-Ovulatory oocyte on release from the follicle. f) Cumulus free pre-ovulatory oocyte.
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The cumulus masses were transferred to hyaluronidase solution (150 mg/ml in PBS) to dispersed the cumulus cells and the ova were then removed and washed three times in PB1 (Fig. 14c). It has been reported that the cumulus mass offers little more resistance to water movement than the zona (Powers and Tupper, 1974). It was thus only necessary to remove the cumulus cells surrounding the oocyte to allow the unobstructed observation of the shrinkage response in the microscope diffusion chamber. A clear view of the plasmamembrane was required since the membrane water permeability coefficient was calculated from measurements of the cross sectional area of the oocyte. Care was taken when handling the oocytes that they were kept at 37°C, and this was achieved by holding the dishes containing the ovaries and oviducts on a heated plate set at 37°C. Thereafter, all manipulations were performed on the heated stage.

2.5.2 Collection of Pre-Ovulatory Oocytes.

F₁ virgin hybrid C57Bl x CBA females were stimulated as described for the production of ovulated oocytes. The oocytes were collected 8h post hCG injection using the same dissection techniques as for removal from the oviducts in the collection of the ovulated oocytes, but now the ovaries were included in the tissue mass excised (Fig 14, 15e). The majority of oocytes remained in the follicles at this
point, although a small number of blood filled follicles were visible, suggesting ovulation of some of the mature follicles had already begun. Bloody fluid or clots indicated ovulation had occurred a short time prior to harvesting i.e. within the last 12 h. The follicles were punctured using the needle of a fine insulin syringe, the oocytes were released into PB1 (Fig 15f, 15g), and placed into hyaluronidase until the cumulus cells dispersed. The oocytes were removed and washed three times in PB1.

2.6 Classification and Recognition of Abnormal Morphology in Mouse Oocytes and Embryos.

Prior to experimentation and *in vitro* culture used to test cell viability, it was necessary to assess the oocytes *in vitro* for normal appearance since damage resulting from experimental treatments needed to be accurately assessed and not confused with degeneration naturally occurring *in vivo*. To this end, morphological criteria were used to grade the oocytes. Immediately after release from the cumulus those oocytes lacking a zona, empty zona, and atretic oocytes which were dark, granular, and often shrunken in appearance (Fig 16a, 16b) were discarded as this suggested degeneration had commenced.

A second criterion of abnormal configuration or shape of the oospheres was used for oocyte exclusion from I.V.F. culture. When culturing the oocytes after experimentation
Figures 16a, 16b, 16c and 16d: Magnification Factor x 400.

Figure 16 a) and b) Atretic oocyte recovered from the ampulla. c) and d) Fragmented oocyte released from the ampulla.
the fact that each cleavage stage has a characteristic blastomere size and tiered arrangement was utilized as proof of normality. Although there are variations in blastomere shape, gross deviations from the expected shape suggest an abnormal embryo. The most difficult criterion to judge was fragmentation which was a disorganized cell division resulting in blastomeres of uneven sizes. It was difficult to distinguish between fragmentation and normal embryonic development at late stages (Fig 16c, 16d). Misjudging early stages of development as fragmented was less likely than discriminating later cleavage stages and fragmentation. If the embryo appeared to be at a developmental stage inappropriate to the length of time in culture it could be called fragmented.

2.7 _In Vitro Fertilisation of Mouse Oocytes_

Spermatozoa were collected from two different C57Bl x CBA F₁ hybrid males aged 12 wks which were sacrificed by cervical dislocation and then placed on absorbent paper. The abdomen was soaked with 70% alcohol to reduce the risk of contamination from the hair as the skin was removed. Forceps were used to grasp the skin and a small cut made at the midline, the skin was pulled sharply backwards towards the head while holding the skin below the cut firmly to expose the body wall.
A fine pair of forceps was used to hold the peritoneum and fine scissors used to make two lateral cuts to reveal the organs in the body cavity. The testes of the male were located in the scrotal sac or in their retracted position in the canal which contained fatty tissue (Fig 17a). The fatty tissue was dislodged and used to pull the testis and cord free by manipulation with a pair of blunt forceps.

The epididymis was located by following the vas deferens to the head and the whole epididymis removed and placed into 1 ml of T6-16 medium prepared for the mouse I.V.F. (Fig. 16b). The cauda epididymis and vas deferens are naturally used to store large numbers of sperm which allowed collection of a semen sample free from seminal fluid and avoided coagulation of the spermatozoa in the presence of air. The vas deferens was stroked along its' entire length to assist extrusion of a dense pencil of sperm. The epididymis was repeatedly punctured with a fine gauge needle to allow the sperm to enter into suspension. The spermatozoa rapidly became motile and formed an homogeneous suspension. The testes from two different C57Bl x CBA F1 hybrid males aged between 10 - 12 weeks were used in each fertilisation dish to ensure a mixed population of sperm as a check against the chance of a single male producing abnormal sperm.

After incubation at 37°C in an atmosphere of 5% CO2, 5% O2, and 90% N2 for 1 ½ h to allow capacitation, the sperm were diluted 1 : 10, (0.1 ml of outer well added to centre
Dissection of Reproductive Organs of a Male Mouse
A) Alimentary Tract displaced to reveal reproductive organs
B) Reproductive organs

Figure 17 a) Dissection of a male mouse showing b) the reproductive system.
Figures 18a, 18c, 18d, and 18e Magnification Factor x 600.

Figure 18b : Magnification Factor x 750.

Figure 18f : Magnification Factor x 300.
Figure 18. Developmental stages of the mouse embryo. a) 2-cell  b) 4-cell  c) 8-cell  d) morula  e) blastocyst  f) hatching blastocyst.
well of 0.9 ml T6). A number of samples of the outer well sperm solution from multiple fertilisation dishes were counted on a Horwell Fertility Semen Counting Chamber to determine the final concentration which was approximately 2 x 10^6 / ml. Oocytes were released directly from the oviducts (refer to section 2.5.1) into the sperm suspension immediately on collection. Following an incubation period of 5 - 6 h the oocytes were recovered, transferred through a series of droplets to remove excess sperm, and cultured in T6 4 under paraffin oil (refer to section 2.4.1). The oocytes were examined 24 h post insemination for fertilisation as determined by development to two-cell embryos, and they were then observed over a 4 - 5 day period to observe whether development continued to the hatching blastocyst stage (Fig 18a - 18f).

2.8 Human Oocyte Collection and Culture

2.8.1 Media for Human In Vitro Fertilisation

For each patient 25 ml of stock Earles Balanced Salt Solution (EBSS) was decanted and bubbled with 5% CO₂, 5% O₂, and 90% N₂ until the pH stabilized at pH 7.2 - 7.3 as in mouse IVF media preparation. To each 25 ml of EBSS 0.5 ml of Human Serum Albumin was added (HSA, Blood Products Laboratory, Herts., UK.). The solution was filtered as for the mouse I.V.F. and 1 ml pipetted into 5 ml culture tubes
(Falcon, Becton Dickinson & Co, UK.) for incubation at 37°C. The HSA-supplemented medium was also used for sperm preparation (refer 2.10.1).

2.8.2 Isolation of Fresh Human Oocytes

Fresh human oocytes were donated following informed patient consent from the Royal Free Hospital human I.V.F. programme. Multiple follicular growth was achieved following an initial period of down-regulation and suppression of the endogenous pituitary gonadotrophins with administration of Buserelin (D-Ser tBu6 LH-RH 1-9 ethylamide, Hoechst UK Ltd.) for approximately 15 days prior to the onset of stimulation. The ovary was then stimulated by injections of HMG, (Pergonal, Serono Laboratories Ltd, UK), 300 iu days 1-5 followed by 150 iu days 6-10. The follicles were monitored using ultrasound scanning on days 6, 8, and 10 until at least three follicles measured 18mm (Figs, 19a - 19d). The endometrium was monitored to assist judgement of readiness of the patient for oocyte recovery. In addition serum hormonal assays were used for oestrogen, progesterone and LH from day 8 of the treatment cycle to monitor for LH surges and pre-operative ovulation. The oocytes were then recovered 34-36 h after 5000 iu of hCG, (Profasi, Serono Laboratories Ltd, UK.), using either vaginal ultrasound or laparoscopy.
For ultrasound, vaginal egg collection using a DRF 250 scanner (Diasonics U.K. Ltd) and transvaginal probe (7.5 MHz Transducer), the patient was under intravenous sedation using of 100mg Pethidine (Roche Products Ltd, U.K.), 10 mg of Diazimuls (Dumex Ltd, U.K.), and 12.5 mg of Stemetil (May & Baker Ltd, UK). The vaginal probe was covered with a sterile sheath and non-spermicidal condom (Casmed UK Ltd) which was filled with sterile contact jelly ensuring no air was trapped between the transducer and the cover which would result in interference in the scan picture. The pelvis was scanned and the plane of the follicles identified prior to puncturing the lateral vaginal fornix for access to the follicles. A needle guide was attached to the probe and a double lumen aspiration needle (Casmed UK Ltd) passed down the side of the guide and through the vagina and follicle wall. Weak suction was applied, (200 mmHg pressure) to avoid damaging the oocyte on removal from the follicle (Suction Unit, Rockett of London Ltd, UK.), and the needle gently rotated. The follicular fluid was collected in a pre-warmed tube (Falcon, Becton Dickinson & Co, UK.) and the tubes stored in a heated test-tube rack (Grant Instruments Cambridge Ltd, U.K.) for examination of the fluid by the embryologist. The follicle was then flushed with EBSS media and again suction was applied and the flush collected for
Figure 19.
Ultrasound monitoring a) the endometrium and the human ovary b) quiescent c) day 8 d) day 14 (pre-operatively).
examination. The follicle was flushed until an oocyte was identified or up to a maximum of 5 times. The fluids from the follicle and flushes were examined under a dissection microscope using sterile conditions to identify granulosa cells or cumulus mass which appeared as transparent areas in the coloured fluid. Oocytes were then transferred to clean flush medium and washed three times before being placed in 1ml of EBSS supplemented with HSA, and incubated at 37°C in an atmosphere of 5% CO₂, 5% O₂, 90% N₂ for 5 - 6 h. Following this, in those oocytes donated for research, the cumulus mass was removed using hyaluronidase and gentle agitation with a micropipette (Fig 20a - 20d). For routine I.V.F., the oocytes were identified from the follicular aspirations, graded and stored in 1 ml in EBSS supplemented with 4 mg / ml HSA at 37°C for 5 - 6 h to complete the maturation process prior to insemination. The oocytes were examined for pronuclei 16 - 20 h post insemination and after 48 h for cleavage and cell division.

2.8.3 Failed-to-Fertilise Human Oocytes

In addition to fresh excess oocytes, failed to fertilise oocytes (Ff) were donated for research. After collection, the oocytes were placed with sperm and incubated for 20 & 48 at 37°C in an atmosphere of 5% CO₂, 5% O₂, 90% N₂ after which they were examined for signs of fertilisation or development. Those oocytes that showed
no signs of fertilisation but retained a normal morphology and were perfectly spherical (Fig 21a, 21b) were used for permeability studies.

2.8.4 Maturation of the Human Oocyte

The technique of human I.V.F. necessitates the removal of pre-ovulatory oocytes directly from the follicles. This necessarily results in removal of the oocytes from exposure to follicular fluid and granulosa cells. The constituents of the intrafollicular environment may influence the stage of maturity and thus the ability to fertilise the oocyte. Additional time is allowed in I.V.F. for the human oocyte in culture to complete nuclear and cytoplasmic maturation. The aim of this is to avoid polyspermia and increase the fertilisation rate (Trounson et al, 1982).

Oocytes were matured for 5 - 6 h prior to use in permeability studies. A second group of oocytes were matured for a prolonged period of time (24 h) to determine whether a prolonged maturation time affected cell membrane water permeability characteristics. Thirdly, a group of oocytes were examined immediately after collection to see if in vitro maturation had any effect on the membrane permeabilities.
2.8.5 Oocyte Grading

The maturity of the human oocytes was assessed immediately on collection by studying the morphological characteristics of the cumulus / corona cell complex. The grade given to the oocyte allowed decisions concerning the timing of insemination and of any experimental study on the membrane permeability to be made rapidly with as little stress to the oocyte as possible. The criteria used were;

i) Expanded cumulus mass - were the cells compact or a loose aggregation?

ii) Coronal layer - were the cells radiant, expanded, or compact?

iii) Ooplasm - were the germinal vesicle or the first polar body visible, was the cytoplasm granular or dark in colour?

Mature oocytes had an expanded cumulus mass and corona radiata, the first polar body was extruded (Fig 20a, 20b) whereas immature oocytes had compact cumulus and the corona cells were compact and attached to the zona pellucida, and the germinal vesicle was still visible (Fig 20c, 20d). Microscopic observations of the morphology of the oocytes were recorded using a scale of 1 to 3, with 1 being equal to an immature oocyte and 3 a mature oocyte.
Figure 20a: Magnification Factor x 210.

Figure 20b: Magnification Factor x 310.

Figure 20c: Magnification Factor x 250.

Figure 20d: Magnification Factor x 400.
Figure 20: a) Fresh human oocyte, expanded cumulus cell complex. b) Mature oocyte with 1st polar body visible. c) Immature human oocyte, cumulus mass compact. d) Immature human oocyte with germinal vesicle visible.
Figure 21 Failed-to-fertilise human oocyte a) Morphologically normal oocyte. b) Abnormal non-spherical oocyte. Magnification Factor x 400.
2.9 Human I.V.F. and Culture

2.9.1 Preparation of Spermatozoa

The male partner involved in the IVF treatment was asked to abstain from ejaculation for 3 days prior to production of the semen sample since the length of abstinence alters both the number and motility of sperm (Mortimer et al, 1985). The sample was produced by masturbation into a sterile specimen jar (Sterilin, Middlesex, U.K.) to provide an uncontaminated semen sample. The semen sample for insemination was produced 2 h after oocyte collection, and allowed to liquefy for 40 minutes at room temperature. A 1 ml sample of the whole semen was then diluted 1:4 with EBSS supplemented with 4 mg/ml HSA, mixed thoroughly and spun at 600 x g for 10 minutes to remove the seminal fluid. The supernatant was aspirated to leave a pellet of sperm which was then resuspended in EBSS and centrifuged for a further 10 minutes. After the second wash, the supernatant was again removed and 1 ml of the modified EBSS was layered onto the remaining pellet of sperm. The motile sperm were allowed to "swim-up" after incubating the spun cells at 37°C in a CO₂ incubator. The migrated sample was then assessed for sperm quality by placing a drop of the swim-up on a Makler Counting Chamber with a 10 x 10 grid used to measure the following parameters:
2.9.2 Assessment of Sperm Parameters

a) Sperm Count:

Both the count per ml and the number in the total semen specimen were recorded. The number of spermatozoa in 10 of the counting chamber squares was used to indicate the number of sperm in 1 ml of semen. A number of grid square rows was counted and an average taken to account for spermatozoa moving into and out of squares. The total sperm count was the number of sperm / ml x volume of semen.

Normal estimations of spermatozoa / ml were considered to fall within the range 20 - 200 x 10^6 / ml. In cases of less than 20 x 10^6 / ml, the patient was classified as oligozoospermic, a complete absence of sperm was azoospermic, and more than 350 x 10^6 / ml was polyzoospermic.

b) Motility (Quantitative)

Sperm showing any movement were said to be motile and this was expressed as a percentage of the total sperm count:

\[
\% \text{ Motile sperm} = \frac{\text{No. motile sperm / ml}}{\text{Total no. / ml}} \times 100
\]
Normal sperm motility parameters were taken as 50 % or above.

c) Progression ( Qualitative )

Progression was used as a subjective indication of sperm quality and was graded on a scale of 1 - 4 by the type of movement shown.

Grading of Sperm Progression

<table>
<thead>
<tr>
<th>Definition</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Poor</td>
<td>Weak, slow forward movement</td>
</tr>
<tr>
<td>2 Moderate</td>
<td>Slow but definite progression observed</td>
</tr>
<tr>
<td>3 Good</td>
<td>Relatively fast movement</td>
</tr>
<tr>
<td>4 Excellent</td>
<td>Very rapid, vigorous movement</td>
</tr>
</tbody>
</table>

Normal analysis was a count of 3 or above.

d) Sperm Morphology ( Abnormal forms )

Sperm were observed for head and tail abnormalities such as a reduced or enlarged head size or duplicate heads. Tail abnormalities included coiled tails preventing movement, double or short, truncated tails, all of which prevent fertilisation. The abnormal forms were expressed as a percentage of the sperm density. A normal semen analysis was considered to have less than 40 % of abnormal sperm.
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In addition, the number of white cells were noted since their presence in a semen sample suggested inflammation and possible infection.

For I.V.F., the sample was diluted to a concentration of $1 \times 10^6$ / ml. From the $1 \times 10^6$ / ml stock solution 0.1 ml was used for insemination to produce a final concentration of $\approx 100,000$ / ml in the tubes containing the oocyte.

2.10 Microscope Diffusion Chamber

To determine the dynamic osmotic response of the oocyte, a diffusion chamber (Fig 22, 23), (McGrath, 1985) was developed allowing direct observation of volumetric alterations following changes in the external solution. The chamber was fitted to a Nikon inverted microscope capable of taking both still pictures using a Nikon FE2 camera and video pictures using a Panasonic camera and U-matic Sony Video Cassette Recorder. Built into the chamber body were two flow channels running independently of one another. The outer flow channel ran around the edge of the chamber surrounding the inner channel and contained fluid from a circulating water bath. The chamber body was copper to facilitate heat transfer between the sample / outer heat exchanger compartment, and the outer heat exchanger fluid / pre-cooled water which was pumped to control the temperature of the stage. The inner flow channel ran across the centre of the chamber.
over a clear plastic coverslip and through an outlet pipe providing the bathing medium for the oocyte.

The sample region of the diffusion chamber was a circular brass top fitting on to which a glass coverslip was fixed to allow visualisation of the sample. The top fitting was placed in an oven, a layer of melted wax placed on the coverslip and then left to solidify on a flat surface. A Pasteur pipette was used to bore a hole in the wax to contain the sample.

Prior to an experimental run the temperature of the controlling water bath was set at the desired value and then allowed to equilibrate. The perfusing system was pre-flushed with isotonic medium since this minimized the chance of incorporating air bubbles in the system, reduced the friction and thus the likelihood of tearing the membrane on lowering the sample region into the bulk flow area. The oocytes ( one human or several mouse ) were pipetted into the sample region of the diffusion chamber ( Fig 24 ) and a dialysis membrane ( Cuprohan M80, Enka AG, FDR ) which had been soaked in distilled water for 24 h ( to remove the glycerol used for the preservation of the membrane ) was positioned over the oocytes. The dialysis membrane absorbed the shear stresses of the bulk flow, keeping the oocytes stationary for accurate observation. The solute diffusivity for the dialysis membrane was reported by the manufacturers to be one tenth that of the
CHAPTER 2 - MATERIAL AND METHODS

diffusion coefficient in free solution. The sample chamber was inverted into the bulk flow area and the pump system started for continuous flow with isotonic medium at a rate of 20 ml / min. The position of an oocyte in the sample region was determined by calculating the sample region thickness using fine focusing, and measuring the depth from the glass coverslip to the top of the wax. The oocyte's position was assigned depending on its distance from the base of the coverslip and was then assumed to be constant throughout the run. The isotonic medium was changed to a hypertonic solution of known osmolality ( Table 4 ) and the osmotic response of the oocyte was monitored using video microscopy which recorded digital time and date using a Video Timer ( VTG - 12, E. S. Video, London. ) and the diameter change. The start of hypertonic perfusion was signified by the passage of a bubble across the sample region which was introduced between the isotonic and hypertonic media. This was taken as time 0 for measurements of diameter change in response to hypertonic exposure. A number of thermocouples ( NiCr / NiAl, Comark Ltd, U.K. ) connected to a digital temperature display unit were used to monitor the temperature at various points in the experimental perfusion system ( Fig 25 ). These included the temperatures of the fluid in the circulating water bath, of the fluid at the centre of the sample region and of the fluid leaving the bulk flow region ( Table 5 ).

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The diffusion chamber system was set up to allow either multiple experiments with different cells at the same experimental conditions or for a single cell experiment for observation under different conditions such as multiple-step changes in the extracellular concentration or multiple changes in temperature. Using these variations of experimental conditions, the normalized osmotically inactive volume of the cell, the cell membrane water permeability, and the Arrhenius activation energy for water transport were determined as described in section 2.10.4.

After each set of experiments data measurements were made by replaying the video recording. The "pause" function was used to allow the cell diameter to be measured directly across three axes from the TV. screen (Video Monitor 5470, Panasonic, UK). The magnification factor was calculated by focusing on an eye piece graticule (Graticule Ltd, England), a recording made and the distance between the centre of adjacent divisions measured. The distance measured on the video monitor was converted to a magnification factor:

Graticule Scale - 100 divisions = 5 mm

1 division = 0.05 mm

Microscopic Measurements -

2 divisions = 126 mm
1 division = 63 mm
CHAPTER 2 - MATERIAL AND METHODS

Conversion - 0.05 = 1 division = 63 mm

0.05 = 63

magnification factor

63 / 0.05 = 1260 x

2.10.1 Non - Equilibrium Experiments

Determination of the Membrane Water Permeability (Lp).

The membrane water permeabilities of individual oocytes were obtained from measurements of changes in the cell diameter (Table 6). The bathing medium of the oocyte was changed from the isotonic baseline (PBS; 290 mOsm) to a hypertonic solution (0.5 M NaCl; 920 mOsm) and the diameter history recorded over a 5 minute period at 20°C with measurements being taken every 5 seconds.

The parameters of the system and the diameter measurements were entered into a computer model (SENS) used to predict the diffusion of a binary solution (NaCl) across the dialysis membrane. Changes in the extracellular solute concentration in the sample region were then calculated over time and used with the Kedem-Katchalsky membrane transport model (Kedem and Katchalsky, 1958, Sherban, 1987) to produce a best estimate of the cell membrane hydraulic conductivity based on the predicted and experimental data. An ordinary least squares method was used to minimise the error between the functional values.
Figure 22a: Chamber base measures 11.0 cm.

Figure 22b: Brass ring measures 3.0 cm.
Figure 22 a) Microscope diffusion chamber used for the derivation of the permeability parameters of the oocyte. b) The sample region of the microscope diffusion chamber.
Figure 23 Schematic representation of the microscope diffusion chamber.
Legend to Figure 25.

A - Sample Chamber.
B - Brass Ring.
C - Dialysis Membrane.
D - Sample Region.
E - Bulk Flow Perfusate.
F - Thermocouple.
G - Outer Temperature Regulating Chamber.
Figure 24 Representation of the sample region of the microscope diffusion chamber.

Figure 25 Representation of the microscope diffusion chamber showing thermocouples for monitoring the temperature.
### Table 4 Concentration of Hypertonic Solutions in Non-Equilibrium and Equilibrium Experiments

<table>
<thead>
<tr>
<th>Molar Concentration</th>
<th>Osmolality (mOsm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M NaCl</td>
<td>0.410</td>
</tr>
<tr>
<td>0.3 M NaCl</td>
<td>0.600</td>
</tr>
<tr>
<td>0.4 M NaCl</td>
<td>0.790</td>
</tr>
<tr>
<td>0.5 M NaCl *</td>
<td>0.980</td>
</tr>
<tr>
<td>0.7 M NaCl</td>
<td>1.395</td>
</tr>
</tbody>
</table>

* 0.5 M NaCl was used for both the non-equilibrium and equilibrium experiments.
### Table 5  Temperature of the Diffusion Chamber: Overall System

<table>
<thead>
<tr>
<th></th>
<th>Circulating Water Bath</th>
<th>Media Input Point</th>
<th>Centre of Sample Chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td>40.3</td>
<td>37.1</td>
<td>37.4</td>
<td></td>
</tr>
<tr>
<td>30.8</td>
<td>30.0</td>
<td>30.0</td>
<td></td>
</tr>
<tr>
<td>18.9</td>
<td>20.2</td>
<td>19.8</td>
<td></td>
</tr>
<tr>
<td>6.3</td>
<td>10.1</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>-4.9</td>
<td>0.1</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

Room Temperature 22°C - 25°C

All temperatures recorded as °C
CHAPTER 2 - MATERIAL AND METHODS

created by the mathematical model and the experimental measured values. An example of the computer input data is listed for information purposes.

2.10.2 Computer Input: Sens Parameters for Lp estimation

1) Dialysis membrane thickness (RL1)
2) Cell chamber thickness (RL2)
3) Cell position (Lip)
4) Diffusivity in cell chamber (D2)
5) Diffusivity in dialysis membrane (D1 = D2/10)
6) Mass transfer coefficient (Hd = 1000*D2)
7) Initial concentration - osmolality mOsm (CINIT)
8) Final concentration - osmolality mOsm (CINF)
9) Inactive volume % (VINA)
10) Magnification factor (R MAG)

1) Time step - sec (DT)
2) Time range - sec (TM0, TM1)
3) Permeability step - μm/sec (DP)
4) Permeability range (P0, P1)
5) Investigating permeability (PSENS)

2.10.3 Normalized Inactive Volume (Vb).

For this measurement an oocyte was initially perfused with isotonic medium and the diameters recorded. It was then perfused for a 10 minute period with several step -
wise changes of media of increasing concentration and allowed to come to equilibrium cell volume at each and the diameter again recorded (Table 7). The osmolality of each solution was measured with a freezing point osmometer (Table 6). The oocyte went through a total of five increases of medium and the diameter taken at the end of each step. The normalized inactive volume was obtained by a Boyle - Van't Hoff plot from the equilibrium volumes at the various concentrations versus \( \frac{1}{\text{solute concentration}} \).

2.10.4 Activation Energy. (\( \text{E}_a \))

The temperature - dependence of the movement of water across the cell membrane was studied by changing the temperature at which the membrane water permeability values were calculated. The temperature of the circulating water bath and thus the heat exchange system was set over a range of values from 37°C to 0°C (including 37°C, 30°C, 20°C, 10°C, and 0°C). Additional methodology concerning the investigation specifically at 0°C which caused an unusual cell response can be found in Chapter 5. Both equilibrium and non - equilibrium experiments were carried out at all the temperatures. The relationship which expressed the temperature dependent nature of the movement of water was derived from the classic Arrenhius equation.

\[
P = K \exp \left[ - \frac{\text{E}_a}{RT} \right]
\]

equation 2.1
CHAPTER 2 - MATERIAL AND METHODS

where

\[ K \text{ is a constant} \]
\[ \text{Ea is the activation energy} \]
\[ T \text{ is temperature } ^\circ\text{K} \]
\[ R \text{ is the gas constant} \]
\[ P \text{ is the membrane water permeability} \]

This can be re-written as:

\[
\ln (P) = \ln (K) - \frac{\text{Ea}}{RT}
\]

\[ \text{equation } 2.2 \]

and by plotting \( \ln (P) \text{ v.s. } \frac{1}{T} \) the activation energy can be determined from the slope of the graph produced.
CHAPTER 2 - MATERIAL AND METHODS

Table 6

**Computer Input Data File : Sample**

The values entered were:

1) **Dialysis membrane thickness** 0.160E+02 microns
2) **Cell chamber thickness** 4.000E+02 microns
3) **Cell position (5 - 11)** 5
4) **Diffusivity in cell chamber** 0.780E-10 M*M/sec
5) **Diffusivity in dialysis membrec** 0.780E-09 M*M/sec
6) **Mass transfer coefficient** 0.780E-05
7) **Initial concentration** 0.28
8) **Finial concentration** 1.00 Osmoles
9) **Inactive volume %** 39
10) **Magnification factor** 1260

The values entered were:

1) **Time step** 5.00 sec
2) **Time range** 0.0 - 310.00 sec
3) **Permeability step** 0.25 microns/sec
4) **Permeability range** 0.00 microns/sec to 40.00 microns/sec
5) **Investigating permeability** 15.00 microns/sec
Table 6 (continued) The data points entered were:

(including the time delay of .000 sec)

<table>
<thead>
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<th>Time (J) (sec)</th>
<th>Diameter (J) (cm)</th>
</tr>
</thead>
<tbody>
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<td>5</td>
<td>6.07</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>6.05</td>
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<td>5.07</td>
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<tr>
<td>44</td>
<td>300</td>
<td>5.07</td>
</tr>
</tbody>
</table>
## Table 7

**Values for a Boyle Van't Hoff Plot**

<table>
<thead>
<tr>
<th>C⁻¹</th>
<th>0.2 M</th>
<th>0.3 M</th>
<th>0.4 M</th>
<th>0.5 M</th>
<th>0.7 M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(0.72)</td>
<td>(0.48)</td>
<td>(0.36)</td>
<td>(0.28)</td>
<td>(0.21)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diameter (cm)</th>
<th>5.62</th>
<th>5.52</th>
<th>5.10</th>
<th>4.98</th>
<th>4.67</th>
</tr>
</thead>
</table>

Diameter in Isotonic Media 6.07cm

when a value of C⁻¹ = 1.00

the value for V = 1.00
CHAPTER 3 - PERMEABILITY PARAMETERS

CHAPTER 3

The Membrane Water Permeability and Related Coefficient of Temperature Dependence for Unfertilised Oocytes.

3.1 Aims

The experiments presented in this section were concerned with the derivation of the Lp, and Vb of murine ovulated oocytes, fresh human oocytes, and failed-to-fertilise human oocytes over a range of temperatures including 37°C, 30°C, 20°C, 10°C and 5°C. From the calculated Lps, the coefficient of the temperature dependence (Arrhenius activation energy, Ea) of Lp was determined.

3.2 Introduction

The passive movement of water across biological membranes and in particular the equilibrium and non-equilibrium responses of cells to osmotic stress have been indicated as important predictors of the response of a cell to freezing (Mazur 1963, 1977). There have been a number of studies investigating the osmotic properties of a wide range of cell types including tumour cells (Hempling, 1960), red blood cells (Savitz et al, 1964), mouse oocytes (Leibo, 1980), corneal cells (Armitage, 1983),
liposomes (Callow and McGrath, 1984), and hamster ova (Shabana and McGrath, 1988). These studies utilized a number of techniques including stop flow spectrophotometry (Savitz et al., 1964, Hempling, 1960) and a diffusion chamber (Callow and McGrath, 1984, Shabana and McGrath, 1988).

The development of a microscope diffusion chamber and associated computer programme for data analysis (McGrath, 1985) overcame problems associated with determining the transient osmotic response of individual cells by techniques such as light scattering, coulter counter analysis, optical techniques or diffusion chambers developed for the observation of tissues (Spring and Hope, 1978). The microscope diffusion chamber allowed the continuous observation of an individual cell or a small number of cells in microvolumes. By recording the volumetric alterations of the cell directly using real time, the properties of each cell, rather than an average of the population, were available providing information concerning the inherent diversity within a population. Since repeated measurements could be easily performed on individual cells, the microscope diffusion chamber created the opportunity for calculation of the $L_p$, $V_b$ and the $E_a$ of unfertilised human oocytes which were impossible to obtain in large enough numbers to use other techniques such as coulter counter systems.
The membrane permeability parameters (Lp and Ea) dictate the response of the cell to changes in the concentration of the extracellular environment, such as would be experienced during cryopreservation, by determining the rate at which water could leave the cell. Both the Lp and Ea were described for the mouse oocyte by Leibo in 1980, Bernard et al 1988, and Toner in 1990. However for the unfertilised human oocyte these basic measurements have not been made. Since the Lp and Ea for the mouse oocytes had been established it was possible to use these values as a comparison for the human oocyte. In addition to the membrane permeability characteristics, it should be pointed out that the surface area/volume ratio differs between mouse and human, which may affect freezing responses. The mouse oocyte is approximately 85 µm in diameter with a surface area of \(2.3 \times 10^{-2}\) mm\(^2\) and a volume of 270 pl and thus has a larger surface area to volume ratio than a single cell human oocyte which measures 150 µm in diameter and has a surface area of \(7.07 \times 10^{-2}\) mm\(^2\) and a cell volume of 800 pl.

The Boyle - Van't - Hoff Law states that if a cell behaves as an ideal osmometer it will exhibit an inverse relationship between the external osmotic pressure and cell volume when in an equilibrium state. Since the SENS permeability estimation was dependent upon this relationship it was necessary to calculate the inactive volume of oocytes for both mouse and human to ensure the
assumption was correct. The \( V_b \) was measured by equilibrium perfusion experiments with a range of hypertonic sodium chloride solutions as described in Chapter 2, Section 2.11 and the volume at each concentration plotted in a Boyle–van't Hoff form. The cell diameters were transformed into a measure of the relative cell volume using equation 3.1.2.

From the ratio of the diameter:

\[
\hat{d} = \frac{d_1}{d_o}
\]

3.1.1

the ratio for volumes can be derived:

\[
\hat{V} = \frac{(d_1)^3}{(d_o)^3}
\]

3.1.2

where

- \( d_1 \) is the diameter in hypertonic saline
- \( d_o \) is the diameter in isotonic PBS

The relative volume is plotted against the inverse concentration ratio:

\[
\hat{C} = \frac{C_1}{C_o}
\]

3.1.3

where

- \( C_1 \) is the concentration of the hypertonic saline
- \( C_o \) is the concentration of isotonic PBS
3.3 The Cell Membrane Water Permeability

The $L_p$ was determined from the change in the cell volume of the oocyte with respect to time when subjected to an impermeable solute of known osmolality. The rate at which the water left the cell could be seen from the computer generated plots of the experimental and predicted normalised cell volume (Fig 26a, 26b). The initial steep section of the slope indicated the immediate response of the cell to hypertonic perfusion when the differences between the extracellular and intracellular concentrations were large and water efflux rapid. As the cytoplasm became increasingly concentrated and the differential between the osmotic potentials of the solutions approached equilibrium, the rate of water loss slowed as was reflected in the slope of the graphs of cell volume which gradually plateaued. A comparison of the experimental and predicted cell volumes indicated, from the extent to which the two plots diverged, the accuracy of the membrane water permeability value generated by the computerised Kedem-Katchalscky equations (Appendix 1).

The parameter estimation programme SENS was used to generate theoretical values for the cell volume and to compare these predicted values to the experimental data (Table 7). Appendix 1 gives a full explanation of the
Figure 26 Representative plots of change in volume with time on hypertonic (0.5 M NaCl) perfusion of mouse oocytes at 20°C showing kinetics of water transport for a) $L_p = 0.48$ and b) $L_p = 0.76$
method used by the SENS computer programme to calculate these values. From the best fit curve generated for the shrinkage response, the cell membrane water permeability could be calculated for both mouse and human oocytes over a temperature range 37°C to 5°C (Tables 8, 9, 10). The temperatures at which the Lp's were investigated were 37°C, 30°C, 20°C, and 10°C. An additional temperature, 5°C, was used when determining the Lp for fresh human oocytes. In addition, failed-to-fertilise human oocytes were examined at the same temperatures as the murine ova and a comparison made with the fresh human oocyte. This was important because if the Lp for failed-to-fertilise and fresh oocytes were similar, investigations of Lp could be made from oocytes that were of no use to the patient and allow fresh ova to be used for other purposes.

By plotting the relative volume against the inverse of the concentration and extrapolating the resulting best fit straight line to an infinite concentration, the Vb was derived. The Vb's of both individual ovulated unfertilised murine and human oocytes were calculated at each temperature. Values for the mouse oocytes were pooled and a mean taken, whilst the large standard deviations and wide range of values determined for the human oocytes meant a mean value had little significance and individual values must be used in the SENS programme.
3.4.1 The Permeability Parameters of the Ovulated Mouse Oocyte.

A preliminary group of 20 ovulated mouse oocytes were examined at 20°C and the Lp for each individual oocyte determined from the volumetric change in response to hypertonic perfusate (Table 8, 28). The Lp data were pooled for the derivation of a mean value and standard deviation which was then compared to the permeability values generated, using a variety of methods, for mouse oocytes in previous studies (Leibo, 1980, Bernard et al, 1988, Toner, 1990). The mean value of 0.48 ± 0.18 μm / atm / min compared favourably with that determined by Leibo of 0.44 ± 0.03 μm / atm / min for the unfertilised mouse oocyte at 20°C determined using the difference in the density of cytoplasm as the oocyte dehydrates and measurements of the cross sectional area (Leibo, 1980).

A preliminary report in which Lp values were determined using both the microscope diffusion chamber (Bernard et al, 1988) and the associated SENS software (Sherban, 1987) calculated a mean Lp of 0.36 ± 0.07 μm / atm / min. Again, this corresponded well with that predicted in my present study. It was therefore assumed, due to the comparable nature of the Lp data, that the microscope diffusion chamber permitted the accurate determination of oocyte membrane transport parameters.

Subsequent to the derivation of Lp at 20°C, the
investigative temperature was altered within the range 37°C to 10°C including 37°C, 30°C, 20°C, and 10°C. Again the individual Lps were calculated for each oocyte and the values pooled to derive a mean value (Table 8). A one-way analysis of variance was used to determine whether any differences in the Lp values calculated for the mouse oocyte occurred over the temperature range investigated. A value of $P < 0.0001$, indicating a high level of significance was determined. Further analysis using a least difference test calculated a significant difference between the Lps for mouse oocytes between each of the temperature groups ($P < 0.05$). Finally, a high level of significance was assigned ($P < 0.0001$) to the probability of the Lp values decreasing with decreasing temperature using the linear contrast test. As the temperature was decreased the mean Lps calculated exhibited a decline indicating a temperature dependence for Lp. At 37°C the mean Lp was 1.24 ± 0.29, at 30°C 0.80 ± 0.33, 0.48 ± 0.18 at 20°C, and at 10°C 0.28 ± 0.06 (µm / atm / min). The fall in the mean Lp values was duplicated by a fall in the range of values derived (from 1.75 - 0.84 µm / atm / min at 37°C to 0.41 - 0.19 µm / atm / min by 10°C) indicating a trend towards slower movement of water across the membrane barrier.
3.4.2 The Arrhenius Activation Energy of the Ovulated Mouse Oocyte.

The temperature coefficient of the cell membrane water permeability was calculated from the Lp data accumulated at each temperature (Appendix 2). The natural logs of the mean Lp values were plotted against the inverse of the temperature (°K) using the value determined at 20°C as a reference temperature (Tg). A best fit line was plotted using a Curfit computer programme (Apple, MacKintosh), and from the slope of the line the Arrhenius activation energy (Ea) was calculated (Fig 27). An Ea of 9.48 KCal/mol calculated for the ovulated mouse ova which was comparable to those previously published (Leibo, 1980, Toner, 1990). It should be noted that the Curfit Programme calculates a coefficient of determination (R² value) for the least squares plot of the straight line fitted through the data points. The R² value was an indication of the accuracy of the best fit line with 1.00 corresponding to an ideal fit and 0.00 meaning the line did not fit the data at all. For the Arrhenius plot of Ea for the mouse an R² of 0.99 was calculated suggesting a close linear fit between the line applied and the experimental data.

In the study by Leibo (1980) both fertilised and unfertilised mouse oocytes were investigated and found to have Ea values of 13.0 KCal/mol and 14.5 KCal/mol respectively. Toner (1990) investigated the Lp and Ea of
unfertilised ovulated mouse ova at subzero temperatures in the presence of ice using cryomicroscopy and determined a value of 13.3 ± 2.5 KCal / mol. Both of these values correlate favourably with that of 9.48 KCal/ mol calculated in my study, thus again establishing the Lp and Ea values obtained with the microscope diffusion chamber (McGrath, 1985) in comparison to other authors' work and validating the use of the diffusion chamber for permeability parameter analysis.

3.4.3 The Inactive Volume of the Ovulated Mouse Oocyte

The Vb values for the unfertilised mouse oocytes fell within a narrow range and thus although the individual values were derived (Table 8, 29) the data calculated at each temperature were pooled and mean values determined for use in the SENS parameter estimation (Fig 28). The mean Vb values determined varied little with respect to temperature ranging between 18.50 ± 3.30 % and 20.50 ± 3.83 %. The results for the mouse oocytes compared favourably to the Vb values calculated by Leibo (1980), Bernard et al (1988), and Toner (1990) which were 18 %, 20 % and 21 % respectively.

3.5.1 The Membrane Water Permeability of Fresh Human Oocyte.

The mean Lp value of 0.39 ± 0.12 μm / atm / min
calculated for fresh pre-ovulatory human oocyte at 20°C was similar to the Lp values determined using the microscope diffusion chamber for the ovulated mouse oocyte of 0.48 ± 0.18 μm / atm / min ( Fig 29 ). In addition, this value compared well to those determined in other studies for the mouse oocyte at 20°C ( Leibo, 1980, Bernard et al, 1988 ). However there was only a slight change in the mean Lp values with respect to an alteration in temperature, indicating that the Lp for the human oocyte may be less dependent on temperature than in the mouse, or alternatively, the temperature dependence may be masked by the large standard deviations ( Table 8, 9 ). However, using a one-way analysis of variance the overall difference determined was statistically significant at P < 0.05. This difference between the Lp values detected using the one-way ANOVA test, on analysis with the least significant difference test, was due to differences between the groups 37°C and 5°C and secondly 30°C and 5°C ( P < 0.05 ). Lastly, analysis using a linear contrast test to determine the likelihood of Lp decreasing as temperature decreased in a correlative way calculated a high level of significance at P < 0.005 for the decrease in values. The decrease in the mean Lps observed with decreasing temperature was not
### Table 8 The Permeability Parameters of Murine Ovulated Oocytes

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Mean Lp (µm / atm / min)</th>
<th>Number (n)</th>
<th>Mean Vb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>1.24 ± 0.29</td>
<td>22</td>
<td>19.30 ± 3.65</td>
</tr>
<tr>
<td>30</td>
<td>0.80 ± 0.33</td>
<td>24</td>
<td>20.50 ± 3.83</td>
</tr>
<tr>
<td>20</td>
<td>0.48 ± 0.18</td>
<td>20</td>
<td>19.40 ± 2.70</td>
</tr>
<tr>
<td>10</td>
<td>0.31 ± 0.11</td>
<td>20</td>
<td>18.50 ± 3.30</td>
</tr>
</tbody>
</table>
Figure 27. An Arrhenius plot of the mean Lp with respect to temperature to calculate the activation energy for the unfertilised mouse oocyte.

A Representative Plot of the Inactive Volume of an Ovulated Mouse Oocyte

Figure 28. A representative Boyle Van't Hoff plot of the osmotically inactive volume of the mouse oocyte.
accompanied by a change in the spread of Lp values recorded for the individual oocytes, and at all temperatures a wide variation in Lp values was evident (Table 9, 30). At 37°C there was a mean Lp of 0.58 µm / atm / min, while the values estimated for the individual oocytes were within a broad range of 0.16 – 1.07 µm / atm / min. Lowering the temperature to 30°C caused a concomitant decrease in the mean Lp to 0.53 µm / atm / min whilst the range increased to 0.14 – 1.22 µm / atm / min. By 5°C the mean had fallen to 0.25 ± 0.09 µm / atm / min with a range of between 0.14 – 0.40 µm / atm / min.

3.5.2 The Arrhenius Activation Energy of The Fresh Human Oocyte.

Plots of the Arrhenius activation energy were made from the mean Lp data derived for the human ova (Fig 30). The Ea values established from the slope of the least square fit were extremely low, at 3.73 KCal / mol for fresh human oocyte with a coefficient of determination (R² value) of 0.77. Although the R² value was relatively high, the standard deviations around the predicted means of the Lp at each temperature were large and made the statistical significance of the apparent decline less certain.
### Table 9 The Permeability Parameters of the Fresh Human Oocyte

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Mean Lp (μm / atm / min)</th>
<th>Number (n)</th>
<th>Mean Vb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>0.58 ± 0.32</td>
<td>10</td>
<td>34.00 ± 10.2</td>
</tr>
<tr>
<td>30</td>
<td>0.53 ± 0.39</td>
<td>10</td>
<td>28.29 ± 5.47</td>
</tr>
<tr>
<td>20</td>
<td>0.39 ± 0.12</td>
<td>10</td>
<td>25.10 ± 7.95</td>
</tr>
<tr>
<td>10</td>
<td>0.43 ± 0.21</td>
<td>10</td>
<td>25.38 ± 8.23</td>
</tr>
<tr>
<td>5</td>
<td>0.25 ± 0.09</td>
<td>10</td>
<td>30.70 ± 5.31</td>
</tr>
</tbody>
</table>
Chapter 3 - Permeability Characteristics

Figure 29. Shrinkage kinetics of two representative fresh human oocytes at 20°C in 0.5 M NaCl solution.

Figure 30. An Arrhenius plot of the mean Lp values calculated for the fresh human oocyte to determine the activation energy.
3.5.4 The Inactive Volume of Fresh Pre-Ovulatory Human Oocytes.

Although the Vbs calculated for the individual mouse oocytes both within each set temperature and between different temperature groups were similar, the values for the fresh human oocytes varied widely within each group (Fig 31a, 31b). Although the mean Vb values at the different temperatures varied (34.0 ± 10.2% at 37°C, compared to 25.10 ± 7.95% at 20°C) no significance could be attached to these differences since the values derived at each temperature were so diverse (Table 8, 31). The extremes of the values derived for the fresh human oocyte were a minimum of 14% and a maximum of 48%.

3.5.1 The Permeability Parameters of Failed-to-Fertilise Human Oocytes.

The mean Lp of 0.62 ± 0.32 μm / atm / min determined for the Ff human oocytes at 20°C was larger than that for both the ovulated mouse oocytes and the fresh pre-ovulatory human. In addition, the individual Lp values derived for the Ff oocytes within a temperature group were extremely varied ranging between 2.19 - 0.28 μm / atm / min at 37°C and 1.17 - 0.19 μm / atm / min at 10°C (Table 10, 32). In comparison to the fresh human ova, which exhibited a slight decline in mean the Lp values with a decrease in the investigative temperature, the failed-to-fertilize human
ova showed an even greater degree of scatter, with values showing little significant relationship to the temperature. Although at 37°C the mean Lp of 0.96 μm / atm / min was estimated which was much greater than that for the fresh ova and comparable to the mouse Lp at 37°C an extremely large standard deviation (0.65 μm / atm / min) was calculated. The Lp fell to 0.37 ± 0.14 at 30°C only to rise again at 20°C to 0.62 ± 0.32 μm / atm / min and remain at this value even though the temperature was reduced to 10°C (0.62 ± 0.30 μm/atm/min). The inconsistency of the results for the mean Lp value (Table 10) in conjunction with the large standard deviations made the temperature dependency of the Lp ambiguous.

3.5.2 The Arrhenius Activation Energy of the Failed-to-Fertilise Human Oocyte

The Arrhenius activation energy for the failed to fertilize oocyte was 1.03 KCal / mol with an R² value of 0.03 which suggested a poor correlation for the temperature dependence for the permeability of those oocytes classified as failed-to-fertilize (Fig 32). The difficulty associated with fitting a straight line to the mean Lp data for both the Ff and fresh human oocyte combined with the large standard deviations at each temperature made the application of a single Ea value from pooled data unreliable.
Figure 31 Boyle Van't Hoff plots of fresh human oocytes, a) Vb of 16 % and b) Vb of 48 %.
Table 10 The Permeability Parameters of the Failed-to-Fertilise Human Oocytes

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Mean Lp (μm / atm / min)</th>
<th>Number (n)</th>
<th>Mean Vb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>0.96 ± 0.65</td>
<td>11</td>
<td>30.00 ± 8.98</td>
</tr>
<tr>
<td>30</td>
<td>0.37 ± 0.15</td>
<td>10</td>
<td>35.17 ± 9.24</td>
</tr>
<tr>
<td>20</td>
<td>0.62 ± 0.32</td>
<td>10</td>
<td>26.62 ± 6.00</td>
</tr>
<tr>
<td>10</td>
<td>0.62 ± 0.30</td>
<td>10</td>
<td>25.00 ± 4.85</td>
</tr>
</tbody>
</table>
Figure 32 The Arrhenius activation energy of the failed-to-fertilise human oocyte.
3.5.3 The Osmotically Inactive Volume of the Failed-to-
Fertilise Human Oocyte.

The individual Vb values calculated at each temperature for the Ff oocytes were as equally varied as those
determined for the fresh human material (Table 10, 33). However, as was the case with the fresh oocytes, the
differences exhibited at each temperature for mean Vbs, which ranged between 25.00 ± 4.85 % and 35.17 ± 9.24 %,
were not statically different. Due to the variation in the Vb of both fresh and Ff human oocytes the value correlating
to the individual cell was used for the permeability calculations of its' own Lp. This was in contrast to the
mouse experiments.

Summary

i) The Lp values of 1.24 ± 0.29 at 37°C, 0.80 ± 0.33 at
30°C, 0.48 ± 0.18 at 20°C, and 0.28 ± 0.06 at 10°C
were calculated for the mouse oocyte. An Ea for Lp of
9.48 KCal / mol was derived with a good linear fit of
the data.

ii) For the fresh human oocytes these values were 0.58 ±
0.32 at 37°C, 0.53 ± 0.39 at 30°C, 0.39 ± 0.12 at
20°C, 0.43 ± 0.21 at 10°C, and 0.43 ± 0.09 at 5°C. The
standard deviations of Lp at each temperature were
large. An Ea value of 3.73 KCal / mol with a less
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clear, but still significant, linear relationship, was calculated.

iii) The Lp values of 0.96 ± 0.63 at 37°C, 0.37 ± 0.15 at 30°C, 0.62 ± 0.32 at 20°C, and 0.62 ± 0.30 at 10°C were determined for Ff human oocytes. Again, standard deviations were large, and when an Arrhenius plot was constructed to yield a value for Ea, a poor linear relationship between Lp and temperature was produced. Under these circumstances the Ea value of 1.03 KCal/mol has little statistical validity.

From the results in this chapter, my work demonstrated that the microscope diffusion chamber yielded values for Lp and Ea in mouse oocytes comparable to previous reports. The values obtained with Ff human oocytes were so variable that these oocytes may be of little practical use in studying the membrane permeability characteristics. For fresh human oocytes, values of Lp and Ea were of the same order as the mouse, but there were greater individual variations between oocytes at a given temperature. Considering these facts, I decided to investigate the temperature-dependent alterations in Lp in the same, individual fresh human oocyte in the following chapter. Studies were also designed to address the possible reasons for the variability in the fresh human oocytes.
CHAPTER 4

The Membrane Water Permeability and the Activation Energy of Pre-Ovulatory Murine Oocytes, Fertilised and Unfertilised Human Oocytes.

4.1 Aims

The results presented in this chapter aim to elucidate the cause of the variability observed in Chapter 3 for the $L_p$ and $V_b$ values calculated for the fresh and $F_f$ human oocytes by examining:

i) pre-ovulatory mouse oocytes collected 8 h post hCG.

ii) fresh human oocytes immediately after collection (5.5 h) or prolonged culture for 24 h.

iii) pronucleated human oocytes after culture with sperm for 19 h.

iv) individual fresh human oocytes at each of the temperatures investigated for calculation of the $E_a$. 

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4.2 Introduction

It was apparent from the Lps and the value for Ea calculated from the mean Lp values over the range of investigative temperatures for the fresh human oocytes that the variability within the population was large. Protocols for egg collections in human I.V.F. required the recovery of ova immediately prior to ovulation when they were still retained in the follicles. When collecting the mouse oocytes for experimental investigations of Lp and Ea, in my study, and those by Leibo (1980), Bernard et al (1988), and Toner (1990), the usual method for oocyte retrieval was to liberate the cumulus masses from the ampulla after ovulation had been induced by the administration of exogenous hCG. Thus while human oocytes were pre-ovulatory, mouse oocytes had undergone ovulation and exposure to the oviductal environment.

To investigate the cause of the greater variability in the human oocyte data it was necessary to ascertain the membrane water transport properties of pre-ovulatory mouse ova and to compare this to the response of both ovulated mouse oocytes and the pre-ovulatory human material.

A second area of interest in my studies was the maturation period which is normally built into I.V.F. protocols between the egg collection and the addition of sperm. The lag incorporated in the procedure is to take into account the additional time the oocyte would have
remained in the follicle before being released into the oviduct following ovulation and the time spent in this environment prior to insemination. To allow for this extra time required to complete maturation, after collection the fresh human oocytes in our programme were matured for 5 \( \frac{1}{2} \) h at 37°C in EBSS medium and a humidified atmosphere of 5 % CO\(_2\), 5 % O\(_2\), 90 % N\(_2\) (as described in section 2.9.1) prior to incubation with sperm. This culture period was replicated in my membrane permeability studies of the fresh human oocytes in which the oocytes were incubated for 5 \( \frac{1}{2} \) h prior to removal of the cumulus cells and observation of the dynamic response to hypertonic perfusion. The degree of scatter in the Lps calculated for the fresh human oocytes led us to be interested in the influence of the time spent in culture between the collection of the oocyte from the follicle and the hypertonic perfusion for Lp analysis. The effects of varying this culture period on the Lp of the fresh human oocyte were studied by determining the permeability parameters of fresh human oocytes at 20°C and 10°C. Oocytes were studied either immediately following recovery from the follicular environment or alternatively after an extended period of culture in EBSS at 37°C and an atmosphere of 5 % CO\(_2\), 5 % O\(_2\), 90 % N\(_2\).

A third approach to investigating the variability within the fresh human oocyte population was to use oocytes in which maturity was indicated by the ability of a
spermatozoa to fertilise the egg. Since it has been suggested (Lopata and Leung, 1988) that although sperm may penetrate the zona at any point during development, entry through the vitelline membrane increases with maturity. In addition to an increase in receptivity, the oocyte becomes better able to incorporate the sperm chromatin. Extrusion of the second polar body was observed in those oocytes which were fully mature to meiotic metaphase II. It was possible therefore by studying ova in which two pronuclei had been identified to study only those that were fully mature and fertilised. In addition, I was interested to investigate whether fertilisation itself could lead to a change in the Lp and Ea values for the human oocyte. Although Leibo (1980) had found that the Lp and Ea for the pronucleated single cell mouse ova were not significantly different from that in the unfertilised ovulated oocytes no data was available for the human oocyte. This information was of value as a high survival rate has been shown following cryopreservation of pronucleated human eggs (Testart, 1987). If Lp and Ea were similar for pronucleate fertilised and unfertilised eggs the extent to which the rate of water loss contributed to cell death during oocyte cryopreservation would be in question. To elucidate the cause of mortality, other factors would need to be studied such as the permeability coefficient of the unfertilised oocyte to cryoprotectants.
Permeability to glycerol has been shown to increase 3 x on fertilisation and continue to increase with increasing time following fertilisation (Jackowsi, 1977, Jackowsi et al, 1980).

Fourthly, since calculation of a mean value for Ea using populations of fresh human oocytes was unsatisfactory due to the large standard deviations about the mean Lp at each temperature, individual Ea values for a single fresh human oocytes were derived. The Lps at different temperatures for the same oocyte were measured and an Arrhenius plot made to determine a value for the Ea. If Eas could be calculated for each oocyte the range of values produced could give an indication of the temperature dependence of human ova and the variability inherent in the Ea values.

4.3 Materials and Methods

4.3.1 Investigation of Pre-Ovulatory Murine Oocytes.

The ovaries were isolated from female hybrid mice (C57Bl x CBA) 8 h post hCG administration as described in section 2.5.2 and the pre-ovulatory oocytes released into PB1 by puncturing the follicles visible on the ovary surface. After washing the oocytes to remove the blood released along with the ova, they were transferred into hyaluronidase (150 iu / ml) and manipulated to remove the
surrounding cumulus cells. Those oocytes from which the cumulus cells could not be removed or were denuded on release and thus difficult to locate were discarded. It has been suggested that these oocytes were immature or in the early stages of degeneration (Swartz and Schuetz, 1977). The oocytes were pipetted into the sample region of the microscope diffusion chamber and perfused with hypertonic saline solutions of various known osmolalities in equilibrium and dynamic shrinkage experiments to determine $L_p$ and $V_b$ (2.11.3). The values calculated at $20^\circ C$ and $10^\circ C$ were then compared to those measured for the fresh mouse oocytes at the corresponding temperatures and the results summarised in Table 11.

4.3.2 Investigation of the Influence of Culture Time on the $L_p$ and $V_b$ of Fresh Unfertilised Human Oocytes.

Two approaches were used to investigate the influence of the culture period on the membrane transport properties of the fresh human oocyte:

i) The cumulus cells were removed immediately on collection of the oocyte and the $L_p$ calculated by hypertonic perfusion as described in section 2.11.3.
ii) Alternatively a group of fresh oocytes were stored in culture media at 37°C in a CO\textsubscript{2} incubator for 24 h prior to removal of the cumulus mass and calculation of the Lp.

The Lp and Vb values were calculated at 20°C for both treatments and the results compared to those for fresh human oocytes which had been cultured for the standard 5 ½ h (Fig 35).

4.3.3 Investigation of the Effect of Fertilisation of Human Oocytes on Lp and Vb

In order to investigate further the heterogeneity of the values for the Lp and Vb for the fresh human oocyte, a number of oocytes were co-incubated in 1 ml of EBSS at 37°C in a humidified atmosphere of 5 % CO\textsubscript{2}, 5 % O\textsubscript{2}, and 90 % N\textsubscript{2} with approximately 0.1 ml of sperm (concentration 1 x 10\textsuperscript{6} ml) as for I.V.F. (described previously, section 2.10.1). After incubating of the oocytes for 16 – 19 h the cumulus cells were removed using hyaluronidase and manipulation with a micropipette (section 2.9.1). The ova were then examined for the presence of two pronuclei which usually indicated a single sperm had penetrated the zona and the vitelline membrane and that the chromatin of the sperm head had decondensed forming the male pronucleus. Although 2 pronuclei normally suggested fertilisation has
taken place, they may occasionally be exhibited with retention of both sets of female chromosomes. Those oocytes used in my investigations of Lp had at least 1 polar body, the other may breakdown prior to extrusion of the 2nd. Those oocytes in which fertilisation was verified were investigated for their water transport parameters using the diffusion chamber (section 2.11.3). The Lp was studied at 10°C and 20°C and comparisons made to fresh oocytes at the same temperature.

4.3.4 Membrane Transport Properties of Individual Fresh Human Oocytes.

During cryopreservation the rate of water loss from a cell is dictated by the permeability characteristics, Lp and Ea, of that particular cell. The maintenance of equilibrium water content with the extracellular environment avoids intracellular ice formation and in part determines cell survival. The Lp values for fresh human oocytes over the temperature range investigated were extremely variable and the Ea determined from the Arrhenius plot of the mean Lp values was low with a poor correlation coefficient (Chapter 3). Studies of the Lp and Ea of individual fresh human oocytes were performed in an attempt to investigate the cause of the heterogeneity. The Lp of a particular oocyte was studied at 4 different temperatures including 37°C, 30°C, 20°C, and 10°C and from this the
temperature dependence of the Lp was measured using an Arrhenius plot.

Fresh human oocytes were collected by vaginal ultrasound approximately 36 h after the administration of hCG (as described in section 2.9.1). The oocytes were washed and transferred for storage into EBSS at 37°C in a gaseous atmosphere of 5% CO₂, 5% O₂, and 90% N₂ and cultured with the cumulus cells intact. The cumulus cells were removed after 5 1/2 h using hyaluronidase and manipulation with a micropipette to allow accurate observation of the cell volume changes on transfer to the microscope diffusion chamber (Section 2.11.1). The oocyte was then pipetted into the sample region of the microscope diffusion chamber, a dialysis membrane positioned over, placed in the chamber body, and perfused with isotonic media (as in Section 2.11.2). The temperature of the stage was initially set at 37°C and controlled by water moving through the outer temperature jacket from a circulating water bath (Fig 24). The cell shrinkage in response to non-equilibrium hypertonic perfusion over a 5 min period was recorded at 37°C using video microscopy (Section 2.11.2). The Lp was analysed using the computer formulation of the Kedem-Katchalsky equations (Refer to Appendix 1) in the SENS programme which required input of the cell radius. Thus measurements of the oocyte across three axes were made directly from the screen and
allowances made for the magnification factor to allow input of diameter values.

Since, to derive a value for $E_a$, the $L_p$ must be measured at a number of different temperatures, the oocyte was re-perfused with isotonic PBS and allowed to return to isotonic cell volume while the temperature of the stage was reduced. When the stage had equilibrated at 30°C the oocyte was again perfused with hypertonic 0.5 M NaCl for 5 min and the response recorded for later analysis. The $L_p$s were measured at successive decreasing temperatures since the time required for the stage temperature to decrease and stabilize was less than that necessary for the reverse process of raising the temperature. Lowering the temperature of the stage took between 5 and 15 min, the longer time correlating to decreasing the temperature to 10°C.

The duration of the investigation was kept to a minimum to avoid complications from the increasing time after oocyte retrieval or hypertonic exposure. Determining the osmotically inactive volume of the oocyte necessitated the equilibration of the oocyte with 5 changes of NaCl saline solution of known osmolalities (Section 2.11.4). Due to the need to minimise exposure times, in conjunction with the knowledge that the inactive volume was not a temperature dependent variable (refer to Chapter 3, Results), the $V_b$ was taken at the end of the non-
equilibrium perfusion at 10°C rather than at each
temperature, which would have prolonged the experiment to
unacceptable lengths of time.

The values for the Logn Lp of the Lps predicted by the
SENS programme from the cell measurements were plotted
against the inverse of the temperature (1/T K⁻¹) and the
Arrhenius activation energy, Ea, determined from the slope
of the graph. A linear regression analysis was applied to
fit a straight line to the data points and the correlation
coefficient, R², recorded (Curfit, Apple MacKintosh).

4.4 Results
4.4.1 The Lp and Vb of the Pre-ovulatory Mouse Ova

The Lp values estimated for the pre-ovulatory murine
oocytes at both 20°C and 10°C were more variable than those
predicted for the ovulated ova (Fig 34). At 20°C a mean
Lp of 0.59 ± 0.33 μm / atm / min was determined for the
pre-ovulatory material while at the same temperature a mean
value 0.48 ± 0.18 μm / atm / min was recorded for the fresh
mouse ova (Table 8, 11). While the mean value was larger
than that for the ovulated oocytes at 20°C, due to the
large standard deviation, these values were not
significantly different. The range of Lp values for pre-
iovulatory ova of 0.10 to 1.48 μm / atm / min was 2 x
greater than that for the ovulated material of 0.28 to 0.81
μm / atm / min.
CHAPTER 4 - PRE-OVULATORY AND FERTILISED OVA

At 10°C the increased variability was again apparent in the values calculated for the pre-ovulatory oocytes from both the standard deviation about the mean and the range of individual values calculated (Table 11, 34). A mean of 0.37 ± 0.23 μm / atm / min was calculated for pre-ovulatory as opposed to 0.31 ± 0.11 μm / atm / min for ovulated ova. However, as at 20°C, due to the large standard deviation for the pre-ovulatory material the differences between these values were not statistically significant. The Lp values calculated using the SENS software programme for pre-ovulatory material were even more diverse than those at 20°C varying within the range 0.04 to 1.04 μm / atm / min whilst for ovulated oocytes Lp values fell in the narrow band of between 0.19 and 0.41 μm / atm / min.

For the pre-ovulatory mouse oocyte the osmotically inactive volumes for each oocyte was determined as in my study on ovulated mouse ova and the values found to be more diverse for the pre-ovulatory material (Table 35). The mean values for the Vb at both 20°C and 10°C of 25.00 ± 4.83 % and 22.00 ± 6.81 % respectively were greater than for fresh ovulated oocytes of 19.40 ± 2.70 % at 20°C and 18.50 ± 3.3 % at 10°C. Although the mean Vb values determined for the pre-ovulatory oocytes tended to be larger than those for the ovulated oocytes, the difference did not reach statistical significance. This resulted from the larger variability in the Vb for the pre-ovulatory ova.
4.4.2 The influence of culture time on the Lp and Vb of the human oocyte

A mean Lp of $0.47 \pm 0.25 \mu m / atm / min$ was determined for the "immature" ova at 20°C while for the fresh oocytes at the same temperature this value was $0.39 \pm 0.12 \mu m / atm / min$ (Table 12). The increased variability for those oocytes examined on retrieval was apparent from both the standard deviation about the mean Lp and the range of values. The individual Lp values predicted for those cells examined immediately after collection from the follicle were more varied than those oocytes cultured for a 5½ h period. The Lp values fell within the range of 0.16 to 0.52 \mu m / atm / min for the fresh ova while for "immature" oocytes also at 20°C the Lp values determined were between 0.23 and 1.10 \mu m / atm / min. The Lps ranged between 0.23 and 0.67 \mu m / atm / min for the "matured" ova which is much less than the spread for those examined immediately on collection of 0.23 to 1.10 \mu m / min. However the values for these matured ova did not exhibit a greater degree of consistency than the fresh material which ranged between 0.04 and 0.52 \mu m / atm / min.

Increasing the culture time from 5½ h to 24 h between the collection of the ova and the initiation of hypertonic perfusion did not increase the degree of conformity in the Lp values. The standard deviation about the mean was not significantly different for the "matured" and fresh ova at
## Table 11 The mean $L_p$ and $V_b$ of Pre-Ovulatory Mouse Oocytes

<table>
<thead>
<tr>
<th>Temperature</th>
<th>20°C</th>
<th>10°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
<td>( n )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean $L_p$</td>
<td>0.59 ± 0.33</td>
<td>0.37 ± 0.23</td>
</tr>
<tr>
<td>( $\mu m$ / atm / min )</td>
<td>( 0.48 ± 0.18 )</td>
<td>( 0.31 ± 0.11 )</td>
</tr>
<tr>
<td>Minimum $L_p$</td>
<td>0.10</td>
<td>0.04</td>
</tr>
<tr>
<td>( $\mu m$ / atm / min )</td>
<td>( 0.26 )</td>
<td>( 0.19 )</td>
</tr>
<tr>
<td>Maximum $L_p$</td>
<td>1.48</td>
<td>1.04</td>
</tr>
<tr>
<td>( $\mu m$ / atm / min )</td>
<td>( 0.81 )</td>
<td>( 0.72 )</td>
</tr>
<tr>
<td>Mean $V_b$</td>
<td>25.00 ± 22.00 ±</td>
<td></td>
</tr>
<tr>
<td>( % )</td>
<td>4.83</td>
<td>6.81</td>
</tr>
</tbody>
</table>

( ) are values for ovulated mouse oocytes at the corresponding temperature. Results presented in Chapter 3.
Figure 33 Plots of the Lp for pre-ovulatory and ovulated mouse ova.
0.42 ± 0.13 μm / atm / min for the matured and 0.39 ± 0.12 μm / atm / min for the fresh. The decrease in the variability observed with increasing time in culture (Fig 35) was not statistically significant either for the values calculated for those examined immediately on recovery, the fresh, 5 ½ h culture, human oocyte or those examined 24 h after collection. However, it should be noted that the trend visible from the individual values (Table 36) was towards a more homogenous population.

The mean values for the osmotically inactive volumes calculated for the "immature" (30.55 ± 7.69%), fresh (25.10 ± 7.95%), and "matured" (25.69 ± 11.69%) human oocytes (Table 12, 37) were only marginally different with increasing culture time. When the values for each oocyte within the three groups were compared to the overall range of values calculated for the population of fresh human ova at 20°C no significant alterations were observed from prolonging the culture period.

4.4.3 The Effect of Fertilisation on the Lp and Vb of Human Oocytes.

The mean Lp calculated for the pronucleated egg at 20°C of 0.55 ± 0.29 μm / atm / min was not significantly different from the fresh, non-matured human oocytes at the same temperature of 0.34 ± 0.12 μm / atm / min (Table 13). A large standard deviation was determined for the
Table 12 The Mean Lp and Vb at 20°C of Human Oocytes Immediately Following Collection and After Storage for 5 ½h and 24 h

<table>
<thead>
<tr>
<th>Culture Time</th>
<th>0</th>
<th>5 ½h (fresh)</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>11</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Mean Lp</td>
<td>0.47 ± 0.25</td>
<td>0.39 ± 0.12</td>
<td>0.42 ± 0.13</td>
</tr>
<tr>
<td>(μm / atm / min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum Lp</td>
<td>0.23</td>
<td>0.04</td>
<td>0.23</td>
</tr>
<tr>
<td>(μm / atm / min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum Lp</td>
<td>1.10</td>
<td>0.52</td>
<td>0.67</td>
</tr>
<tr>
<td>(μm / atm / min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Vb</td>
<td>30.55 ± 7.69</td>
<td>25.10 ± 7.95</td>
<td>25.69 ± 11.69</td>
</tr>
<tr>
<td>(μ% )</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values for "fresh" human oocytes at the corresponding temperature are presented in Chapter 3.
The Lp at 20°C for human oocytes cultured for varying times.

Figure 34 Plots of the Lp data for human ova to investigate the influence of culture time on the permeability parameters.
### Table 13 The Mean Lp and Vb for Pronucleated Human Oocytes

<table>
<thead>
<tr>
<th>Temperature</th>
<th>20°C</th>
<th>10°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>Mean Lp</td>
<td>0.55 ± 0.29</td>
<td>0.34 ± 0.12</td>
</tr>
<tr>
<td>(μm / atm / min)</td>
<td>(0.39 ± 0.12)</td>
<td>(0.43 ± 0.21)</td>
</tr>
<tr>
<td>Ratio of St.</td>
<td>0.53</td>
<td>0.35</td>
</tr>
<tr>
<td>Dev : Mean</td>
<td>(0.31)</td>
<td>(0.50)</td>
</tr>
<tr>
<td>Minimum Lp</td>
<td>0.11</td>
<td>0.13</td>
</tr>
<tr>
<td>(μm / atm / min)</td>
<td>(0.16)</td>
<td>(0.17)</td>
</tr>
<tr>
<td>Maximum Lp</td>
<td>1.19</td>
<td>0.55</td>
</tr>
<tr>
<td>(μm / atm / min)</td>
<td>(0.52)</td>
<td>(0.96)</td>
</tr>
<tr>
<td>Mean Vb</td>
<td>39.31 ± %</td>
<td>37.18 ± %</td>
</tr>
<tr>
<td>( % )</td>
<td>11.37</td>
<td>11.15</td>
</tr>
</tbody>
</table>

Values for fresh human oocytes at the corresponding temperature from Chapter 3.
fertilised ova compared to that predicted for the fresh human oocytes. The individual Lp's determined for the ova at 20°C for the fresh material ranged between 0.16 - 0.52 μm /atm / min. However, for the pronucleated ova there was a 2 fold increase in the range of Lp values calculated from 0.11 and 1.19 μm / atm / min suggesting a greater heterogeneity in the population of fertilised ova studied. However, at 10°C the pronucleated ova had a mean of 0.34 ± 0.12 μm / atm which was lower than that calculated for the fresh oocytes of 0.43 ± 0.21 μm / atm / min, the reverse of the situation at 20°C (Table 38). The decreased variability at 10°C for the pronucleated cells was also apparent from the range of Lp values derived which varied between 0.15 - 0.55 μm /atm / min as opposed to those of the fresh ova which ranged between 0.17 and 0.96 μm /atm / min.

The mean Vb's determined from the pooled data derived from the Boyle Van't Hoff plots for the fertilised ova at both 20°C and 10°C of 39.31 ± 11.37 % and 37.18 ± 11.14 % respectively were higher than those calculated for the unfertilised oocytes cultured for 5 ½ h at the same temperatures of 25.10 ± 7.95 % and 25.38 ± 8.23 %.
4.4.5 The Lp, Vb, and Ea of Individual Fresh Human Oocytes.

The range of Lp values predicted for the 10 fresh oocytes examined individually at each temperature were equally as diverse as those calculated for the groups of fresh ova at the equivalent temperatures (Table 14) which were then used to generate the mean Lps for the derivation of Ea in sections 3.5.1, and 3.5.2. The Lp values determined for each individual oocyte at each of the temperatures investigated and the extent to which the Lp each decreased for that oocyte as the temperature was lowered, was specific for each oocyte. This was shown most clearly by considering a selection of the 10 oocytes examined such as Oocytes 4, 8, and 10 (Table 15). At 37°C an Lp of 1.80 μm / atm / min was calculated for Oocyte 4, which was greater than for those determined for Oocyte 8 and 10 at the same temperature of 0.35 and 1.28 μm / atm / min respectively. By 10°C the Lp for Oocyte 4 had decreased to 0.23 μm / atm / min which was only marginally different from that of Oocyte 8 of 0.19 μm / atm / min. However, the Lp value at 10°C derived for Oocyte 10 was now much greater than the other 2 ova at 0.66 μm / atm / min. It was apparent even from the 3 oocytes considered that the individual Lp values measured at the same temperatures varied widely.

It was also obvious that the rate at which the Lp decreased with temperature, and thus Ea, was specific to
### Table 14 The Mean Ea for Individual Human Oocytes

<table>
<thead>
<tr>
<th>Number</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Ea</td>
<td>$8.07 \pm 5.06$ (Kcal/mol)</td>
</tr>
<tr>
<td>Minimum Ea</td>
<td>3.60 (Kcal/mol)</td>
</tr>
<tr>
<td>Maximum Ea</td>
<td>18.95 (Kcal/mol)</td>
</tr>
</tbody>
</table>
Table 15 Membrane Water Permeability (μm / atm / min) for Individual Human Oocytes over the Temperature Range 37 - 10°C.

<table>
<thead>
<tr>
<th>Oocyte</th>
<th>37°C</th>
<th>30°C</th>
<th>20°C</th>
<th>10°C</th>
<th>Vb</th>
<th>Ea</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.57</td>
<td>0.58</td>
<td>0.32</td>
<td>0.24</td>
<td>23</td>
<td>6.27</td>
<td>0.93</td>
</tr>
<tr>
<td>2</td>
<td>0.26</td>
<td>0.20</td>
<td>0.36</td>
<td>0.33</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>3</td>
<td>0.65</td>
<td>0.68</td>
<td>0.60</td>
<td>0.22</td>
<td>18</td>
<td>6.64</td>
<td>0.73</td>
</tr>
<tr>
<td>4</td>
<td>1.80</td>
<td>1.58</td>
<td>1.09</td>
<td>0.24</td>
<td>30</td>
<td>12.73</td>
<td>0.86</td>
</tr>
<tr>
<td>5</td>
<td>0.76</td>
<td>0.59</td>
<td>0.56</td>
<td>0.14</td>
<td>23</td>
<td>10.16</td>
<td>0.80</td>
</tr>
<tr>
<td>6</td>
<td>0.33</td>
<td>0.23</td>
<td>0.16</td>
<td>0.13</td>
<td>30</td>
<td>5.92</td>
<td>0.96</td>
</tr>
<tr>
<td>7</td>
<td>1.10</td>
<td>1.08</td>
<td>0.51</td>
<td>0.14</td>
<td>20</td>
<td>18.95</td>
<td>0.95</td>
</tr>
<tr>
<td>8</td>
<td>0.35</td>
<td>0.33</td>
<td>0.23</td>
<td>0.18</td>
<td>38</td>
<td>4.20</td>
<td>0.97</td>
</tr>
<tr>
<td>9</td>
<td>0.57</td>
<td>0.56</td>
<td>/</td>
<td>0.20</td>
<td>48</td>
<td>3.60</td>
<td>0.28</td>
</tr>
<tr>
<td>10</td>
<td>1.29</td>
<td>0.91</td>
<td>0.80</td>
<td>0.66</td>
<td>29</td>
<td>4.02</td>
<td>0.92</td>
</tr>
</tbody>
</table>
Figure 35 Three representative plots of the Arrhenius activation energy determined from the Lp for individual oocytes over the entire range of temperatures, a) Oocyte 5, b) Oocyte 7, c) Oocyte 9.
each particular oocyte (Table 15). This fact could only be uncovered when considering a range of Lp's measured at the different temperatures, but could be accurately determined for different oocytes which were used to determine a single Ea. Variability in the temperature dependence of the Lp could only be substantiated when a single ovum was considered at all of the temperatures over which Ea was calculated. The values derived for the Arrhenius activation energy for each separate oocyte were calculated from the linear regression plots for those particular oocytes. Figure 36 shows a selection of plots for 3 ova (Oocytes 5, 7, and 9); for clarity the other oocytes were not included. The Ea values were extremely diverse, ranging between a maximum of 18.95 Kcal/mol and a minimum of 3.60 Kcal/mol with a mean of 8.06 ± 5.07 Kcal/mol compared to the value of 3.33 Kcal/mol determined using the meaned Lp values (Figure 30). The correlation coefficients (Table 15) ranged between 0.73 and 0.97 indicating that in the majority of cases the mathematically predicted best fit straight lines exhibited good linear correlations between Lp values and temperature.

An exception to the high values of $R^2$ was that determined for Oocyte 2 of 0.45 which was excluded from analysis. Although 10 fresh oocytes are described in Table 15, all of the results presented for the individual oocytes were for a group of 9 oocytes. Fitting a best fit line to
the Arrhenius plot for Oocyte 2 revealed a positive slope which was totally anomalous and it may be that the membrane of Oocyte 2 was damaged at some point during the experiment although there was no absolute proof of this. Injury may have become more apparent as the time during which that oocyte was exposed to hypertonic solutions increased, and the oocyte aged. This may have led to an anomalous and apparently increasing permeability of the membrane as perfusion with hypertonic 0.5 M NaCl was repeated at lower temperatures.

Summary

It was found that:

i) pre-ovulatory mouse oocytes exhibited a greater variability in Lp and Vb than those determined for the ovulated mouse oocyte, although the mean values for each parameter were not statistically different at the temperatures studied.

ii) increasing the time for which the human oocytes were cultured in EBSS had no significant effect on the observed variability.

iii) Fertilisation of the human oocyte did not
significantly reduce the heterogeneity shown in the permeability characteristics.

iv) Studying individual human oocytes at each of the chosen temperatures confirmed the inherent biological variability. However, each oocyte showed its own distinctive relationship between temperature and Lp, with good linear relationships in the linear regression analyses used to plot Ea values. A mean value for Ea of 8.06 ± 5.07 for the 9 oocytes studied was similar to values determined for other mammalian cells, but lower than the values reported for ovulated mouse oocytes. These results clearly identify the fact that human pre-ovulatory oocytes cannot be considered as a single uniform population of cells with respect to membrane Lp values. Nevertheless each oocyte exhibits an expected relationship between Lp and temperature on cooling.
The Morphological Response of Fresh Human and Mouse Oocytes to Hypertonic Perfusion at Low Temperatures.

5.1 Aims

The osmotic behaviour of both murine and human oocytes over a range of temperatures was examined in Chapter 3 to determine values for Lp and Ea. During the course of this study it was revealed that the response of fresh oocyte to hypertonic exposure at 0°C was unlike the dynamic behaviour at higher temperatures and was characterised by atypical and non-spherical shrinkage at 0°C. The experiments presented in this chapter were designed to investigate further the responses of the oocyte to an osmotic stress at low temperatures and determine whether this had a bearing on the overall response of oocytes to cryopreservation. In addition, the influences of equilibrating the oocytes with various additives known to be successful cryoprotective agents prior to osmotic stress at low temperatures were examined using the microscope diffusion chamber. The cryoprotectants chosen were those previously used with some success in murine oocyte cryopreservation (DMSO - Whittingham, 1977; glycerol - Fuller and Bernard, 1984). The chosen osmotic stress (0.5M NaCl) was one known from
CHAPTER 5 - DEFORMATION OF THE OOCYTE

previous studies (Bernard et al, 1988) and my own work (Chapters 3 and 4) to produce extensive and repeatable oocyte volume reduction without causing gross disruption of the oocyte morphology.

5.2 Introduction

The majority of studies investigating the membrane water permeability of a particular cell, and the influence of temperature on this, have done so within the temperature range of 5°C to 37°C (Hempling, 1973, and 1977; Leibo, 1980; Sherban, 1987; Myers et al, 1987). There is little information available concerning the permeability characteristics of cells below this range, including high subzero temperatures (McCaa et al, 1986, Aggarwal et al, 1988). In a study by Toner (1990) unfertilised mouse ova were examined at subzero temperatures in the presence of ice and the Lp and Ea were determined. In my study, fresh mouse and human ova were observed over a range of temperatures from 0°C to 37°C to calculate values for both the Lp and Ea. Results for the Lp and thus Ea were presented in Chapter 3. However, due to an unusual shrinkage response on perfusion with hypertonic NaCl, the change in the cell volume with respect to time could not be measured accurately and a value for the Lp could not be determined at 0°C.

The responses of both the mouse and human oocytes to an
imposed osmotic stress were examined at 0°C in an attempt to characterise the abnormal shrinkage and observe whether the behaviour was common to both cells. In addition, I was interested to discover whether shrinkage would be altered in the presence of CPA's, which are known in the case of unfertilised mouse ova (Whittingham, 1977; Fuller and Bernard, 1984) to protect against damage during cryopreservation, in which low temperatures and high concentrations are inevitable.

Materials and Methods
5.3 Hypertonic Exposure at 0°C Using the Microscope Diffusion Chamber

Human pre-ovulatory and ovulated mouse oocytes were collected as previously described (section 2.5.1, 2.9.1). The human oocytes were cultured for 5 1/2 hrs in EBSS at 37°C in a gaseous atmosphere of 5 % CO₂, 5 % O₂, and 90 % N₂ prior to removal of the cumulus cells using hyaluronidase and agitation with a micropipette. The ovulated mouse ova from which the cumulus mass had been dispersed were transferred into PB1 for storage at 37°C.

A refrigerated cooling bath containing ethylene glycol as diluent was set at -5°C and allowed to equilibrate. The temperature was set below that desired for investigation of the oocyte membrane water permeability to compensate for heat loss between the circulating fluid, the chamber body
and the perfusing medium. In addition, using this set temperature for the water bath allowed the temperature of the stage to be easily maintained at 0°C - 0.5°C during the investigation. The perfusate was stored in a refrigerator at 4°C until required when it was placed in the main body of the circulating fluid in the water bath. This ensured the temperature difference between the set temperature and the bathing medium was as small as possible. A fluid damper was fitted to the perfusing system at a point just prior to the inlet pipe of the bulk flow channel to minimize the transfer of vibrations from the water bath and pump mechanism to the sample. This reduced the extraneous oscillations of the oocytes, ensuring a sharp image for videomicroscopy.

The oocytes were pipetted into the sample region of the diffusion chamber and a dialysis membrane placed over the chamber body to maintain the position of the oocyte. The sample region was then inverted into the bulk flow region of the stage and perfused with isotonic PB1 to allow the cells position to become stable while the depth of sample region was calibrated as described previously (Section 2.11.1). After the cells were located the video recorder was started and hypertonic 0.5 M NaCl introduced into the bulk flow channel (refer to sections 2.11.1 - 2.11.3). The osmotic response of each oocyte was recorded for 5 minutes, after which still photographs were made. The
CHAPTER 5 - DEFORMATION OF THE OOCYTE

Oocytes were then either re-equilibrated with isotonic medium (PB1) at 0°C, or the stage warmed to 20°C whilst still exposing the cells to hypertonic 0.5 M NaCl, after which they were returned to isotonic conditions.

5.4 Equilibration with CPA's Prior to Hypertonic Exposure Using the Microscope Diffusion Chamber.

Oocytes were equilibrated with cryoprotectants prior to study of the osmotic response at 0°C to examine whether their addition altered the behaviour of the oocytes to this osmotic stress at low temperatures. Two cryoprotectants were chosen for investigation on the basis that they had been used with some success for the preservation of mouse oocytes. The equilibration regimes were those used in the published cryopreservation protocols:

i) Equilibration with 0.625 M glycerol at 37°C (Fuller and Bernard, 1984).

ii) Equilibration with 1.5 M DMSO at 0°C (Whittingham, 1977).
5.4.1 Equilibration with Glycerol and Hypertonic Perfusion.

Due to the long periods of time necessary for glycerol to penetrate the oocyte fully, it was decided to equilibrate the ova outside of the microscope diffusion chamber. In the study by Fuller and Bernard (1984) up to 60 minutes were allowed for full equilibration of mouse ova prior to cryopreservation. However, this period was extended to over 120 minutes in some preliminary investigations into equilibration at 37°C using the microscope diffusion chamber. The mouse oocytes were therefore placed in 0.5 ml of 0.25 M glycerol in an incubator at 37°C and allowed to equilibrate for 20 minutes, after which 0.5 ml of 1.0 M glycerol was added. The oocytes were returned to the incubator at 37°C and thorough mixing of the two solutions ensured and the oocytes left to equilibrate with the final concentration of 0.625 M glycerol for 40 minutes. Human ova were allowed to equilibrate with the initial 0.25 M glycerol for 25 minutes prior to the addition of 0.5 ml of 1.0 M glycerol in which they were equilibrated for approximately 50 minutes.

After full equilibration the oocytes were pipetted into the sample region of the diffusion chamber, inverted into the bulk flow channel as described for the experiments to derive the Lp (section 2.11.1, 2.11.2). The oocytes were then perfused with 0.625 M glycerol in PBS at 0°C to
maintain the osmotic potential. When the temperature of the fluid and the position of the oocyte were stable, the bathing medium was changed to 0.625 M glycerol plus 0.5 M NaCl solution and the shrinkage response monitored using videomicroscopy and still photography.

5.4.2 Equilibration with DMSO and Hypertonic Perfusion.

Although equilibration with 1.5 M DMSO was faster than with glycerol in the microscope diffusion chamber (occurring in 45 minutes), equilibration outside of the chamber was still achieved in a much shorter time period of 15 minutes. Oocytes were pipetted into 1.5 M DMSO which had been placed on ice in a refrigerator and cooled to approximately 0°C. The cells were allowed to equilibrate with the cryoprotectant until re-expansion to isotonic volume was complete as assessed by visual observation, after which they were placed in the diffusion chamber and inverted into the bulk flow region. The oocytes were then perfused with 1.5 M DMSO in PBS, to maintain osmotic equilibrium in the CPA-loaded oocytes, and the cell position allowed to stabilise whilst the original diameters were recorded. The video recording was started, the bathing medium altered to 1.5 M DMSO in 0.5 M NaCl solution and the osmotic response of each oocyte monitored.
5.4.3 Experimental Groups.

Microscopic observations were made with respect to the morphology of the oocytes after the experimental procedures, and assigned to a scale of 1 to 4, where 1 was taken to be equal to a perfectly spherical cell and 4 was a severely deformed, non-spherical cell. Three groups of oocytes were studied for both fresh human and mouse oocytes, and compared to the response at 20°C:

i) oocytes exposed to isotonic PB1 and hypertonic 0.5 M NaCl at 0°C.

ii) equilibration with 0.625 M glycerol in PBS and subsequent exposure to 0.5 M NaCl + 0.625 M.

iii) equilibration with 1.5 M DMSO in PBS and subsequent exposure to 0.5 M NaCl + 1.5 M DMSO.

For human oocytes a minimum of ten oocytes were used in each group, while for murine the group sizes ranged between 15 and 25 oocytes.
Results

5.5 Hypertonic Exposure at 0°C Using the Diffusion Chamber.

The responses of oocytes to the hypertonic perfusion at 0°C was abnormal, and was seen with both murine and human oocytes. At all temperatures of 5°C and above both the human and mouse oocytes responded to hypertonic perfusion by loosing water in an even, spherical shrinkage as the cells attempted to equilibrate with the extracellular NaCl solution. Although this resulted in a volume reduction the cells shrank in a regular fashion across their whole surface, maintaining their spherical appearance throughout the 5 minute exposure. Likewise, all oocytes in isotonic medium which were examined at 0°C in the microscope diffusion chamber appeared spherical. It was only when the osmotic stress was imposed on the ova at low temperatures that an abnormal morphology was observed.

During the hypertonic perfusion for a five minute period (as used previously in Chapter 3), measurement of the diameter changes could not be made with any accuracy due to the uneven collapse of the oocytes. Thus Lp values could not be calculated as the computer programme SENS relied upon the sphericity of the cell to predict volume change in response to changes in the extracellular concentration. (The derivation of the Lp by the computer programme SENS is described in Appendix 1). In addition,
it was not possible to calculate the inactive volume as described in section 2.11.4 as the Boyle van't Hoff relationship also requires that the cell remain spherical in order to be able to relate the cell volume to the external concentration.

The 10 fresh human oocytes and 25 fresh ovulated mouse oocytes examined at 0°C exhibited a consistently different response to perfusion with 0.5 M NaCl compared to that recorded for exposure to hypertonic solution for the same period of time but at higher temperatures. After only a short exposure to 0.5 M NaCl (of between 2 - 3 minutes at 0°C) the ova lost their spherical appearance and became extensively deformed or crumpled (Fig 36a - 36f). All of the ova at 0°C, both mouse and human, crumpled on perfusion with hypertonic saline.

Since "crumpling" was, by its nature, an uneven occurrence, using the scale described in section 5.4.3 it was possible only to semi-quantitatively designate a mean score for the morphology of the ova at each of the temperatures investigated. At temperatures of 5°C and above in both isotonic and hypertonic media the oocytes approached perfect sphericity, as was the case, for ova in isotonic media at 0°C (Table 16, 17). Although the oocytes were generally spherical, measurements of the diameter across 3 axes revealed that in some cases this was not perfect sphericity, and thus the mean scores of grading
did not correspond to a perfect sphere.

Using the mean radius in the computer calculations could only make allowances for small deviations from perfect sphericity, and the "crumpling" effect was too drastic to be treated in this way or even make predictions of a diameter. Following hypertonic perfusion at 0°C mean scores of 3.10 ± 0.90 and 3.40 ± 0.50 (Table 16, 17) were recorded for human and mouse oocytes respectively, demonstrating generalised gross deformities in the normally spherical ova.

When the oocytes were re-perfused with isotonic medium at 0°C they gradually regained a smooth, spherical form. Oocytes warmed while still in hypertonic 0.5 M NaCl also regained their sphericity, only the osmotic stress and cold exposure combined caused "crumpling".

5.5.1 Exposure to CPA's and Subsequent Hypertonic Exposure at 0°C Using a Diffusion Chamber.

In all of the oocytes (both mouse and human) fully equilibrated with 1.5 M DMSO at 0°C prior to perfusion with hypertonic 1.5 M DMSO in 0.5 M NaCl, the "crumpling" response was still observed (Fig 37a, 37b). The severity of the deformation observed was only slightly reduced with the mean score decreasing from 3.10 ± 0.90 to 3.00 ± 1.00 for the fresh human oocytes and from 3.40 ± 0.50 to 3.20 ± 1.10 for the mouse (Table 16, 17).
A greater reduction in the degree of "crumpling" was observed when the oocytes had been previously equilibrated with 0.625 M glycerol prior to hypertonic perfusion. The oocytes seemed not to crumple and although the plasma membrane appeared to be distorted, the extreme deformation observed in unprotected oocytes was avoided. Rather than crumpling the oocyte plasma membrane took on a "ruffled" appearance while the oocyte assumed a more typical spherical appearance (Figures 38a, 38b). The mean score for the fresh human oocytes was reduced from 3.10 ± 0.90 to 2.00 ± 0.30 and for the mouse a reduction from 3.40 ± 0.50 to 2.10 ± 0.50 was estimated.

The scores assigned to the oocytes to assess the severity of the deformation were analysed using a non-paired students t-test. The differences between the scores attributed to the different morphologies of the ova in isotonic media at 0°C and those exposed at 0°C to hypertonic NaCl solution alone or following prior equilibration with a cryoprotectant were statistically significant (P < 0.005). However, it should be stressed that an exact degree of crumpling was difficult to assign in a totally objective way, and these crumpling indices should only be taken as a very generalised indication of the events taking place. Never-the-less it should be emphasised that all oocytes at 0°C in hypertonic solution exhibited this deformation.
### Table 16 Response of Mouse Oocytes to Hypertonic Exposure

<table>
<thead>
<tr>
<th>Temp</th>
<th>Isotonic n=25</th>
<th>0.5M NaCl n=25</th>
<th>1.5M DMSO + 0.5M NaCl n=20</th>
<th>0.625M Glyc + 0.5M NaCl n=15</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>1.3 ± 0.3</td>
<td>1.3 ± 0.3</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>30°C</td>
<td>1.0 ± 0.0</td>
<td>1.1 ± 0.2</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>20°C</td>
<td>1.2 ± 0.4</td>
<td>1.4 ± 0.4</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>10°C</td>
<td>1.4 ± 0.3</td>
<td>1.6 ± 0.5</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>0°C</td>
<td>1.3 ± 0.3</td>
<td>3.4 ± 0.5</td>
<td>3.2 ± 1.1</td>
<td>2.1 ± 0.5</td>
</tr>
</tbody>
</table>

Fresh mouse oocyte scored on a scale of 1 to 4, 1 perfect sphericity, 4 severe deformation. Values are averages for groups of 15 or more.
Table 17 Deformation of the Human Oocyte on Hypertonic Exposure at Low Temperatures.

<table>
<thead>
<tr>
<th>Temp</th>
<th>Isotonic n=10</th>
<th>0.5M NaCl n=10</th>
<th>1.5M DMSO + 0.5M NaCl n=10</th>
<th>0.625MGlyc +0.5M NaCl n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>1.1 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>30°C</td>
<td>1.3 ± 0.4</td>
<td>1.3 ± 0.4</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>20°C</td>
<td>1.1 ± 0.2</td>
<td>1.3 ± 0.3</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>10°C</td>
<td>1.5 ± 0.3</td>
<td>1.7 ± 0.3</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>0°C</td>
<td>1.4 ± 0.2</td>
<td>3.6 ± 0.9</td>
<td>3.3 ± 1.0</td>
<td>2.0 ± 0.3</td>
</tr>
</tbody>
</table>

Fresh human oocyte scored on a scale of 1 to 4, 1 perfect sphericity, 4 severe deformation. Values are averages for n=10.
Figures 36a and 36b: Magnification Factor x 450.

Figures 36c and 36d: Magnification Factor x 300.
Figure 36 Fresh human oocyte at 37°C a) in isotonic media b) after 5 minutes in hypertonic saline solution. Fresh human oocyte at 0°C c) in isotonic media, d) after 5 minutes in hypertonic 0.5 M NaCl solution.
CHAPTER 5 - DEFORMATION OF THE OOCYTE

Figure 37 Fresh human oocyte at 0°C a) after full equilibration with 1.5 M DMSO b) perfusion for 5 minutes with 1.5 M DMSO in 0.5 M NaCl. Magnification Factor x 400.

Figure 38 Fresh human oocyte a) fully equilibrated with 0.625 M glycerol at 37°C and b) after perfusion with 0.625 M glycerol in 0.5 M NaCl at 0°C.

Figures 38a and 38b: Magnification Factor x 400.
CHAPTER 5 - DEFORMATION OF THE OOCYTE

Summary

i) The response of ova to hypertonic perfusion at 0°C was unlike that at temperatures of 5°C and above. However the abnormal morphology was universal to both ovulated mouse and fresh pre-ovulatory human oocytes.

ii) Equilibration with cryoprotectant significantly altered the degree of deformation observed although it did not completely prevent. Equilibration with glycerol changed the shrinkage response from gross deformation to minor distortion of the plasma membrane in cells retaining an overall spherical shape.

iii) Since both glycerol and DMSO have been used to successfully preserve mouse ova, the crumpling on the application of an osmotic stress at low temperatures, which was observed even after equilibration, with CPA's can not be the only reason for cell injury during cryopreservation. However, cryoinjury may result from a multiplicity of interrelated responses, and crumpling may be one change which potentiates the overall damage. In view of this, I felt that it was necessary to investigate the crumpling response, and the changes to this response brought about by prior equilibration with CPA's, in relation to possible
alterations in fertilisation and subsequent development of oocytes. This formed the basis of my studies in the following chapter.
6.1 Aims

This chapter examines the response of unfertilised oocytes to a reduction in temperature to elucidate whether as has been suggested (Pickering and Johnson, 1987, Johnson et al., 1988, Sathananthan, 1988) the mouse and human oocyte were damaged by exposure to low temperatures. It may be possible to separate damage which occurs as a result of cooling to temperatures of 0°C and above, and the resulting loss of viability, from injury caused by freezing during cryopreservation. In addition, and following the observations in chapter 5, the effect of imposing low temperatures in conjunction with increased hypertonicity on the fertilisation rate and subsequent development were considered to investigate whether these were additive to cooling damage. Finally the effects of prior equilibration with cryoprotectants before cooling and hypertonic stress were studied to see if these would modulate subsequent fertilisation and development.
6.2 Introduction

Oocyte cryopreservation, which combines a temperature reduction with an inevitable osmotic stress as a result of an increase in the extracellular concentration, caused either by the formation of ice crystals or the addition of cryoprotectants, has been difficult to achieve successfully, especially for the unfertilised human oocyte. Indeed, there is evidence to suggest that merely reducing the temperature from 37°C to 4°C has adverse effects upon the unfertilised mouse and human oocytes, without the additional problems of ice crystal formation and toxicity of the cryoprotectant. During cooling of oocytes, it has been suggested that the arrangement of the microtubular elements of the cytoskeleton are disrupted leading to dislocation of the chromosomes from the meiotic spindle (Magistrini and Szollosi, 1980, Johnson and Pickering, 1987, Sathananthan et al, 1988). The inability to reorganize the chromosomes on rewarming and microtubule repolymerisation would result in an abnormal chromosomal compliment in the embryo on fertilisation. This temperature reduction or "cold shock" is also known to cause loss of the selective permeability of the plasma membrane (Watson, 1981), separation of the lipid components of the membrane into gel phase domains accompanied by the exclusion of membrane proteins (Crowe and Crowe, 1984, Sillerud and Barnett, 1982), the release of membrane proteins and
polysaccharides (Quinn et al., 1969).

6.3 Materials and Methods

Mouse ova were released from the ampullae into hyaluronidase at 37°C at 13 h post hCG injection, the cumulus cells were removed and the oocytes were transferred to PB1 at 37°C prior to use as described in sections 2.5.1. Fresh human ova were collected pre-ovulatory from the follicle approximately 36 h post hCG administration and incubated in 1 ml of EBSS in an atmosphere of 5% CO₂, 5% O₂, 90% N₂ at 37°C for 5 ½ h prior to removal of the cumulus mass as described in section 2.9.1. Following the removal of the cumulus cells, a series of experiments were then carried out to investigate the effects of cooling to 0°C - 0.5°C on the potential for fertilisation and development of the oocyte. Oocyte viability, subsequent fertilisation and on-growth following hypertonic exposure, and a combination of cooling and osmotic stress was also studied.

6.4 Experimental Groups
6.4.1 I.V.F. Control.

An IVF control group of mouse oocytes which had undergone no experimental treatment were cultured to the hatching blastocyst stage to allow comparisons of fertilisation rate as described in section 2.7. The number
of human ova fertilised following investigation were compared to the overall fertilisation rate and number of cell divisions that could normally be expected from the remainder of the cohort of the oocytes undergoing I.V.F. treatment.

6.4.1 Isotonic Exposure at 37°C

Groups of 20 mouse oocytes were exposed to isotonic PB1 in a 37°C incubator for 30 minutes, after which they were pipetted into T6 culture media, left for 60 minutes and fertilised (section 2.7). Individual human oocytes were pipetted into 1 ml of PB1 at 37°C, incubated for 30 minutes after which they were removed and returned to EBSS for culture for 60 minutes prior to addition of sperm (as described in section 2.10.1).

6.4.2 Hypertonic Exposure at 37°C

In an attempt to differentiate between a reduction in the fertilisation rate caused by an osmotic stress as opposed to a reduction in temperature, oocytes were exposed to hypertonic NaCl solution at 37°C. Tubes containing 1 ml of hypertonic 0.5 M NaCl were held in a 37°C incubator. After the temperature of the saline solution had stabilised at 37°C, fresh ovulated mouse and pre-ovulatory human oocytes were pipetted into the media and allowed to equilibrate with the hypertonic environment for 15 minutes.
CHAPTER 6 - COOLING AND OSMOTIC STRESS

The ova were removed and placed in culture media ( T6 or EBSS ) and left to re-equilibrate for 60 minutes. Sperm were subsequently added to the oocytes and 24 h later they were examined for signs that fertilisation had taken place.

6.4.3 Isotonic Exposure at 0°C - Rapid Cooling

Tubes containing 1 ml of PB1 were placed on ice in a refrigerator for 15 minutes and the temperature allowed to equilibrate at 0°C. A NiCr / NiAl thermocouple ( Comark, UK Ltd ) was used to monitor the temperature of the medium and when it had stabilised at 0°C - 0.5°C, groups of 20 mouse oocytes or a single human ovum were pipetted directly from isotonic media at 37°C into the PB1 at 0°C resulting, in a very rapid rate of cooling. After 30 minutes at 0°C the oocytes were placed in a 37°C and allowed to return to physiological temperature prior to return to culture conditions and co-incubation with sperm as described in section 2.7 and 2.10.1.

6.4.4 Hypertonic Exposure at 0°C - Rapid Cooling.

As with exposure to low temperature in isotonic media tubes containing 1 ml of PB1 were cooled for 15 minutes and allowed to equilibrate at 0°C. In addition tubes of 1 ml of 0.5 M NaCl solution were also incubated on ice in a refrigerator for approximately 15 minutes until the
Figure 39 Plot of the rate of temperature decrease for oocytes in media on incubation on ice in a refrigerator.
temperature recorded was stable at 0°C. Again cumulus free oocytes, both mouse and human, were pipetted into PB1 at 0°C - 0.5°C resulting in rapid cooling. However, after 15 minutes the oocytes were removed from the isosmotic environment and placed in hypertonic 0.5 M NaCl at 0°C for a further 15 minute period. The oocytes were subsequently returned to isotonic conditions at 0°C, followed 5 minutes later by removal from the cold environment and incubation at 37°C for a 1 h period to allow the complete re-expansion and return to physiological temperature. The oocytes were then inseminated and cultured for 24 and 48 h as described in sections 2.7. and 2.10.1.

6.4.5 Hypertonic Exposure Prior to Cooling to 0°C.

Instead of placing the oocytes directly into pre-cooled medium, they were placed in either PB1 or 0.5 M NaCl at 37°C and left for 15 minutes to equilibrate and dehydrate. The oocytes were then placed on ice in a refrigerator and cooled down to 0°C - 0.5°C. A thermocouple was used to monitor the temperature and from a plot of the temperature decrease a cooling rate of 2°C / min determined ( Fig 39 ). The temperature had equilibrated at ≈ 0°C within 15 minutes and the oocytes were then left for a further 15 minutes to ensure the exposure time to 0°C was equal to that in the previous experiments. The tubes containing the oocytes were then removed, placed in an incubator at 37°C and allowed to
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...equilibrate at 37°C (approximately 5 minutes) prior to re-equilibration with isotonic media for 1 h. After this, they were inseminated.

6.4.6 Parthenogenic Activation.

In order to determine the influence of exposure to low temperatures, a hypertonic environment, and a combination of these stresses, on parthenogenic activation, oocytes were exposed to either isotonic or hypertonic conditions at both 37°C and 0°C, and re-equilibrated with isotonic media at 37°C as in procedures 6.4.1 - 6.4.5. Although cultured in T6 as for I.V.F. (section 2.7, 2.10.1) the oocytes were not incubated with sperm but observed for signs of spontaneous parthenogenic development at 24 h and 48 h.

Fertilisation in the mouse following all of the above procedures was determined by division to the 2-cell stage, after 24 hrs in culture. Fertilisation in the human was indicated by the presence of 2 pronuclei approximately 16 to 19 h post insemination. The developmental potential of those oocytes successfully fertilised was followed by observing the embryos through to the hatching blastocyst stage in the case of the mouse, and in the human, through 1 or more cell divisions.
6.5 The Influence of equilibration with cryoprotectants prior to exposure to a hypertonic environment or low temperatures.

During cryopreservation cells undergo an inevitable exposure to high concentrations of both electrolytes and cryoprotectants at low temperatures. It was decided, to investigate the influence of prior equilibration with cryoprotectants on the adverse affects of hypertonic exposure at low temperatures. As in chapter 5, the cryoprotectants chosen for study were glycerol and DMSO.

6.5.1 Equilibration with Glycerol.

Cumulus free human and mouse oocytes were obtained as previously described in sections 2.5.1, 2.9.1. Groups of 20 mouse ova were pipetted into 0.5 ml of 0.25 M glycerol at 37°C and allowed to equilibrate for 20 minutes, after which 0.5 ml of 1.0 M glycerol was added. Thorough mixing of the two solutions was ensured and the oocytes returned to a 37°C incubator and left to equilibrate with the final concentration of 0.625 M glycerol for 40 minutes. Human ova were allowed to equilibrate with the initial 0.25 M glycerol for 25 minutes prior to the addition of 0.5 ml of 1.0 M glycerol in which they were equilibrated for approximately 50 minutes. Following full equilibration of the cells with 0.625 M glycerol in PB1 at 37°C (signified by the shrink/swell response) and complete re-expansion
of the oocytes to their original diameters, their response to exposure to an hypertonic environment and low temperatures were investigated. A number of conditions were imposed on both the mouse and human oocytes including:

i) Exposure to 0.625 M glycerol at 37°C.

ii) After equilibration at 37°C the ova were removed from and placed directly into 0.625 M glycerol previously cooled to 0°C on ice in a refrigerator and exposed to 0°C for 30 minutes. The oocytes were then placed in 0.625 M glycerol at 37°C for 5 minutes to return to and stabilise at physiological temperatures.

iii) Following complete re-expansion in glycerol at 37°C, the oocytes were removed and placed in 0.5 M NaCl + 0.625 M glycerol at 37°C for 15 minutes. The ova were then transferred back into 0.625 M glycerol at 37°C for 5 minutes to allow return to isotonic cell volume.

iv) Oocytes were removed from glycerol at 37°C, pipetted into 0.625 M glycerol at 0°C, left for 15 minutes and then transferred to 0.5 M NaCl + 0.625 M glycerol at 0°C for a further 15 minutes after which they were again placed in 0.625 M glycerol in PB1 and incubated at 37°C for 5 minutes.
When the oocytes had returned to physiological temperature the intracellular glycerol was removed by transferring the cells to 0.5 M sucrose in PB1 at 37°C for 60 minutes. Subsequently the oocytes were cultured at 37°C for 60 minutes and then fertilised as in sections 2.7, 2.10.1.

6.5.2 Equilibration with DMSO

Mouse and human oocytes from which the cumulus cells had been removed (sections 2.5.1, 2.9.1) were again investigated under different conditions including:

i) Ova were transferred to tubes containing 1 ml of DMSO in PB1 pre-cooled to 0°C on ice in a refrigerator and equilibrated for 15 minutes prior to return to physiological conditions.

ii) After 15 minutes in 1.5 M DMSO at 0°C and full re-expansion to isotonic cell volume, the ova were transferred directly into hypertonic 0.5 M NaCl in 1.5 M DMSO at 0°C and allowed to dehydrate and equilibrate with the extracellular medium for 15 minutes. The cells were then returned to 1.5 M DMSO at 0°C for 10 minutes to re-expand to isotonic cell volume.

The intracellular DMSO was removed by transferring the
oocytes to 1 ml of PB1 at 37°C and incubation for 15 minutes. After the oocytes had re-equilibrated at 37°C they were transferred to IVF culture conditions for 60 minutes prior to insemination as in section 2.9.

6.5 Statistical Evaluation

Comparisons were made between the various treatment groups to determine the influence of exposure to low temperatures and/or an osmotic stress on developmental capacity of the mouse oocyte. To determine the significance of any alterations in fertilisation and development the results were analysed using a $X^2$ test with a $P$ value of $P < 0.01$ as the cut off level for establishing significance. Since the chance of arriving at a significant value with multiple comparisons is greater than when analysing a single result, a $P$ value of 0.01 was chosen for statistical significance rather than 0.05. In line with the Bonferroni method for multiple comparisons (Gower et al, 1989).

Results

6.6 The Influence of Osmotic Shock and Low Temperatures on the Cell Function of the Mouse Oocyte.

Exposing mouse oocytes to isotonic PBI and hypertonic 0.5 M NaCl for 30 minutes and 15 minutes respectively at 37°C had no adverse effects on cell viability or function as determined by fertilisation and development to
blastosysts (Table 18). In the two treatment groups at 37°C fertilisation rates of 90% for PBI exposure, and 89% for 0.5 M NaCl, were achieved as determined by the numbers dividing to the two cell stage. These compared favourably with the results achieved for the I.V.F. control group of 95%. Although a slight decrease in the development to hatching blastocyst was recorded for those ova exposed to isotonic media at 37°C (80%) and following an imposed osmotic stress (79%), this was not significantly different from the development in the control group (92%). (All values are expressed as a percentage of the original number of oocytes treated rather than of those fertilised).

Although culturing the mouse oocyte in isotonic media at 37°C for 30 minutes produced no significant decreases in either the fertilisation rate or the development to hatching blastocysts, exposing ova to isotonic media at 0°C significantly decreased cell viability. A 95% fertilisation rate was achieved in the I.V.F. controls which fell to 75% following cooling to 0°C. A further slight decrease was observed in the numbers of ova progressing to the hatching blastocysts stage (at 67%) which again was lower than in the control group at 92% (Table 18).

Imposing an additional osmotic stress onto mouse oocytes cooled to 0°C caused a further reduction in both
the fertilisation rate and the number continuing to develop after successful insemination. The fertilisation rate of 56% for hypertonic exposure at low temperatures (compared to 95% for the control rate) was significantly lower within the limits of $P < 0.01$. However of those oocytes developing to the 2-cell stage, a large number failed to continue through the normal developmental processes to blastocyst stage. Although 56% of the oocytes fertilised, only 26% of these retained the ability to continue normal development (Table 18). Of the oocytes successfully fertilised but which did not continue dividing past the 2-cell level 30% fragmented and began to degenerate.

Imposing an osmotic stress at 37°C, prior to cooling to 0°C resulted in significantly reduced rates ($P < 0.01$) of fertilisation (26%) and lowered capacity for development to hatching blastocysts (15%) (Table 19). Many of the oocytes after incubation with spermatozoa were penetrated by multiple sperm heads (Fig 40a, 40b). Unlike those oocytes cooled and subsequently dehydrated, a number of these oocytes were observed to have an abnormal appearance on re-equilibration with isotonic media at 37°C. Many of the cells lost the normal refractile quality of the cytoplasm, which in some cases was accompanied by an increase above isotonic cell volume. Of those oocytes undergoing equilibration with 0.5M NaCl and subsequent
Table 18 Fertilisation and Development of Mouse Oocytes Following Hypertonic Exposure at Low Temperatures

<table>
<thead>
<tr>
<th></th>
<th>37°C</th>
<th>0°C</th>
<th>0.5M NaCl</th>
<th>Isotonic</th>
<th>0.5M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Number</td>
<td>200</td>
<td>120</td>
<td>174</td>
<td>202</td>
<td>278</td>
</tr>
<tr>
<td>No. Fert.</td>
<td>190</td>
<td>108</td>
<td>154</td>
<td>151</td>
<td>156</td>
</tr>
<tr>
<td>% Fert.</td>
<td>95</td>
<td>90</td>
<td>89</td>
<td>75 *</td>
<td>56 *</td>
</tr>
<tr>
<td>No. H.B.</td>
<td>184</td>
<td>102</td>
<td>143</td>
<td>135</td>
<td>72</td>
</tr>
<tr>
<td>% H.B.</td>
<td>92</td>
<td>85</td>
<td>82</td>
<td>67 *</td>
<td>26 *</td>
</tr>
</tbody>
</table>

Fertilisation based on the number of oocytes at 2 cell stage 24h post insemination as a percentage of the original number.

* Significant at P < 0.01 X² to control rate
Table 19 Fertilisation of Mouse Oocytes Following Slow Cooling to 0°C and Parthenogenic Activation.

<table>
<thead>
<tr>
<th></th>
<th>Indirect cool to 0°C</th>
<th>Parthenogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Isotonic 0.5M NaCl</td>
<td>Isotonic 0.5M NaCl</td>
</tr>
<tr>
<td>Number</td>
<td>200 124 *231</td>
<td>53 57</td>
</tr>
<tr>
<td>No Fert.</td>
<td>190 103 61</td>
<td>0 0</td>
</tr>
<tr>
<td>% Fert.</td>
<td>95 83 26 **</td>
<td>0 0</td>
</tr>
<tr>
<td>No H.B.</td>
<td>184 85 34</td>
<td>0 0</td>
</tr>
<tr>
<td>% H.B.</td>
<td>92 69 15 **</td>
<td>0 0</td>
</tr>
</tbody>
</table>

* 38% of ova became fragmented on rewarming and incubation at 37°C
** Significant at P < 0.01 X² to control rate
Cooling to 0°C, 38% appeared to be abnormal upon return to 37°C.

A check was run on the treatments performed on the murine oocytes to examine whether the various regimes of exposure to a cold environment, either with or without an imposed osmotic stress, resulted in spontaneous parthenogenetic activation. Although none of the conditions caused parthenogenic development to occur, exposure to hypertonic saline at 37°C followed by cooling to 0°C resulted in many of the oocytes appearing morphologically abnormal on return to 37°C, with many rapidly becoming fragmented. An oocyte in the process of degeneration can take on the appearance of a dividing embryo and only by judging the number of cell divisions appropriate for the length of time in culture could the fragmenting oocyte be distinguished from the normally developing diploid embryo. All of the oocytes examined for spontaneous activation after being osmotically stressed at 37°C and then cooled were fragmented rather than dividing parthenogenically, since at 24 h post insemination many appeared to have divided to the 8 cell plus with uneven blastomere size (Figures 41a, 41b).

6.7 Equilibration of Mouse Oocytes with Glycerol

Equilibrating mouse oocytes with 0.625 M glycerol at 37°C led to a reduction per se in the number of oocytes
 CHAPTER 6 - COOLING AND OSMOTIC STRESS

fertilising (57%) and developing to the hatching blastocyst stage (52%) which in comparison to the control IVF rate of 94% fertilisation and 90% development to hatching blastocyst was significantly different at P < 0.01 (Table 20). Although equilibration with glycerol at 37°C appeared to be detrimental to oocyte viability, cooling the cells to 0°C following full re-expansion and equilibration at 37°C caused no further loss. At 0°C in glycerol 50% of the ova divided to the 2 cell stage and 49% continued to the hatching blastocyst stage. This was contrary to the situation when ova were exposed to 0°C in PB1 alone when fertilisation fell from 90% at 37°C to 75% with 67% progressing to the hatching blastocyst level (Table 18).

Although survival following an imposed osmotic stress at 37°C in glycerol equilibrated ova was significantly lower (P < 0.01) than those exposed to hypertonic 0.5 M NaCl at 37°C alone it caused no further reduction in viability from that recorded after glycerol equilibration at 37°C. This was as expected from earlier results since fertilisation and development following exposure to a hypertonic environment at 37°C were the same as in the control culture conditions. In addition however, equilibration of ova with 0.625 M glycerol followed by exposure of the cells to a hypertonic solution at low temperatures did not significantly lower cell viability.
Figure 40: Magnification Factor x 400.
Figure 40 Morphology of the oocyte following insemination after cooling and shrinkage showing multiple sperm penetration.

Figure 41 Morphology of the oocyte on return to physiological conditions following dehydration and cooling to 0C.

Figure 41: Magnification Factor x 600.
compared to glycerol exposure at 37°C. However the number of ova developing to the hatching blastocyst stage (41%) was significantly higher ($P < 0.01$) than the rate for an imposed osmotic stress at 0°C without the presence of glycerol (26%).

6.8 Equilibration of Mouse Oocytes with DMSO

Exposing the unfertilised mouse oocyte to 1.5 M DMSO at 0°C and subsequent dilution caused a significant reduction in the fertilisation rate compared to the I.V.F. control rate of 93% (Table 21). However both the number of oocytes developing to 2-cell (73%) and to hatching blastocysts (65%) were not significantly different from those merely cooled in isotonic media to 0°C in which 75% fertilisation and 67% development to hatching blastocyst were recorded (Table 18). Development to 2-cell (73%) after equilibration with 1.5 M DMSO at 0°C was not significantly different to that observed following exposure to 0.625 M glycerol at 37°C and subsequent cooling to 0°C (52% and 49% respectively). Although there was a disparity between the numbers fertilising after glycerol exposure (50%) and DMSO (73%) the difference at the hatching blastocyst stage (60%) was not statistically significant.

The application of an osmotic stress of 0.5 M NaCl at 0°C following prior equilibration with DMSO was not as
detrimental to oocyte development as exposure to a hypertonic environment without the presence of cryoprotectant. A fertilisation rate of 60% with 42% continuing development to the hatching blastocyst stage was achieved after DMSO exposure as opposed to 56% (fertilisation) and 26% (hatching blastocysts) without CPA. While equilibration with DMSO at 0°C appeared not to have as adverse an effect on oocyte viability as exposure to glycerol, imposing an osmotic stress on the ova while at low temperatures in DMSO reduced survival to the level recorded when ova were exposed to a hypertonic stress after glycerol equilibration (42% and 41% respectively, Table 20, 21).


It was impossible to perform statistically valid tests on the fertilisation rate and cell division stage to which the embryos progressed for the human material due to small numbers involved. The only comparisons that could be made were to the overall fertilisation rate in the IVF unit and the average development stage reached at 48 hours prior to replacement. All cell division stages cited in Table 22 were for experimental embryos examined 48 hours post insemination.

As with mouse oocytes equilibration with isotonic media
at 37°C had no deleterious effects on the development capacity of the human oocyte, with 8 out of 10 oocytes fertilising normally and continuing to divide. Fertilisation was indicated by the presence of 2 pronuclei at approximately 19 h post insemination. The number of cell divisions through which each embryo proceeded was within the normal spread for I.V.F. treatment (Table 22). In addition, as again was the case with the mouse oocytes, exposure to hypertonic saline at 37°C did not cause any decrease in fertilisation, with 8 out of 9 oocytes exhibiting two pronuclei, 6 of which continued to develop through 1 or more cell divisions and once again development was as expected for untreated controls (Table 22).

Unlike the mouse however, exposure to PBI at 0°C appeared to have no effect on the capacity of the human oocyte to fertilise and divide. Of the oocytes incubated at 0°C in isotonic media, 8 from a group of 9 developed 2 pronuclei, and of these 5 continued to develop with 1 embryo progressing through three cell divisions to the 8 cell stage within 48 hours of insemination. The combined effects of osmotic stress and low temperatures resulted in a decrease in both in the ability of the oocytes to fertilise and the developmental capacity. Only 3 of the 10 oocytes in which the oocytes experienced temperature stress and subsequent dehydration developed pronuclei. Of those
Table 20  Fertilisation and Development of Mouse Oocytes Following Equilibration with Glycerol and Subsequent Hypertonic Exposure at Low Temperatures

<table>
<thead>
<tr>
<th></th>
<th>0.625M Glycerol</th>
<th>Glycerol + 0.5 M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
<td>0°C</td>
</tr>
<tr>
<td><strong>Number</strong></td>
<td>150</td>
<td>118</td>
</tr>
<tr>
<td><strong>No. Fert</strong></td>
<td>141</td>
<td>67</td>
</tr>
<tr>
<td><strong>% Fert.</strong></td>
<td>94</td>
<td>57 *</td>
</tr>
<tr>
<td><strong>No. H.B</strong></td>
<td>137</td>
<td>61</td>
</tr>
<tr>
<td><strong>% H.B.</strong></td>
<td>91</td>
<td>52 **</td>
</tr>
</tbody>
</table>

* Statistically different at \( P < 0.01 \) from the control IVF fertilisation rate.
Table 21  Fertilisation and Development of Mouse Oocytes Following Equilibration with DMSO and Subsequent Hypertonic Exposure at Low Temperatures

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>1.5M DMSO</th>
<th>1.5M DMSO 0.5M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number(n)</td>
<td>103</td>
<td>145</td>
<td>141</td>
</tr>
<tr>
<td>No. Fert.</td>
<td>97</td>
<td>103</td>
<td>85</td>
</tr>
<tr>
<td>% Fert.</td>
<td>94</td>
<td>73 *</td>
<td>60 *</td>
</tr>
<tr>
<td>No. H.B.</td>
<td>95</td>
<td>94</td>
<td>59</td>
</tr>
<tr>
<td>% H.B.</td>
<td>92</td>
<td>65 **</td>
<td>42 **</td>
</tr>
</tbody>
</table>

* Statistically different from the control IVF fertilisation rate
oocytes in which fertilisation was confirmed, 1 was a triploid which was indicative of abnormal fertilisation. This may be as a result of failure to extrude one of the polar bodies leading to the retention of a set of female chromosomes. Alternatively, polyspermy may have resulted from a failure of the mechanisms of depolarisation of the oocyte plasma membrane and release of the cortical granules involved in blocking penetration by more than one spermatozoa. Of the 2 remaining oocytes in which fertilisation was normal, cell division failed to proceed past the first cell division and the 2-cell stage. After 48 h extensive fragmentation was observed which suggests a poor quality embryo and the beginning of degeneration (Table 22).

The adverse effects of hypertonic exposure prior to cooling the oocytes to 0°C in the mouse, in which large numbers of abnormal forms were observed with an extremely low rate of fertilisation (26%) and development (15%), were repeated with the human material (Table 23). None of the oocytes experiencing an osmotic stress prior to incubation at 0°C for 30 minutes developed pronuclei (0/7) and several had lost the refractile quality of the cytoplasm, had been penetrated by multiple spermatozoa and had numerous sites of sperm head decondensation.
6.10 The Developmental Potential of the Human Oocyte Following Equilibration with Glycerol

The decrease in the fertilisation rate observed following exposure of mouse oocytes to glycerol was repeated when fresh human oocytes were equilibrated with 0.625 M glycerol at 37°C, irrespective of the subsequent temperature to which the oocytes were exposed. Following incubation of ova at 37°C in isotonic PB1, 8 ova developed 2 pronuclei, while 9 of the oocytes exposed to 0°C in isotonic media fertilised. Exposure of fresh oocytes to glycerol at 37°C resulted in a decrease in the numbers fertilising with only 5 from a group of 9 developing pronuclei (Table 24). Similarly after glycerol exposure and cooling to 0°C only 4 of the 10 oocytes examined developed 2 pronuclei (Table 24).

Equilibrating human ova with glycerol, while causing a decrease in the fertilisation rate, appeared to be even more detrimental to the developmental capacity of those cells that had successfully fertilised. All of those...
Table 22 Fertilisation of the Human Oocyte Following Cold Exposure

<table>
<thead>
<tr>
<th></th>
<th>37°C Isotonic</th>
<th>37°C 0.5M NaCl</th>
<th>0°C Isotonic</th>
<th>0°C 0.5M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (n)</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Pronucleated</td>
<td>8</td>
<td>8</td>
<td>9 2 PN.</td>
<td>2 2 PN.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 3 PN.</td>
<td></td>
</tr>
<tr>
<td>Developmental Stage</td>
<td>2 2 cells</td>
<td>3 2 cells</td>
<td>2 2 cells</td>
<td>2 2 cells</td>
</tr>
<tr>
<td></td>
<td>1 3 cell</td>
<td>4 4 cells</td>
<td>4 4 cells</td>
<td>+ fragments</td>
</tr>
<tr>
<td></td>
<td>4 4 cells</td>
<td>3 4 cells</td>
<td>2 6 cell</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 6 cell</td>
<td></td>
<td>1 8 cell</td>
<td></td>
</tr>
</tbody>
</table>

235
### Table 23: Fertilisation of the Human Oocyte Following Slow Cooling to 0°C.

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Isotonic</th>
<th>Hypertonic</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Pronucleated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2x2 cells</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>3x4 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1x6 cell</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Number (n)**

<table>
<thead>
<tr>
<th></th>
<th>Isotonic</th>
<th>Hypertonic</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x2 cells</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>3x4 cells</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1x6 cell</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
oocytes in which pronuclei were observed after exposure to PB1 either at 37°C or 0°C proceed through a minimum of 1 cell division and some progressing to the eight cell stage by 48 h after insemination. However, when the human oocytes were equilibrated with glycerol, they all remained arrested at the pronuclei stage, failing to continue to divide by 48 h (Table 24).

As previously stated hypertonic exposure per se did not appear to be deleterious to either fertilisation or development in the human as 8 from a group of 9 fertilised and divided to the 2 - 4 cell stage. However, following glycerol equilibration at 37°C in conjunction with a hypertonic stress at 0°C the fertilisation rate was reduced (Table 24) although as with the mouse this was not below the numbers recorded following exposure to glycerol alone. Combining the affects of hypertonic exposure and low temperatures was detrimental to both fertilisation and development with only 3 from 10 developing pronuclei when cooling was followed by hypertonic exposure, and 0 from 7 when cooling was preceded by hypertonic exposure (Table 24). The addition of glycerol to oocytes prior to the combined treatment reduced the detrimental effects by increasing the numbers successfully fertilising to 7 from a group of 12 ova. However as with all other treatments in which the oocytes were equilibrated with glycerol, none of the fertilised ova continued to divide.
6.11 The Developmental Potential of the Human Oocyte Equilibrated with DMSO Prior to Imposing an Osmotic Stress at 0°C.

Following incubation of fresh human oocytes with 1.5 M DMSO at 0°C, 8 from a group of 10 oocytes successfully fertilised developing 2 pronuclei, a rate which was comparable to those incubated in isotonic PB1 at 37°C (Table 25). Of those fertilising, 4 continued to develop, 3 to the 2 cell stage and 1 to the 4 cell stage, a result which was in contrast to the effects of equilibration with glycerol at 37°C, as described in section 6.10, in which both the potential to fertilise and to continue normal development past the pronucleate stage was reduced after CPA exposure.

Equilibrating fresh human oocytes with DMSO at 0°C prior to exposure to hypertonic 0.5 M NaCl resulted in 5 oocytes developing pronuclei (5/12). The number of ova fertilising was less when DMSO was used as the protective substance as opposed to glycerol (7/12). However, 2 from the 5 pronucleated ova, after exposure to hypertonic medium and low temperatures in the presence of DMSO, progressed through one cell division on incubation for a further 24 h. This was in contrast to culture following the removal of the glycerol, which revealed that all the embryos were arrested at the pronucleated stage. In addition, the ensuing embryos produced following DMSO
Table 24 Fertilisation and Development of Human Oocytes Following Equilibration with Glycerol and Subsequent Hypertonic Exposure at Low Temperatures

<table>
<thead>
<tr>
<th></th>
<th>0.625M Glycerol</th>
<th>Glycerol + 0.5 M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
<td>0°C</td>
</tr>
<tr>
<td>Number(n)</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>No.Fert.</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Devel. Stage</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td>0°C</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>
Table 25  Fertilisation and Development of Human Oocytes Following Equilibration with DMSO and Subsequent Hypertonic Exposure at Low Temperatures

<table>
<thead>
<tr>
<th></th>
<th>1.5M DMSO</th>
<th>1.5M DMSO + 0.5M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number(n)</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>No.Fert.</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Devel. Stage</td>
<td>3 x 2 cell</td>
<td>2 x 2 cell</td>
</tr>
<tr>
<td></td>
<td>1 x 4 cell</td>
<td></td>
</tr>
</tbody>
</table>
exposure showed no signs of fragmentation at 48 h post insemination. However on subsequent observation at 72 h or longer, no further cell divisions had occurred.

6.12 Summary

i) Cooling unfertilised ovulated mouse oocytes to 0°C in an isotonic environment caused a reduction in both the fertilisation rate and the developmental potential of the resulting embryos.

ii) Cooling fresh human pre-ovulatory oocytes in a isotonic media had no affect on cell function either in numbers fertilising or progressing through a minimum of 1 cell division, although numbers were necessarily small.

iii) Imposing an additional osmotic stress in the form of a hypertonic environment after the oocytes, both mouse and human, had been rapidly cooled to 0°C, significantly reduced the developmental potential of the cell.
iv) When the cells were osmotically stressed prior to cooling the numbers of morphologically abnormal oocytes increased in addition to which oocyte viability was reduced below that for shrinkage after cooling.

v) The addition of glycerol to mouse ova prior to hypertonic exposure significantly increased development to the hatching blastocyst stage over that seen after hypertonic exposure of unprotected cells.

vi) The number of mouse embryos developing to the blastocyst stage after equilibration with DMSO and exposure to 0.5 M NaCl at 0°C was not significantly different to that achieved following equilibration with glycerol and hypertonic exposure at 0°C.

vii) For the human oocyte, although equilibration with glycerol increased the number of oocytes fertilising following hypertonic exposure at 0°C, there appeared to be a prohibitive effect on further cell division.
viii) Equilibration with DMSO prior to hypertonic exposure at low temperatures resulted in an increase in the number of human oocytes fertilising in comparison to an osmotic stress at low temperatures. In addition at 48 h incubation the pronucleated ova had undergone cell division.
CHAPTER 7

Cryopreservation of the Unfertilised Oocyte.

7.1 Aims

In Chapter 6 it was shown that cooling both the mouse and human oocytes to 0°C, in conjunction with exposure to a hypertonic environment, significantly reduced cell viability. Cell function was affected to a greater extent when the oocytes were exposed to hyperosmotic conditions prior to cooling. Equilibrating human and mouse oocytes with glycerol or DMSO prior to treatment appeared to protect the oocytes as indicated by the increased fertilisation rate compared to cooling accompanied by an osmotic stress alone. Thus I decided to use these two cryoprotectants effects of cryopreservation on mouse and human oocytes this would allow assessment of the effects of ice formation and very low temperatures as additional factors from those studied in chapter 6.

7.2 Introduction

Cryopreservation of unfertilised oocytes has become an increasingly attractive proposition as part of treatments for infertility, overcoming some of the ethical and religious objections to embryo storage. During cryopreservation it is essential to avoid the formation of intracellular ice crystals (I.I.F.) if the cell is to
survive the procedure. This requires that the intracellular water content be known and accurately predicted during cooling. During cryopreservation as a result of the reduction in temperature ice nucleates in the extracellular medium causing an increase in the solute concentration of the remaining solution, which causes development of a differential in the chemical potential of the intracellular and extracellular water. Water tends to move down the chemical potential gradient which causes the cell to dehydrate. Equilibrium cooling, in which the differences between intra- and extracellular osmotic pressures are eliminated as water leaves the cell to maintain an equilibrium with the extracellular water potential, avoids intracellular ice nucleation and thus lethal cell damage. However, prolonged exposure of cells to high concentrations of solutes during such slow cooling can be harmful per se. On the other hand "rapid" cooling, while avoiding the toxic affects of high concentrations of cryoprotectants and damage from high concentrations of electrolytes allows insufficient time for cell dehydration, resulting in a tendency towards intracellular ice crystal nucleation.

Predicting the rate of temperature decrease which allows sufficient cell dehydration and avoids I.I.F. necessitates the investigation of the parameters which dictate the rate at which water can leave the cell. The membrane water permeability and activation energy were
known for the mouse oocyte (Leibo, 1980, Bernard et al, 1988, Toner, 1990) but remained unknown for the human oocyte. This thesis involved the derivation of the permeability parameters of fresh human oocyte using the microscope diffusion chamber (McGrath, 1985). The values predicted for the human oocyte in my investigation were in the same range as those for the mouse both in previous studies. Since $L_p$ and $E_a$ were similar, this indicated that the rate of water loss was similar to the mouse. Thus the cooling rates already established for producing viable mouse ova following cryopreservation may also produce viable human oocytes. It was therefore decided to investigate the survival of the human oocytes using cooling conditions similar to those in cryopreservation protocols derived for murine oocytes using DMSO (Whittingham, 1977) and glycerol (Fuller and Bernard, 1984).

**Materials and Method**

7.3 Protocol 1: Glycerol as CPA

7.3.1 Glycerol Addition

Groups of 20 murine oocytes or 1 human oocyte were transferred into a 50μl droplet of 0.25 M glycerol in PBS in a culture dish (Becton & Dickinson, UK) and held at 37°C for 20 and 25 minutes respectively. The oocytes were transferred to droplets of 0.625 M glycerol and allowed to
equilibrate at 37°C for 40 minutes for the mouse and 50 minutes for the human oocyte. After equilibrating the ova with glycerol they were pipetted into sterile plastic insemination straws (L'AIGLE, France) containing 45 μl of 0.625 M glycerol and separated by an air bubble 100 μl of 0.5 M sucrose.

7.3.2 Cryopreservation

The straws with the oocytes were transferred to a programmable freezing machine (P.T.C. 200, Planar Products Ltd, Middlesex.) and cooled at 2°C/min to -7°C as indicated by a thermocouple in a dummy straw. Once the temperature of the straws had reached -7°C, ice nucleation was induced by touching the surface of each straw at the level of the meniscus of the medium with forceps cooled in liquid nitrogen. The temperature was held at -7°C for 15 minutes to allow dissipation of the latent heat of ice crystallisation, after which the straws were cooled at -0.3°C/min to -35°C (Fig 35). The samples were then transferred directly to liquid nitrogen at -196°C and stored for 1 to 7 days.

7.3.3 Glycerol Removal

The samples were warmed rapidly at a rate of 200°C - 300°C/min by agitation in a 37°C water bath. The glycerol was removed by expelling the ova into 1.0 ml of 0.5 M
sucrose which, due to the fact that the cell membrane was impermeable to sucrose, provided as osmotic buffer to prevent the cell from over-expanding initially due to a rapid influx of water into the cell during dilution. It also allowed control of cell volume during diffusion of glycerol out of the cell. After exposure to sucrose for 1 hr at 37°C the oocytes were washed in PB1 three times prior to transfer to T6 medium for fertilisation and on-growth as described in sections 2.7 and 2.10.

7.4 Protocol 2: Dimethylsulphoxide as CPA.

Straws containing 45 µl of 1.5 M DMSO and 100 µl of PB1 were cooled to 0°C and left for 5 minutes at this temperature. Then, 20 mouse oocytes or a single human oocyte were pipetted into the straws and 15 minutes allowed for equilibration with the cryoprotectant at 0°C. The straws were then cooled to -7°C at -2°C / min, and seeding was induced by touching the outside of the straw with forceps pre cooled in liquid nitrogen. The straws were left for 15 minutes for dissipation of the latent heat of ice crystallisation, after which they were cooled at 0.3°C / minute to -65°C, and then plunged into liquid nitrogen at -196°C as described by Whittingham, 1977. The oocytes were stored for 1 - 7 days and then rewarmed at a rate of 8°C / minute to 0°C in the programmable freezer, following which the oocytes were expelled into 1.0 ml of PBS to remove the
cryoprotectant. The oocytes were then washed three times in PB1 and incubated at 37°C in T6 for 1 hr prior to addition of sperm for fertilisation and subsequent culture as for I.V.F. (section 2.9).

Both mouse and human ova were exposed to cryoprotectant without freezing as CPA controls and fertilised and cultured as for I.V.F. Mouse and human oocytes were equilibrated with 0.25 M glycerol for 20 minutes and 25 minutes respectively. This was followed by equilibration with 0.625 M glycerol for 40 and 50 minutes respectively after which the glycerol was removed by incubating the oocytes with 0.5 M sucrose for 60 minutes at 37°C. Mouse and human ova were equilibrated with DMSO at 0°C for 15 minutes after which they were pipetted into PB1 at 37°C to remove the cryoprotectant and allowed to equilibrate for 60 minutes prior to insemination and culture.

Unfertilised mouse oocytes were used as a model system against which the success of the cryopreservation procedure could be measured. The numbers of viable mouse oocytes could be compared directly to those achieved in previous studies using identical protocols. From these comparisons, conclusions could be drawn concerning the survival of human oocytes using the same techniques.
Results

7.5 Mouse Oocyte Cryopreservation.

In my investigation, the morphological survival rate was 90% & 87% following exposure to glycerol at 37°C & cooling to 0°C respectively and 84% after cryopreservation (Table 26) indicating losses of between 10 - 16% of the original number of oocytes. These losses were either as a result of difficulties locating the oocytes when expelled from the straws and thus failure to find the original number in the media, or retention in the straws, or severe damage to individual oocytes, resulting in cell disintegration. Development to the hatching blastocyst level was 50%. All values expressed in my study are a percentage of the original number of oocytes investigated rather than of the number of morphologically normal oocytes on thawing or those that fertilised. In a study by Fuller and Bernard, 1984 in which 0.625 M glycerol was used as a cryoprotectant for unfertilised mouse ova, the authors achieved a morphological survival rate of 82% following glycerol exposure (CPA controls) and 89% during cryopreservation. Subsequent development to hatching blastocyst was 55% which was similar to that achieved in my study.

Cryopreservation of unfertilised mouse oocytes using DMSO as a cryoprotectant was also found to produce survival at rates similar to those recorded in the original report.
(Whittingham, 1977). A fertilisation rate of 53% was derived for my study, the majority of which continued to develop to the hatching blastocyst stage (47%) (Table 26) while a fertilisation rate of 61% and 50% (with and without cumulus cells respectively) was achieved by Whittingham, 1977. Fertilised 2-cell embryos were transferred to pseudo pregnant females in the original study and normal foetuses resulting in live born young.

7.6 Human Oocyte Cryopreservation

The number of morphologically normal oocytes and those developing on after fertilisation suggested that, for the murine system, both glycerol and DMSO were achieving reasonable rates of survival following cryopreservation and thus the protocols were applied to the fresh human oocytes. Exposure of the human oocytes to both glycerol and DMSO in the two different protocols caused a reduction in the fertilisation rates compared to those for normal I.V.F. which were used as a comparative "control". Under the comparable I.V.F conditions, fertilisation and cell division past the pronucleate stage was 72%. However, only 4/10 and 5/10 oocytes developed pronuclei at 24 hrs post insemination for glycerol and DMSO exposure respectively (Table 27). No further cell development was observed with oocytes equilibrated with glycerol as a cryoprotectant. However, 4 of 5 fertilised following
Table 26 Fertilisation and development of mouse ova following cryopreservation.

<table>
<thead>
<tr>
<th></th>
<th>GLYC. CONTROL</th>
<th>GLYCEROL FREEZE</th>
<th>DMSO 0°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
<td>0°C</td>
<td>CONTROL 0°C</td>
</tr>
<tr>
<td><strong>Number</strong></td>
<td>135</td>
<td>190</td>
<td>230</td>
</tr>
<tr>
<td><strong>No Norm</strong></td>
<td>121</td>
<td>166</td>
<td>195</td>
</tr>
<tr>
<td><strong>% Norm</strong></td>
<td>90</td>
<td>87</td>
<td>84</td>
</tr>
<tr>
<td><strong>No. Fert</strong></td>
<td>85</td>
<td>119</td>
<td>154</td>
</tr>
<tr>
<td><strong>% Fert</strong></td>
<td>63</td>
<td>63</td>
<td>67</td>
</tr>
<tr>
<td><strong>No. H.B.</strong></td>
<td>75</td>
<td>101</td>
<td>114</td>
</tr>
<tr>
<td><strong>% H.B.</strong></td>
<td>56</td>
<td>53</td>
<td>50</td>
</tr>
</tbody>
</table>
Table 27 Fertilisation and cell division following cryopreservation of human ova using either glycerol or DMSO as the cryoprotective agent

<table>
<thead>
<tr>
<th></th>
<th>GLYC. CONTROL</th>
<th>GLYcerol Freeze</th>
<th>DMSO 0°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
<td>0°C</td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>10</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>No. Morp Norm.</td>
<td>10</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>No. Fert 2 PN</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Final Devel. Stage</td>
<td>4x2PN</td>
<td>4 x 2PN</td>
<td>3 x 2 PN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
exposure to DMSO continued to develop and progressed through at least 1 cell division. Both equilibration with and freezing in the presence of DMSO resulted in cell division, 4 / 10 from the CPA control group and 1 / 15 from those frozen and thawed continued through 1 or more cell divisions. While 4 / 10 oocytes fertilised (as signified by the presence of 2 pronuclei) following equilibration with glycerol alone and 4 / 9 following cryopreservation, further development was arrested, in all cases that glycerol was slightly more damaging to unfertilised human oocytes than DMSO.

7.7 Discussion

The number of ovulated mouse oocytes fertilising and developing following cryopreservation in my investigation and in other studies (Whittingham, 1977, Fuller and Bernard, 1984, Schroeder et al, 1990) show that while banking of a variety of genetic strains is possible, there is still considerable room for improvement using both protocols. In particular, it would be useful to direct investigations towards elucidating the reasons which caused some oocytes, which on thawing appeared morphologically normal, to resist fertilisation. While there is only a small rate of loss of viability between 2 - cell and development to blastocyst, the major area of loss in the mouse oocytes was in those ova which failed-to-fertilise.
CHAPTER 7 - CRYOPRESERVATION

Any alterations to the techniques to increase fertilisation may be reflected in an increased development to blastocyst. Alternatively, new procedures using different cryoprotectants or cooling rates, such as vitrification (Rall and Fahy, 1985, Nakagata, 1989), may ultimately produce higher survival. However, at present these techniques are adequate methods for preserving mouse oocytes producing approximately 50% development to hatching blastocyst.

There has been concern in the past that cryopreservation may result in an increase in the number of abnormal embryos produced. Indeed cooling oocytes, both mouse and human, to 4°C caused the disassembly of the meiotic apparatus and possible chromosome abnormalities (Pickering and Johnson, 1987, Johnson and Pickering, 1988, Sathananthan et al, 1988). Studies by both Whittingham, 1977 and Schroeder et al, 1990 investigated transfer of embryos to pseudo-pregnant females following insemination of oocytes cryopreserved using DMSO. These resulted in normal foetuses and live young. These observations suggest that, while the developmental potential of cryopreserved mouse oocytes is reduced, those that do fertilise have no gross abnormalities.

Fertilisation, as signified by the formation of 2 pronuclei, of the human pre-ovulatory oocytes was not prohibited by exposure to glycerol or DMSO (Table 27).
However, only a small number of oocytes exposed to DMSO continued to divide and none of those which had been equilibrated with glycerol developed past the pronucleate stage. Subsequent to freezing, only a single oocyte frozen using DMSO progressed to the 2-cell stage before development was arrested. None of those equilibrated with glycerol progressed beyond 2 pronuclei. This result is encouraging as it shows that human oocytes have the potential to survive cryopreservation, fertilise and develop further. However it is disappointing in that it indicates that I was still some way from defining optimal conditions for human oocyte cryopreservation. The fact that over 60% of the human oocytes cryopreserved with either glycerol or DMSO were morphologically normal, and more than 30% subsequently fertilised, suggested that the conditions chosen have avoided gross damage via intracellular ice formation. Thus the predictions I made about choice of cooling conditions, based on my measurements of $L_p$ and $E_a$ appear essentially correct. Other factors maybe influencing survival, such as susceptibility of pre-ovulatory oocytes to freezing damage.

While the survival of ovulated mouse oocytes is reasonably high, reports attempting to cryopreserve pre-ovulatory murine oocytes (Deansley, 1957, Schroeder et al, 1990) have had little success. In the investigation by Schroeder only 17% of the pre-ovulatory oocytes survived
and produced 2 cell embryos and only 2 % developed to the blastocyst stage. The numbers fertilising increased to 55 % following in vitro maturation with added FSH, but in both systems survival was low compared to that of ovulated oocytes, from which 88 % fertilised. However, no account of those excluded for morphological abnormalities appeared to have been taken when calculating their figures, as they were expressed as a percentage of the numbers inseminated. If these values are considered in conjunction with the poor fertilisation and small numbers of human oocytes undergoing cell division in my study it may be that pre-ovulatory ova are more susceptible to freezing damage than ovulated ova.

Summary

i) Both glycerol and DMSO equilibration prior to cryopreservation produced adequate rates of survival and development to the blastocyst stage for the mouse oocytes using the two chosen cooling and warming regimes.

ii) Successful fertilisation of human oocytes, and further development of the embryos produced, was achieved following equilibration with DMSO at 0°C.
iii) Although fertilisation of fresh human ova previously exposed to glycerol was recorded, all oocytes with 2 pronuclei failed to progress through a single cell division by 48 hrs in culture.

iv) Following cryopreservation, human oocytes were successfully inseminated after equilibration with both glycerol and DMSO. However, cell division to the 2 cell stage was recorded in only 1 oocyte which had undergone exposure to DMSO, freezing and thawing. No cell divisions were recorded after glycerol cryopreservation. These studies are the first, to my knowledge, to compare different techniques for cryopreservation of human oocytes under carefully controlled conditions. The results suggest that DMSO and slow cooling may be the best protocol to evaluate further.
Discussion

8.1 Principal Findings of my Studies

i) The measurements made for Lp, Vb and Ea utilising the microscope diffusion chamber were reproducible.

ii) The permeability parameters determined for the fresh ovulated murine oocyte by my technique were similar to those produced by other investigators.

iii) The Lp and Vb values derived for the human pre-ovulatory oocytes, fresh and failed-to-fertilise, were much more varied than those for the mouse ova. Thus the Ea's calculated from the mean Lp values were difficult to interpret due to the large standard deviations for the Lp.

iv) Neither fertilisation nor in vitro maturation had a significant effect on the variability of the Lp or Vb values recorded for human ova.
v) Pre-ovulatory mouse oocytes were found to exhibit a greater heterogeneity in both Lp and Vb compared to those examined following ovulation.

vi) Although the range of Lp values determined at each of the investigative temperature for individual fresh human ova remained large, it was possible using these data to determine a more realistic value for Ea.

vii) Whilst measuring the Lp for the mouse and human oocytes at low temperatures (0°C) it became apparent that the cells did not shrink in a spherical manner as had occurred at temperatures > 5°C, questioning the advisability of extrapolating data derived assuming spherical shrinkage to subzero temperatures.

viii) A combination of cooling oocytes to low temperatures and osmotic stress was found to have a deleterious effect on cell function, reducing fertilisation and the subsequent embryonic developmental potential. By equilibrating the oocytes with CPA's prior to exposure to these adverse conditions the viability of the oocytes was protected to a degree.
ix) Having established that the values for $L_p$ and $E_a$ for the fresh human oocytes were in the same order as those for mouse oocytes, an attempt to cryopreserve the fresh human oocyte was made using regimes successfully applied to the mouse. Although fertilisation following both equilibration with the CPA and cryopreservation was achieved, the oocytes progressed through only limited cell division.

8.2.1 Assessment of the Microscope Diffusion Chamber

The passive movement of water across the cell membrane is of particular importance in cryobiology due to the dependence of the optimum rate of cooling and thawing on the cell water content. Since water permeability has been demonstrated previously to be a governing factor in determining the speed of water loss, the extent of supercooling, the likelihood of intracellular ice formation and thus cell death (Mazur, 1963, 1973, 1977), it has been extensively studied for numerous cell types employing many different techniques. By elucidating the dynamics of water transport in the unfertilised oocyte in response to a hypertonic extracellular environment such as experienced during freezing, cell survival following cryopreservation may be improved. Cell death is intimately associated with intracellular ice formation and using the derivation of the water permeability and its temperature coefficient, the
cooling rate that avoids intracellular ice crystallization can be calculated (Mazur, 1963). Knowledge of this optimum cooling rate would contribute towards the development of a cryopreservation protocol for unfertilised human oocytes.

The experiments in my study were designed to determine the osmotic properties of human oocytes and to supplement the knowledge already available for murine oocytes. However, prior to the development of the microscope diffusion chamber (McGrath, 1985), experiments on the transient osmotic response of cells to perfusion with an anisomotic solution required that large numbers of cells be available for analysis. Techniques such as the coulter counter exploited differences in the electrical conductivity of cells and pure medium to determine volumetric fluctuations. By measuring the increased resistance to an electric current applied to a cell suspension passing through a small orifice in a constant current system, and adjusting the threshold level above which the increased voltage was recorded and calibrating with latex beads of a known size, the cell volume could be measured (Hempling, 1977).

An alternative method for measuring volume changes in cell suspensions was developed by measuring the amount of light scattered and absorbed in a stop-flow spectrophotometer (Sha'afi and Gary-Bobo, 1973). However
the accuracy of such methods is debatable particularly when applied to small numbers of cells. In a study by Tate (1989) sea urchin eggs were used to calibrate equipment used for the derivation of the permeability parameters for bovine ova since they were cheap and abundant. Although the Coulter Counter technique was accurate at predicting the peak value for the volume, the distribution was consistently skewed towards smaller values. In conjunction with this the large numbers of cells (approximately 100 cells as a minimum) required for a single analysis meant that this system was unsuitable for use in determining the volume of cells in relatively limited supply (such as ova, and in particular human oocytes). In addition, since only mean values for a large number of cells are provided by Coulter Counter or stop-flow techniques, the lack of information concerning individual cells in a population is a major omission for specimens in which variability could dictate the type of regime imposed during cryopreservation.

In my study, the microscope diffusion chamber allowed monitoring of the response of individual cells in microliter volumes in real time, permitted repeatable changes of the extracellular solution to be made with ease during the course of a single investigation, and accurately controlled the temperature of the system. The osmotic response of cells to hypertonic perfusion could be recorded visually on video tape, which gave access to detailed information
pertaining to changes in both the volume and morphology of the oocytes. In addition, as the cells were visualized throughout the entire procedure, the initial period of volume fluctuation (which is critical for permeability studies of both water and coupled flow of water and solutes such as cryoprotectants) could be chronicled.

The diffusion chamber has been used in the past to accurately measure both equilibrium and non-equilibrium responses of a wide range of cells, including liposomes (Callow and McGrath, 1984, Sherban et al, 1985), lymphocytes (Sherban, 1987), mouse ova (Bernard et al, 1988), and hamster ova (Shabana and McGrath, 1988). These investigations using the microscope diffusion chamber to elucidate the membrane water permeability and inactive volume for other cell types (including the preliminary study by Bernard et al, 1988 on mouse and human ova) demonstrated the ability of the chamber and the associated software to generate realistic and reliable measurements of the permeability parameters. This justified the application of this method when attempting to determine the Lp, Ea, and Vb for fresh human ova.

8.2.2 Membrane Permeability Parameters of the Ovulated Mouse Oocyte

The mean Lp values derived for murine unfertilised oocyte at 20°C was directly comparable to those estimated
by Leibo (1983) and Bernard et al. (1988). In the study by Leibo a mean Lp value of 0.44 ± 0.03 μm / atm / min was derived by measuring the change in the cell diameter following immersion of the ova in a hypertonic environment. Leibo utilised the difference in the density of cell cytoplasm in isotonic and hypertonic solutions. In isotonic media the cell density is less than in hypertonic saline and by transferring an oocyte into a column of hypertonic solution it dehydrates until it achieves equilibrium density rising up the column. From photographs of the oocyte at various times the cross sectional area was determined and the Lp calculated. Even though the method used by Leibo differed from my study the value calculated correlated well with the Lp at 20°C of 0.48 ± 0.18 μm / atm / min for the mouse oocyte determined using the diffusion chamber.

Although the individual Lp values were not presented in the study by Leibo (1980), it was stated that the water permeability for fertilised and unfertilised mouse ova were not significantly different. For the 18 fertilised eggs observed, 5 were found not to fit the curves calculated for water loss for any Lp, 8 corresponded to Lp = 0.43, 4 to Lp = 0.38, and 1 to Lp = 0.48 μm / atm / min. Even though the majority of the ova fell within the limits of 0.38 - 0.48 μm / atm / min, it was important to note that 5 of 18 oocytes did not fit any of the predicted curves. It was
unlikely that these cells were abnormal, and suggests rather that the Lp's corresponding to the water loss in these particular cells was higher or lower than those of the predicted range. Thus the actual range of Lp values was probably larger than that recorded in the study (Leibo, 1980). In my study of the Lps for mouse oocytes at 20°C, 10 from a group of 20 ova fell between the limits 0.33 - 0.49 μm / atm / min. However, it should be noted that I have included all values in my calculations of mean values for Lp, Ea and Vb, because I did not consider it valid to select only values within pre-determined limits.

In the investigation by Bernard et al, 1988 using the microscope diffusion chamber a best estimate for the mean Lp of a group of 7 mouse oocytes was 0.36 ± 0.07 μm / atm / min which correlated well with the data in the study by Leibo, 1980 and that in my study. An investigation into the Lp of unfertilised mouse ova at subzero temperatures in the presence of ice (Toner, 1990) calculated a value of 0.044 μm / atm / min at 0°C. By extrapolating the Lp value determined at 20°C in the studies by Leibo, 1980 and Bernard et al, 1988 to 0°C, the author found the measured Lp at 0°C correlated relatively closely with the predicted values. The Lp's estimated at 20°C for the mouse ova were consistent with those obtained for other animal cells with the exception of erythrocytes which are thought to be highly permeable (Levin et al, 1980).
The study by Leibo (1980) also estimated the affect of temperature on the Lp for both the fertilised and unfertilised mouse ova between 0°C and 30°C. Although data including means for Lp's were not recorded the activation energies determined from the Arrhenius plot of Ln Lp vs 1/T could be compared to those derived from the Lp's in the present investigation. The Lp's predicted from the microscope diffusion chamber in my study decreased with decreasing temperature and from the Arrhenius plot an Ea of 9.48 Kcal/mol was calculated for the mouse. The Ea value calculated in my study correlated well with the values determined by Leibo (1980) for unfertilised (14.5 Kcal/mol) and fertilised ova (13.3 Kcal/mol) or Toner, 1990 of 13.3 ± 2.5 Kcal/mol.

8.3 The Membrane Permeability Parameters of Human Oocytes.

The Lps for the fresh and failed-to-fertilise human ova at 20°C described previously (Bernard et al, 1988) exhibited extreme variability, which is in agreement with the observations made in my investigation. The mean value of Lp calculated for the Ff ova of 0.84 ± 0.39 μm/atm/min correlate well with that predicted in my current study of 0.62 ± 0.32 μm/atm/min but were much higher than the Lp for the mouse stated in all reports to date (Leibo, 1980, Bernard et al, 1988, Toner et al, 1990, Table 10). Even though this suggested that the Ff ova were more
permeable to water than the murine oocytes, this was not corroborated by the values calculated at the other investigative temperatures. At 37°C and 30°C the mean Lp's while lower than those obtained for the mouse the difference was not statistically significant. The most striking point was the large standard deviations for the Lp values at temperatures studied, and these led to an overlap in values over the temperature range, even between those estimated for Ff at 37°C and 10°C.

The measurements made on the fresh human ova were similar to those for the Ff material. Once again the mean Lps show a general decrease as the temperature decreased, even though the ranges for the different temperatures were coincidental. The rate of change was extremely small with the mean Lp decreasing from 0.58 ± 0.32 μm / atm / min at 37°C to 0.25 ± 0.09 μm / atm / min at 5°C. This is reflected in the Ea value which was low in comparison to those determined for other cells. Unlike the Ff ova the mean Lps were consistently lower than those for murine ova at the equivalent temperature, but again the standard deviations were extremely large and the difference was not statistically significant.

In order to assess the consistency of the response of the fresh human ova, a number of fresh human oocytes were investigated for repeatability of both Lp and Vb measurements. From the fresh ova at 20°C, 7 underwent two
consecutive exposures to 0.5 M NaCl with return to isotonic media in between for Lp calculation. The repeat values for Lp fell within 0.01 - 0.13 μm / atm / min of the initial value. Repeat Vb values were determined by exposing the oocyte to 5 increases in NaCl concentration from 0.2 M NaCl to 0.7 M NaCl, returning the oocyte to isotonic medium and exposing the oocytes to a second set of increasing concentrations of NaCl. The cell volume was measured at each concentration and a Boyle Van't Hoff plot made. For the 4 fresh human ova investigated for repeat Vb values all were within 0 - 3 % of the original value. The consistency of these values indicated that the ova retained the ability to respond to perfusion with hypertonic salt solutions even after exposure to saline solutions between 0.2 - 0.7 M NaCl for 10 minutes at each concentration ( totalling over 60 minutes of perfusion ). If NaCl exposure per se was damaging to the plasm membrane of the ova this would have been reflected in an alteration of the permeability parameters and the independent calculations would not have been as closely repeatable as they were. Damage resulting in the ova becoming permeable to NaCl would have caused the volume of the ova to increase above the original value on return to isotonic or a less concentrated solution of NaCl, a response which was not observed during my investigation. Thus I feel confident that the oocyte plasma membrane retained functional selective permeability during the
studies.

The variability of both the Lp and Vb values apparent for the human ova is unlikely to be attributed to the quality of the donated ova. Fresh human ova used in the study were chosen at random from the cohort of oocytes collected in each treatment cycle. The oocytes were morphologically normal, with typical spherical shape, clearly-defined plasma membrane and granular refractive cytoplasm. The material was donated from a group of 69 patients, 85.5% of whom underwent embryo transfers indicating fertilisation of 1 or more of the remaining ova. A fertilisation rate of 72% (number of ova fertilised / number of ova collected x 100) was calculated overall. Of those patients failing to proceed to the embryo transfer stage (10 / 69), a further 7 (10% of the original number) fertilised ova with therapeutic donor sperm, while 3 were not placed with test sperm due to a smaller number of oocytes or previous therapeutic testing and failed to fertilise any oocytes. Only 1 patient, diagnosed as unexplained infertility, failed to fertilise any of the ova collected for reasons unconnected with sperm parameters. The most reliable test of oocyte quality is the ability to fertilise and produce embryos after culture. When taken in conjunction with the random nature of the sample allocation, since 72% of the total number of ova fertilised, this data suggests that the oocytes were of
good quality.

8.3.2 The Temperature Dependence of the Human Oocyte

When Ea values were determined for the human ova from Arrhenius plots of Lp and inverse temperature, it was evident that the human and murine oocytes responded in a different fashion. The Ea for the Ff oocytes was only 1.98 Kcal / mol and for the fresh oocytes it was 3.43 Kcal / mol as opposed to 9.48 Kcal / mol for the mouse. The values obtained for the human oocytes are extremely low, since the Ea for free diffusion of water isotopes through water without the presence of a membrane is approximately 3 Kcal / mol. It appears that from the variability of the results and the large standard deviations around the mean Lp values, the calculation and application of a single Ea for a group of either fresh or Ff human ova is unreliable. Indeed, values in the same order as those for free diffusion are indicative of water moving through pores in the membrane as was suggested for erythrocytes (Sha'afi & Gary - Bobo, 1975). Alternatively, although very unlikely, low Ea values could signify facilitated transport. The reliability of the results obtained using mean values of Lp for groups of human oocytes are questioned further by the favourable comparison between the mouse data in my study and values for other animal cells. Excluding erythrocytes, in which the membrane is highly permeable to water, as
described previously, several different cell types including mouse tumour cells (Ea 9.60 Kcal / mol; Hempling, 1960), bovine oocytes (Ea 12.11 Kcal / mol; Myers et al, 1987), chondrocytes (Ea 8.06 Kcal / mol; McGann, 1988), and keratinocytes, (Ea 10.70 Kcal / mol; Aggarwal et al, 1988) have remarkably similar Ea values. Thus my studies suggest that it is unwise to view groups of human oocytes as homogeneous populations. Following from this it is not sensible to assign a single mean value for Ea to the human oocyte.

8.4 Possible Sources of Variability in the Membrane Water Permeability of the Human Oocyte

One of the most conspicuous differences between the human and mouse ova was the time of oocyte collection. All of the studies on mouse ova were performed on ovulated oocytes collected from the oviduct, whereas a human IVF programme requires that oocytes were collected directly from the ovarian follicles. Although human egg collections were timed to coincide as closely as possible with ovulation, this can only be judged by the length of time following hCG injection. Since the majority of patients were down-regulated, no endogenous LH surge should have occurred under these circumstances. Ovulation naturally occurs within 24 h of the measured LH surge, but by implementing a regime of down-regulation, this period is
extended to 36 - 41 hrs post hCG injection. Collection routinely occurred at 36 hrs post hCG. The ova were then stored in culture medium for an additional 5 hrs to bring their biological development time closer to that for expected ovulation. Although the timing was chosen to mimic the expected time of release, obviously the follicular environment is not reproduced by in vitro culture. In addition, the mouse ova were isolated from the ampulla and had been exposed to oviductal fluid for a period of up to about 3 hrs, since the process of ovulation is not synchronous and some ova are released immediately prior to dissection of the ovary, whilst others have spent longer in the ampulla. These differences raise the question of the importance of maturity on oocyte plasma membrane permeabilities.

Studies by Steponkus and co-workers (Lin et al, 1987, and Myers et al, 1987) and Tate (1989) on bovine ova have elucidated the permeability parameters for ova at various stages of maturity. Using a microscope diffusion chamber to investigate water permeability at 20°C of immature bovine ova aspirated from ovarian follicles Lin et al, 1987, and Myers et al, 1987 determined mean Lp values of 0.54 ± 0.16 μm/atm/min and 0.29 ± 0.06 μm/atm/min respectively. A recent study also on bovine ova by Ruffing et al, 1990 predicted an Lp of 0.45 ± 0.20 μm/atm/min for immature ova, and 0.80 ± 0.47 μm/atm/min for in vitro matured with a mean
Vb of 32 % ± 6.6 % for the in vitro matured ova. In the study by Tate (1989) in which ova were classified as being in metaphase II or immature germinal vesicle stage, Lps were estimated within a range of 0.27 - 210.00 µm / atm / min with values for Vb falling within 0.1 - 57.0 %. Direct comparisons between the work of Tate to that of Steponkus' group and my studies for pre-ovulatory human ova are difficult since the Lp for the bovine oocytes (Tate, 1989) were determined with respect to time and a best fit single value predicted from the conglomerate. However, what is obvious from all these studies on pre-ovulatory bovine ova is the wide scatter of values for Lp and Vb, a phenomenon which was not apparent from the investigations on the ovulated mouse ova.

The variability observed in the pre-ovulatory ova was reflected in the investigations using murine oocytes collected from the ovary in my study. The range of Lp's were greater and thus the standard deviations about the mean were much larger, with the maximum Lp's being 2 - 3 x greater than measured for the ovulated oocytes. Since the distribution of permeability values in the cells isolated from the ampulla exhibited a greater degree of conformity, this suggested that ovulation may cause alterations in the oocyte which resulted in a more homogeneous population with respect to the permeability characteristics.

The follicular environment is known to be influential
on the maturation process of the oocyte. It has been known for a number of years that pre - ovulatory ova will spontaneously resume meiosis on release from the follicle ( Edwards, 1965 ). It is thought that c.AMP at basal levels maintains the chromosomes in meiotic arrest while at the higher, LH-stimulated, level it acts as a promoter of development mediated through the follicle cells ( Dekel, 1989 ). Prior to ovulation, as a response to the elevated LH, c.AMP production increases which interrupts the connections between the oocyte and the cumulus mass, decreasing the c.AMP concentration and removing the inhibition on the resumption of meiosis. Retention of the cumulus cells surrounding the immature oocytes following mechanical release from the ovary results in a high percentage successfully undergoing maturation and fertilisation ( Schroeder and Epigg, 1984 ).

Although in our I.V.F. programme, after being graded for maturity at collection, the human ova were cultured for a period of time under the assumption that maturation will continue, no specific follicular factors such as FSH were added to the culture medium. FSH has been found to be advantageous in maturation of murine ova ( Epigg and Schroeder, 1989, Schroeder and Epigg, 1989, Schroeder et al, 1988 ). Culturing the human ova for periods of time in vitro may not completely mimic the changes in the chemical environment occurring naturally in the follicular fluids.
This may account for the greater variability observed for both the Lp and Vb values for both the human and murine pre-ovulatory ova compared to the ovulated material. Although the surge of endogenous gonadotrophins present in the follicular environment are responsible for triggering the resumption of meiotic division in vivo, they appear not to be essential for attaining the ability to complete the maturation process in vitro. Recent work on the mouse has found that the addition of FSH is advantageous, increasing fertilisation and normal development to the blastocyst stage (Epigg and Schroeder, 1989, Schroeder and Epigg, 1989, Schroeder et al, 1988). In the rat, the addition of LH to culture medium for immature pre-ovulatory ova may result in an increase in the number of morphologically normal oocytes, the ability of sperm to penetrate the cumulus complex and the overall fertilisation rate (Shalgi et al, 1979). The action of the LH may be mediated through the oocyte itself or the surrounding cumulus cells, although these effects have been disputed in a study by Fleming et al, 1985. Administration of the gonadotrophin FSH is known to override the inhibitory affects of purines (Epigg et al, 1985).

The mammalian oviduct does not merely act as a tube for the transfer of the oocyte from the ovary to the uterus. The components of the oviductal fluid are modified along its length with both ionic and macromolecular changes. It
has been suggested that, after a variable length of time following ovulation into the bursal sac, the oocyte is moved into the ampulla. The composition of these fluids is very different with a higher concentration of Na⁺ and lower concentrations of K⁺, Mg⁺, and P⁺ in the ampulla possibly resulting in a hyperpolarisation of the oocyte membrane (Borland et al, 1977). The epithelium has a secretory function releasing proteins (Marcus and Saravis, 1965, Mastroianni et al, 1970) and antigens (Fox and Shivers, 1975 a, b, Gaunt, 1985). These macromolecules present in the oviductal fluid may be passively acquired by the ovulated ova, binding to the zona pellucida or accumulating in the early embryo. They may influence the completion of maturation, development of competence for fertilisation and early embryonic development. It has been suggested that the appearance of antigens on the surface of an oocyte, rather than being attributable to the synthesis of new membrane components, may be a result of the binding of factors from the fluid in the oviduct (Fox and Shivers, 1975 a, b). In a study using in vivo and in vitro cultured eggs and embryos the surface antigen 2B5 was detected 5 - 6 hrs following ovulation only in those cells isolated from the oviduct. Later developmental stages of both culture systems, in vivo and in vitro, developed the capacity to synthesise the antigen. In addition, a 215 kD glycoprotein secreted by the murine oviduct epithelium which is found in
the oviductal fluid associates with the ovulated oocyte and early cleavage stages of the embryo. The glycoprotein associates in a selective manner only in the perivitelline space until the blastocyst hatches (Kapur and Johnson, 1985, 1986, 1988).

Quinn et al (1985) found that a significantly higher number of mouse zygotes develop to blastocysts, and a higher number of pregnancies were initiated after the transfer of human embryos cultured in media based on the composition of human tubal fluid than in T6. It may be an accumulation of one or more of the components of the oviductal fluid rather than the additional time per se spent in the follicular fluid that is responsible for the discrepancies observed between the properties of ovulated and pre-ovulatory oocyte. These may include permeability parameters such as Lp.

Meiotic maturation has a number of recognizable morphological changes initiated by the breakdown of the germinal vesicle membrane and ending with polar body extrusion. In the mouse, the ability to undergo germinal vesicle breakdown appears to be independent of the size of the oocyte and is not necessarily accompanied by the capacity to complete maturation to metaphase II which is achieved subsequently (Epigg and Schroeder, 1989). It is not known whether the altered developmental potential as maturation proceeds is as a result of intrinsic changes in
the oocyte or the influence of changed signals received from the somatic cells. However it is possible that as part of the overall process other characteristics such as the permeability properties of the plasma membrane may change at the same time. Maturation is known to be accompanied by a gradual increase in membrane depolarisation from $-41\,\text{mV}$ to $-17\,\text{mV}$ up to polar body formation, possibly as a result of a decrease in the permeability to $K^+$ (Powers, 1982). In addition it should be noted that during maturation there is a 1.6 fold change in the surface area of the oocyte. Changes in the number and size of microvilli resulting from altered cytoskeletal organisation were measured using both scanning electron microscopy and transmission electron microscopy and geometric formulas to determine the total surface area (Longo and Chen, 1985).

The investigation into the permeability parameters of the pre-ovulatory oocytes of the mouse out of necessity did not include a study of the primary oocytes. This was due to the difficulty encountered when attempting to remove the tightly bound cumulus cell layers, preventing accurate measurement of the volume changes due to lack of visual clarity of the membrane. The adult mouse ovary, from which the pre-ovulatory ova were mechanically released, contains various classes of oocyte at assorted stages of development including primary, immature germinal vesicle, and ova at various stages of maturity through to completion.
of metaphase II with extrusion of the first polar body (Swartz and Schuetz, 1975). The study with pre-ovulatory bovine ova (Tate, 1989), while emphasizing the disparity of the permeability properties, stressed that it was not necessarily accounted for by the stage at which the oocytes were investigated. Both those eggs designated as being immature with a germinal vesicle and those in metaphase II as assessed by cytogenetic staining techniques were equally variable in terms of Lp. The Vb values for the immature ova ranged between 0.1 and 57% while for the mature cells this was 0.1 - 41%.

The ability of sperm to penetrate the oocyte appears to exist at any stage of maturity, with sperm attaching to the zona at equivalent rates from germinal vesicle stage onwards (Lopata and Leung, 1988). Sperm penetration of the vitelline membrane increases with meiotic maturation, reaching a maximum at metaphase II. This was a stage-dependent change rather than relating to time spent in culture. Although sperm will interact with germinal vesicle stage oocytes, the chromatin will only partially decondense if development has proceeded to the point of meiotic metaphase I. An increased receptivity of the plasma membrane and ability of the cytoplasm to incorporate the sperm to produce the male pronucleus appears to coincide with germinal vesicle breakdown. Complete breakdown of the acrosome membrane, release of the cortical granules and
chromatin decondensation only occur at the metaphase II stage (Lopata and Leung, 1988). Maturation to the end of metaphase II must occur before mammalian oocytes can be fertilised to yield viable embryos (Moor and Trounson, 1977).

In my study on pronucleated ova, these facts suggested that all of the pronucleated human ova had undergone complete meiotic maturation to the end of metaphase II. In addition it was known that Leibo (1980) had found no significant difference between fertilised and unfertilised mouse ova with respect to either Lp or Ea. Considering the mouse membrane water permeability data, and the knowledge that in order to fertilise the ova had all developed to comparable states of maturity, it was tentatively hypothesised that by investigating the pronucleated human ova a greater degree of conformity in Lp values may be confirmed and in addition allowed the calculation of a single realistic Ea value. Although the mean Lp was lower at 10°C and the standard deviation determined was smaller than that for the fresh ova at the corresponding temperature, the reverse is true for the pronucleated ova at 20°C. In addition, when the standard deviations were considered, the differences were not significant. The mean Lp values predicted for pronucleated human ova at 20°C and 10°C of 0.55 μm / atm / min and 0.34 μm / atm / min respectively were extremely close to those for the ovulated
mouse oocytes at the same temperatures of 0.48 μm / atm / min at 20°C and 0.28 μm / atm / min at 10°C. This suggested a greater agreement between the permeability parameters of the human pronucleated ova and the ovulated mouse oocytes than the fresh human ova, which may be expected if the Lp for fertilised and unfertilised oocytes are similar and the major sources of variability within the investigation were as a result of differences in cell maturity. However, it is important to note that the Lp values were still very varied and standard deviations large indicating a population that was not homogeneous. The heterogeneity in the pronucleated ova may well be on account of the complex time dependent events that occur following fusion of the sperm which could alter the properties of the zygote including those of the plasma membrane. In a study investigating the permeability of mouse ova to glycerol, Jackowski (1977) found that following penetration, there was a progressive increase in the speed of CPA permeation which was exhibited by those with and without a zona pellucida. This, in conjunction with the fact that it was a gradual change, suggested it was in response to a cellular event associated with fertilisation.

Not only is there an immediate deplorisation of the oocyte membrane from the point of sperm entry and release of the cortical granules to block further sperm penetration, but the activation of development at
fertilisation is accompanied by numerous cytoskeletal changes. Although the exact timing of fertilisation was difficult to determine, all of those used in the study had both male and female pronuclei present. In the unfertilised oocyte, a continuous network of cortical filamentous actin is found associated with the microfilament-containing microvilli. Following fertilisation, due to a concentrated area of actin above the spindle in the mouse, the spindle rotates and a cleavage furrow forms between the actin-rich shoulder and the less concentrated region. In the human, an increased concentration of actin forms in the cleavage constriction between the ovum and the second polar body while the female chromosomes complete metaphase II and the second polar body is extruded. Submembraneous actin remains concentrated around the pronuclei and cytoplasmic microtubules become evident. Although all of the fertilised ova had progressed to the pronucleate stage, the exact stage of pronuclear migration or the imminence of pronuclear membrane breakdown could not be accurately predicted. As migration proceeds, the microtubular elements of the cell develop to produce a dense complex meshwork, whilst by the time of nuclear membrane breakdown these are present only as microtubule-organising centres around the chromosomes from which the spindle forms for the onset of mitosis (Maro et al, 1984, 1986, Pickering et al, 1988). The cytoskeletal changes which occur in the cell after
fertilisation are accompanied by alterations of the molecular processes of the oocyte. Although transcription of the embryonic mRNA only occurs subsequent to the 2-cell embryo stage, with early embryonic development being controlled by the maternal genome, some limited mRNA activation not involved in embryonic development has been recorded (Moore, 1975). Changes in the polypeptide synthesis dependent on or accelerated by fertilisation occur in the ova which is expressed in a manner dependent on the time following penetration as a result of differential mRNA activation or post translational modifications (Howlett and Bolton, 1984). These changes in the cell components may be responsible for the spread of the Lp values calculated since not all ova develop pronuclei at the same time and a window, in which 2 pronuclei are visible, exists.

Alternatively, it is known that although the water content of fertilised ova remains unchanged, the structure is modified throughout the first cell cycle. In a study utilizing fertilised sea urchin ova, the change in the proton NMR T1 relaxation time during the cell cycle was calculated. The T1 time is a measure of the speed at which the hydrogen proton on a water molecule disturbed by an electromagnetic pulse can return to equilibrium. The motional state of water is determined by its' interactions with macromolecules as compared to water in the bulk and
can be detected from the T1 time. The more structured or constrained the water molecules, the shorter the time period required to return to equilibrium. During the first cell cycle following fertilisation the T1 relaxation time was modified, even after the hyaline layer and fertilisation membrane were removed, indicating that the changes must be due to intracellular events in the egg proper rather than the accumulation of water in the perivitelline space (Cameron et al, 1987).

Additional evidence from this study for changes occurring in the water and macromolecular arrangement in the fertilised egg come from the pattern of ice crystal growth following freezing. Interactions with proteins allows more rapid diffusion of water along a protein surface creating larger ice crystals. The differences observed agree closely to the T1 times, suggesting that there is a change in water structure which may possibly be linked to a modification of the amount of polymerised actin in the cell (Cameron et al, 1987).

It was apparent from the values predicted for the permeability parameters Lp and Vb that the human material was extremely variable. It may not be accurate to use the values calculated for groups of oocytes at each temperature in the form of a mean to derive characteristics (such as Ea) for application to human oocytes as a single cell type.
CHAPTER 8 - DISCUSSION

The investigation of the Lp for a single fresh human oocyte at each of the temperatures of observation for determining Ea allowed both the variability of Lps within a temperature group and that between oocytes at different temperatures to be investigated. By predicting individual Ea's, a greater accuracy could be assigned to those values given to the ova. The Lp's at each temperature fell within the range expected from my earlier work on fresh human ova with similar maximum and minimum values. The same degree of variability was present in the Ea's of individual oocytes which ranged between 3.60 Kcal / mol and 18.95 Kcal / mol with a mean of 8.06 ± 5.07 Kcal / mol. By attempting to describe a mean Lp and Ea value for a "typical" oocyte, the real inter-cell variability of Lp was disguised. By taking individual Lp and Ea, values a more realistic impression was gained.

8.5 Possible Mechanisms for the Altered Morphology of the Oocyte in Response to Low Temperatures and an Osmotic Stress.

It was evident from the Lp investigations with the microscope diffusion chamber in which it was possible to record the response of a cell to an anisosmotic environment in its entirety, that perfusion with a hypertonic medium per se does not produce obvious morphological changes. In addition the oocyte remained smooth and spherical in
appearance when perfusion with isosmotic medium was performed at low temperatures. From observations performed between 37°C and 10°C on changes in the cell radius in response to exposure to 0.5 M NaCl, it was obvious that the oocyte shrank in a uniform manner retaining its sphericity. However the combination of an imposed osmotic stress and low temperatures caused gross deformation of both mouse and human ova. On rewarming, or removal of the hypertonic perfusion, the deformation was reversible indicating the changes in structure occurring in response to cooling are reversible.

A wide range of cells have been shown to be sensitive to cold shock including ciliated protozoan species (Morris et al., 1984), amoebae (McLellan, 1984), spermatozoa (Wales and White, 1959) and embryos (Polge et al., 1974, Wilmut et al., 1975). Cold shock injury is damage to cell structure and function resulting from rapid cooling and is quickly apparent following exposure to low temperature. The rate of temperature reduction, the final temperature attained and the duration of the exposure all contribute towards the expression of damage and differ with different cell types and species (Wales and White, 1959, Watson, 1981).

The biochemistry of cold shock is not well understood, although disruption of metabolic pathways does not appear to be the primary cause since damage is greatest at fast
cooling velocities. During rapid cooling, the exposure time to low temperatures is less and metabolic damage resulting from differences in the temperature coefficients of individual steps in a pathway which may cause a build up of toxic products should be at a minimum. The amount of damage resulting from cooling is dependent on the rate of temperature decrease but is virtually independent of the rate of warming, only very slow velocities causing further decreases in viability, suggesting the injury occurs during cooling.

The plasma membrane has been suggested as the primary site of injury in cold shock. Biological membranes are composed of a mixture of phospholipids and proteins, the former being composed of varying fatty acid chain length, number of double bonds or degree of saturation and type of head group. Within the bilayer, the phospholipids are arranged with their hydrophilic or polar groups at the intra and extracellular surfaces. Thus the interior is hydrophobic, non polar, and transport of polar molecules is energetically demanding.

The phospholipids respond to a temperature reduction by altering their organisation. Studies on experimentally-controlled phospholipid bilayers showed a transition from the disordered fluid (liquid crystalline) to hexagonal lattice (gel phase) as the temperature was lowered with a well defined mid point transition temperature (Tc). Due
to the heterogeneous nature of natural cell membranes, the phase separation is unlikely to be sharp. Lateral phase transitions are more likely to occur as the temperature falls below the Tc of any one of the components with the phospholipids in mixed compositions separating out into their respective crystalline forms. Cholesterol, a neutral lipid component of many membranes, decreases fluidity by ordering the acyl chains on the fatty acids above the temperature of Tc. Below Tc, it confers an increased fluidity by decreasing the cooperative nature of crystalline lattice formation.

Slow cooling rates create fewer nucleation sites for lipid transition, larger gel-state domains and allow lateral protein diffusion, reducing damage. During slow cooling as the lipid crystal lattice grows, intramembranous proteins are excluded along lines of dislocation, forming particle free patches of lipid and domains of high concentrations of proteins. Rapid cooling, responsible for cold shock damage as may be experienced by the ova in the diffusion chamber on perfusion with fluid at 0°C, results in numerous nucleation sites for formation of a crystalline lattice, the growth of which proceeds rapidly. This allows insufficient time for membrane proteins to diffuse laterally as revealed by combinations of differential scanning calorimetry and X-ray diffraction (Melchior et al 1982). Rapid growth of the lipid crystal lattice causes
numerous packing faults, with adjacent domains showing packing faults, and these imperfections are made worse by the presence of proteins ( Hui et al, 1974, Chapman et al, 1979 ). Since the two layers of the bilayer nucleate independently, the coincidence of two such faults would allow the leakage of small molecules ( Sillerud and Barnett, 1982 ).

Fourier transformation infrared spectroscopy ( FTIR ) has been used on intact cells to detect transition temperatures of phospholipids in biological membranes as opposed to isolated or manipulated bilayers. Tm temperatures ( melting temperatures ) for sperm from three different species correlate well to the sensitivity they exhibit to cooling, with goat sperm ( which can not be cooled below room temperature without damage ) having the highest Tm of 21°C, human at 13.6°C followed by shrimp at 0°C. This may indicate that lipid phase transitions contribute towards damage resulting from cooling ( Crowe et al, 1989 ). In Tetrahymena reversible phase transitions and particle free patches have been identified using freeze etching ( Speth and Wunderlich, 1973a, 1973b ) and the temperature of these lipid phase separations in the membrane are closely related to those at which viability decreases ( Morris et al, 1984 ).

It has been proposed that among the numerous lipids constituting a biological membrane some may be non-
bilayer forming lipids exist in lamellar configurations as a result of interactions with the integral proteins (Quinn, 1985). Due to the higher Tc temperature of hexagonal II structures, which form on cooling, further cooling causes the separation of the non-bilayer lipids which on rewarming may not allow the reassembly of the original membrane structure. The inclusion of the non-bilayer lipids in the form of hexagonal structures such as micelles may destroy the membrane's function as a permeability barrier. CPA's may act by altering the water content of the lipids, thus lowering the Tc of the hex II lipids to that of the lamellar forming lipids. This would conserve the original structure until protein aggregation occurs and the non lamellar lipids accompany them and prevent non bilayer structures from forming (Quinn et al, 1985).

The temperature-induced events occurring in the oocyte observed in the microscope diffusion chamber in response to perturbations of the external environment of "crumpling" may be as a result of the altered arrangement of the components of the membrane. Membranes act as a barrier to passive diffusion of solutes, thus keeping the cell contents intact from the environment. Both the function and behaviour of the cell membrane is thought to be dependent on the distribution of its constituents and the external environment. Separation of the membrane into different
domains at low temperatures may not produce visible changes in the cell's appearance at the light microscopy level. However, imposing an additional stress in the form of hypertonic perfusion may result in the altered structure becoming apparent as the regions of different composition (with different physical characteristics such as viscosity) respond separately and the cell becomes unable to respond to dehydration by uniform shrinkage as it could at higher, more physiological temperatures.

It has been suggested that for small changes in the surface area caused by shrinkage over a limited time period, membrane material can remain in the plane of the membrane merely causing an increase in the surface tension. However, if the surface alteration is of larger proportions or for longer periods of time, membranous material may be forced to leave the membrane to a reservoir, from which it may be reincorporated on return to isotonic conditions (Steponkus et al, 1981, Wolfe and Steponkus, 1981). In oocytes, there are large numbers of microvilli which are thought to allow changes in the cell volume without rupture, possibly by altering the number or length of microvilli present. The oocyte membrane may be unable to remove excess material, incorporate it in the microvilli, or increase the surface tension following lateral phase transitions at the rate required during equilibration with the hypertonic solution at low temperatures. Following this
the oocyte plasma membrane may be unable to maintain its spherical appearance and thus the cells respond to the decrease in volume by seeming to "crumple".

While the crumpling response of the oocyte to osmotic stress at 0°C may be as a result of lateral lipid phase transitions, alternatively it may be due to changes in the viscosity of the membrane. Membrane viscosity of the cell determines the speed at which it can respond to an applied stress. Decreasing the temperature causes a concomitant increase in the viscosity (in red blood cells a tenfold increase in the viscosity has been measured following a temperature decrease from 37°C to 6°C, Houchmuth and Waugh, 1987). If a similar increase in the viscosity of the oocyte were to occur over the investigative temperature range it may alter the ability of the cell to respond to an osmotic stress. At 0°C the surface area of the oocyte membrane may be unable to decrease at an equivalent rate to the change in the volume as the cell dehydrates in order to attain equilibrium with the hypertonic environment. The increased viscosity may lead to a decrease in the speed at which the membrane alters to maintain the spherical nature of the cell creating a "crumpled" appearance. Alternatively it has been postulated that plasma proteins may interact with the cortical actin component of the cytoskeleton, influencing the shape and elasticity of the oocyte (Longo and Chen, 1985). Temperature-induced
changes in the cytoskeleton may cause the ova to change shape and only when either the cytoskeletal elements or the connecting proteins have been restored to the normal distribution can the cell regain a spherical morphology.

It is known that DMSO causes a reduction in the water permeability of the cell plasma membrane (in the presence of other permeating solutes water permeability is designated as $K$) possibly as a result of an increase in the structuring of the intracellular water (Rule et al, 1980, Toupin et al, 1989, Hempling and White, 1984). A study investigating the $K$ value for hamster lung fibroblasts over the temperature range 0 - 37°C in the presence of 0.5 M DMSO, found the water permeability to be reduced by a factor of 2 compared to that determined in the absence of CPA. In addition to this, glycerol was found to reduce $K$ by an amount comparable to DMSO (Rule et al, 1980). It is possible that the reduction in the severity of the crumpling observed on hypertonic perfusion at low temperatures is as a result of the lowered $K$ value. At lower temperatures the membrane may be destabilised by alterations in the osmotic pressure, rendered susceptible to mechanical damage and thus be unable to withstand rapid changes in the cell volume. A lower $K$ value may prevent or reduce damage by decreasing the rate at which water can leave the cell and thus the rate at which the cell volume and surface area change, which may be apparent as a
decrease in the extent of deformation or crumpling.

8.6 Possible mechanisms involved in damaging the oocyte resulting in a decrease in the fertilisation capacity and developmental potential following cold hypertonic exposure.

8.6.1 Metabolic Imbalances

In conjunction with the deformation of oocytes following cold exposure to a hypertonic environment a concurrent decrease occurred in the capacity of oocytes to fertilise. As with "crumpling", the decrease in fertilisation was only apparent following the combined treatment regime. The separation of membrane lipids into gel-state zones and the accompanying protein aggregation as temperature falls, when superimposed onto the change in surface area during dehydration, may prevent the resumption of a normal distribution of membrane components on return to physiological conditions. At higher temperatures the membrane lipids may still be fluid and on return to isosmotic volume may resume a normal arrangement. Likewise at low temperatures in PBS, the cells were not subjected to perturbations of the osmotic conditions so the dehydration-induced re-arrangement of the proteins and lipids in the bilayer may not have been so severe as to influence with subsequent fertilisation.
A permanent change in the membrane structure following osmotic stress and cooling may have numerous repercussions on the cell viability. The formation of particle free patches due to the separation of lipids into their respective zones and protein rich domains results in packing faults in the bilayer. The coincidence of faults in the individual lipid layers may disrupt the selective permeability function of the plasma membrane and allow leakage of molecules into and out of the cell. Much work has been conducted on mammalian spermatozoa the plasma membrane of which appears to be particularly susceptible to damage with initial disruption being shown by the loss of selective permeability leading to an eventual decrease in metabolic activity. Cooling species of mammalian spermatozoa to 0°C results in an irreversible loss of motility, the loss of both low and high molecular weight substances and the accumulation of sodium and calcium with a concurrent loss of potassium and magnesium (Quinn and White, 1966). Ram spermatozoa have been shown to exhibit phase transitions at approximately 17°C (Holt and North, 1986).

In conjunction with the loss of selective permeability capacity membrane material may actually be lost from the plasma membrane of spermatozoa (Quinn et al, 1968). Phospholipids are thought to be lost from the acrosomal membrane of sperm which becomes severely
disrupted, the anterior becoming swollen and detached as a result of the temperature reduction. Cold shocked spermatozoa release proteins and polysaccharides from the mid-piece where they must permeate the double membrane of the mitochondrion in addition to the plasmamembrane (Quinn et al, 1969). The alteration of the composition of a membrane as a result of lipid phase transitions, changes in membrane viscosity, or the loss of components of the bilayer may have profound effects on the composition of the cytoplasm and thus the cell viability.

The properties of membrane bound enzymes are thought to be dependent on the surrounding lipids and may be altered on lowering the temperature (Sinensky et al, 1979). It has been suggested that enzymes may be denatured not only by increased temperatures but also by exposure to low temperatures (Franks, 1982, Bock and Friedman, 1978). The hydrogen and hydrophobic bonds that confer stability on proteins respond to cooling in different ways and alter the structure which in turn may cause unfolding and denaturation if it is part of a multiple subunit. This dissolution into biologically inactive species, which may not be reversible on rewarming, would be disruptive to metabolic pathways. In addition a variety of enzymes are released on cooling which necessarily disturbs the biochemical processes and metabolic activity (Moore et al 1976, Harrison and White 1972, Pursel et al 1968, 1970).
CHAPTER 8 - DISCUSSION

Disturbances of the intracellular environment for any reason would lead to a decrease in the viability of the oocyte, which would become apparent as a lowering of the fertilisation rate. The detrimental effect of exposure to hypertonic saline may be a result of exacerbating damage occurring during cooling. Although metabolic imbalances or damage to the plasma membrane may be responsible for the fall in the number of oocytes being successfully fertilised, it is unlikely to be the cause of subsequent losses. Following the first cell division to the two cell stage embryonic RNA is transcribed and the maternal enzymes are replaced by those of embryonic origin. The small number of mouse ova developing to the hatching blastocyst stage and pronucleated human progressing through one or more cell divisions following osmotic stress at low temperatures, may be due to chromosomal abnormalities leading to a decrease in the developmental potential.

8.6.2 Cytoskeletal Damage

Cell shape, motility and division are controlled by the cytoskeleton which is a dynamic system and sensitive to a reduction in temperature. Since the oocyte is ovulated arrested in metaphase II and the events following sperm fusion involve major spatial reorganisation of the cell, the presence of a normal cytoskeleton is extremely important. In the oocyte there is a matrix of actin microfilaments and discrete tubulin-containing
microtubules. Both the microfilaments and microtubules are responsible for the maintenance of chromosomal organisation, polar body formation and extrusion, and the complex movements involved in cleavage after meiosis and mitosis are resumed.

The metaphase II chromosomes are able to alter the cytoskeletal organisation of the cell and induce polymerisation of both actin and tubulin. Immediately prior to the breakdown of the germinal vesicle, the metaphase II chromosomes develop the ability to alter the actin organisation in their vicinity (Van Blerkon and Bell, 1986). After the germinal vesicle breakdown, the chromosomes affect the tubulin polymerisation, causing microtubule and spindle formation. Movement of the spindle to the cell periphery is dependent on microfilaments (Longo and Chen, 1985). When the chromosomes have migrated, they bring about the production of an actin rich region. The chromosomes are thought to promote microtubule formation by reducing the concentration of tubulin required for microtubule polymerisation.

The microfilaments are located in the cortex in a continuous band of filamentous actin. Mature mouse ova have a concentrated region of microfilaments overlying the spindle which is not present in immature mouse ova or human oocytes (Longo and Chen, 1985, Maro et al, 1984, Pickering et al, 1988). This concentration of actin is thought be
due to the presence of the chromosomes since it is found only after the breakdown of the germinal vesicle when the chromosomes have become localised at the cortex. In addition, it is thought to restrict the chromosomes and meiotic spindle to the cortical position and be responsible for normal polar body formation (Maro et al, 1986). Polar body extrusion in the human is thought to be the result of a dense ring of actin microfilaments in the cleavage furrow (Pickering et al, 1988). There are few microfilaments in the area of the oolemma to which sperm normally fuse although after fusion microfilaments form a cone near the decondensed sperm head.

Microtubules are only found in the meiotic spindle their presence being dictated by the location of the chromosomes. The mouse spindle is barrel shaped eccentrically placed with the longitudinal axis parallel to the membrane (Maro et al, 1985). The human spindle is peripheral and thought to be orientated radially to the membrane (Pickering et al, 1988, Szollosi et al, 1986).

The microtubules and microfilaments thus influence a variety of events in the cell division. Spindle rotation and polar body formation and extrusion both require the presence of microfilaments. Both microtubules and microfilaments are necessary for the migration of the pronuclei from a cortical position to the centre. After the migration of the pronuclei the nuclear membrane breaks down
and the microtubules are responsible for the mixing of the male and female haploid sets of chromosomes. Microtubule organising centres found around the chromosomes converge to form the poles of the mitotic spindle and the microtubules produce chromosome separation.

The microtubules of a cell are composed of protofilaments which consist of polymers of doublets of tubulin subunits. Microtubules are sensitive to calcium, high pressure, temperature, and drugs such as colchicine which binds to the tubulin dimers and prevents polymerisation, and taxol which promotes tubulin assembly. A pool of tubulin monomer exists in cells allowing rapid polymerisation of the tubules. Inhibition of either microtubule depolymerisation or polymerisation is extremely detrimental to their function. There is evidence that there are several classes of microtubules all of which may respond to temperature reduction in different ways (Behnke and Forer 1967). Cytoplasmic microtubules are known to be highly labile, and exhibit thermal sensitivity depolymerising on exposure to low temperatures. The mitotic apparatus is one of the most unstable of the microtubule structures. The microtubules of the meiotic spindle of the oocyte are known to become disorganised or completely depolymerise on cold treatment, a response which is reversible on return to normal physiological temperatures (Lambert and Bayer, 1977; Magistrini and Szollosi, 1980;
The decreased fertilisation rate observed in my study following cooling from 95 % in the controls to 75 % in PB1 at 0°C was similar to that reported by Glenister et al, 1987 in which a reduction in fertilisation from 68 % to 53 % was recorded. However, it was not as extreme as that reported following cooling to 4°C in which the fertilisation rate after cooling was 50 - 60 % compared to 93 % and removal of the zona restored to the control rate of > 90 %. It is possible that alterations to the zona pellucida occurred caused by premature release of the cortical granules ( Johnson et al, 1988 ). An earlier study found that disruption of the meiotic spindle occurred on cooling to 4°C which was not completely reversible on warming ( Pickering and Johnson, 1987 ). Spindle abnormalities as a result of microtubule clumping and depolymerisation leading to spindle disassembly may cause the chromosomes to disperse.

Pre-ovulatory human ova are known to undergo spindle damage when cooled slowly to 0°C and although no displacement of the chromosomes was exhibited, this may have been related to short culture periods subsequent to exposure ( Sathananthan et al, 1988 ). The dissolution of the meiotic spindle may be deleterious to oocyte fertilisation since this leads to chromosomal clumping and
their dispersal from the spindle equator. Although the microtubules exist in equilibrium with a pool of tubulin monomer and will reassemble on warming the ability of the chromosomes to reorientate correctly on the equator is unknown.

The activity of the other component of the cell cytoskeleton, the actin system, is determined by regulatory proteins responsible for actin cross linking. Although actin is not known to be sensitive to depolymerisation on exposure to low temperatures, the regulatory proteins acting on polymerised F actin are susceptible to alterations of the temperature and may cause alterations in crosslinks between the filaments causing shape changes. In amoeba, one protein is active and calcium sensitive at 28°C and not at 0°C while others are calcium active at 0°C (Hellewell and Taylor 1979, McLellan 1984). Alterations of the polymerised actin could result in the crumpling response of the oocytes at low temperature as the cell dehydrates in the hypertonic saline solution.

Oocytes and embryos have been found to contain a further cytoskeletal element composed of cytokeratin and ordered in sheets in the cytoplasm. Although a function has not been definitively assigned to the structures, they are known to be salt labile and on their removal the periphery of the cell takes on an irregular shape loosing its spherical appearance (McGaughey and Capco, 1989). It may
be that the combined effects of cold exposure and hypertonic salt treatment disrupt the organisation of the cytokeratin sheets which may be influential in the future development of the oocyte. A tentative role in development for the sheets has been suggested associated with the alterations in their spatial arrangement during early embryogenesis. If these cytokeratin sheets are involved in development, alteration of their structure may in addition to shape changes, become obvious as poor fertilisation rates and continued development (McGaughey and Capco, 1989).

The organisation of the cytoskeleton is of key importance for the production of a diploid gamete and the continuation of normal cell division. Defects in either of the systems could result in abnormalities in the embryo. Faults in the microtubule functioning could cause alterations in the number of chromosomes each cell received due to retention or loss of a number of chromosomes. On the other hand, defects of the microfilaments could result in malfunction of the extrusion process for the polar body and create an embryo that was triploid, containing two sets of the maternal chromosomes. Triploidy results in the formation of numerous spindles and the allocation of uneven numbers of chromosomes. There is contradictory evidence with respect to chromosomal abnormalities following cooling. Kola et al, (1988) published data suggesting a
2 - 3 fold increase in the rate of aneuploidy, whereas Glenister et al, (1987) reported contradictory findings suggesting that there was no increase in the incidence of aneuploidy. The effect of cooling on the cytoskeleton of the unfertilised oocyte is extremely important since any chromosomal imbalances would be detrimental to both the fertilisation rate and the continued development of any subsequent embryos. This is particularly pertinent as cooling to low temperatures in anisosmotic conditions is unavoidable in the initial stages of a freezing protocol.

8.7 The Possible Role of CPA Equilibration in Protecting Ova from Damage at Low Temperatures.

The protective effects of cryoprotectant addition, which are reflected in the higher numbers of mouse ova developing to blastocyst and the increased numbers of human ova fertilising normally are possibly mediated through the stabilising effects on proteins and the cytoskeleton. As previously discussed CPA's are preferentially excluded from the hydration shell of protein molecules lending additional stability to the native form as opposed to the denatured structure. The CPA exclusion is energetically unfavourable and both the native form of the protein and the polymer have smaller surface contact than the denatured form of the polymer (Arakawa et al, 1990). It may be the prevention of cold denaturation and depolymerisation of the protein
reduce the extent of damage by avoiding the disruption of metabolic pathways or by maintaining the plasma membrane structure.

It is well known that *in vitro* DMSO can cause polymerisation of tubulin into microtubules without the presence of microtubule-associated proteins, and once formed appears to retard their disassembly at low temperatures (Himes et al, 1976, 1977, Filner and Behnke, 1973). *In vivo*, the addition of DMSO to unfertilised ova causes the retention of the meiotic spindle apparatus during exposure to low temperatures for up to 45 minutes (Magistrini and Szöllösi, 1980; Johnson and Pickering, 1987; Johnson, 1989). However, prolonged exposure of 60 minutes or longer results in disruption of the spindle. Cytoplasmic asters form as the critical concentration for microtubule polymerisation is attained in areas removed from the metaphase II chromosomes. On removal of the DMSO and rewarming, the effects on the oocyte are reversed and normal fertilisation and development recorded (Magistrini and Szollosi, 1980; Johnson, 1989). It may be that by stabilising the meiotic spindle, DMSO reduces the dispersal of chromosomes, increasing the number of ova that fertilise and which have a normal chromosome compliment and can therefore progress to hatching blastocysts. It has however been suggested that disruption of microfilaments, such as occurs in mouse oocytes following prolonged DMSO exposure.
and cold treatment, is beneficial in rabbit ova during freezing, possibly by altering tolerance to shape changes caused by osmotic pressure (Vincet et al, 1989, 1990).

Although the stabilising effects of DMSO on proteins have been well documented, glycerol while having some similar cryoprotective properties to DMSO, has been studied less. Both CPA's are known to promote microtubule assembly in vitro (Himes et al, 1977, Shelanski et al, 1973). It is possible that glycerol may act in a similar manner to DMSO in vivo and preserve the integrity of the meiotic spindle, and thus the chromosomal organisation on the equator. If chromosomal abnormalities in those that fertilise are reduced, this may account for the similar rates of development to hatching blastocyst following equilibration with cryoprotectant prior to cold exposure and hypertonic treatment.

It may be that in addition to the changes which occur to the oocyte as it matures to completion of metaphase II and following ovulation, other changes such as an altered permeability to ions (Borland et al, 1977), accumulation of proteins (Fox and Shivers, 1975 a, b), and the ability to incorporate the δ pronucleus (Lopata and Leung, 1988), reduce the freezing sensitivity. The low levels of fertilisation and development may also be as a result of an increased sensitivity to non-ideal culture conditions. As observed with the mouse in vitro, culture for maturation
may be unable to completely mimic the normal in vivo environment and the addition of specific factors such as FSH (Schroeder et al, 1990) may be required to reduce susceptibility to damage.

8.8 Possible Factors Influencing Survival of Cryopreservation Techniques.

A number of factors are known to influence the response of cells to a reduction in temperature including Lp, Ea, the surface area to volume ratio and, the permeability coefficient to cryoprotectants (Mazur, 1977, 1984). These factors are all influential in determining the residual water content of the cell during freezing and thus the likelihood of intracellular ice formation as the temperature is reduced. Provided the Lp and Ea are the same oocytes will lose water at slower rates than a multicellular embryo due to the smaller surface area to volume ratio and, thus will in theory, require slower rates of cooling to avoid intracellular ice crystal formation. However differences in surface area / volume ratio and the ability to lose up to 50% of the constituent blastomeres comprising an embryo without affecting the developmental potential, can not be the only factor involved in the discrepancies between survival of embryos and oocytes following freezing and thawing. The successful cryopreservation of single cell pronucleate human embryos
(Testart et al, 1987) indicates other factors may be involved.

The concentration of CPA's needs to be high enough to protect the cell from freezing damage while avoiding toxic injury. This creates problems when designing protocols for their addition and removal. The differentials in permeabilities for water and cryoprotectant that exist across the cell plasma membrane result in a number of volume fluctuations during addition and removal of CPA. When the cell is initially placed in the CPA solution, it loses water until the cryoprotectant begins to permeate. The movement of CPA, accompanied by water causes the cell to return to normal isotonic volume once osmotic balance has again been regained. Additional volumetric excursions can be caused by abrupt, single step, dilution of the cell suspension following a freeze/thaw protocol which permits a rapid uptake of water due to the fact that the intracellular compartment is hypertonic relative to the extracellular solution and water enters as the cell attempts to maintain osmotic equilibrium. The CPA diffuses out much slower than water enters the cell. To avoid excessive swelling, procedures for the addition and removal of CPA's often require progressive step-wise increases and decreases in the extracellular concentration to limit the volume excursions. The limits over which the cell volume can be altered and still be tolerated can be
calculated by perfusion with non-permeating solutes of various osmolalities, and subsequent protocols designed to stay within these limits (Armitage and Mazur, 1984).

From these discussions it will be obvious that the membrane permeability to the CPA solutes is of extreme importance in developing cryopreservation protocols. Although many of the CPA's are of low toxicity in the concentrations used in cryopreservation, as the temperature is lowered the CPA becomes increasingly concentrated as water freezes and the toxicity may increase. In general, the shorter the exposure time to the CPA at high temperatures, the less toxic is the CPA to the cell. However, permeability increases with temperature, reducing the equilibration period required for individual cryoprotectants. Thus benefits at the higher temperatures of increased permeability and faster equilibration times must be balanced against increased toxicity. This is extremely important for glycerol, since it is much less permeable to cells than either DMSO or propanediol and to achieve high survival rates full equilibration is required (Smith, 1952, Ashwood-Smith, 1986).

Few studies have investigated the permeability coefficient of the oocyte plasma membrane to cryoprotectants (Jackowski et al, 1980, Schneider and Mazur, 1987) and virtually no information concerning permeation of water alone at subzero temperatures exists
for any cell type (McCaa et al, 1986, Aggarwal et al, 1988, Toner, 1990). However, although studies on cryoprotectant permeability on different cells types are limited (Mazur et al, 1974, Jackowski et al, 1980, Arnaud and Pegg, 1986), the permeation of CPA's across a cell membrane appears to be more sensitive to alterations in temperature than Lp. This indicates that at low temperatures an influx of cryoprotectants will be less probable than water efflux. This may be important in cryopreservation protocols requiring equilibration with a cryoprotectant at low temperatures such as that for mouse oocytes using DMSO (Whittingham, 1977) since the exposure time required for full equilibration will be increased and the volume excursions may be greater.

A study investigating the membrane permeability to cryoprotectants of the mouse ova (both fertilised and unfertilised) found that a change in $P_{glyc}$ occurred following fertilisation (Jackowski et al, 1980). The permeability of fertilised ova was found to be 3 x greater than that of the unfertilised oocytes, a change which occurred over a time span corresponding to the time of fertilisation (the $P_{glyc}$ increase occurred $\approx 16$ hrs post hCG, fertilisation $\approx 12 - 18$ hrs). Arrhenius plots of glycerol permeability showed significant differences in the temperature dependence of glycerol transport with Ea values of 28 Kcal / mol for unfertilised oocytes and 19 Kcal / mol
for the early zygote. The increase in the rate of glycerol permeation was continued through pre-implantation embryo development and it has been suggested this indicates a change in mode of transport of glycerol across the cell membrane (Jackowski et al, 1980, Mazur et al, 1976). The change in permeability to glycerol was not related to the alterations to the zona pellucida, which occur on fertilisation due to the release of the cortical granules, since its removal did not affect the permeability values calculated. In addition the altered $P_{glyc}$ was not merely a time dependent modification, since unfertilised oocytes of the same age as the fertilised ova had equivalent values to the fresh unfertilised oocytes. Although the permeability parameters of ova to other cryoprotectants have not been investigated, except in my present study for DMSO if they are found to exhibit similar differences on fertilisation it may offer one explanation to the relative success of cryopreservation of one cell pronucleate embryos when compared to results for the unfertilised oocyte. It may be that the oocytes require longer to equilibrate fully with a given concentration of cryoprotectant than the fertilised single cell ova. In addition it may also be the case that greater care will be needed to avoid osmotic damage during stages of addition and removal of the CPA from unfertilised oocytes. Studies on membrane transport properties of ova to different CPA's, similar to the initial work reported in my
thesis, should help to answer some of these questions.

8.9 Proposals for Future Work

i) Studies on the permeability parameters of human oocytes cultured in the presence of FSH and LH.

ii) Fluorescent dye studies to determine the affect on the microtubule and microfilament cytoskeletal elements of oocytes during crumpling after hypertonic exposure at low temperatures.

iii) Equilibration with other cryoprotectants such as propanediol, prior to imposing an osmotic stress at $0^\circ$C, to investigate the ability to protect the oocytes from the deleterious effects on both morphology and cell function as determined by fertilisation and on growth.

iv) Due to the possibilities of chromosome abnormalities in embryos resulting from fertilisation of oocytes after exposure to a hypertonic environment and low temperatures it would be informative to perform chromosome analysis on both human and mouse embryos. It would also be useful in the mouse to transfer the embryos formed after cold hypertonic treatment.
v) Chromosome analysis of ova, both mouse and human, fertilised after cryopreservation. In addition, embryo transfer in animals following cryopreservation and fertilisation of oocytes.

vi) Investigation of the permeability coefficients of unfertilised, fertilised, and pronucleate, mouse and human oocytes to cryoprotectants using the microscope diffusion chamber and amended computer software.
APPENDIX 1 - KEDEM - KATCHALSKY

APPENDIX 1

Derivation of the Membrane Water Permeability
Kedem-Katchalsky Equations

The movement of molecules can usually be expressed as Fick's Law:

\[ J_s = -\left(\frac{D}{L}\right) \Delta C_s \]

equation 9.1.1

where

- \( J_s \) is the flux of species \( s \)
- \( D \) is the diffusivity
- \( L \) is the membrane thickness
- \( \Delta C_s \) is the concentration gradient

but

\[ -\left(\frac{D}{L}\right) = P \]

equation 9.1.2

when

- \( P \) is the permeability coefficient

When the molecular flux is complicated by the movement of other species through the membrane as a result of the initial movement, Fick's Law is no longer appropriate for describing the flow. The Kedem-Katchalsky equation for
coupled flow must be used. The basis of the Kedem - Katchalsky equation is that the flow is a function of all the driving forces present in a system, and if small, this dependence is linear. For free diffusion, solvent and solute move relative only to one another and resistance to diffusional flow is due to friction between the solute and solvent molecules only. When diffusion is through a membrane, two additional frictions are imposed, those between the membrane and the solute and the membrane and the solvent. Flow will depend upon the interaction of these forces. If the membrane has large pores, the permeability value will approach that for free diffusion, whereas if the solute is less permeable to the membrane, the values deviate from free diffusion.

A thermodynamic description of a non - equilibrium system shows the total volume flow is:

$$J_v = L_p \Delta P + L_{p0} \Delta \pi$$

equation 9.1.3

and the differential flow ( the velocity of the solute relative to the solvent ) is:

$$J_D = L_{p0} \Delta P + L_D \Delta \pi$$

equation 9.1.4
where

\( J_v \) is the total volume flow
\( J_D \) is the differential volume flow
\( L_d \) is the diffusional coefficient
\( L_p \) is the hydraulic coefficient
\( L_{pd} \) is the coupling coefficient between osmotic and diffusional flow
\( \Delta P \) is the difference in hydrostatic pressure
\( \Delta \pi \) is the difference in osmotic pressure

( when \( \Delta \pi = R T \Delta C_s \) which is the driving force in Ficks equation )

9.2 Calculation of the Cell Membrane Permeability

The microscope diffusion chamber was designed to allow the calculation of the membrane permeability of cells from real time direct observations of small numbers of cells. A computer programme (SENS) was developed to solve the permeability calculations using a form of the Kedem-Katchalsky equations 9.1.3 and 9.1.4. Initially a number of assumptions were made concerning the system including:

i) there is no net volume flow in the sample region.

ii) the chamber is isobaric and isothermal.

iii) the mass diffusivities are constant.
iv) solute velocities are negligible.

v) the presence of cells is taken as inconsequential.

vi) the bulk flow is a step change at zero time.

vii) the dialysis membrane is non-selective although diffusion of the solute is slower than through free solution. Compensation can be made for this since the solute diffusivity through the membrane ($D_1$) is known from the manufacturers to be $D_2 \times 0.1$ where $D_2$ is the solute diffusivity in free solution and can be found in published tables (CRC Handbook of Chemistry and Physics).

The computer programme divides the processes occurring in the diffusion chamber into two constituent parts. When the bulk flow region of the chamber is flushed with hypertonic solute a concentration boundary layer develops at the base of the dialysis membrane. Solute diffuses through the dialysis membrane into the sample region and the cell responds osmotically. SENS considers this as:

i) the diffusion of solute into the sample region and

ii) the response of the cell.

It is of greater use when studying the permeability of
a cell experimentally to consider the solute flow \((J_s)\) rather than the differential flow \((J_D)\).

\[
J_s = \frac{(J_v + J_D) \bar{C}_s}{(\bar{v} C_s + 1)}
\]

\text{equation 9.1.5}

If both solutions are considered to be dilute \(v C_s \ll 1\) so

\[
J_s = (J_v + J_D) \bar{C}_s
\]

\text{equation 9.1.6}

From equations 9.1.3 and 9.1.4 the phenomological coefficients \(L_p, L_D,\) and \(L_{PD}\) can be transformed to \(w_s, \sigma_s\) and \(P\) where:

\(w_s\) is the solute permeability and can be determined experimentally

\[
w_s = \left[ L_p L_p - L_{PD}^2 \right] \bar{C}_s \left[ L_p \right]
\]

\text{equation 9.1.7}

\(\sigma_s\) is the reflection coefficient and ranges between 1 and 0 with 1 corresponding to an ideal semipermeable membrane and all the solute is reflected.
\[ \sigma_s = \frac{\Delta P}{R T \Delta C_s} \]  
\text{equation 9.1.8}

\( P \) is the membrane permeability

\[ P = \frac{L_p R T}{\bar{v}_w} \]  
\text{equation 9.1.9}

where

\( \bar{v}_w \) is the molar volume of solute

There is unlikely to be a hydrostatic pressure difference across the membrane of the diffusion chamber system so \( \Delta P \) is assumed to be 0.

The cell membrane is assumed to be an ideal semipermeable membrane and has a reflection coefficient of 1.

The volume flux can be written in terms of the change in the cell volume and cell surface area:

\[ J_v = \frac{1}{\Lambda_t} \frac{d V_t}{dt} \]  
\text{equation 9.1.10}

where
At is the change in the cell surface area with time. For spherical systems this is:

\[ 4 \pi R_t^2 \]

equation 9.1.11

\( V_t \) is the change in cell volume with time. For spherical systems this is:

\[ \frac{4}{3} \pi R_t^3 \]

equation 9.1.12

so substituting equations 9.1.11 and 9.1.12 into 9.1.10 the volume flux can be written as:

\[ \frac{d}{dt} \frac{R_t}{\sqrt{P}} = \frac{1}{\sqrt{P}} \left[ C_{\text{ext}} - C_{\text{int}} \right] \]

equation 9.1.13

where

\( C_{\text{ext}} \) is the extracellular concentration found from the values D1, D2 and Hd.

\( C_{\text{int}} \) is the concentration inside the cell and is found using

321
equation 9.1.14.

\[ \frac{C_{s_{int}}}{V_t - V_b} = \frac{N_{s_{int}}}{V_0 - V_b} \]

where

\( N_{s_{int}} \) is the number of moles of solute inside the cell
\( V_t \) is the total cell volume
\( V_b \) is the inactive volume of the cell which is found experimentally from the equilibrium experiments described in section 2.11.2.

At time 0

\[ C_i = \frac{N_{s_{int}}}{V_0 - V_b} \]

where

\( C_i \) is the initial concentration

so substituting 9.1.15 into 9.1.13

\[ \frac{d R_t}{dt} = -P \frac{v_w}{v} \left[ C_{s_{ext}} - C_i \left\{ \frac{V_0 - V_b}{V_t - V_b} \right\} \right] \]

\[ \left\{ \frac{V_0 - V_b}{V_t - V_b} \right\} \]

equation 9.1.16

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The volume flow is in a form appropriate for practical calculations since it is described by the rate of change of the cell radius and hence, by computation, the cell volume. The partial molar volume of water, \( v_w \), is a constant. The concentration in the sample region is known as a function of the position and time. \( V_b \) is calculated from the Boyle van't Hoff formulation. Thus the only unknown is the \( P \) the permeability value which must be solved for.

### 9.2.2 Parameter Estimation

SENS uses equation 9.1.16 to solve for \( P \), which if the only species to flow across the membrane is water, is equivalent to the membrane water permeability, \( L_p \). The technique used in SENS for calculating the permeability value is a parameter estimation ordinary least squares method which attempts to minimise the error between the functional values generated from the mathematical model and the experimental measured values. The difference between the predicted values and the experimental data is summed and then squared. When the predicted data points correspond to the experimental values the equation is solved. SENS achieves this by generating theoretical data sets for the cell radius over a range of permeability values supplied by the operator. The data that fits the measured cell radii, \( S_{min} \), is the best fit curve for the radius with respect to time. This provides the best estimate of the cell membrane
PERMEABILITY $P_{est}$.

\[ S = \sum_{i=n}^{n} \left[ Rm_i(t) - Rc_i(t) \right]^2 \]

\textit{equation 9.1.17}

where

$Rm_i(t)$ is the measured cell radius at time $t$

$Rc_i(t)$ is the calculated cell radius at time $t$

$n$ is the number of data points

Given a set of experimental data points $(t_i, Rm_i \text{ to } n)$ $S$ can be minimised by SENS to generate theoretical sets of data for a likely range of $P$ values. The standard deviation of $P_{est}$ is then found to calculate the accuracy of fit using the sum of the squares.
Appendix 2

Derivation of the Activation Energy Using the Membrane Water Permeability Values of a Selected Individual Fresh Human Oocyte.

10.1 Introduction

In 1884 Van't Hoff emphasised the temperature dependence of biological reactions in the theoretical equation:

\[ \frac{d (\ln K)}{dT} = \frac{a + b}{T^2} \]

where

- \( K \) is the velocity of the reaction (reaction rate)
- \( a, b \) are constants
- \( T \) is the absolute temperature

This relationship was described further by Arrhenius in 1889 and 1915 as:

\[ \frac{d (\ln K)}{dT} = \frac{\mu}{RT^2} \]
where

$\mu$ is a constant (the Arrhenius activation energy)

$R$ is the Universal gas constant ($8.3 \, \text{J} \, \text{K}^{-1} \, \text{m}^{-1}$)

or

$$v = a \exp\left(-\frac{\mu}{RT}\right)$$

equation 10.1.3

where

$v$ is the reaction rate

$$\frac{d}{dT} (\ln K) = v \quad ( \text{the reaction rate})$$

equation 10.1.4

$$\mu = E_a \quad (\text{the Arrhenius activation energy})$$

equation 10.1.5

For a simple reaction with a single rate limiting step, a graph of the logarithm of the reaction rate against the inverse of the absolute temperature yields a straight line with a slope of the activation energy $/ 2.3 *$ the universal gas constant. This was relatively accurate at describing simple chemical reactions. However, the $E_a$ value can not exhibit a simple relationship in many biological reactions.
APPENDIX 2 - ARRHENIUS ACTIVATION ENERGY

due to the numerous steps involved in an individual process. Non-linear Arrhenius plots are difficult to interpret and it has been suggested that curvilinear plots are in fact made of numerous intersecting straight lines intimating changes in the reaction processes. However, only a few plots show the sharp discontinuities or breaks required to indicate they are actually composed of two different mechanisms. In biological membranes composed of lipid components which may exist in either liquid crystalline or gel states depending on temperature, it has been argued that cooling will result in phase transitions to yield gel state membrane lipids. However, this has only clearly been demonstrated in artificial membranes composed of a few lipid classes. Biological membranes are composed of a complex mixture of lipids and proteins which separate into domains over a wide range of temperatures and fail to exhibit sharp phase transitions.

Arrhenius introduced the concept of absolute reaction rates in which all basic processes such as diffusion or hydrolysis can be thought of as equilibrium states between molecules in the reactive and normal state. During activation the molecules become altered in a manner that makes the constituent bonds susceptible to modification. The activation energy of the process is related to the rate limiting step. In the particular case of molecules diffusing through a membrane, the probability that a
molecule will have sufficient energy to permeate, is exponentially related to the temperature. This can be related to the absolute reaction rate, and the calculated activation energy can suggest the possible mechanisms involved in transport of the molecule.

For example, the activation energy for the free diffusion of an isotopically labelled water molecule through bulk water is approximately 3 K Cal / mol. The calculated Ea value for water movement through the membrane of red blood cells is similar to free diffusion, suggesting water moves unhindered through pores in the erythrocyte membrane (Sha'afi and Gary - Bobo, 1973). However, other values for cell water diffusion are much higher than that for free diffusion of water. This suggests that the membrane in some way hinders water movement.

The absolute reaction rate for molecular diffusion through a membrane can be expressed as:

\[ P( T ) = P_g( T_g ) \times \exp \left[ \left( \frac{-E_a}{R} \right) \times \left( \frac{1}{T} - \frac{1}{T_g} \right) \right] \]

\( \text{equation 10.1.6} \)

where

- \( P( T ) \) - is the permeability at temperature \( T \)
- \( P_g \) - is the permeability at reference temperature \( T_g \).
- \( E_a \) - is the activation energy
- \( R \) - is the Universal gas constant
The activation energy is obtained experimentally from the related permeability parameters, calculated with respect to temperature which requires calculation of the movement of molecules through the membrane.

From the Lp values calculated for a single oocyte and the temperature co-ordinates at which they were generated the Ea was calculated.

Using the Arrhenius Equation 10.1.6 data was transformed for plotting a straight line form of the Arrhenius graph.

\[ Lp = Lp_0 \times \exp \left[ \left( - \frac{Ea}{R} \right) \times \left( \frac{1}{T} - \frac{1}{T_g} \right) \right] \]

\text{equation 10.1.6}

where

Lp \quad \text{is the membrane water permeability}

Lp_0 \quad \text{is the membrane water permeability at reference temperature (20°C)}

Ea \quad \text{is the activation energy}

R \quad \text{is the Universal gas constant}

T \quad \text{is the temperature (°K)}

T_g \quad \text{is the reference temperature (°K) - 20°C.}
For example the Lp for a selected individual human oocyte at different temperatures are displayed here:

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Lp (µm / atm / min)</th>
<th>Ln Lp</th>
<th>1 / T (°K)</th>
<th>1/T - 1/T₀</th>
</tr>
</thead>
<tbody>
<tr>
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<td>3.2258 E⁻³</td>
<td>-1.8710 E⁻⁴</td>
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<td>3.3003 E⁻³</td>
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<td>- 1.43</td>
<td>3.5335 E⁻³</td>
<td>+1.2060 E⁻⁴</td>
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</tbody>
</table>

From these measured values the activation energy could be calculated as it is known to be the slope of the plot of the Lp against the inverse of the temperature * the gas constant.

A best fit straight line was plotted for the data using the Straight Line Equation:

\[ y = mx + C \]

where

- \( m \) is the slope of the line
- \( C \) is a constant
- \( y \) and \( x \) are the co-ordinates (variables)
APPENDIX 2 - ARRHENIUS ACTIVATION ENERGY

the best fit straight line generated using Curfit Programme was

\[ y = -6412.5743 \times x + 0.922 \]

therefore using

\[ \text{Ea} = m \times R \]

\( m = 6412.5743 \)

\( R = 1.985 \text{ cal} / \text{mol} \)

\[ \text{Ea} = 12.73 \text{ KCal} / \text{mol}. \]
APPENDIX 3

11.1 Membrane Cryoprotectant Permeability Coefficient

Very little information is available concerning the permeability coefficients of cryoprotectants for mammalian ova. The only reported studies investigated the permeability of fertilised and unfertilised murine ova, (Jackowski et al, 1980) and fertilised bovine ova (Schneider and Mazur, 1987) to glycerol at temperatures above 0°C. The permeability of the oocyte plasma membrane to other CPA's including 1 - 2 propanediol and DMSO have not been studied in ova.

The ability to calculate the permeability coefficients for various cryoprotectants would provide information concerning the degree of expected permeation as a function of the equilibration time and temperature of exposure. These parameters could be translated for use in cryopreservation protocols, indicating the time required for equilibration with a known concentration of CPA. Detailed information concerning the membrane permeability to CPA's and their affect on water permeability would provide a basis for calculating the osmotic fluxes into and out of cells during cryopreservation, allowing the volume excursions and their possible lethal consequences to be minimised.

Transient volume excursions experienced during
shrinkage as a result of cryoprotectant addition may be large enough to cause the cell to collapse or, conversely, during swelling on cryoprotectant removal, to cause cell lysis (Levin and Miller, 1981). In addition, cellular components such as proteins and membranes may be adversely affected by exposure to high or low concentrations of electrolytes caused by water efflux or influx. In conjunction with determining the CPA permeability, knowledge of the membrane water permeability in the presence of additive would be influential in dictating the optimum cooling rate. Alterations to the membrane water permeability following equilibration with CPA's could change the predicted content of intracellular water remaining within the cell at any time during cooling, and thus the likelihood of intracellular ice formation. In this respect the addition of 5% DMSO to rat megakaryocytoperiopic cells has been found to reduce the membrane water permeability value recorded in the presence of impermeable solutes (Hempling and White, 1984). Similarly, the addition of glycerol reduces the optimal cooling rate for erythrocytes, from what would be expected in the absence of CPA if intracellular ice formation was to be avoided (Rapatz et al, 1980).

The ability to observe the osmotic response of a small number or individual cells during anisosmotic perfusion using the microscope diffusion chamber has permitted the
APPENDIX 3- CRYOPROTECTANT PERMEABILITY

derivation of the membrane water permeability of the oocyte. Recent amendments to the associated software have made it possible to investigate the kinetics of permeability of the oocyte to a variety of cryoprotectants taking into account the coupled flow of CPA and water across the cell membrane. Some preliminary studies investigating the permeability coefficient of a small number of unfertilised mouse ova using the microscope diffusion chamber have been performed. I choose to study DMSO since this was the most promising CPA in my cryopreservation experiments (Chapter 7).

11.2 Materials and Methods

Oocytes were collected as described in section 2.5.1, placed in the sample region of the microscope diffusion chamber, held in place with a dialysis membrane, and inverted into the stage at 20°C, perfused with isotonic medium, and the isotonic cell volume recorded as described for derivation of the membrane water permeability parameters with an impermeable solute (section 2.11.1). The bathing medium was rapidly altered to 1.5 M DMSO in PB1 and the change in the cell diameter recorded using video microscopy until the oocytes had returned to isotonic volume. Measurements of the cell diameter were taken at regular intervals and entered into the amended computer
APPENDIX 3- CRYOPROTECTANT PERMEABILITY

programme. Values for water permeability (K), solvent permeability (Cs), and the reflection coefficient (σ) were derived for a group of 10 fresh murine ova.

11.3 Results

On perfusion with the penetrating cryoprotectant, a time-dependent sequence of events occurred. The oocytes initially responded in a similar way to hypertonic perfusion with an impermeable solute such as NaCl. The oocytes lost water in an attempt to maintain osmotic equilibrium with the extracellular medium, causing the cell volume to decrease. However, since the cell membrane was permeable to the cryoprotectant and the extracellular concentration of DMSO was greater than in the intracellular compartment, CPA molecules entered the cells, accompanied by water to maintain equilibrium. Thus the oocyte volumes regained isotonic values.

The value derived for the DMSO permeability for the mouse oocyte plasma membrane at 20°C was 0.19 μm / sec. This was similar to those for megakaryocytopoietic cells, of 0.15 μm / sec (Hempling and White, 1984), of 0.36 μm / sec for human granulocytes (Toupin et al, 1989), and of 0.13 μm / sec for erythrocytes (Naccache and Sha'afi, 1973). The σ value of 0.67 in my study also correlated well with that reported for DMSO in megakaryocytopoietic
APPENDIX 3- CRYOPROTECTANT PERMEABILITY

cells of 0.65, and 0.48 - 0.58 for granulocytes (Hempling and White, 1984, Toupin et al, 1989). However it should be noted that these studies both used coulter counters to measure cell volume changes, which measure mean values. Also the volumes of both cell types are approximately \( \times 3 \) smaller than the mouse ova.

Finally, the values for \( K \), which is the membrane water permeability or \( L_p \) in the presence of CPA, were larger than those determined for other cell types in which the permeability coefficients in the presence of DMSO has been investigated. A value of 10.5 \( \mu m / sec \) for rat megakaryocytopoietic cells (Hempling and White, 1984) and 11.9 \( \mu m / sec \) for human granulocytes (Toupin et al, 1989) have been calculated as opposed to 27 \( \mu m / sec \) for the mouse ova in my study. It may be relevant that the study on megakaryocytopoietic cells involved prior equilibration with 1.5 M DMSO followed by perfusion with impermeable solvent, and thus the conditions may not be directly comparable to the present investigation.

These preliminary results for DMSO suggest that the microscope diffusion chamber may be a very useful method for determining the permeability coefficient for cryoprotectants of both mammalian oocytes and embryos. Further studies at other temperatures and with other CPA species are required to elucidate the response of oocytes to cryoprotectant addition and removal. Also it will be of
APPENDIX 3- CRYOPROTECTANT PERMEABILITY

great importance to investigate the effects of CPA's on the movement of water across the plasm membrane, and again the diffusion chamber will be of value in this.
APPENDIX 4

The Individual Values for the Lp and Vb of the Oocytes Investigated
Table 28 Membrane Water Permeabilities for Fresh Murine Oocytes (μm / atm / min)

<table>
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<th>30°C</th>
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APPENDIX 4 - INDIVIDUAL DATA

Membrane Water Permeabilities for Fresh Murine Oocytes
(continued)

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APPENDIX 4 - INDIVIDUAL DATA

Table 29  Inactive Volume ( % ) for Fresh Murine Oocytes

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**APPENDIX 4 - INDIVIDUAL DATA**

**Inactive Volume for Fresh Murine Oocytes**

(continued)

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**Table 30 The Membrane Water Permeabilities for Fresh Human Oocytes (μm / atm / min)**

<table>
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<td>0.56</td>
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</table>

* Represents those oocytes that underwent duplicate shrinkage experiments
### Table 31: Inactive Volume (%) of Fresh Human Oocytes

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<thead>
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<td>/</td>
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<td>/</td>
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Table 32 The Membrane Water Permeabilities for Failed-to-Fertilise Human Oocytes (µm/atm/min).

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<td>1.75</td>
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<td>0.38</td>
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<td>0.66</td>
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</table>
Table 33 Inactive Volume for Failed to Fertilise Human Oocytes

<table>
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</table>
Table 34 Lp values for Pre-Ovulatory Murine Oocytes (μm / atm / min)

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<td>0.58</td>
<td>0.31</td>
</tr>
<tr>
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<td>0.44</td>
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</table>
Membrane Water Permeabilities (µm / atm / min) for Pre-Ovulatory Murine Oocytes (continued)

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<tr>
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<td>1.18</td>
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<td>0.51</td>
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<tr>
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<tr>
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<tr>
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Table 36 Membrane Water Permeability (µm / atm / min) of Human Oocytes Matured Overnight or Examined Immediately On Collection.

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<th>20°C Examined Immediately</th>
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<tbody>
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</tr>
<tr>
<td>0.49</td>
<td>0.29</td>
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<tr>
<td>0.23</td>
<td>0.56</td>
</tr>
<tr>
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<td>0.61</td>
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<td>1.10</td>
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<td>0.67</td>
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<td>0.31</td>
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<td>0.49</td>
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<td>0.48</td>
<td>0.35</td>
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<tr>
<td>0.40</td>
<td>0.36</td>
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<td>0.47</td>
<td></td>
</tr>
<tr>
<td>0.26</td>
<td></td>
</tr>
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</table>
Table 37 Inactive volumes (% ) of Human Oocytes Matured Overnight or Examined Immediately On Collection.

<table>
<thead>
<tr>
<th>20°C Matured overnight</th>
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<td>22.00</td>
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<tr>
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Table 38 Membrane Water Permeabilities (μm / atm / min) for Pronucleated Human Oocytes

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<td>0.27</td>
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<tr>
<td>0.69</td>
<td>0.13</td>
</tr>
<tr>
<td>1.17</td>
<td>0.55</td>
</tr>
<tr>
<td>0.11</td>
<td>0.41</td>
</tr>
<tr>
<td>0.48</td>
<td>0.48</td>
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<tr>
<td>0.40</td>
<td>0.33</td>
</tr>
<tr>
<td>0.16</td>
<td>0.25</td>
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<tr>
<td>0.53</td>
<td>0.36</td>
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<tr>
<td>0.46</td>
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<tr>
<td>0.39</td>
<td></td>
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<tr>
<td>0.47</td>
<td></td>
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</table>
Table 39 The Inactive Volume ( % ) of Pronucleated Human Oocytes

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<tr>
<td>35.00</td>
<td>/</td>
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<tr>
<td>37.00</td>
<td>/</td>
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<td>/</td>
<td>/</td>
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</table>
Table 40 Repeat Lp values (μm/ atm/ min) for Fresh Human Oocytes

<table>
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<tbody>
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<td>0.38</td>
<td>0.51</td>
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<tr>
<td>0.50</td>
<td>0.60</td>
</tr>
<tr>
<td>0.56</td>
<td>0.57</td>
</tr>
<tr>
<td>0.48</td>
<td>0.40</td>
</tr>
<tr>
<td>0.10</td>
<td>0.12</td>
</tr>
<tr>
<td>0.58</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Mean Lp Run 1 | Run 2
0.44 ± 0.16 | 0.48 ± 0.18

Paired T - Test
6° Freedom  P = 0.146

Repeat Vb values Fresh Human Oocytes

<table>
<thead>
<tr>
<th>Vb1</th>
<th>Vb2</th>
</tr>
</thead>
<tbody>
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<td>27 %</td>
<td>24 %</td>
</tr>
<tr>
<td>20 %</td>
<td>17 %</td>
</tr>
<tr>
<td>33 %</td>
<td>34 %</td>
</tr>
<tr>
<td>16 %</td>
<td>16 %</td>
</tr>
</tbody>
</table>
Background Information Concerning Fresh Human Oocytes

i) Oocytes were donated from 69 patients 85.5% of whom fertilised 1 or more of the rest of the cohort oocytes collected and had embryos replaced (59 patients had an embryo transfer).

ii) Of those patients who did not fertilise any of the oocytes collected 60% were due to male factor infertility such as 0% Hamster Egg Penetration Test, Sperm Antibodies, or an abnormal semen analysis (6 out of 10 patients with nil fertilisation).

iii) Of the remaining patients with nil fertilisation 1 was classified as Tubal infertility, 1 Polycystic Ovary Syndrome, and the other two were unexplained.

iv) The fertilisation rate for the oocytes collected and fertilised for replacement for the patient, excluding those donated for research, was 72% (586 oocytes collected 366 fertilised).

v) The pregnancy rate for patients undergoing an embryo transfer after taking into account the fact that 8 patients returned for a second treatment cycle was 16%.
Table 41 Details Fresh Human Oocytes: Fertilisation Rates

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Diagnosis</th>
<th>No. Oocytes Collected</th>
<th>No. Oocytes Fertilised</th>
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</thead>
<tbody>
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<td>6 / 7</td>
</tr>
<tr>
<td>2</td>
<td>Tubal</td>
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<td>7 / 9</td>
</tr>
<tr>
<td>3</td>
<td>Tubal + Endo.</td>
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<td>Unexplained</td>
<td>12</td>
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APPENDIX 4 - INDIVIDUAL DATA

Details of Fresh Human oocytes: Fertilisation Rates (cont.)

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<td>13</td>
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<td>11 / 11</td>
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<td>19</td>
<td>Tubal</td>
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</tr>
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<td>* 20</td>
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<tr>
<td>* 21</td>
<td>Tubal</td>
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<td>7 / 11</td>
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<td>8 / 8</td>
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## Details on Fresh Human Oocytes: Fertilisation Rates (cont.)

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<td>8</td>
<td>3 / 10</td>
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<tr>
<td>31</td>
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<td>8</td>
<td>0 / 6</td>
</tr>
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<td>Tubal</td>
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<td>12 / 12</td>
</tr>
<tr>
<td>* 33</td>
<td>Tubal</td>
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<td>6 / 10</td>
</tr>
<tr>
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</tr>
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<td>*</td>
<td>11</td>
<td>0 / 8 **</td>
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<td>15</td>
<td>9 / 12</td>
</tr>
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</tr>
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Details on Fresh Human Oocytes: Fertilisation Rates (cont.)

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### Details on Fresh Human Oocytes: Fertilisation Rates (cont.)

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<td>58</td>
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<td>59</td>
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<td>3 / 12</td>
</tr>
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<td>60</td>
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<td>11</td>
<td>0 / 8 **</td>
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</table>
Legend

* Indicates a pregnancy resulting from the treatment cycle from which the oocytes were retrieved.

Indicates a pregnancy resulting from a treatment other than that from which the oocytes were donated.

** Although there was no fertilisation with the partners sperm the oocytes inseminated with therapeutic donor sperm fertilised and developed.
### Table 42 Details of Donated Failed to Fertilise Oocytes

<table>
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<tr>
<th>Patient No.</th>
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<td>3</td>
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<td>4</td>
<td>δ</td>
<td>9</td>
<td>0 / 9</td>
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## Details of Donated Failed to Fertilise Oocytes

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## APPENDIX 4 - INDIVIDUAL DATA

### Details of Donated Failed to Fertilise Oocytes

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</table>
APPENDIX 4 - INDIVIDUAL DATA

i) The failed to fertilise oocytes were donated from 43 different patients 53% of whom were diagnosed as male factor infertility (23 patients out of 43).

ii) Of the oocytes used 52% were from male factor infertility patients, 22% from tubal, 19% unexplained and 7% endometriosis infertility patients.
ACKNOWLEDGEMENTS

I would like to thank Dr B. J. Fuller and Mr A. G. Bernard for all of their advice, encouragement, and time.

I am also grateful to Professor R. W. Shaw for his support and to Professor J. J. McGrath for help with the microscope diffusion chamber, designing the computer software and analysis of the results.

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I wish to thank the I.V.F. team:
Dr B. Bentick
Dr C. Iffland
Dr W. Reid
Dr N. Amso
Dr P. Curtis

Dr G. Burford
Sister B. Chander
Mrs K. Gleeson
Mrs E. Keith

Lastly I would like to thank both my family and friends for their support.
References


References


References


References


References


References


References


References


References


References


References


References


References


References


References


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