# EARLY HUMAN FOLLICLE ULTRASTRUCTURE COMPARISON AFTER SLOW CRYOPRESERVATION IN TWO DIFFERENT CRYOPROTECTANTS

By

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***Declaration***

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: ……

4 March 2008

## SUMMARY

**BACKGROUND**: The cryopreservation and transplantation of ovarian tissue have been shown to restore ovarian function temporarily and may also preserve the fertility of young female cancer patients until after their sterilizing cancer treatment. Since tissue samples are large and morphologically complex, the cryopreservation methodology is difficult to optimize and standardise. Ovarian tissue cryopreservation is therefore, still in its experimental stages and is not a routine option offered to cancer patients.

**OBJECTIVES**: Our main aim was to initiate, develop and implement a practical ovarian tissue cryopreservation and re-transplantation protocol which would restore ovarian function, and possibly fertility, in young female cancer patients undergoing sterilizing cancer therapies in South Africa. The objective of this study was to improve the slow cryopreservation protocol for human ovarian tissue. The ultrastructural effects after cryopreservation with two well-known cryoprotectants, dimethyl sulfoxide (DMSO) and 1,2-propylene glycol (PROH), on early human follicles were investigated and compared to identify and the better cryoprotectant.

**MATERIALS AND METHODS**: A single group experimental study design was used. The participants consisted cancer patients of the Gynaecological Oncology Unit of Tygerberg Hospital who entered on a basis of voluntary informed consent. Ovarian tissue was obtained by laparoscopic oophorectomy. After dissection of the ovary(ies), some fresh cortical tissue was sent for metastatic analysis and a few strips taken as fresh control. Remaining dissected ovarian cortical tissue sections of each patient were equally divided into the two cryoprotectant groups. Five resulting groups could be compared: i) fresh tissue (control group); tissue equilibrated in ii) DMSO; or iii) PROH and tissue equilibrated and cryopreserved in iv) DMSO or v) PROH. Five tissue samples per patient were therefore fixed for standard histological haematoxylin and eosin (HE) staining and transmission electron microscopy (TEM). Tissue samples showing early follicles on HE slides were sent for TEM processing. Ultrastructural studies on micrographs of primordial and primary follicles were assessed according to a scoring system which gave an indication of follicular health. Appropriate statistical tests were applied to analyse the mean scores where $P \leq 0.05$ was considered as statistically significant.

**RESULTS**: No significant overall cryopreservation treatment effect was evident in any of the follicular ultrastructures evaluated. This result indicated that the cryopreservation protocol used did not induce significant damage to the cortex tissue compared to the fresh control group. Comparison of the effect of PROH and DMSO on the follicular ultrastructures showed that PROH tend to induce more extensive damage, especially after cryopreservation. Correlation studies showed significant positive relationships between the majority of the evaluated ultrastructures, especially between the oocyte and granulosa cell layer and basal membrane. The stromal cells and extracellular matrix did not correlate well with other structures. Correlations indicated that the granulosa cells, oocyte and basal lamina are affected similarly and that the damage in one of these structures may be representative of the damage in the other structures.

**CONCLUSION**: The main aim of the study was achieved since results showed that no significant damage was induced by the cryopreservation protocols. Ovarian tissue cryopreserved in this study has shown to restore endocrine function temporarily after heterotopic autotransplantation in menopausal patients. From the electron microscopy evaluations, DMSO showed better cryopreservation results. The DMSO cryopreservation protocol was also more time efficient and has shown the most successful outcomes in the literature. The stromal tissue seemed to be affected differently by cryopreservation compared to the primordial follicle ultrastructures. Younger patients are needed for future studies since a larger initial follicular reserve may allow for larger follicle sample sizes.

## OPSOMMING

**AGTERGROND**: Vriesbewaring en terugplanting van ovariële weefsel het in gevalle-studies getoon dat ovariële endokriene funksie en fertiliteit tydelik herstel kan word na steriliserende kankerbehandeling van jong vroue. Die tegniek word egter nog nie as ‘n roetine opsie aan kanker pasiënte gebied nie aangesien die vriesmetodiek steeds in die eksperimentele- en verfyningsfase is. Die metodiek word gekompliseer deur die multisellulêriteit en grootte van ovariële weefselmonsters.

**DOELWITTE**: Die oorhoofse doelwit was om ‘n praktiese ovariële vriesbewaring- en terugplantings-prosedure te ontwikkel, inisieër en implementeer wat moontlik gebruik kan word om ovariële funksie te herstel en selfs fertiliteit te bewaar van jong vroue wat steriliserende kankerbehandeling in Suid-Afrika oorleef. Die doelwit van hierdie studie was om ‘n bydrae te lewer in die verbetering van die stadige vriesbewaringsmetode vir menslike ovariële weefsel. Twee bekende vriesmediums, dimetielsulfoksied (DMSO) en 1,2-propanediol (PROH), se effek op die ultrastruktuur van menslike vroëe follikels in ovariële weefsel is bestudeer om ten einde die beter vriesmedium te identifiseer en te implenteer.

**MATERIALE EN METODES**: ‘n Enkelgroep eksperimentele studie is uitgevoer. Die studiegroep het bestaan uit pasiënte van die Ginekologiese Onkologie Eenheid van Tygerberg Hospitaal wat ten volle ingelig is en vrywilliglik skriftelike toestemming gegee het vir deelname aan die studie. Die ovariële weefsel is deur middel van ‘n laparoskopiese ooforektomie verwyder. Na disseksie van die ovarium(s), is vars korteks weefsel gefikseer vir metastatiese analise. Vars korteks repies is ook gefikseer as kontrole groep. Oorblywende voorbereide korteks repies is gehalveer om een helfde in DMSO en die ander helfde in PROH te vries volgens die stadige vriesbewaringsmetode. Vyf groepe weefselmonsters is dus geëvalueer vir vergelyking: i) vars weefsel (kontrole groep); weefsel ge-ekwillibreer in ii) DMSO, of iii) PROH; weefsel ge-ekwillibreer en dan stadig gevries in iv) DMSO, of v) PROH. Die vyf weefselmonsters van elke pasiënt is gestuur vir standaard heamotoksilien en eosien (HE) kleuring. Weefselmonsters met vroëe follikels op HE-skyfies is gestuur vir transmissie elektronmikroskopie (TEM). Die ultrastruktuur van primordiale en primêre follikels is geëvalueer en ‘n puntestelsel is toegepas om die mate van oorlewing aan te dui. Gemiddeldes van toegekende puntetellings is met gepaste statistiese metodes verwerk waar $P \leq 0.05$ aanvaar was as statisties betekenisvol.

**RESULTATE**: Vegelyking van die vars weefsel met die ekwilibrasie en gevriesde weefsels het geen betekenisvolle oorhoofse behandelingseffek uitgewys nie. Dit het daarop gewys dat die vriesbewaringsmetode nie verdere betekenisvolle skade veroorsaak het in vergelyking met die vars weefsel nie. Vergelyking van die vriesmediums het gewys dat PROH geneig het om erger skade aan die follikel ultrastrukture aan te rig as DMSO, veral gedurende vriesbewaring. Verskeie ultrastrukture het sterk positiewe liniêre korrelasies getoon, veral diè van die granulosa selle, die oösiet en die basale membraan. Die sterk liniêre korrelasies het daarop gewys dat die granulosa selle, oösiet en basale lamina moontlik dieslfde skade ly en dus kan die een struktuur se skade verteenwoordigend wees van die skade in die ander. Die stromale selle en ekstrasellulêre matriks het nie korrelasies met ander strukture getoon nie.

**GEVOLGTREKKING**: Die oorhoofse doelwit van die studie is bereik aangesien die vriesbewaringsmetode nie betekenisvolle addisionele skade aan die korteksweefsel en follikels aangerig het nie. Sommige deelnemers van die studie wat terugplantings van gevriesde en gestoorde weefsel ontvang het, het tydelike endokriene herstel getoon. DMSO is uitgewys as die beter vriesmedium aangesien dit minder skade aan die follikels gerig het as PROH. DMSO het ook ‘n tydsvoordeel in die vriesprotokol en het ook in die literatuur die beste uitkomste getoon. Die stroma van die korteks weefsel word moontlik anders geaffekteer as die follikels. Verdere navorsing in hierdie gebied benodig jonger deelnemers, met ‘n groter follikulêre reserwe, om ‘n groter steekproef te bewerkstellig vir meer akkurate uitkomste.

*Opgedra aan my ouers,*

*Alet & Basie Els*

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**CHAPTER THREE**

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# INTRODUCTION

Scientific research on ovarian cryopreservation and transplantation started in the 1950s and clinical studies followed in the 2000s (Hovatta, 2003).

As radiotherapy and chemotherapy treatments improve, more young women survive cytotoxic cancer treatment but are left menopausal and infertile. The research indicated the potential of ovarian cryopreservation and transplantation to restore endocrine function and fertility in women facing premature ovarian failure due to chemotherapy, radiotherapy and/or surgery. Four published case studies have recently reported pregnancies (Donnez *et al*., 2004; Meirow *et al*., 2005a, 2007; Silber *et al*., 2005) of which some are controversial. Currently, studies with larger patient numbers are needed to determine longevity of grafts, hormone production, follicle development, and possibilities for pregnancy as well as improvement of cryoprotectant media and cryopreservation techniques (Buyuk & Oktay, 2002).

In a study of the effect of cryopreservation on human ovarian tissue and also the use of thawed tissue for fertility and/or endocrine function in especially cancer patients, it is important to review all the different aspects that may play a role. These aspects include the normal histology and physiology of the ovary, basic cryobiology principles, effects of cancer therapies on the ovary and treatment options available and current methods and results achieved in ovarian cryopreservation and transplantation studies published in the literature.

In **Chapter 1** the above mentioned aspects are therefore reviewed and compiled as a **Literature Review**.

In **Part 1** the physiology and histology of the healthy human ovary is discussed with focus on the ovarian cortex.

In **Part 2** the principles of cryobiology and cryopreservation are reviewed, including strategies to avoid intracellular ice formation, cooling rates, thawing rates, the role of cryoprotectants, seeding and sample complexity.

In **Part 3** the effect of cancer therapies on ovarian function is discussed as well as current options available to these patients to preserve their fertility and/or endocrine function, i.e. oophoropexy, gonadal shielding, embryo cryopreservation, oocyte cryopreservation, and pharmacological agent administration.

In **Part 4** the development of different human and animal ovarian cryopreservation methods and protocols are reported and compared. Issues that are addressed are size of tissue sample, size and type of cryo containers, toxicity of different cryoprotectants, equilibration times and temperatures, cooling rates, seeding method and thawing rate. The current published results in human and animal ovarian cryopreservation are reviewed. Transplantation results and evaluation methods are also included.

In **Chapter 2** the **Aims and Objectives** and **Materials and Methods** of the study are detailed.

In **Chapter 3** the **Results** of the study are presented.

In **Chapter 4** the results are evaluated in the **Discussion**.

In **Chapter 5** a **Conclusion** is formulated.

In **Chapter 6** possible **Future Prospects** are discussed.

In **Chapter 7** all relevant and quoted **References** are alphabetically listed.

In **Chapter 8** all **Appendices** and **Abbreviations** are included.

# CHAPTER ONE

# LITERATURE REVIEW

## 1. NORMAL HISTOLOGY OF THE OVARIAN CORTEX

### 1.1. Ovary development and folliculogenesis

Human folliculogenesis begins during foetal life. Primordial germ cells in the yolk sac are distinguishable at the 4$^{th}$ week of embryonic development and settles in the gonadal ridge at 5 to 6 weeks of gestation to differentiate into oogonia by the 7$^{th}$ week. By active mitosis, these germ cells give rise to about 6 to 7 million early oogonia, which are diploid cells at this stage. At 13 weeks of gestation, mitosis rate slows down, meiosis initiates to produce haploid gametes and oocytes are arrested at the diplotene stage of the first meiotic division. Primordial follicle formation commences between 16 and 18 weeks of gestation in the inner part of the ovary (Sadeu, 2006). Each oocyte is surrounded by a single layer of pregranulosa cells, transforms into a primary oocyte and continues the process of meiosis until completion of the first prophase (Gougeon, 1996; Sadeu, 2006). At 5 to 6 months of foetal life, an estimated 7 million oogonia are present in the ovaries (Sadeu, 2006). Oogonia that do not acquire granulosa cells or do not undergo meiosis, undergo apoptosis (programmed cell death). The surviving primordial follicles constitute the resting follicle reserve from which follicles enter the growth phase by folliculogenesis (Gougeon, 1996).

Some primordial follicles initiate folliculogenesis to preantral stage, but undergo atresia because of absent gonadotropin support, resulting in a total of about 1 (Buga, 2007) to 2 million (Sadeu, 2006) or 266 000 to 472 000 viable resting follicles per ovary left in reserve at time of birth (Gougeon, 1996) (*see Fig.1.1.*).

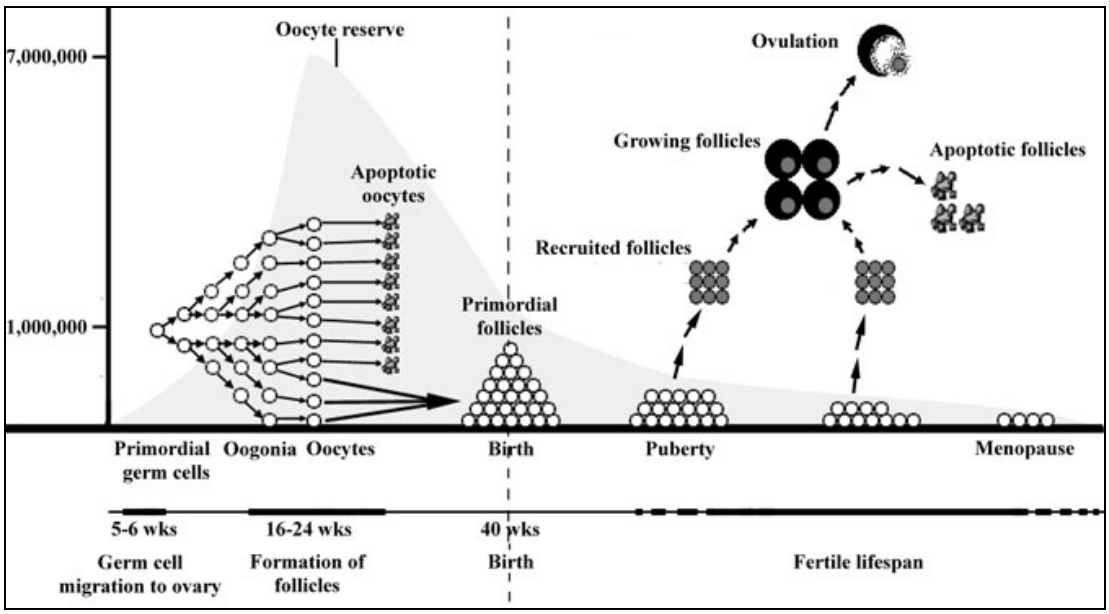



*Figure 1.1.* Germ cell attrition and follicular atresia. Germ cells migrate to the ovary during early embryonic development. Their number increases through mitotic divisions but most of the oocytes formed during development do not survive to the time of birth [http://herkules.oulu.fi/isbn9514266676/html/i267605.html].

In the adult ovary the primordial follicles are located in the outermost part of the ovarian cortex (Sadeu, 2006; Shaw, 2003a).

The population of resting follicles decreases with increasing age (see *Fig.3*.). Depletion of follicles continues during childhood and by menarche, only 300 000 to 500 000 are left. Follicle reserve depletion continues henceforward at 1 000 per month, either by programmed atresia or entry into growth phase, of which only 400 follicles are destined to be ovulated in a woman's reproductive life (Gougeon, 1996; Buga, 2007; Sadeu, 2006).

At approximately the age of 35 (Shaw, 2000) or 37 (Buga, 2007) or 38 (Gougeon, 1996), the rate of follicle depletion accelerates until menopause, when the resting follicle reserve reaches between less than a hundred (Buga, 2007) and a 1000 (Gougeon, 1996).

Data of Gougeon (1998) showed that the mean number of follicles in all stages from preantral to selectable (0.15mm to 2mm in size), were significantly less in the 41-45 years old age group (n=32) compared to the 36-40 years old group (n=13). The age group of 46 years and older (n=36) showed another significant decrease in mean preantral and small antral follicle numbers. The acceleration in decay rate of resting

follicles after 38 years may be linked to the significant increase in circulating FSH levels in women older than 35 years (Gougeon, 1996).

The start of follicular growth is characterized by three main events, namely change in shape of granulosa cells from flattened to cuboidal, proliferation of granulosa cells and oocyte growth (Sadeu, 2006). Initiation of basal follicular growth is continuous, independent of gonadotrophins and is uninterrupted by childhood, pregnancy or lactation. Follicles appear to enter the next growth phase in the same order as they entered meiosis, based on the production-line hypothesis of 'first-in-first-out' (Buga, 2007).

The mechanism of recruitment of primordial follicles to initiate folliculogenesis is unknown. The first stage of folliculogenesis, preantral development, takes 120 days and commences with the increase in size of the primary oocyte without resumption of meiosis. Granulosa cell proliferation by mitosis follows, converting the primordial follicle to a primary and subsequent pre-antral follicle. At this stage, the follicle contains a fully grown oocyte, a zona pellucida, secreted by the oocyte, and two or more layers of granulosa cells and a basement membrane, separating it from the stroma. The surrounding stroma condenses around the growing follicle to form a vascular theca (Buga, 2007).

About 15 to 20 of all the follicles initiating gonadotropin-independent development are recruited for the next stage of folliculogenesis, namely antral development, i.e. follicle growth from preantral to before selectable stage. Survival and maturation is highly dependent on the function of various oocyte-mediated events as well as gonadotropin support from both follicle stimulating hormone (FSH) and luteinising hormone (LH) (Buga, 2007).

The pre-antral follicle takes about 85 days to reach pre-ovulatory size and will therefore, experience a fluctuating hormonal environment. These cyclical changes in circulating levels of steroid hormones and gonadotrophins will have a variable influence on the developmental capacity of the follicle and together with intra-ovarian factors, will determine its fate towards either growth or atresia. Atresia causes the elimination of >90% of follicles entering the growth phase and affects follicles at all stages of development, making it a significant factor in determining the precise number of follicles that will enter the next growth phase (Gougeon, 1986).

From the follicles completing antral development, normally only a single dominant Graafian follicle is selected around day 5 to 7 of the menstrual cycle destined for ovulation (Buga, 2007) (*see Fig. 1.2*.)

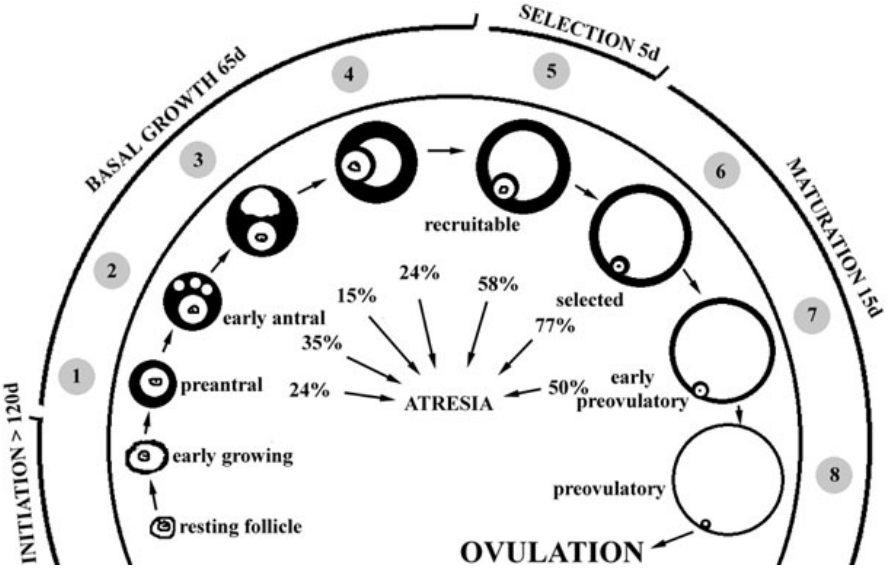



*Figure 1.2.* Folliculogenesis and classification of growing follicles in the human ovary. Growing follicles usually enter the early antral stage (2) in the late luteal phase, antral stage (3) between late luteal and early follicular phases, late antral stage (4) during late follicular phase and become recruitable (5) follicles during late luteal phase (Gougeon, 1996).

## 1.2. The ovary

The ovarian surface is covered with a single layer of simple cuboidal-to-columnar mesothelium, usually called the germinal epithelium although it does not contain any germ cells (*see Fig.1.4.*). The ovary consists largely of stroma with scattered follicles and abundant vasculature. The stroma is highly cellular, consisting of spindle-shaped stromal cells with inconspicuous supporting fibres, i.e. reticular fibres and collagen. The outer stroma, i.e. cortex, of the ovary contains the follicular reserve. The inner stroma, i.e. medulla, contains the larger follicles and blood vessels (King, 2002) (*see Fig.1.3.)*.

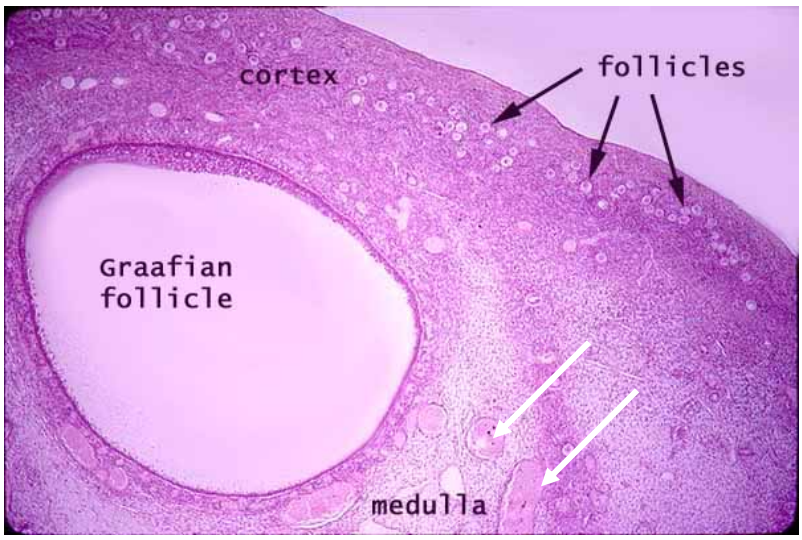



*Figure 1.3.* Histological haematoxylin and eosin stain slide showing the location of the cortex containing early follicles (black arrows) and the medulla of the ovary, containing larger blood vessels (white arrows) (King 2002).

## 1.3. Histology of the ovarian cortex

Primordial follicles are most abundant in the outermost 1-2mm of the ovary, the cortex. The ovarian cortex, which is rich in primordial follicles, can easily be recognised macroscopically as the white, tough collagenous surface layer. Individual primordial follicles cannot be seen and is not evenly distributed through the cortex (Shaw, 2003a, 2003b).

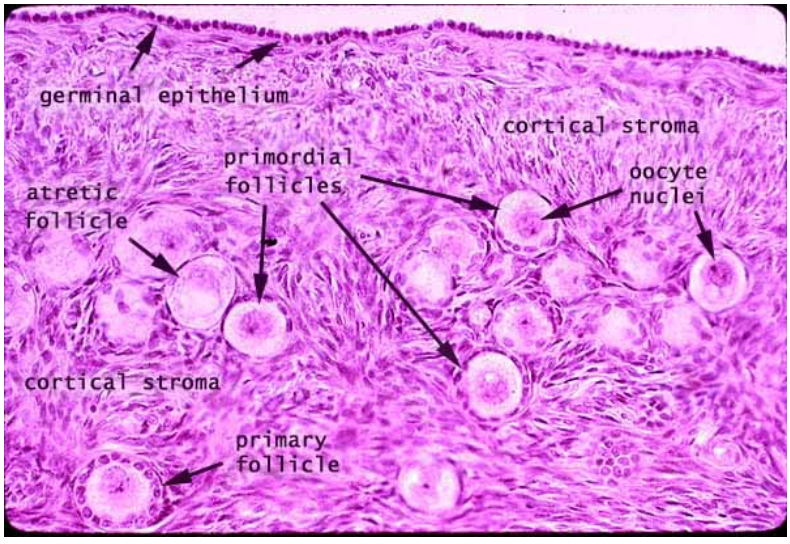



*Figure 1.4.* Histological haematoxylin and eosin stain slide showing the cortical stroma surrounded by the germinal epithelium, containing several primordial, primary and atretic follicles (King, 2002).

## 1.4. Histology of follicle development

While the majority of follicles remain in resting primordial condition in the cortex, follicles in different developmental stages can be recognised under light microscopy. The classification of growing follicles is based on morphological aspects and the total number of granulosa cells present in the follicle. Most easily recognised are the primordial follicles and the mature Graafian follicle (Gougeon, 1996).

Primordial, intermediary and primary follicles differ in diameter due to difference in number and size of granulosa cells, while the oocyte and nucleus diameter is unchanged. Primordial follicles consist of a primary oocyte surrounded by a single layer of flat mitotically inactive granulosa cells resting on a thin basal lamina. These flat granulosa cells are also referred to as follicular cells. An intermediary follicle is slightly larger than a primordial follicle and is surrounded by a mixture of mitotically active, flat and cuboidal granulosa cells. A primary follicle is surrounded by a single layer of cuboidal granulosa cells (Gougeon, 1996).

The secondary follicle is about 80-125µm in size and is at least partly surrounded by one or more layers of cuboidal granulosa cells with stromal cells surrounding the basal membrane aligned parallel to each other. The connective tissue within the stroma differentiates into two parts, namely the theca externa and theca interna. The theca externa do not differ from the surrounding stroma, but one or two small arterioles may be visible, terminating in an anastomotic network within the theca interna, just outside the basal lamina. When epithelioid cells appear in the theca interna and three to six granulosa cell layers are visible, the follicle has reached pre-antral stage (Gougeon, 1996).

In the early antral stage, antrum development starts with appearance of small fluid-filled cavities within the granulosa cell layer. When the follicle acquires a single antral cavity, the granulosa cells that border the basement membrane change from cuboidal to columnar shape and constitute the cumulus oophorus. The progress of the follicle through subsequent stages of development is related to the accumulation of antral fluid and the proliferation of granulosa and theca interna cells, until reaching the recruitable stage (Gougeon, 1996).

The selected pre-ovulatory follicle or Graafian follicle consists of the maturing oocyte, an antrum, several layers of differentiating granulosa cells and theca layers. These differentiated preovulatory follicular cells acquire the ability to perform endocrine functions under primary control of FSH and LH (Gougeon, 1996; Bedaiwy *et al*., 2006).

### 1.5. Atresia

It has been recognized that more than 99% of follicles present at birth become atretic, while only less than 1% reach ovulation. Thus, it could be considered 'normal' for a follicle to disappear by atresia, allowing the ovary to cyclically produce the ovulatory quota (Gougeon, 1996).

Histological studies use morphological criteria to assess the health of follicles, whether the individual follicle is actively growing or degenerating. Most common descriptions of atresia are oocyte involution, irregular follicle and/or oocyte shape and pyknosis in the granulosa cell layers. The first morphological signs of apoptosis are the condensation of nuclear chromatin into crescentric caps in the periphery of the nucleus and cytoplasmic condensation. This is associated with nucleolar disintegration, reduction in nuclear size and reduction of cell volume and increase in cell density (Gougeon, 1996).

In early antral and smaller follicles, atresia is characterized by an early and fast oocyte degeneration and disappearance with subsequent hypertrophy of the theca interna cells. In larger follicles, atresia happens in four successive stages of degeneration and the oocyte persists long after the granulosa cells have disappeared. The first stage entails pyknosis and fragmentation of 20% of the granulosa cells. In the second stage, up to half of the granulosa wall has disappeared. The third stage is recognized by a shrunken follicle, disappeared granulosa cells, a thickened basal lamina and the thecal interna has changed into an interstitial gland. The fourth and final stage of atresia constitutes a very thick basal lamina, a collapsed follicle, an antrum filled with fibroblasts and an absent cumulus oophorus and theca-interstitial gland (Gougeon, 1996).

The study of Bedaiwy *et al.* (2006) used the following histological criteria to assess follicular health within ovarian tissue under light microscopy. Characteristics of healthy follicles included an intact oocyte, an intact granulosa membrane, the

absence of necrosis and the presence of less than 5% pyknotic nuclei. Atretic follicles present an attenuated granulosa membrane with loosely attached granulosa cells and more than 5% pyknotic nuclei. Atretic follicles also have a thin inner layer of granulosa cells and outer theca layer (*see Fig.1.4.)*.

# 2. CRYOPRESERVATION PRINCIPLES

## 2.1. Introduction to cryobiology, anhydrobiology and freezing damage

Cryobiology is defined as the study of life or cellular systems at low temperatures. Anhydrobiology is the study of life at low water content, of which some principles are used in cryobiology for cryopreservation purposes, since freezing damage in cells are partly caused by the dehydration effects of freezing (Wolfe & Bryant, n.d.).

Three ranges or stages of low temperature are useful in cryobiology. The first stage is cold temperatures down to 0ºC, which is damaging if exposure is sustained but survived by a wide variety of cells and species. The second stage is between 0ºC and -40ºC where freezing and most of the freezing damage occur. Sustained exposure in this stage could be survived only by a few species. The third stage is temperatures beyond -40ºC to around the boiling point of nitrogen (-195.8ºC). These cold temperatures are known as cryogenic temperatures. In this stage, sustained exposure is not damaging since biochemistry and physiological processes are effectively zero and if no mechanical damage is incurred or temperatures stay constant, little or no damage occurs in this cryopreserved state. Cooling to these low temperatures and warming or thawing are the damaging stages (Wolfe & Bryant, 2001).

Freezing damage is largely caused by intracellular ice formation (IIF), which initiates two types of damage, namely mechanical stress and dehydration. IIF is widely used as an indicator of cell death in vitro (Wolfe & Bryant, 2001).

Ice crystals are directly physically damaging to the cellular ultrastructure. The growth of ice crystals through the cell membranes and macromolecules as well as the volumetric expansion that accompanies freezing, cause permanent and fatal mechanical damage to the cell membrane and inner structure (Wolfe & Bryant, 2001). Rupture of the cell plasma membrane is one of the most common indicators of cell death (Wolfe & Bryant, 1999).

Secondly, the propagation of ice crystals can indirectly cause severe and fatal cellular dehydration. As only pure water freezes, solute concentration increases as more solutes are diluted in less and less water. As more water freezes, pure water molecules are drawn out of the intracellular solution to initially reduce the solute concentration, but eventually also crystallize (*discussed in 2.2.2*).

The increasing high solute concentration can reach toxic levels. The change of water into ice changes the electrolyte concentrations and hydrophobic and hydrophilic interactions which can affect ionic interactions to such extent that biochemical compounds are permanently destabilized. In this environment, enzymes, for example, unfold and denature permanently (Wolfe & Bryant, 2001).

The crystal formation of freezing starts with nucleation of water molecules. In a pure aqueous liquid at very low temperatures, before freezing occurs, water molecules have a lower kinetic energy, slowing Brownian motion. This increases the probability of water molecules to spontaneously arrange into a lattice formation resulting in clusters of ice that dissipates rapidly. When this crystal lattice reaches a critical size, crystallization is energetically favoured and freezing is initiated. This process is called nucleation and can be homogeneous, as described above, or heterogeneous when initiated by an impurity or container wall (*see Fig.1.5.*) (Wolfe & Bryant, 1999).

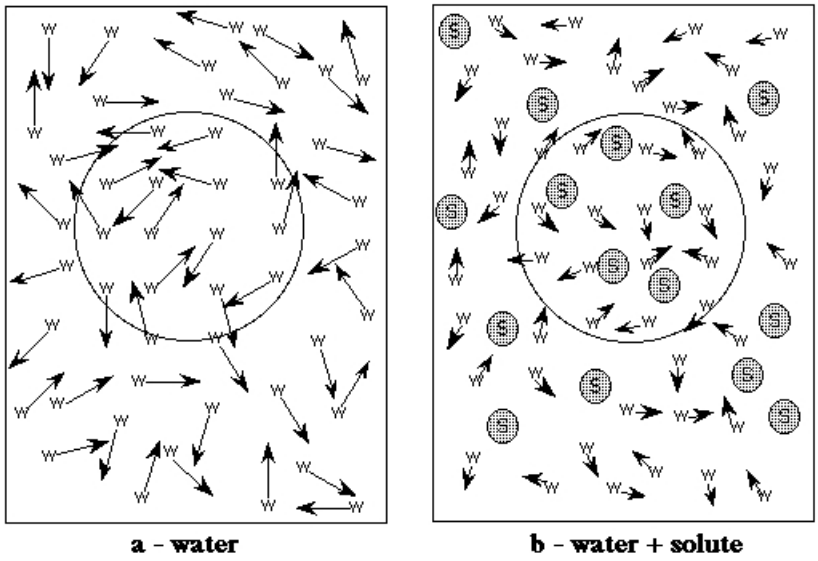



*Figure 1.5*. Nucleation initiation. a) In pure water at near freezing temperature, water molecules (indicated by W) while in Brownian motion (indicated by arrows) must spontaneously arrange in crystal lattice formation larger than critical size (circle area) for nucleation to start freezing. b) The probability of ice nucleation is reduced when solutes (S) are present since diffusion is reduced (smaller arrows than in Fig.1.5.a.) and the probability of the critical area (encircled area) being filled with only water molecules (indicated by W) which are needed for regular lattice formation are reduced. This effect becomes stronger as solute concentration increases (Wolfe & Bryant, 1999).

## 2.2. Strategies in cryopreservation to avoid IIF

For the artificial process of cryopreservation to succeed, cryogenic temperatures must be achieved without incurring fatal freezing damage by IIF. Also, an intact cell membrane is required to maintain the different compositions of intra- and extracellular solutions as well as prevent the extracellular ice from nucleating the intracellular solution (Wolfe & Bryant, 2001).

Four strategies can be followed in cryopreservation to avoid IIF, namely freezing point depression, dehydration, supercooling and vitrification. In the cooling of biological tissues, these strategies are not separate, but exist in balance, one leading to the other (Wolfe & Bryant, 2001).

### 2.2.1. Freezing point depression

The main function of freezing point depression in cryopreservation is the avoidance of nucleation in relatively high sub-zero temperatures, for example during initial phases of slow freezing. Freezing point depression is mostly achieved by the accumulation of solutes in the intra- and/or extracellular solutions, to increase the viscosity (Wolfe & Bryant, 1999). The higher viscosity reduces diffusion, which has several advantages. Firstly, nucleation probability is reduced thus impeding ice crystal growth (*see Fig.1.5.*). Secondly, dehydration is moderate, allowing non-fatal osmotic cell contraction (*discussed in 2.2.2.)*, and thirdly, slows down cell metabolism, reducing cryoprotectant toxicity (Wolfe & Bryant, n.d.).

In biological samples, high concentrations of extracellular solutes are needed to lower the equilibrium freezing point by only a few degrees (Wolfe & Bryant, n.d.). For example, in a study of Demirci *et al.* (2001) on slow freezing of sheep ovarian tissue, equilibrium freezing temperatures were determined for samples with increasing concentrations of cryoprotectants. Dimethyl sulfoxide (DMSO) and 1,2-propylene glycol (PROH) were compared at concentrations of 1M, 1.5M and 2M in the freezing media. Freeze point depression was achieved by increasing DMSO or PROH concentration resulting in concurrent decreasing seeding temperatures of -7ºC, -8ºC and -11ºC for DMSO and -6ºC, -8ºC and -11ºC for PROH. Equilibrium freezing temperature determination was necessary for seeding purposes (*discussed in 2.4*.).

Other solutes that can be tolerated in high concentrations in biological tissues are sugars (Wolfe & Bryant, 2001). The addition of certain sugars to cryomedia thus acts as a non-permeable cryoprotectant in cryopreservation (*discussed in 2.3.3.*).

### 2.2.2. Dehydration

Dehydration of cells and tissues can be caused or induced by solute concentration differences across cell membranes or by ice crystal growth during freezing.

Dehydration caused by high solute concentration differences across cell membranes is well known. The extracellular osmotic pressure is increased when a non-permeable solute are added to the extracellular solution. Water thus moves osmotically out of the cytoplasm until equilibrium is reached, resulting in cellular dehydration (Wolfe & Bryant, 1999).

The extracellular solution volume is usually higher than the intracellular volume and freezing, being a probalistic process because of nucleation, would most likely start in the extracellular solution. The freezing causes the extracellular solutes to become more and more concentrated as they are diluted in less liquid water, since only the water crystallize and are effectively removed from solution. The osmotic pressure difference causes water to leave the cells. Freeze-induced dehydration is dependent on cooling rate: the slower the cooling rate, the more osmotic equilibration time, the more crystallization can take place and the more severe the dehydration, resulting in possibly damaging osmotic contraction of cells (Wolfe & Bryant, 2001; n.d.) (*see Fig.1.6.*).

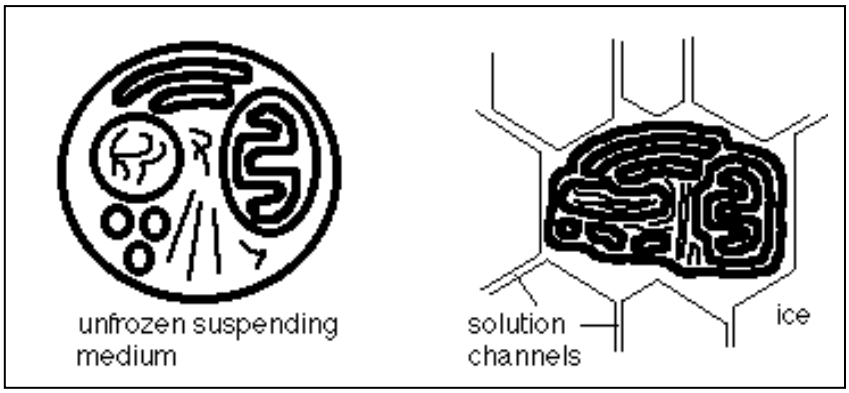



*Figure 1.6.* Illustration of dehydration caused by freezing. A cell before (left) and after (right) crystallization of extracellular medium. The elevated osmotic pressure causes a large reduction in the aqueous volume of the cell. Consequently, all of the non-aqueous components are brought into close proximity. In this state, stacks of membranes begin to resemble lamellar phases (Wolfe & Bryant, n.d.).

If dehydration is moderate, however, it can effectively protect against IIF. Moderate dehydration and moderate non-fatal cell contraction can be achieved by either controlling cooling rate protocol or with the addition of a non-penetrating cryoprotectant, like hydroxyethyl starch (HES) or dextran. The cells may be cooled to just above freezing temperature and then allowed to contract to equilibrium prior to final fast cooling phase. A non-permeable cryoprotectant increases the osmotic pressure of the cytoplasm, promoting freezing point depression, supercooling and vitrification inside the cell. Detailed mechanisms of polymer cryoprotection are still unclear (Wolfe & Bryant, 2001; n.d.).

### 2.2.3. Supercooling

For an organism or cell, supercooling is a freezing avoidance mechanism where the solutions are cooled below its equilibrium freezing point while remaining in liquid state, allowing a life-sustaining, slower than normal, metabolism (Wolfe & Bryant, 2001).

High sample volume, low solute concentration and increased number of impurities contribute to a greater probability of nucleation in a supercooled liquid. A small volume (micro litres) of a pure liquid can supercool tens of degrees Celsius below its equilibrium freezing point, while biological solutions in situ can usually supercool only a few degrees Celsius because it contains many possible ice nucleators which may initiate nucleation either inside or outside the cell (Wolfe & Bryant, 2001).

Biological tissues are at risk when supercooled because of its instability. If one ice crystal is introduced, the solution may freeze immediately. In nature, some arctic fish have a special protein in their blood serving as a natural “anti-freeze” which impedes the growth of ice crystals when entering colder waters (Wolfe & Bryant, 2001).

In cryopreservation, supercooling is important as it allows vitrification (Wolfe & Bryant, 2001) (*discussed in 2.2.4.*).

### 2.2.4. Intracellular vitrification

It is the primary goal of cryopreservation to achieve intracellular vitrification, while avoiding IIF and membrane damage.

As a liquid is cooled to very low temperatures, the viscosity rises sharply, reducing molecular diffusion. With less Brownian motion, nucleation probability is reduced and

freezing point depressed. If a liquid is cooled sufficiently fast, the viscosity becomes so large, nucleation is almost negligible and it will vitrify, forming a vitreous solid. This amorphous, liquid phase has such a large viscosity ($10^{14}$Pascals) and is by definition in a stable, long-lived state of non-equilibrium with the mechanical properties of a solid, e.g. glass (Wolfe & Bryant, 2001).

## 2.3. Determining factors in cryobiology

### 2.3.1. Cooling rate

Cooling rate is one of the critical factors determining vitrification because it plays a major role in determining osmotic or volumetric contraction of cells, which in turn, influences intra- and extracellular concentrations of water and ions (Wolfe & Bryant, 2001; Woods *et al*. 2004).

In nature, cooling rates are usually slow, allowing the distribution of water to reach equilibrium. The extracellular ice concentrates the extracellular solutes, causing water to leave the cells osmotically. Water content of cells can reach less than 10%, at which stage all of the non-aqueous components are crushed very close together. Electron micrographs of freeze induced cells with 10 to 20% water content has shown stacks of membranes lacking intramembrane macromolecules resembling lamellar phase lipid bilayers, which may be more accurately predictable when using theoretical models. This demixing of macromolecules and membranes into different domains in the cell are topological features correlated with cell damage (Wolfe & Bryant, 1999). At slow cooling rates, the longer time allows for substantial osmotic contraction of cells. This may inhibit IIF because of the resulting higher intracellular concentration but the contraction and/or severe dehydration of the cells may cause fatal damage (Wolfe & Bryant, 2001). Slow cooling rates over long periods of time however can lead to “solution injury” or toxicity from the high external concentrations of solutes, by affecting the membrane directly or by mechanical stress due to dehydration (Woods *et al*., 2004).

At fast cooling rates, dehydration and resulting volumetric contraction is reduced, not increasing the intracellular concentration sufficiently, making vitrification less likely (Wolfe & Bryant, 2001).

Therefore, an optimum cooling rate exists, ruled by the osmotic equilibration time of the cell and the tendency of the cytoplasm to nucleate ice. This optimum rate is

usually phased because most cells surviving cryopreservation respond to cooling rates in an inverted “U” shape pattern. Slow at first to allow non-fatal contraction to osmotic equilibrium to ensure sufficient intracellular concentration, followed by a fast cooling rate to allow vitrification and avoid IIF. This critical cooling rate for maximal survival may vary greatly between cell types. High survival rates usually demand avoidance IIF, where some cells survive or even benefit from otherwise fatal IIF (Wolfe & Bryant, 2001; Woods *et al*., 2004).

When cooling rate control is limited, e.g. larger sample size, freezing point depression is applied to assist in the control of osmotic contraction. In a larger sample size, e.g. tissues, where the osmotic equilibrium time is longer and the control of cooling rate are limited, freezing point depression (by adding a non-permeable cryoprotectant) is important for tissue survival by slowing dehydration and metabolism (Wolfe & Bryant, 2001) (*discussed in 2.2.2.*).

### 2.3.2. Thawing rate

Thawing rate can influence survival as much as cooling rate because thawing rate and effects of thawing often depend on cooling rate and events that occurred during cooling. If the cooling rate was too high, initiating some IIF, the surviving cells or tissue may be surrounded by clusters of smaller, thermodynamic unstable ice crystals with the potential to continue crystallization upon warming. This can induce the fatal damage, implicating thawing as a determining factor in the survival of the cells. But if the rate of cooling was optimal to allow sufficient dehydration and cell contraction and avoided IIF, the warming rate can vary depending on cell type, in some making no difference, in others rapid or slow thawing are critical. Empirical trials are still needed to develop methods (Woods *et al*., 2004).

### 2.3.3. Cryoprotectants

Glycerol was introduced as a permeable cryoprotective agent in the late 1940s and the discovery of other cryoprotectants quickly followed. DMSO is widely used because it permeates cell membranes rapidly (Woods *et al*., 2004).

Permeating cryoprotectants are usually permeable to both the membrane and water resulting in a continuous coupled flow between the intra- and extracellular solutions. This flow controls the cell volume during dehydration and the intracellular concentrations throughout the cryopreservation process, i.e. the cryoprotectant

addition, freezing, thawing and cryoprotectant removal steps. Cryoprotectants have dramatic osmotic effects on cells during their addition and removal, and the higher the concentration required for protection, the greater the risk of damage. To prevent IIF, a critical intracellular concentration of cryoprotectant must be met before the plunging step. The initial exposure to the high concentration causes extensive contraction because of severe dehydration followed by rehydration swelling when removed. This contraction followed by re-swelling can cause cell death (Woods *et al*., 2004). Permeating cryoprotectants are also toxic because being membrane-soluble means that they partition into the membrane, changing the structure, causing possible fatal damage. DMSO, for example, has been shown to reduce the lamellar repeat spacings in lipid bilayers, reducing the membrane thickness. This effect can protect the membrane from freezing damage. The critical nucleation area is larger than the space available in the lamellar spaces, thereby reducing the probability of ice nucleation (Wolfe & Bryant, 1999).

Macromolecules and inert polymers are extracellular solutes that act as non-permeating cryoprotectants. The macromolecules include protein, hydroxyethyl starch (HES), dextran and sugars. The polymers include Ficoll, Poly Vinyl Pyrrolidone (PVP) and Poly Vinyl Alcohol (PVA). Non-permeable cryoprotectants protect cells firstly, against fatal dehydration, and secondly, enhances the stability of cryoprotectant solutions in higher subzero temperatures (Shaw, 2000; Wolfe & Bryant, n.d.).

The addition of a non-permeable cryoprotectant to the extracellular medium increases the extracellular osmotic pressure, lowering extracellular water concentration. This leads to a moderate non-fatal osmotic contraction of cells in the initial dehydration process, in presence or absence of a permeable cryoprotectant. This ensures survival of the cell by avoidance of IIF during the initial cooling. The higher extracellular osmotic pressure cause the vitrification temperature to raise, in other words, vitrification is more likely at relatively higher temperatures (Wolfe & Bryant, n.d.). During the post-thaw cryoprotectant removal and rehydration process of cells, non-permeating cryoprotectants prevent excessive osmotic swelling by keeping the coupled flow moderate, protecting the plasma membrane against rupture (Woods *et al*., 2004). The details on the mechanisms of cryoprotection of these polymers are still unclear (Wolfe & Bryant, 2001).

Addition of a non-permeable cryoprotectant increases the temperature of the solution at which it will vitrify, or form a 'glass'. Therefore, the cryoprotectant solution would be in a stable glass phase at temperatures higher than -196ºC liquid nitrogen. This would protect the sample against IIF during cooling and especially thawing as well as during transfer to and from storage. In light of the possibility of pathogenic transmission when samples are stored in liquid nitrogen, addition of non-permeable cryoprotectants to cryoprotectant solutions, samples may be stored at -140ºC to -150ºC in the vapour phase of liquid nitrogen or ultra cold freezers. The composition of such cryoprotectants, however, still needs to be developed (Shaw, 2000).

Addition of viscous polymers also reduces the brittleness of frozen solutions and reduces ice crystal growth during thawing (Shaw, 2000).

A compromise must thus be made between toxic cellular effects, parameters of concentration, duration of exposure and temperature of exposure to types of cryoprotectants to meet the optimal intracellular concentration as close as possible to prevent IIF and structural damage as far as possible (Wolfe & Bryant, 2001).

Cryoprotectants are now being relied upon in most cryopreservation protocols and their use in different molar concentrations has added flexibility as well as complexity to cryopreservation methods, especially in the optimisation of these methods for specific cells and tissues (Woods *et al*., 2004).

### 2.3.4. Sample complexity

During cooling, optimal thermal and hydraulic equilibrium times varies greatly among cells of different type and size (Wolfe & Bryant, 1999).

For single cell suspensions, such as spermatozoa, oocytes and embryos, a description of the cell's membrane permeability, cell volume, osmotic tolerance limits, cold shock tolerance, hydraulic conductivity, cryoprotectant permeability and its affect can be compiled converging theoretical and empirical approaches. These methods of mathematical predictions can be used to develop optimal cryopreservation conditions, such as cooling rate and the transition from extracellular to intracellular ice, for single cell samples (Woods *et al*., 2004).

Thermodynamic and mechanical effects of freezing have mostly been conducted on model systems consisting of only several chemical components, which is known and controlled, necessary for theoretical analysis. Models of lamellar phase lipid

membranes or macromolecules in regular hexagonal arrays are not entirely predictable for biological tissues (Wolfe & Bryant, 1999). The challenge of cryopreserving biological tissues lies in the scaling of these theories and models up to the multi cellular level. Single cells can be considered two compartment systems, water moving in and out of the cell to the medium outside. As compartmentalization becomes more and more complex, to the order of tissues or organs, for dehydration to occur, water must move through many different cell layers before reaching the outside medium. This compartmentalization and the sheer size of the tissue, pieces or whole, can result in damaging chemical and thermodynamic gradients (Woods *et al*., 2004).

Cooling rate is greatly limited by the sample size because of heat conduction. Cell suspensions containing a population of cells in minimal media can be placed in containers with at least one very small dimension e.g. straws or cryo loops used for single oocyte or blastocyst vitrification (Wolfe & Bryant, 2001; Mukaida *et al.*, 2005). These tiny volumes allow rapid cooling and vitrification. Thus for high volume macroscopic tissues the addition of cryoprotectants, permeable and impermeable, is necessary to increase the solute viscosity sufficiently to allow vitrification (Wolfe & Bryant, 2001).

As the complexity of the sample increases, so does the difficulty to achieve optimal cooling to survivable vitrification. Thus most cryopreservation methods of tissues to date have resulted from empirical trials (Woods *et al*., 2004). Ovarian tissue is also heterogeneous where each of the cell types has its own optimal speed, making it difficult to select an appropriate cooling speed for the whole tissue (Lornage & Salle, 2007).

Models and theories could be combined to successfully cryopreserve and even though the laws of thermal physics and mechanics are difficult to apply quantitively to cells, they are not necessarily violated and qualitive behaviour could be similar (Wolfe & Bryant, 1999).

### 2.4. Seeding

Seeding is the initiation of nucleation and ice crystal growth during cryopreservation to reduce temperature fluctuations within the sample. Seeding is usually done at the equilibrium freezing temperature which may be different for each type of sample,

depending on volume, purity, solute concentration and type and cryoprotectant concentration, but is usually between -5ºC and -11ºC (Demirci *et al*., 2001).

Seeding can be done in several ways. Manual seeding is achieved by touching the container wall at the upper meniscus of the sample with swab or forceps cooled in liquid nitrogen (see *Fig. 1.7.*). Seeding is successful when a propagating ice crystal front can be observed (Freeze Control® User's Guide, CryoLogic, Victoria, Australia). Semiautomatic seeding is done by the freezer system used where a sudden increase in cooling rate at the seeding temperature is set in the cooling protocol (*see Fig. 1.8.*) (Demirci *et al*., 2001).


*Figure 1.7.* Illustration of manual seeding of cryo vials. The upper meniscus of the container wall is touched by the tip of forceps cooled in liquid nitrogen until ice crystal growth can be observed.

In a study on sheep ovarian tissue by Demirci *et al.* (2001), the seeding temperature of different concentrations of different cryoprotectants was determined. During a constant slow cooling rate freezing protocol of the samples, temperature fluctuations within the sample were recorded. Recorded temperatures showed a sudden spontaneous increase in temperature in the sample when nucleation initiated. This temperature increase at nucleation initiation is due to the release of negative calories when water changes from liquid to solid state as is called the latent heat of fusion (Wolfe & Bryant, 1999; Freeze Control® User's Guide, CryoLogic, Victoria, Australia).

The sample cooled quickly as energy is released during ice crystal growth, followed by a return of the sample temperature to protocol values as set in the freezing protocol and cooling continued without further temperature fluctuations (Demirci *et al*., 2001). The temperature at which the temperature spontaneously increased is the equilibrium freezing temperature and is also the seeding temperature of that sample. When ice nucleation is initiated by seeding, in the case of this study semiautomatic seeding, at the predetermined seeding temperature, temperature variations in the sample is controlled (Demirci *et al*., 2001) (see Fig.1.8).

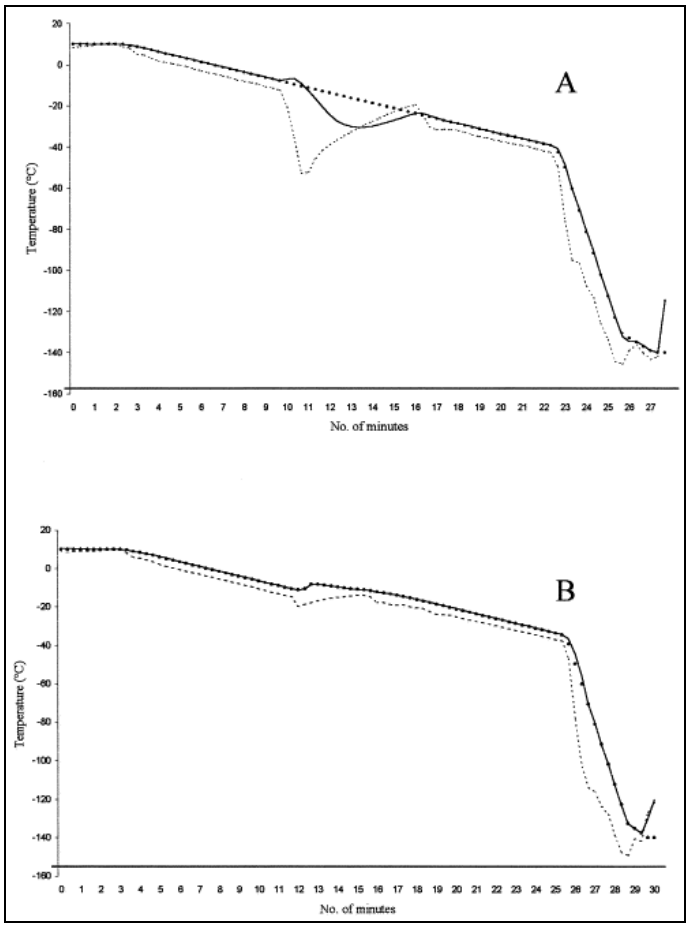



*Figure 1.8.* Seeding temperature determination. When nucleation starts, temperature in the sample fluctuates as heat of fusion is released, affecting the cooling protocol. A) During the freezing without seeding protocol, seeding temperature was determined for each freezing medium. As the cryoprotectant concentration increased, equilibrium freezing temperature decreased (freeze point depression): −7°C, −8°C, and −11°C with DMSO and −6°C, −8°C, and −11°C with PROH at concentrations of 1 M, 1.5 M, and 2 M, respectively. After a slight increase in temperature, the temperature of the medium decreased, then increased to reference levels and followed the 2°C/minute descent curve once again. B) During the freezing with seeding protocol, negative calories were released at the ice nucleation temperature, which was previously determined for each freezing medium. With seeding, the temperature variations in the medium were constantly controlled (Demirci *et al.*, 2001).

Samples that are manually seeded may be kept at the seeding temperature for five minutes to allow absorption of the heat of fusion, also reducing temperature fluctuations (Freeze Control® User's Guide, CryoLogic, Victoria, Australia).

# 3. OVARIAN FUNCTION AND/OR FERTILITY PRESERVATION OPTIONS FOR WOMEN WITH CANCER

## 3.1. The effects of cancer treatments on ovarian function and fertility

The long term survival rates and life expectancy of young female cancer patients, including women with squamous cervical carcinoma, have improved significantly. This is due to improved chemotherapy and/or radiotherapy treatments (Fabbri *et al.*, 2003) as well as early detection (Gook *et al.*, 1999; 2000; 2004a; 2004c). According to Kim (2006), the five-year relative survival rate of women, in all cancer types combined, has improved from 56% to 64% in the past 25 years, even though the number of young women diagnosed with cancer has increased. Some estimate that by the year 2010 about 1 in 250 people of reproductive age would be a childhood cancer survivor. Moreover, cancer is not rare in younger women and chemotherapy, radiotherapy and bone-marrow grafting are now curing over 80% of young female patients (Lornage & Salle, 2007).

The risk of ovarian failure does not depend on type of malignancy but rather on the therapeutic protocol used for each disease and according to initial response to treatment (Meirow, 2000).

Aggressive chemotherapy is cytotoxic and therefore also gonadotoxic, which may lead to permanent early infertility (Fabbri *et al.*, 2003; Gook *et al.*, 1999; 2000; 2004b; Oehninger, 2005). The ovaries are especially sensitive to cytotoxic treatments, leading to both endocrine and gametogenic function (Lornage & Salle, 2007). Ionizing radiation affects gonadal function at all ages. Ovaries are exposed to when total body irradiation is used to treat cervical cancer, rectal cancer, central nervous system cancer and haematological cancer, like Hodgkin's disease (Abdallah & Muasher, 2006).

The degree of ovarian damage depends on type of drug (alkylating agents, anti-metabolites and vinca alkaloids) and dosage used, length of treatment and the age of the patient (Fabbri *et al.*, 2003).

The gonadotoxicity of different chemotherapeutic agents vary. Alkylating agents such as cyclophosphamide, chlorambucil, melphelan, busulfan, nitrogen mustard and procarbazine are most toxic to the ovaries. Cisplatin and adriamycin are less toxic alkylating agents, while methotrezate, 5-fluorouracil, vincristine, bleomycin and actinomycin D have the least effects on ovarian follicle reserve (Akar & Oktay, 2005). Platinum compounds produce a similar but less intense result. Methotrexate, 5-fluorouracil, etopside and doxoruicin have a milder effect as they are cell-cycle specific (Abdallah & Muasher, 2006).

Follicular loss will increase as dosage increases. This also imply that, as the amount of chemotherapy courses increase, the cumulative dosage also increases and with each chemotherapy course, the residual ovarian reserve is smaller and consequently the ovarian reserve is lost quicker (Akar & Oktay, 2005; Kim, 2006). The age of a patient will therefore, also affect the time frame in which ovarian failure sets in. An older woman will show ovarian failure sooner than the younger patient only because of a lower initial ovarian reserve, while the younger patient will show delayed ovarian failure and infertility, hence early menopause (Akar & Oktay, 2005). A mathematical model showed that a 90% reduction in germ cell population by 14 years of age would result in permanent ovarian failure after 13 years (Kim, 2006).

Radiation particularly affects the less differentiated germinal cells causing potential irreversible germ cell depletion (Oehninger, 2005). Radiation therapy to the pelvis would lead to ovarian damage and/or failure without preventative intervention (Scott & Schlaff, 2005). Meirow (2000) showed that chemotherapy followed by bone marrow transplantation induced ovarian failure in 92% of patients (n=63). An epidemiological study by Stovall *et al*. (2004) of more than 25 000 childhood cancer patients, could detect, with a >90% power, a 1.3 fold risk of an adverse pregnancy outcome associated with radiation exposure to the gonads.

The degree of ovarian damage depends on the site and dosage of ionizing radiation (Oehninger, 2005; Fabbri *et al.*, 2003). Radiation also induces gonadal damage indirectly through oxygen radical formation which is cytotoxic to DNA (Akar & Oktay, 2005). According to Kim (2006), a dose of more than 300cGy delivered to the ovaries can result in loss of ovarian function. Patients with Hodgkin's disease generally receive 2000-4000cGy pelvic radiation which usually leads to premature ovarian failure.

According to Akar and Oktay (2005), a mere 20Gy can induce ovarian failure in woman under 40 years old and 6Gy in woman older than 40 years. Patients whose menstrual cycles continue after radiation still develop early menopause (Akar & Oktay, 2005). Long-term survivors of childhood, adolescent and adult cancers can present a range of ovarian failure, from complete and irreversible to partial and recoverable (Oehninger, 2005).

In addition to effects on ovarian reserve, uterine irradiation at an early age reduces uterine volume in adulthood (Donnez *et al.*, 2004). An exposure of the uterus to a mere 10Gy radiation could affect uterine vascular and muscular structures. This compromises uterine elasticity associated with higher rates of pregnancy loss and uterine factor infertility (Akar & Oktay, 2005).

The age at which cancer treatment starts, is a major risk factor in ovarian failure after cancer treatment since the lower follicular reserve in the older patient will be more quickly destroyed. A younger patient may recover ovarian function after cancer treatments because the follicular loss are less and the damage to the ovary concealed until ovarian failure sets in prematurely while still of supposed reproductive age. Ovarian failure was significantly increased when chemotherapy commenced at age of 34 years compared to 27 years Meirow (2000). Some female childhood cancer survivors, treated with the most common chemotherapeutic agents and/or radiotherapy, have, however, demonstrated no adverse pregnancy outcomes. The outcomes investigated included live births, still births, miscarriages, congenital anomalies, neonatal death and incidence of cancer (Oehninger, 2005). It was not reported whether these women conceived naturally or with assisted reproduction procedures.

In consequence, cancer treatments generally result in loss of both reproductive and endocrine function. Premature menopause due to ovarian failure has shown to increase the risk for osteoporosis and cardiovascular morbidity in young women (Gallagher, 2007; Imhof *et al.*, 2004; Vehmanen *et al*., 2001; Gorodeski, 1994).

## 3.2. Current strategies available to preserve ovarian function and/or fertility

The options currently in practice for reproductive protection for woman undergoing cancer treatment are few. These options also depend on the type of cancer therapy and the patient's needs, whether for fertility preservation and/or early menopause prevention. Other than oocyte donation and/or surrogacy, the most trusted options are oophoropexy (i.e. ovarian transposition), gonadal shielding, and embryo cryopreservation. New and/or experimental stage strategies include oocyte cryopreservation, ovarian tissue cryopreservation, whole ovary cryopreservation, hormonal protection with gonadotropin-releasing hormone (GnRH) analogues and pharmacological anti-apoptotic agents (Imhof *et al.*, 2004; Kim, 2006; Oehninger, 2005).

### 3.2.1. Oophoropexy

Oophoropexy, also called ovarian pinning (Stovall *et al.*, 2004), is the transposition of the ovaries to a site outside the radiation area and is usually done during laparotomy for staging for Hodgkin's disease or radical hysterectomy for cervical cancer (Abdallah & Muasher, 2006). It has been done for decades to preserve ovarian function for after radiation therapy and is considered relatively safe, simple and effective (Kim, 2006). Scott and Schlaff (2005) reported a case study of a 19-year-old patient who underwent a laparoscopic medial oophoropexy prior to radiation therapy. Both ovaries were repositioned behind the uterus. The patient regained ovarian function 22 months after completion of radiation therapy and conceived spontaneously. The study of Huang *et al*. (2007) investigated ovarian recovery after laparoscopic oophoropexy to above the umbilical line. Two of the 14 patients received only radiation therapy after oophoropexy. The 43-year-old patient showed ovarian failure during three years follow-up, while the 30-year-old patient showed normal ovarian function during 12 years follow-up after radiation therapy. Of the 12 patients who received additional chemotherapy, 50% recovered ovarian function. Five of the six patients were aged between 30 and 38 years compared to the ovarian failure group who were all aged 38 to 42 years. Age was therefore concluded to be the most important determining factor of success of this procedure.

The disadvantages of oophoropexy are, firstly, that it does not protect ovarian function of patients who are undergoing chemotherapy. Secondly, radiation is then usually delayed to allow recovery after the oophoropexy. Thirdly, the procedure is not always reliable since the ovaries could migrate back into original position during the recovery period. Furthermore, oophoropexy can carry the risk of injury to the vasculature and ovarian cyst formation (Kim, 2006; Scott & Schlaff, 2005, Abdallah & Muasher, 2006).

### 3.2.2. Gonadal shielding

Gonadal shielding is the physical protection of a certain body part/area with a lead shield from the harmful effects of radiation during radiotherapy. This procedure is not completely reliable and effective, since radiation leakages do occur. It is most effective when used in conjunction with oophoropexy (Husseinzadeh *et al*., 1984).

### 3.2.3. Embryo cryopreservation

The technique of embryo freezing is clinically well-established and requires an ovarian stimulation cycle, oocyte retrieval and fertilization via in vitro fertilization (IVF) or intra cytoplasmic sperm injection (ICSI) (Kim, 2006). Embryo cryopreservation can be successfully performed at different phases of embryo development, namely the pronuclear, cleaving and blastocyst stages (Oehninger, 2005; Kim, 2006). It allows the storage of surplus or all embryos for transfer at later stage. The number of embryos that are thawed and transferred can be regulated, allowing multiple transfers from one stimulated cycle. In addition, pre-implantation genetic diagnosis (PGD) of embryos can aid in the management of inherited genetic abnormalities (Oehninger, 2005). Reports show post-thaw survival rate of embryos ranges from 35% to 90%, implantation rates from 8% to 30%, cumulative pregnancy rates can be around 60% and the average delivery rate per embryo transfer around 27% (Abdallah & Muasher, 2006).

For embryo cryopreservation, however, cancer treatment need to be delayed for 2-5 weeks per cycle, or more, depending on the number of oocytes retrieved and number of good quality embryos achieved for cryopreservation. This delay may not always be practical for cancer patients.

Stimulation with gonadotropin may be contraindicative for patients with oestrogen-sensitive tumours, such as breast cancer (Kim, 2006; Abdallah & Muasher, 2006). Ovarian stimulation done in conjunction with an aromatase inhibitor, letrozole, with minimal gonadotropin has been successfully used to achieve embryo cryopreservation cycles for patients with oestrogen-sensitive tumours (Kim, 2006).

The fertilization of oocytes is usually not ideal for women without a partner and/or who does not want donor insemination (Kim, 2006; Abdallah & Muasher, 2006). Embryo storage may also raise ethical issues that may end in a court of law since embryos are co-owned by two people. Besides the possibility of separation, in the case of cancer patients where one partner face an uncertain future, the fertilized embryos may face an ethical battle (Kim, 2006; Tucker *et al.*, 2004).

### 3.2.4. Oocyte cryopreservation

Mature (i.e. Metaphase II) oocyte cryopreservation also requires an ovarian stimulation cycle where chemotherapy can be postponed with no contraindications (Akar & Oktay, 2005; Kim, 2006). However, in one induction cycle an average of ten oocytes may be harvested, which may not be sufficient in cancer cases (Lornage & Salle, 2007).

The freezing of mature oocytes is technically complicated by the fact that their structures are extremely sensitive to temperature changes, large cell volume and have limited capacity for repairing cytoplasmic damage (Kim, 2006) and the high degree of specialization, i.e. presence of zona pellucida, cortical granules and meiotic spindle (Abdallah & Muasher, 2006; Lornage & Salle, 2007). It has been shown that even minor changes in ambient temperature may damage the tubulin proteins in the cell spindle. The spindle is a dynamic structure composed of assembling microtubules and even though the chromosomes can reassemble and re-align along the spindle equator during thawing, some risk of chromosomal loss and aneuploidy remain. In addition, the cytoskeleton of mature oocytes is damaged by cryopreservation, leading to significant changes in the organization and trafficking of molecules and organelles. Some studies have also shown that cryopreservation of oocytes may result in hardening of the zona pellucida, if present, preventing natural fertilization (Oktay *et al.*, 2000).

The cryopreservation of oocytes is still under investigation because resulting survival and pregnancy rates reported are inconsistent. This may, in partial, be due to the radically different types of protocols used and persistent concerns that cryopreservation of mature oocytes may disrupt the meiotic spindle via depolymerisation, increasing the potential of aneuploidy and structural defects in embryos. A pregnancy with a woman's own cryopreserved oocytes was first reported in 1986. In 150 offspring so far worldwide, no major abnormalities have been reported (Tucker *et al.*, 2004; Kim, 2006). Lucena *et al.* (2006) has shown recently that 61% (97 viable of 159 thawed) of vitrified and thawed oocytes became viable embryos for transfer after ICSI. A pregnancy rate of 56,5% (13 pregnant of 23 patients) were achieved with an average embryo transfer rate of 4,63 (embryos per intrauterine embryo transfer). Selman *et al*. (2006) reported 58% (14 viable of 24 thawed) viable embryos for transfer after vitrification, thawing and ICSI. Two to three embryos were replaced per intrauterine transfer. Two of the six patients had resulting ongoing pregnancies after a single transfer. A recent report of Barritt *et al.* (2007) showed a unique case where three pregnancies were achieved out of four recipients after donor oocyte vitrification, but the number of embryos transferred per patient was very high, at 5.8±2.1 (Lornage & Salle, 2007).

A disadvantage of vitrification is that the method exposes the oocyte directly to liquid nitrogen and is stored under semi-open conditions where the samples can be contaminated with microorganisms or pathogens or other sample contents, for example, seminal fluid, which may contaminate the oocyte with sperm or bacteria. Aseptic systems and sealing kit have only been developed recently (Lornage & Salle, 2007; Kim, 2006).

In cases where ovarian stimulation is not suitable, for example, ovarian stimulation of pre-pubertal girls is considered unethical (Akar & Oktay, 2005; Kim, 2006), the cryopreservation of unstimulated immature oocytes offers a possible alternative. At the germinal vesicle stage, the meiotic spindle that is vulnerable to cryopreservation damage in the mature metaphase II stage is not yet formed and the chromosomes are protected in a nuclear membrane (Kim, 2006).

In a study by Russell *et al.* (2004) the 24 hour survival rate of three different cryopreservation protocols and cryoprotectants were compared for the cryopreservation of unstimulated immature oocytes. It was shown that vitrification with 40% ethylene glycol plus 1.0M sucrose were superior for survival of immature oocytes immediately post-thaw (56.7%, 51 of 90 oocytes frozen) and after 24 hours (57%, 29 of 51 oocytes survived). Ages of patients and pregnancy figures were not reported.

This option may cut down on administration of gonadotrophins, reduces risk of ovarian hyper stimulation, follicular monitoring and associated costs, but when the oocytes are needed for assisted reproduction, the immature oocytes requires additional in vitro maturation to reach metaphase II stage before fertilization is possible. This strategy may be costly and is also in an experimental stage and not yet reliable (Kim, 2006). The reason may be because cumulus cells have recently shown to be essential in *in vitro* maturation to produce developmentally viable oocytes, and the freezing techniques for oocytes with cumulus must still be investigated (Lornage & Salle, 2007).

### 3.2.5. Hormonal protection with gonadotropin-releasing hormone agonists (GnRH-a)

In early animal studies, a GnRH-a blocked the transition from primordial to intermediary follicles, suggesting that gonadotropins act on the transformation of flat granulosa cells into cuboidal granulosa cells, therefore play a role in the maturation process of folliculogenesis. The initiating signal, however, is still unknown (Gougeon, 1996).

When the maturation stage of the ovary was noted before the start of chemotherapy, prepubertal ovaries have shown to be more resistant to alkylating agents (Kim, 2006; Pacheco *et al.*, 2001). Animal and human studies followed, which focused on the possible protection of primordial follicles when the maturation cycle of oocytes is inhibited during chemotherapy. Immature follicle reserve would therefore be protected and not targeted by the alkylating agent, which targets all cells in mitosis and/or meiosis (Pacheco *et al.*, 2001).

The use of combined hormone contraceptives was the first to be tried and tested to induce anovulation. Some positive results were obtained, but follicular growth was only partially inhibited (Pacheco *et al.*, 2001). Most studies have not demonstrated any protective effects with oral contraceptives (Abdallah & Muasher, 2006).

A GnRH-a was introduced for administration parallel to chemotherapy (Blumenfeld, 2007). A matched control preliminary study by Pacheco *et al.* (2001), showed the potential protection offered with GnRH-a administration before, during and after chemotherapy. The first control group consisted of five prepubertal patients, ages three to seven years, undergoing chemotherapy without GnRH-a. In the follow-up until ages 12 to 18, three of the patients fell pregnant; this resulted in five healthy children. The second control group consisted of four postmenarchal patients, ages 16 to 20 years, with normal menses and ovulatory cycles prior to chemotherapy and no GnRH-a was included. All four patients presented secondary amenorrhea for the six years of follow-up and had to be treated with hormone therapy (HT). The study group consisted of 12 postmenarchal patients, ages 14 to 20 years, who received a GnRH-a (leuprolide acetate) prior to and during chemotherapy and was followed up for five years. All patients recovered menstrual cycles within 45 to 120 days of discontinuing the GnRH-a inhibition that was induced during and after chemotherapy for six to ten months. Two patients conceived spontaneously and the pregnancies resulted in three healthy children. Even though the sample groups were small the studies showed that GnRH-a may play an important role in protecting ovarian function.

A prospective nonrandomized study by Blumenfeld (2007) compared cancer patients, ages 15 to 40, receiving chemotherapy with (n=65) and without (n=46) additional GnRH-a. POF occurred in significantly less patients receiving GnRH-a administration compared to chemotherapy alone (3.1% vs. 37%, $P<0.01$). Cyclic ovarian function returned in significantly more patients receiving GnRH-a compared to patients without (96,9% vs. 63%, $P<0.01$). The average age of POF onset of the GnRH-a group were 36 compared to 26 years in the chemotherapy only group. Blumenfeld (2007) also suggested that inhibition of the pituitary-gonadal axis to reduce the rate of folliculogenesis might also render the germinal epithelium of the ovary less susceptible to gonadotoxicity.

The exact mechanism how GnRH-a may protect the follicular reserve is still unknown. The GnRH-a treatment is limited by the fact that it can only offer ovarian protection for chemotherapy patients and would not protect ovarian function from radiation therapy (Kim, 2006). GnRH-a protection may also be helpful where metastatic transfer risk is too high for ovarian tissue storage and transplantation (Blumenfeld, 2007).

### 3.2.6. Pharmacological agents

Medroxyprogesterone acetate is a pharmacological agent that has shown to protect the primordial follicles against acute injury from chemotherapeutic agents. The quality of the oocyte-follicle complex, however, may still be affected, resulting in atresia and a shortened fertility period (Abdallah & Muasher, 2006).

Chemotherapy has shown to act on primordial follicles through the induction of apoptotic changes in pregranulosa cells that lead to follicle loss (Meirow, 2000). The intrinsic program of apoptosis, or active cell death, possessed by all cells, including oocytes, can be activated by radiation and/or chemotherapy (Kim, 2006). The second type of promising protective pharmacological agents is apoptotic inhibitors (Abdallah & Muasher, 2006). Ceramide is the stress sensor that activates the apoptotic pathway while Sphingosine-1-phosphate (SIP) is the antagonist, deactivating apoptosis and promoting cell proliferation (Kim, 2006). These two molecules exist in a specific balance in cells controlling the cell's fate (Huwiler & Zangemeister-Wittke, 2007). SIP activation has shown to inhibit apoptosis *in vitro* culture and *in vivo* xenografted monkey ovarian cortical tissue (Lee *et al.*, 2005). Promoting SIP activation, may therefore, in theory, be a potentially promising method to preserve immature follicle reserve, but no human studies have been done to show its efficacy in conjunction with cancer treatments (Kim, 2006).

A recent study by Huwiler and Zangemeister-Wittke (2007) investigated the possibility of inducing ceramide production, thereby reducing cell proliferation and activating apoptosis, for use as a cancer treatment and not as a protective agent.

The inhibition of acid sphingomyelinase, another early messenger of apoptosis, has shown encouraging results but no human studies have been done (Abdallah & Muasher, 2006).

The disadvantage of administration of pharmacological agents may be that these molecules are not exclusive to the oocyte, are abundant in many other cells and thus the effects or treatment may not be specifically effective for ovarian protection.

# 4. OVARIAN TISSUE CRYOPRESERVATION AND TRANSPLANTATION

## 4.1. Advantages of ovarian tissue cryopreservation and transplantation

The main goal of ovarian tissue cryopreservation is to save the follicular reserve by means of storing primordial follicles. **Large numbers of resting oocytes in primordial follicles can be preserved in ovarian tissue** (Demirci *et al*., 2001), about 120 follicles in a 4mm ovarian fragment of a 30-year-old patient (Lornage & Salle, 2007). Schmidt *et al*. (2003) showed that 94% of the follicles in the ovary are at primordial stage. A single ovarian tissue graft has the potential to restore normal ovarian cyclicity and may remain functional for an extended period of time (Shaw, 2000).

**Primordial follicles have shown higher rates of survival after cryo-storage** at -196ºC than more mature secondary follicles (Newton *et al.*, 1996; Demirci *et al.*, 2001; Lornage & Salle, 2007). In 1956 oestrous cycles were restored after cryopreservation and transplantation of ovarian tissue in mice for the first time, even when less than 10% of the follicles were still present (Gook *et al.*, 2000). In 1994 Gosden *et al*. achieved the first mammalian birth in sheep following ovarian tissue cryopreservation and transplantation. Several rodent studies have since shown successful whole-organ and foetal ovarian tissue cryopreservation and transplantation, but human ovaries are much larger, more fibrous and primordial follicles are more widely dispersed than in either rodents or monkeys. A more comparable animal model for human ovarian tissue is that of sheep ovaries. The human and sheep ovary are more similar in size, also has a dense fibrous stroma and a relatively high primordial follicle density in the cortex (Newton *et al.*, 1996; Oktay *et al*, 2000; Demirci *et al.*, 2001).

**Primordial follicles can withstand cryo-toxicity and cryo-injury better than growing or mature follicles** since human primordial follicles have shown to survive the triple insult of freeze-thaw-ischeamia in long-term transplants (Oktay *et al*, 2000; Shaw, 2000). The primordial follicles may be more tolerant to the freeze-thaw process because they are consistently small in size (Newton *et al.*, 1996; Demirci *et al*., 2001; Lornage & Salle, 2007), metabolically less active, have no meiotic spindle that can be damaged and also no zona pellucida (Newton *et al.*, 1996; Hreinsson, 2003; Akar & Oktay, 2005).

Problems associated with water and cryoprotectant diffusion are reduced because the smaller cell size has a larger surface to volume ratio and the primordial oocyte is surrounded by only a single layer of follicular cells (Shaw, 2000). The absence of cortical granules and zona pellucida avoids the problem of zona hardening (Shaw, 2000) and facilitates cryoprotectant equilibration (Hreinsson, 2003). In the resting cell cycle stage primordial follicles avoid cryogenetic induced abnormalities or other problems associated with the chromosomes that are arranged on a temperature-sensitive metaphase spindle (Shaw, 2000). Resting follicles are also more likely to survive the ischeamic insult during cryopreservation and transplantation procedures before vascularisation are re-established (Hreinsson, 2003).

No differences in primordial and primary follicle survival were found after slow freezing of bovine ovarian tissue (Horizonte, 2005). A study by Wang *et al.* (2005) compared the cryo-survival of primordial follicles and primary follicles in cryopreserved human ovarian tissue. Histological examination showed that the percentage of morphologically normal primordial follicles did not differ significantly, but the percentage morphologically normal primary follicles were significantly less after cryopreservation compared to fresh control tissue (79.8% vs. 84%, $P>0.05$ and 57.9% vs. 84.6%, $P<0.05$, respectively).

**Ovarian tissue can be collected at any time from patients of any age** by laparoscopy or laparotomy **without delay or expensive hormonal treatments** (Shaw, 2000; Hovatta, 2003). Cryopreservation of ovarian tissue is therefore, especially advantageous for children and/or cancer patients who need immediate cancer therapy (Newton *et al.*, 1996; Gook *et al.*, 2000; Donnez *et al.*, 2004; Akar & Oktay, 2005; Kim, 2006; Abdallah & Muasher, 2006).

Furthermore, **an ovarian graft provides a source of natural sex steroids** in circumstances where HT would otherwise be required (Radford *et al.*, 2001).

Ovarian tissue cryopreservation **allows for adaptability** in endocrine and/fertility preservation treatment because it **can be used in conjunction with other established standard options**. For instance, embryo or oocyte storage could be done before ovarian tissue cryopreservation if circumstances are favourable. If only one ovary is retrieved for cryopreservation in a very young patient, GnRH-a therapy could also be administered during chemotherapy to enhance the chances of possible spontaneous recovery of ovarian function.

Another appealing advantage is that the cryopreserved ovarian tissue **may be used in several ways to restore fertility and/or endocrine function**. This is because the ovarian tissue pieces can be transplanted or grafted onto various sites (*discussed in 4.2.*) and on several occasions, since tissue samples are usually stored in more than one container. Thawed ovarian tissue samples can also be cultured *in vitro* or be used to isolate primordial follicles for *in vitro* maturation, followed by *in vitro* fertilization (ICSI) and embryo transfer (IVF-ET) when these procedures are standardized in future.

For cancer patients, ovarian cryopreservation and subsequent transplantation carries the risk of reintroducing undetected malignant cells to a cured cancer patient. It is therefore highly recommended to use the best possible methods of ovarian metastatic detection before transplantation (Meirow *et al.*, 2005a; Elizur *et al.*, 2004). Ovarian involvement in Wilm's tumour, Hodgkin's lymphoma, Non-Hodgkin's lymphoma, Ewing's sarcoma, uterine sarcoma and squamous cell carcinoma is rare and metastasis is a low risk in these patients (Akar & Oktay, 2005). Blood borne cancers, such as leukaemia, carry the highest risk of transmission since cancer cells are already in the bloodstream and blastic cells will be present in the cryopreserved specimen (Oktay & Buyuk, 2004). Theoretically, the ovarian tissue of leukaemia patients, whose metastasis risk is high, may be cryopreserved after initial chemotherapy when the patient enters remission but the safety of ovarian tissue cryopreservation and transplantation in such cases needs further studies (Oktay & Buyuk, 2004; Akar & Oktay, 2005). To our knowledge, cases of reintroduced cancer from ovarian transplantation have not yet been reported. By maturing the follicles *in vitro*, the risk of malignancy could be avoided. Isolated follicles or the cortex tissue may be cultured. The maturation procedure, however, is more difficult because of the longer time required for maturation of primordial follicles. The *in vitro* culture procedures are still being improved (Hovatta, 2005).

## 4.2. Reported results of human ovarian tissue cryopreservation and transplantation

Ovarian tissue cryopreservation and transplantation may restore 'natural' fertility if grafted orthotopically (Newton, 1998b). Ovarian tissue has been successfully transplanted as an **orthotopic autograft** where the ovarian tissue is transplanted into the original donor and grafted onto the original location or at close proximity to the natural location of the ovaries (Abdallah & Muasher, 2006).

Oktay and Karlikaya (2000) reported the first case of ovarian function restoration after orthotopic transplantation of human ovarian cortex. Radford *et al.* (2001) reported successful restoration of endocrine function in a 36-year-old patient with POF due to high-dose chemotherapy for Hodgkin's lymphoma. One ovary was retrieved for cortical tissue cryopreservation before chemotherapy commenced. Tissue was orthotopically transplanted to native ovary and to original retrieval site after 19 months of POF. Estradiol could be detected 7 months after the transplantation and FSH and LH levels were also decreased. The sex steroid concentrations, however, returned to previous POF levels after 2 months (9 months since transplantation).

Spontaneous fertility recovery has recently been reported after orthotopic autografting. Donnez *et al.* (2004) reported the first case live birth after spontaneous conception in a patient after chemotherapy for stage IV Hodgkin's disease and six years of premature ovarian failure (POF). Ovulatory cycles returned five months after the transplantation procedure and pregnancy were confirmed at 11 months by human chorionic gonadotropin concentration and ultrasound. The pregnancy resulted in the first live birth from an orthotopic autotransplantation of cryopreserved ovarian tissue.

Meirow *et al.* (2005a) reported the first live birth after ovarian tissue cryopreservation, orthotopic transplantation and subsequent IVF-ET in a case study of a patient with POF resulting from chemotherapy. A single mature ovum was retrieved at aspiration which resulted in one 4 cell embryo after IVF (not ICSI) on day 2 and was also transferred on day 2. In both these case reports, the possibility remains that the oocyte could have originated from the native non-cryopreserved ovary's tissue and thus these cases are controversial (Oktay & Oktem, 2005; Oktay & Tilly, 2004; Abdallah & Muasher, 2006).

**Heterotopic autografting** entails transplantation of ovarian tissue into the original

donor but grafted to a different site as the origin, for instance, subcutaneously above either the brachioradialis fascia of the forearm or the fascia of the rectus abdominus muscle. The forearm emerged as a suitable choice because of the simplicity of the grafting technique and ease of subsequent monitoring. The heterotopic approach would then obviously need assisted reproduction techniques if a pregnancy is desired. Oocyte retrieval can be percutaneously and therefore, does not require general anaesthesia and grafting is simplified compared to orthotopic transplantation. Subcutaneous and peritoneal sites do not seem optimal as differences in blood flow and temperature may affect oocyte quality. On the other hand, it can allow an increased number of grafts per transplantation in cases where the ovaries are removed or are atrophic rendering the ovarian volume too low (0.3 to1.3$cm^3$) to support sufficient tissue grafts for adequate hormonal production (Oktay *et al.*, 2004; Demeestere *et al.*, 2006).

Heterotopic transplantation has shown to restore ovarian function temporarily in POF women, with one pregnancy but no live births to date. Oktay *et al*. (2004) reported the first human heterotopic transplantation of cryopreserved ovarian cortex strips which was under the skin of the lower abdominal wall of a 30 year-old patient who had POF for 6 years since completion of chemotherapy for breast cancer. Ovarian function returned after 85 days and was confirmed by increased estradiol levels. In eight consecutive percutaneous oocyte retrievals, 8 of the 20 ova were suitable for IVF. Two oocytes were fertilized by ICSI after IVM and one developed into a high-grade four-cell stage embryo. Although no pregnancy resulted after intrauterine transfer, the study showed that fertility could possibly be restored by oocytes aspirated from the graft with minimal invasion followed by IVF-ET. With follow-up, the patient maintained restored ovarian function for 2 years and continued to develop multiple follicles in the graft (Oktay *et al.* 2004; Oktay & Buyuk, 2004; Oktay & Oktem, 2005; Akar & Oktay, 2005).

**Allogeneic transplantation** involves genetically different members of the same species which is potentially more difficult since the host may reject the graft and may thus need immunosuppressive therapy. Donnez *et al.* (2007) recently reported the first successful orthotopic allotransplantation of ovarian tissue between two genetically non-identical sisters. The healthy twin donated fresh ovarian tissue for her sister who had POF after total body irradiation before bone marrow

transplantation for cancer. The fresh ovarian tissue biopsies from the healthy sister were immediately sutured onto the POF sister's ovary (the two theatre procedures were synchronized). About two thirds of the ungrafted ovarian cortex of the POF sister was also biopsied during the transplantation procedure for histological examination of her follicular reserve. Her ovaries were atrophic and no follicles could be identified on the histological sections. Six months after the graft implantation, the first follicle appeared. The time was consistent with the time needed of primordial follicles to reach ovulatory stage. Twelve months after the transplantation, two mature oocytes could be retrieved for ICSI after a stimulation protocol. Two embryos developed 24 hours post-fertilization, a 2-cell and 3-cell embryo was observed, but no further development occurred. Even though the embryos were not transferred to the uterus due to developmental arrest, ovarian function continued and more stimulation cycles were planned. The study concluded that the functioning ovarian tissue were indeed the graft and not the native ovary. No cryopreservation was done in this study.

**Syngeneic transplantation** involves identical members of the same species (Abdallah & Muasher, 2006). Silber *et al.* (2005) reported the first such case in 24-year-old monozygotic twins. Ovarian tissue of a fertile monozygotic twin was transplanted orthotopically to the ovary of the infertile twin who experienced POF since the age of 14. Fertility was restored spontaneously after 3 months and natural conception was achieved in her second cycle. A live healthy female was born. No cryopreservation was done in this study.

**Xenografting** is tissue grafting across species. Xenografting is usually used for research purposes, for example, human cryopreserved tissue grafted into SCID-mice to evaluate tissue survival. Xenografting are also used to investigate different procedures, like sites of grafting, grafting methods and hormone function restoration before these procedures can be applied in humans (Demirci *et al*., 2001, Lornage & Salle, 2007). SCID-mice have a genetic mutation of T- and B-cell deficiency, resulting in an immunodeficiency that allows xenografts to revascularize and survive in these mice without being rejected. Return of estrogen production, fall of FSH levels and development of antral follicles from xenografting small ovarian cortex strips into oophorectomized SCID mice indicated success and showed that human primordial follicles survive the triple insult of freeze-thaw-ischemia in short- and long-

term transplants (Oktay *et al*., 2000). Ethical and moral constraints complicated xenografting and rendered the method unfeasible for human clinical application (Newton, 1998b).

An account of the surgical and laboratory procedures was given by Martin *et al.* (2007) on a combined approach for fertility preservation in a woman facing possible POF. Ovarian cryopreservation of one ovary and ovarian transposition of the other ovary was done before radiation therapy to maximise the patient's fertility options in the future.

Demeestere *et al.* (2006) has also reported a combined procedure of orthotopic and heterotopic transplantation of slow cryopreserved ovarian tissue in a patient previously treated with bone marrow transplantation for Hodgkin's disease. The patient showed follicular development at all graft sites and after 6 normal menstrual cycles, fell pregnant spontaneously but unfortunately ended in a miscarriage.

## 5. OVARIAN TISSUE CRYOPRESERVATION METHOD DEVELOPMENT

Successful cryopreservation of primordial follicles in ovarian cortex strips requires survival of the oocyte and granulosa cells and the maintenance of gap junctions for metabolic cooperation essential for oocyte growth and development (Demirci *et al*., 2001). Tissue cryopreservation methods require optimization of the cooling and warming rate including the type and concentrations of cryoprotective agents as freezing medium (Newton, 1998b). The main difficulty of whole tissue cryopreservation, however, is to optimize the procedure for each of the tissue components, follicles and vasculature (Akar & Oktay, 2005) since there is a marked difference in cell volume between the oocyte in the primordial follicle and the surrounding granulosa cells (Gook *et al*., 1999). The relative density of packing of cells in tissue and the size of pieces results in thermal gradients across cell which alters cooling rates for individual cells. Cellular density and tissue geometry also affect infiltration of cryoprotectant (Gook *et al*., 1999).

Every aspect of the cryopreservation protocol used for ovarian tissue can have a fatal effect on the physiology of follicles and surrounding supporting cells. It is essential that these follicles and cells survive and are healthy enough in order to reinstate endocrine function and/or fertility successfully in the future.

A summary of the most successful methods used in ovarian tissue cryopreservation is presented in Table 1.

### 5.1. Cooling rate

The cooling protocol is probably the most obvious area of major variation in cryopreservation protocols. Today, cooling rate can either be controlled within tenths of degrees per minute with software programs or, to the other extreme, can be thousands of degrees per second during instantaneous vitrification.

Ovarian tissue is heterogeneous, and each of the cell types has its own optimal cool rate, making it difficult to select an appropriate cooling rate for the whole tissue sample (Lornage & Salle, 2007). According to Gook *et al*. (1999), the granulosa cells and oocyte achieve better survival with a slow cooling rate, where the stromal cells have shown better morphology with an intermediate cooling rate.

The cooling rate for ovarian tissue was initially based on the standard slow cryopreservation protocol for embryos. Gosden *et al*. (1994) achieved the first successful mammal births in ewes with a slightly modified slow embryo-cooling protocol, which is still currently referred to as the 'standard' slow freezing protocol for ovarian tissue because this 'standard' protocol, and variations thereof, has shown the most successful results to date (*see Table 1.*).

Gook et al. (1999) compared different cooling rates in cryopreservation of human ovarian tissue strips. Ovarian biopsies were taken from nine woman between 17 and 40 years old. All groups in the study were cryopreserved in 1.5M PROH with 0.1M sucrose and 10mg/ml HSA cryoprotectant media. Slow cooling (i.e. step wise rate of 2ºC/minute to -8ºC, manual seeding, 0.3ºC/minute to -30ºC, 50ºC/minute to -150ºC and plunged into liquid nitrogen), intermediate cooling (i.e. vials are suspended over nitrogen vapour for >12h and plunged) and rapid cooling (i.e. direct plunging into liquid nitrogen or vitrification) were compared by histological evaluation of follicles. Outcome groups were percentage intact oocytes, granulosa cells and cortex. 'Normal oocytes' were defined as intact oocytes with ≤10% vacuolation. Results were significantly lower in all cooling protocols compared to non-frozen tissue and showed an increase in damage in all groups as cooling rate increased. Slow cooling achieved the best results with the highest proportion of intact normal oocytes (56%) with minimal cytoplasmic damage compared to non-frozen control (96%), intermediate cooling (21%) and rapid cooling (12%). Stromal cells in the cortex, however, showed better survival at the intermediate cooling rate (73%) compared to slow (45%) and rapid (25%) and fresh tissue (92%).

Further TEM investigation of mainly primordial follicles (90%) by Gook *et al*. (2000) showed that non-frozen oocytes have an even distribution of organelles with a few small vacuoles. In comparison, frozen tissue showed oocytes with large proportions of cytoplasmic areas devoid of organelles or consisted of multiple small vacuoles or vesicles. These empty areas could also be observed by light microscopy, eliminating the possibility of being TEM artefacts. In non-frozen tissue, the compact matrix of stroma cells and collagen bundles of the ovarian cortex could be observed and an estimated 92% of the cortex was cellular and contained collagen. In frozen tissue, this proportion was significantly reduced and TEM results showed that collagen bundles were disrupted and stroma cells were lysed. Although the basal lamina of

the oocytes remained complete in the majority of oocytes in slow, intermediate and rapid cooling, the cellular components of the follicle, pre-granulosa cells and oocyte showed a significant reduction in survival. Vacuolation of oocytes increased as cooling rate increased.

Using sheep cortex, Demirci *et al*. (2001) compared the 'standard' slow freezing protocol of Gosden *et al.* (1994) with a constant cooling rate protocol at 2ºC/minute (semiautomatic seeding at -8ºC) in sheep ovarian cortex strips in 1.5M DMSO. Isolated primordial follicle mortality were significantly less with the cooling rate of 2ºC/minute protocol (17.6% vs. 12.0%) but histological follicle morphology results were not shown. The effect of the different seeding protocols could have affected results. Lornage & Salle (2007) have also reported the use of a 2ºC/min cooling rate with 2M DMSO to cryopreserve 2mm$^2$ ovarian fragments in ewes. They achieved four gestations after an orthotopic graft and another four gestations the next year. The graft, however, was exhausted by the next year.

Gandolfi *et al*. (2006) showed that vitrification of small ovarian cortex pieces resulted in significantly lower follicle grading distributions of human primordial and primary follicles, irrespective of cryoprotectant used, compared to control tissue (3.1-17.6% vs. 45%; 0-18.8% vs. 26.1% 'normal' follicles, vitrified vs. control). They also concluded that vitrification caused extensive damage to the follicles and was less efficient than slow freezing for preservation of ovarian follicles.

Vitrification has shown some success with rodent studies, leading to faster rates being intensively investigated currently because of the time-saving advantage from several hours during slow freezing to only a few minutes for vitrification (Lornage & Salle, 2007).

Direct plunging into liquid nitrogen (vitrification) of ovarian tissue has consistently shown low survival results. Histological analysis has consistently shown significant reductions in primordial follicle numbers compared to fresh samples (12% vs. 96%; 25-<10% vs. 96%; <5% (no normal data)) after vitrification and that the slow rate of cooling achieved the highest proportion of oocytes with minimal cytoplasmic damage (Gook *et al*. 1999; 2000, 2004c).

Isachenko *et al.* (2003) showed that irrespective of freeze container or apparatus used, vitrification resulted in >90% atresia after 21 days of *in vitro* culture of isolated

follicles. The control non-frozen group, however, showed 82% atresia. Rahimi *et al.* (2004) also showed that vitrified ovarian tissue samples resulted in more necrotic areas compared the slow-cryopreserved tissue after xenotransplantation.

Lornage and Salle (2007) vitrified ovarian cortex fragments of ewes in a commercial vitrification solution and achieved three gestations and four births, but the graft lasted only one year.

### 5.2. Tissue size and cryo container geometry in slow cryopreservation

According to Newton *et al.* (1996), cryopreservation and revascularization should be equally effective in small and larger slices of tissue if the thickness is similar, i.e. 1-2mm.

Gook *et al*. (1999) suggested that the considerations important for successful organ cryopreservation also apply to tissue cryopreservation. One of these considerations is that the transfer of heat via convection during cooling is rapid in cell suspensions, but is impaired in tissue samples due to the dense packing of cells and the sizes of tissue slices result in thermal gradients across cells which alter the cooling rates for individual cells. Another consideration is that the cellular density and tissue geometry may affect the infiltration of cryoprotectant. Higher cryoprotectant concentrations could overcome some of the problems of infiltration in organ cryopreservation, but tissue studies showed that the aqueous phase within tissue samples is insufficient to balance the high cryoprotectant concentration gradients and cause osmotic shock to cells and disruption of cell-cell communication (Gook *et al*., 1999).

Radford *et al.* (2001) dissected an ovarian cortex of a 36-year-old cancer patient into seven large pieces, sized about 1x10x5 mm (depth x width x length), for slow cryopreservation before chemotherapy. Each piece was cryopreserved in a 1mℓ cryo vial. Two pieces were transplanted orthotopically, one piece into the native ovary and the other onto the excision site of the previously retrieved ovary. The grafts relieved POF and restored normal hormone levels after seven months, but lasted only two months. The study concluded that one of the possible factors influencing the longevity of the graft was the dimensions of the tissue. The size and especially the thickness is relevant when considering that the loss of all follicles, except the primordial follicles are due to hypoxic or ischeamic damage that occurs during the time of re-vascularisation, which is prolonged with increased tissue size.

Oktay (2001) suggested tissue sizes should range around 1-2x5x15mm because excessive damage can be induced during slicing of strips into too small pieces, which are also unmanageable in transplantation.

Meirow *et al*. (2005b) used 2mℓ cryo vials filled with 1.8mℓ cryoprotectant solution for cryopreservation of 1.5 x 0.5 cm cortex strips into the native ovary of a patient with a two year history of amenorrhea since her chemotherapy treatment for Hodgkin's lymphoma. Ovarian function returned spontaneously after eight months since orthotopic transplantation and a pregnancy was achieved with an IVF-ET cycle. The longevity of the graft was not reported.

Dittrich (2006) showed only 12.2% less intact follicles compared to follicles in fresh tissue with LIVE/DEAD fluorescent assays after slow tissue cryopreservation in 2mℓ cryovials containing small 1x1x1mm human ovarian cortex strips. It was also concluded that the use of cryovials provided more storage capacity that improved the routine use in cryopreservation of ovarian tissue with satisfactory results.

Demeestere *et al.* (2006) also achieved ovarian restoration and a pregnancy after cryopreservation of 2x5x5mm cortex pieces in 2mℓ cryo vials containing 1.4mℓ cryoprotectant medium with the 'standard' protocol for cryopreservation of Gosden *et al.* (1994).

Straws have been more successfully used in animal studies of ovarian tissue cryopreservation because ovaries are smaller and young animals have a larger ovarian reserve per cortex piece. In a study by Deng *et al.* (2007) 0.5ml plastic freezing straws were used as containers for small rabbit ovarian cortex pieces for slow cryopreservation in 1.5M DMSO supplemented with 36mg/l pyruvate. Their results showed no significant differences in percentage morphologically normal follicles between fresh and frozen-thawed tissue under light microscopy stained with HE (87.2% vs. 81.8%, respectively). No necrotic follicles were observed in either samples.

The cryopreservation and transplantation of the small tissue samples show some advantages over larger tissue pieces. Firstly, several small tissue pieces can be implanted at a time on several occasions. Secondly, the small size of the tissue pieces facilitates cryoprotectant penetration during cryopreservation which improves tissue viability through cryopreservation and thawing. Thirdly, the small pieces

increase the total area in contact with the surrounding blood supply after implantation, which improves re-vascularisation and thus reduce ischeamic damage. It may therefore, avoid necrosis of the whole transplant during transplantation.

Deng *et al.* (2007) also investigated transplantation success of rabbit ovarian cortex pieces, sized about 1.0mm$^3$ in, into different sites, namely the ovarian bursa, mesometrium and remaining ovary. A larger cortex piece, about a fifth of the whole rabbit ovary, was implanted in the mesometrium. In the small grafts, 78% of follicles were morphologically normal in all three sites. Only two small necrotic areas in the ovarian bursa and mesometrium graft sites were noticed histologically that failed to re-vascularize. The large graft, however, were atrophic and dead within one week after transplantation. This result supported the superiority of small tissue pieces for transplantation.

### 5.3. The permeable cryoprotectants and toxicity

A study by Demirci *et al.* (2001) of sheep ovarian cortex fragments cryopreserved without the supplementation of cryoprotectants showed complete structural disorganization on histological evaluation. Stromal cells were lysed, primordial follicles and connective tissue appeared cleaved, primordial follicles also showed irregular contours, with lysed oocytes and irregular chromatin distribution.

Cryopreservation, therefore, requires the permeation of the tissue sample with a cryoprotectant that moderates the normally lethal effect of IIF and the build up of extracellular solutes. The permeation of isolated cells with a cryoprotectant is relatively fast, but diffusion in multi cellular tissue systems is much slower. Exposure must therefore, be prolonged to reach adequate concentrations in the centre of the tissue, but may cause excessive toxicity to the outer cells. The equilibration time needs to be balanced between overexposure toxicity and sufficient permeation before and after cryopreservation as well as during warming and removal (Newton, 1998a). Equilibration of cells with cryoprotectant prior to cooling may be carried out at a low temperature to minimize damage. The more ideal cryoprotectants are those which penetrate cells rapidly to shorten exposure time (Newton, 1998b).

Incubation time, cryoprotectant concentration, type of cryoprotectant and incubation temperature may affect tissue and follicle survival during cryopreservation.

The first successful pregnancies and live births after ovarian tissue cryopreservation

and transplantation were achieved in mice using a 12% glycerol/saline solution as cryoprotectant and cooled to and stored at -79ºC in the 1950's (Gosden *et al.*, 1994; Newton, 1998b; Gook *et al.*, 2000; Hreinsson *et al.*, 2003).

Gosden *et al.* (1994) achieved the first live mammal births in ewes using 1.5M DMSO.

Newton *et al.* (1996) compared slow cryopreservation of human ovarian tissue using several cryoprotectants at 1.5M concentration, dimethyl sulfoxide (DMSO), ethylene glycol (EG), glycerol (GLY) and propanediol (PROH). The 'standard' slow freezing protocol was used but seeding took place at -9ºC. Tissue was xenografted in SCID-mice under the kidney capsule. Only 35% of the grafts cryopreserved in GLY contained at least one viable primordial follicles compared to the fresh (96%), DMSO (95%), EG (92%) and PROH (84%) grafts. GLY also resulted in significantly less follicle survival (10%) compared to DMSO (74%), EG (84%) and PROH (44%) when follicle numbers were standardized to fresh control tissue (100%). This may be due to the fact that glycerol is relatively more viscous than DMSO, EG and PROH reducing tissue permeation efficiency and therefore, offered lower protection against freezing injury. Glycerol showed better results when equilibration time was increased while the same trend was not seen with DMSO, EG or PROH (Newton, 1998).

Newton *et al.* (1998a) compared the permeation rates of DMSO, PROH, ethylene glycol (EG) and glycerol (GLY) at 4ºC and 37ºC at 5, 10, 20, 40 or 90 minutes by proton nuclear magnetic resonance analysis. Strips were uniform at 2m thick and 4mm wide square disks. Initial permeation rate was rapid followed by a second slower phase. At 4ºC DMSO and EG completed 70% of penetration within the first 10 minutes. At 30 minutes DMSO and EG reached 79% penetration and ±90% after 90 minutes. PROH and GLY had a significantly slower initial rate of permeation compared to DMSO and EG and reached 60% permeation after 10 minutes at 4ºC, 74% after 30 minutes and ±80% after 90 minutes. At 37ºC PROH showed a significantly faster initial permeation rate than at 4ºC and reached ±95% permeation in the first 10 minutes compared to DMSO, EG and GLY; which had no significant differences in rate of permeation. After 30 minutes, PROH had reached almost total equilibration of 99,9% while DMSO, EG and GLY had reached only 81%. At 90 minutes, PROH showed no further change in permeation and DMSO, EG and GLY reached ±84%. PROH, however, showed the most permeation variation between

equilibration runs. The study demonstrated that the temperature of equilibration mostly affect the permeation rate of PROH but not of DMSO, EG and GLY. DMSO and EG permeate human ovarian tissue more efficiently at 4ºC and PROH was more efficient at 37ºC and most of the equilibration takes place in the first 10-20 minutes after which penetration increases are insignificantly low.

Gutiérrez *et al.* (2000) compared DSMO and PROH at 1.5M concentrations for sheep ovarian tissue slow cryopreservation. Light microscopy observations indicated no significant changes in the cortex tissue after cryopreservation in either group compared to the fresh control tissue.

The same cryoprotectant permeation trend was seen in sheep ovarian tissue in a study by Demirci *et al*. (2001). At 37ºC DMSO and PROH penetrated the cells of sheep ovarian tissue at the same rate whereas DMSO penetrated the cells more rapidly than PROH at 4ºC. Cytotoxicity, however, was higher at 37ºC for both cryoprotectants. On histological examination, DMSO appeared to be more toxic even though cell permeation was the same as at 4ºC. According to the review by Wolfe and Bryant (1999), DMSO infiltrates the cell membrane at the lipid-solvent interface, reducing the lamellar repeat spacings, therefore reducing the lipid bilayer thickness. This may protect the cell by preventing nucleation in the membrane, but may interfere with metabolic functions due to alterations of membrane hydration forces. Metabolic rate and diffusion rate decreased as ambient temperature decreased and might be the reason for lower toxicity levels of DMSO during incubation at 4ºC compared to incubation at 37ºC. In comparison, the standard cryoprotectant incubation time of mouse ovarian tissue was five minutes at room temperature. When the incubation temperature was decreased to 0ºC, the incubation time was increased to 15 minutes with similar results. Incubation time for sheep and human ovarian grafts were longer because the tissue was more fibrous which reduces cryoprotectant infiltration (Demirci *et al*., 2001).

Microscopic evaluation of sheep ovarian tissue by Demirci *et al.* (2001) showed that cryoprotectant toxicity causes cytoplasmic vacuoles, pyknotic nuclei and shrinking of follicles due to severe dehydration. After equilibration with different cryoprotectant concentrations of 10M, 9M, 6.5M, 4M and 2M, results showed that the morphology of primordial follicles was better at concentrations lower than 6.5M in both DMSO and PROH. At 2M DMSO and 4M PROH the normal morphology results were lower, but

not significantly different compared to control tissue. Several control fragments also showed disrupted granulosa cells from thecal cells, cytoplasmic vacuolation and nucleic condensation, possibly as a result of ischemia. The morphological structure of ovarian fragments improved when cryoprotectants were added at low concentrations. At 1M DMSO the cytoplasm of primordial follicles was retracted and cytoplasmic micro vacuolation occurred, but the percentage of dead primordial follicles was significantly lower compared to 1M PROH. The significant difference between DMSO and PROH disappeared at 1.5M and the best histological characteristics and rates of follicle viability were obtained after freezing with 2M DMSO or PROH, where DMSO showed the lowest percentage follicle mortality (Demirci *et al.*,2001).

Gook *et al.* (1999 and 2000) compared different dehydration regimens with 1.5M PROH on human ovarian cortex slices after cryopreservation. Histological examination showed that the highest survival rates of cryopreserved primordial follicles were achieved with a single step of 90 minutes incubation. Results showed that 85% 'normal' oocytes vs. 96% in non-frozen tissue were achieved with 90 minutes compared to 44% and 50% 'normal' oocytes with 30 and 60 minutes, respectively. Shortcomings of the study may be that the incubation temperature was not specified and other cryoprotectants were not included. In addition, histological toxicity assessment directly after incubation was not done to control for cooling variables during freezing and thawing. In their follow-up study, Gook *et al.* (2004b) xenografted the same human ovarian strips into immunodeficient female oophorectomized mice (SCID). Antral follicle development was noticed after 20 weeks in 18 of the 24 sites of cryopreserved grafts and the antral development was similar in both cryopreserved and non-frozen tissue. This demonstrated preservation of cellular function with the 90 minute equilibration time protocol.

Gandolfi *et al.* (2006) compared 1.5M PROH and 1.5M DSMO as cryoprotectants in slow cryopreservation of human ovarian tissue. Results showed that both PROH and DMSO caused significant structural damage to primordial follicles compared to control, but did not differ compared to each other. DMSO seemed to cryopreserve primary follicles better since no significant difference was seen between DMSO and the control group (53.7% vs. 69.0%). PROH showed a significantly lower normal follicle grading distribution (16.2% vs. 69.0%). Too few secondary follicles were counted for accurate results (range of 1-4 follicles were counted).

Hovatta *et al.* (1996) compared DMSO and PROH at concentrations of 1.5M. Histological analysis under light microscopy showed 81% viable follicles after thawing in both groups. The fresh control group, however, showed atresia in 27% of the follicles.

Dittrich (2006) cryopreserved rat ovarian tissue pieces in a phosphate-buffered saline solution with 1.5M ethylene glycol and 0.1M sucrose using a slow freezing protocol. LIVE/DEAD fluorescent assay results showed only 12.2% less intact follicles (oocyte and granulosa cells alive) in the freeze-thawed group compared to fresh tissue. Histological slides of grafted cryopreserved and transplanted tissue showed follicles in all developmental stages. This demonstrated that the cryopreserved tissue also retained developmental potential. Actual data were not reported.

Several human studies have used 1.5M DMSO as cryoprotectant solution which showed superior results not yet achieved with other cryoprotectants (*see Table 1.*).

### 5.4. The non-permeable cryoprotectants

#### 5.4.1. Serum

Serum was probably the first non-permeable cryoprotectant used in ovarian tissue cryopreservation. The addition of serum to cryoprotectant media was initially recommended for the use of freezing mouse oocytes. This was mainly to prevent zona pellucida hardening, but is controversial in the case of early follicle freezing as the zona pellucida has not yet been formed. Serum and serum substitutes have long been used in varying concentrations in cryoprotectant media for ovarian tissue to reduce tissue damage in the course of cryopreservation and thawing procedures. The protein macromolecules in serum have been suggested of having protective properties (Hreinsson *et al*., 2003). One of the initial studies in the 1950's contained

88% horse serum where live born mice were achieved after cryopreservation and transplantation. In 1994, Gosden *et al*. achieved live births in sheep with 1.5M DMSO with 10% bovine calf serum supplementation. The use of 20% human serum with PROH-sucrose solution or DMSO has been reported by Hovatta *et al*. (1996) resulting in 81% histological viable follicles after thawing.

Bovine serum is now known to carry the risk of infection and is therefore not suitable for use in cryopreservation of human tissue (Hreinsson *et al.*, 2003). Serum supplementation may therefore cause possible side effects of antigenicity of the tissue at the time of transplantation. It has been suggested that either a protein should be used that will not initiate an antigenic response or no protein should be added to the cryoprotectant solution to avoid these side effects (Shaw, 2003b).

Serum solutions are not subjected to quality control procedures as commercially available media and some effects on human cortical tissue and follicles in vitro have not been established. The effect of commercially available human serum albumin (HSA) in cryoprotectant media for ovarian tissue was recently compared to human serum in a study by Hreinsson *et al.* (2003). An established protocol was used with PROH 1.5mg/ml and 0.1mol/l sucrose cryo-solution supplemented with either 25mg/mℓ commercial HSA or 20%v/v heat inactivated human serum collected from women receiving fertility treatment at their fertility unit. After light microscopy, transmission electron microscopy (TEM) and live/dead assay analysis on control and thawed human ovarian tissue samples, no significant differences on viability of follicles between these two serum supplements were found. The study did not include a control group with no serum or protein supplementation. Gook et al (1999) supplemented cryomedia with 10mg/mℓ commercial human serum albumin (HSA), achieving up to 85% normal cells/oocytes with a PROH-sucrose solution.

According to Shaw, (2003b), the addition of macromolecular substitutes for protein, like synthetic serum, have not shown to have any significant additional protective effects if another macromolecule, like sucrose, has already been added to the cryoprotectant medium. Meirow *et al.* (2007), however, reported supplementation of their cryopreservation media of 1.5M DMSO and 0.1M sucrose with 15% commercial synthetic serum. A pregnancy and live birth was achieved in this case of slow ovarian tissue cryopreservation and orthotopic transplantation after 2 years of POF due to chemotherapy.

According to Oktay (2001) primordial follicles have a higher tolerance for hypoxia when kept in serum supplemented media, therefore, delays during transport form the theatre to the laboratory for cryopreservation can be as long as 4-6 hours without having a significant effect on follicle survival.

### 5.4.2. Sucrose

The beneficial effect of adding a non-permeable sugar such as sucrose, has been shown in single cells and embryos. The mechanism suggested is that it acts as a osmotic buffer against dehydration and rehydration stress incurred by the cells during addition and removal of permeable cryoprotectants (Newton *et al.*, 1998a).

Newton *et al.* (1998a) compared human ovarian tissue survival with lactate dehydrogenase (LDH) assays after cryopreservation in 1.5M DMSO supplemented with different concentrations of sucrose, 0M, 0.05M, 0.1M, 0.25M, and compared to medium with no added cryoprotectant and fresh tissue. Fresh control tissue released the least amount of LDH (2.1IU/g) and the tissue cryopreserved in medium with no cryoprotectant released significantly more than all other groups at 20.8IU/g. Increased sucrose concentration supplementation to 1.5M DMSO from 0M to 0.05M and to 0.1M reduced LDH release but supplementation with 0.25M sucrose showed an increase in LDH release. Although differences in results of sucrose supplemented groups were not significant, the sucrose concentration of 0.1M showed the lowest LDH release.

Gook *et al.* (2000) compared the effect of different sucrose concentrations in different equilibration regimens with 1.5M PROH on human ovarian tissue strips before slow rate cryopreservation. In both dehydration times of 30 minutes and 60 minutes, the standard sucrose concentration of 0.1M resulted in higher oocyte survival in both primordial and primary follicles compared to 0.2M sucrose concentration (56% and 76% vs. 31% and 44%) after histological evaluation. Significance was not calculated or given.

Sucrose supplementation at a 0.1M concentration to cryoprotectant mediums is well-known and has been used in most leading studies (*see Table 1.*).

### 5.5. Seeding

Temperature fluctuations in slow cryopreservation protocols without seeding result in lower primordial follicle survival outcomes in both DMSO and PROH samples when compared to a similar cooling protocol with semi automated seeding where temperature variations are minimal. Cytoplasmic micro vacuolization in primordial follicles and subsequent percentage primordial follicle mortality were decreased in tissue samples that were semi automatically seeded than compared to no seeding during slow cryopreservation (Demirci *et al*., 2001).

### 5.6. Thawing rate

No studies have been done to date to specifically investigate the optimal thawing rate for ovarian tissue cryopreservation. Theoretically, the thawing rate should be as quickly as possible to reduce recurring ice crystallization that may have been initiated during the cooling phase.

The thawing method mostly used in ovarian cryopreservation is the ‘rapid’ thaw method and is not controlled like the cooling rate in a programmable freezing machine. In the rapid thaw method vials are taken out of storage and kept at room temperature (in air) for several seconds to allow the evaporation of excess liquid nitrogen from inside the vials. The vials are then submerged and gently swirled in a water bath at 35-37°C to thaw the tissue samples as quickly as possible, usually within ±3 minutes, until the whole sample is completely thawed (*see Table 1*).

The handling of the vials during thawing should be gentle. Harsh shaking and striking of the vials may cause additional mechanical damage in the cells resulting from vibrations of the ice crystals. The temperature is kept at the physiological body temperature to avoid heat damage. The swirling action maintains a heat gradient across the outside barrier of the vial that allows a more rapid heat transfer to the vials and improves the thawing rate.

The thawed tissue is then transferred from the vials to thawing medium prepared to remove the cryoprotectant from the tissue and rehydrate the tissue cells. This can be done in a stepwise fashion where the tissue is transferred through a series of different thawing mediums each containing a lower cryoprotectant concentration until the cryoprotectant is removed. Alternatively, the tissue is washed several times in a thawing medium with no added cryoprotectant (*see Table 1*).

*Table 1.* Summary of cryopreservation methods used by studies achieving the most successful results after cryopreservation of ovarian tissue.

| Reference | Study Sample | Cryo-preservation Technique | CPA & Base solution | Non-Permeable CPA | Specimen Dimension | Equilibration Time & Temperature | Cooling rate (ºC/min) | Seeding Method & Temperature | Thawing & Wash out | Evaluation Method | Result |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Gosden *et al*., 1994 | Sheep | Slow rate, controlled | 1.5M DMSO in Leibowitz-15 | 0.1M sucrose 10% calf serum | Tissue: 5-15x5mm Vials: 2mℓ | 15min, on ice | 2 to -7, 0.3 to -40, 10 to 140, plunged | Manual, -7ºC, hold 10min after | In air, RT water bath, 10min in Leibowitz-15 | Orthotopic graft | Live births |
| Oktay *et al*., 2000 | Case study: POF patient | Slow rate, controlled | 1.5M DMSO in D-PBS | 0.1M sucrose 20% serum | Tissue: 1-3x3-10mm | 30min 4ºC | From 0, 2 to -7, 0.3 to -40, 10 to -140, plunged | Manual, -7ºC, hold 10min before | In air 30s, 37ºC water bath, Stepwise 4x5min | Orthotopic graft | Ovarian function, ovulation |
| Radford *et al.*, 2001 | Case study: POF cancer patient | Slow rate, controlled | 1.5M DMSO in Leibowitz-15 | 2.5% HSA | Tissue: 1x5x10mm Vials: 1mℓ | 30min, 4ºC | From 0ºC, 2 to -9, 0.3 to -40, 10 to -140, plunged | Manual, -9ºC | In air 30s, 1min 37ºC water bath, 3x 5min in Leibowitz-15 | Orthotopic graft | Ovarian function for 2 months |
| Oktay *et al.*, 2004 | Case study: POF cancer patient | Slow rate, controlled | 1.5M DMSO in D-PBS | 0.1M sucrose 20% serum | Tissue: 1-3x3-10mm | 30min 4ºC | From 0, 2 to -7, 0.3 to -40, 10 to -140, plunged | Manual, -7ºC, hold 10min before | In air 30s, 37ºC water bath, Stepwise ↓CPA 4x 5min | Heterotopic graft | Ovary function, ICSI-ET, No pregnancy |
| Donnez *et al.*, 2004 | Case study: POF cancer patient | Slow rate, controlled | 1.5M DMSO in Leibowitz-15 | 4mg/mℓ HSA | Tissue: 2x2mm Vials: 2mℓ | ? | From 0, 2 to -8, 0.3 to -40, 30 to -150, plunged | Manual, -8ºC | In air 2min, 2min 37ºC water bath, 3x in Leibowitz-15 | Orthotopic graft | Ovary function, Spontaneous Live birth |
| Meirow *et al.*, 2005; 2007 | Case study: POF cancer patient | Slow rate, controlled | 1.5M DMSO in D-PBS | 15% synthetic serum | Tissue: 1-2x5x5mm, Vials: 2mℓ | 30min | 1 to -9, 0.3 to -36, 5 to -140, plunged | Manual, -9ºC | In air 30s, 2min 37ºC water bath, Stepwise ↓CPA 4x 5min | Orthotopic graft | Ovary function, IVF-ET, Live birth |
| Oktay & Oktem, 2005 | 2-year-Follow-up of patient in 2004 | Slow rate, controlled | 1.5M DMSO in D-PBS | 0.1M sucrose 20% serum | Tissue: 1-3x3-10mm | 30min 4ºC | From 0, 2 to -7, 0.3 to -40 10 to -140, plunged | Manual, -7ºC, hold 10min before | In air 30s, 37ºC water bath, Stepwise ↓CPA 4x 5min | Heterotopic graft | Ovary function, Spontaneous pregnancy |
| Demeestere *et al.* 2006 | Case study: POF cancer patient | Slow rate, controlled | 1.5M DMSO in D-PBS | 0.1M sucrose 10% patient's serum | Tissue: 2x5x5mm, Vials: 2mℓ | 30min, 4ºC | From 4ºC, 2 to -7, 0.3 to -40 -10 to 140, plunged | Manual, -7ºC, hold 10min before | In air 2min, 2min 25ºC water bath, Stepwise ↓CPA 4x 5min | Orthotopic and Heterotopic graft | Ovary function, Spontaneous pregnancy |

## 5.7. Evaluation methods of ovarian tissue and primordial follicles

### 5.7.1. LIVE/DEAD assays

Gook *et al*. (2004c) determined with titration studies that follicles could be isolated from ovarian tissue pieces using collagenase type IV at 500U/ml together with deoxyribonuclease at 5U/ml. During cryopreservation, however, collagen bundles within the cortex are disrupted and these enzyme concentrations need to be adjusted to maintain follicle health because inappropriate concentrations of enzymes can also result in inadequate numbers of follicles needed for viability assessments. Besides follicles, naked oocytes were also observed following enzyme digestion, suggesting that enzyme digestion can induce follicle damage, irrespective of whether tissue was cryopreserved or not. Using viability staining, Oktay *et al.* (2000) found that only about 70% of follicles survive the isolation process. Electron microscopic studies showed the digestion process rarely affects the oocyte but may damage the basement membrane (Oktay *et al*., 2000).

It may, therefore, be difficult to ascertain whether follicle damage is due to the cryopreservation and/or follicle isolation process in LIVE/DEAD assays if proper control groups are absent. After LIVE/DEAD assays, isolated follicles cannot be used for further physiological evaluations or treatments.

### 5.7.2. Histological evaluation

Histological examination can establish the numbers of follicles that show normal or abnormal morphology, but lack indications of viability and developmental competence of the follicles examined (Gook *et al.*, 2004b). Long-term survival *in vivo* can therefore not be predicted (Newton, 1998).

Macroscopic histological evaluation (HE slide) of ovarian cortex tissue under light microscopy is a well-known method for the evaluation of follicular structures. Normality of follicles were either scored, classified or graded according to morphological appearance. Normal or degenerate criteria are usually broadly defined. The resulting proportions or percentages of counted follicles are reported.

Gandolfi *et al*. (2006) compared the follicular normality of animal and human models in slow cooling and vitrification of ovarian tissue on HE slides using a grading system. Grade 1 follicle criteria were a spherical oocyte and evenly distributed granulosa

cells, grade 2 follicle criteria were a spherical oocyte and granulosa cells pulled away from the oocyte and grade 3 criteria were granulosa cells with pyknotic nuclei of and a misshapen oocyte with or without vacuolization. Percentages of follicles counted in each criteria of different groups were compared and reported.

The macroscopic criteria used by Lucci *et al*. (2004) to classify bovine preantral follicles as degenerative indicated one or more of the following aspects: condensed oocyte nucleus, shrunken oocyte, non-homogeneous ooplasm, pyknotic bodies in the granulosa cell and low cellular density. Follicles excluded by these criteria were accepted as normal follicles. Rodrigues *et al*. (2004) used other criteria for macroscopic evaluation of caprine preantral follicles. The three specified classifications where the follicles with intact oocyte and granulosa cells without pyknotic nuclei were considered normal. Follicles with pyknosis in the oocyte were considered as a separate degenerative class than follicles with oocyte pyknosis and oocyte shrinkage with or without detachment of granulosa cells from the basal lamina.

Gook *et al*. (1999, 2000, 2004c) used the following criteria to evaluate human primordial and primary follicles under light microscopy. Intact or normal follicles were defined as having a complete basal lamina, intact granulosa cells, circular oocyte with complete cytoplasm and membrane as well as ≤10% vacuolization. Degenerate follicles were defined as having no membrane, irregular and small area of cytoplasm retracted around the germinal vesicle and empty spaces between the oocyte and granulosa layer. Results were reported as percentages of total counted follicles.

Foetal ovarian tissue has shown variable follicular densities within different tissue sections, which mean that the number of follicles and also proportions of viable and atretic follicles, are not evenly distributed across the cortex tissue. These uneven proportions may influence results when follicles are counted macroscopically on a few tissue sections (Sadeu *et al*. 2006).

In a case study of an orthotopic allogeneic transplantation by Donnez *et al.* (2007) the ovarian reserve of the 33-year-old donor sister was shown to be sub-optimal since only two primordial and one primary follicle were observed in 11 sections of a biopsy taken for transplantation. Even though this indicated a very low ovarian reserve, the recipient sister recovered ovarian function and responded to several stimulation cycles with further follicle development. This supported the possibility that primary

ovarian follicles are located in clusters and thus a small fragment may not be a true representation of the whole ovarian reserve in the ovary which would histologically show a normal ovarian reserve

Considering the fact that the follicular density physiologically decreases in parallel with increase in age, the limited amount of follicles available and the heterogeneous distribution of follicles through the cortex may obscure histological evaluation results when investigating adult ovarian tissue (Sadeu *et al.*, 2006). According to *Gook et al.* (1999) the expression of follicle survival as follicle numbers in frozen/thawed tissue pieces relative to numbers in fresh pieces do not account for the wide intra- and inter-patient variation in follicle numbers per piece. Instead, their findings were expressed as proportions of intact follicles, pre-granulosa cells and oocytes versus proportions in fresh control tissue. The same histological criteria for follicle survival and/normality must be used if results of different studies are to be compared.

Transmission electron microscopy (TEM) as an evaluation method for primordial follicle survival has been done in only a few studies. It is usually done on a much lower scale, i.e. only selected tissue samples and/or few selected follicles, compared to light microscopy and mostly serve as a supportive, descriptive section. In these studies the light microscopy follicle counts were reported as the main results since hundreds of follicles can be assessed on fewer slides compared to the small grids used for electron microscopy which contain only a few.

Gook *et al*. (1999) included electron microscopy of some follicles in the non-frozen, and follicles of frozen samples for comparison of different cooling rates and dehydration regimens. 'Normal' primordial follicles (≤10% vacuolization on HE slides) in non-frozen tissue showed even distribution of organelles with a few small vacuoles in the oocytes. The follicles in the frozen tissue showed a large portion of the cytoplasmic area devoid of organelles or consisted of multiple small vacuoles/vesicles. These empty areas were not membrane bound and did not appear to be an electron microscopy artefact, since the areas were also seen on light microscopy slides and were easily be mistaken for vacuoles. Non-frozen follicles also showed dark, round or oval mitochondria with concentric cristae in abundance close to the germinal vesicle. Mitochondria of the oocytes from the slow cooled, one step dehydration method, were absent and the germinal vesicle was surrounded by an

electron dense cytoplasm with many small, relatively empty vesicles. Intermediate cooling resulted in large areas devoid of organelles in the oocyte cytoplasm. No mitochondria were present, only vesicles of similar size and shape to mitochondria but with no structure and reduced electron density could be seen. The oocyte and pre-granulosa cells were also absent. Oocytes cooled by the rapid rate were too severely damaged and excluded from electron microscopy evaluations. Oocytes of the two-step dehydration and slow cooling protocol showed general loss of cellular and matrix structure, lysis of stromal cells with only nuclei remaining. In the follow-up study, Gook *et al*. (2000) suggested that the major form of damage within the oocyte following cryopreservation is an increase in vacuolation which seemed concomitant with damage to the mitochondria. Cortex tissue in the intermediate cooling regimen showed the best morphology but higher magnification revealed loss of the three dimensional structure of the collagen bundles and presumed stromal cells were actually cell remnants. In both these studies, only PROH were used as cryoprotectant and therefore the effects on the ultrastructures may be limited to PROH.

The electron microscopy of bovine preantral follicles in ovarian tissue pieces cryopreserved in different cryoprotectants at different concentrations (Lucci *et al*., 2004) showed that the follicles of the fresh tissue showed oocytes with a large nucleus enclosed by a defined nuclear envelope. The mitochondria were numerous with smooth endoplasmic reticulum, Golgi apparatus and vesicles evenly distributed around the nucleus in a homogeneous cytoplasm. Granulosa cells were tightly connected to the oocyte and showed abundant elongated mitochondria, smooth and rough endoplasmic reticulum and Golgi apparatus. Follicles cryopreserved with 1.5M DMSO showed ultrastructures similar to fresh follicles, but 3M DMSO concentration and both 1.5M and 3M PROH caused loss of granulosa cytoplasm content. Cryopreservation with 10% GLY caused damage to the organelles and organization of the cytoplasm structure of granulosa cells and the oocyte. The organelles in the oocyte were grouped in small areas and most mitochondria lost their cristae and showed a granulated matrix. The ooplasm had abnormal appearance with clusters of cytoplasmic material and large empty spaces. Vacuolization of the oocyte and granulosa cell cytoplasm was common. A study by Rodrigues *et al*. (2004) used the same ovarian tissue cryopreservation protocol, same TEM morphological criteria and

same TEM evaluator, but investigated caprine preantral follicles. Results were very similar to that of the bovine tissue. Follicles frozen in 1.5M DMSO showed ultrastructural characteristics similar to fresh follicles, with only small amounts of empty areas. Again, the 3M DMSO, 1.5M PROH and 3M PROH showed more abnormalities. Follicles were without granulosa cell contents and had greater amounts of empty spaces. These abnormalities were found to be attenuated in 1.5M PROH. These results confirmed the results found by Gook *et al*. (1999, 2000) with the use of 1.5M PROH.

Comparative studies using electron microscopy and evaluating the effect of different cryoprotectants on the ultrastructures have not been done as yet. The extent of damage induced on different ultrastructures has not been reported in human ovarian tissue. It still needs to be elucidated if the amount of damage may be tolerated by the follicle or the ultrastructures before degeneration is caused and which ultrastructures are essential for survival or recovery of the follicle after the cryopreservation and/or ischeamic insult.

### 5.7.3. *In vitro* culture

*In vitro* culture of ovarian tissue or isolated follicles may indicate the viability and ability of a follicle to initiate further development following cryopreservation. Currently, however, difficulties are associated with the maintenance of structural integrity of primordial follicles in extended culture because of the lack of knowledge of triggers required for follicles to initiate growth (Gook *et al.*, 2004c). Neither enzymatic nor mechanical partial isolation appears to improve the survival or growth of the follicles. Isolation caused more atretic follicles in culture compared to intact tissue, suggesting the survival of resting follicle growth require ovarian tissue integrity (Hovatta *et al*., 2004).

Isolated primordial follicles were cultured for 14 days on a synthetic extracellular matrix containing 10% foetal calf serum and 100mIU/ml FSH. The culture system supported attachment and cellular integrity only to a certain extent since 58% of the follicles extruded their oocyte during days 3-14 in culture (Gook *et al.*, 2004c).

Sadeu *et al.* (2006) slow cryopreserved, rapid thawed and cultured human foetal ovarian tissue pieces for 63 days. At day 0 in culture the proportions of primordial, early primary, primary and early secondary follicles were 77.5%, 21.7% 0.8% and

0%, respectively. Morphological signs of atresia were shown in 18% of all the follicles. Tissue pieces cultured for 7-14 and 21-35 days showed similar proportions of follicles as day 0, except atresia were counted as 36% of follicles on days 7-14 and <15% on days 21-35. After 63 days, however, average proportions were very different compared to day 0, at 19.7%, 68.9%, 8.2% and 3.3%, respectively, with <20% atretic follicles. Significance was not given and the amount of follicles decreased with increased days of culture, from 1168 on day 0 to 76 at 63 days. The results did show that the increase in the proportion growing follicles was accompanied by a decrease in primordial follicles, where the majority was at early primary stage. Follicular growth was assessed by measurements of follicular and oocyte diameters of growing follicles. Primordial follicles and early primary follicles showed a significant increase in oocyte and follicle diameter after culture for 7-14, 21-35, and 63 compared to each other and the control day 0 ($P$<0.001). The main findings of the study were that follicles were viable after 63 days in culture and frozen-thawed primordial follicles initiated growth and were grown to early secondary stage. The in *vitro* culture conditions, however, caused massive follicular activation of primordial follicles from the resting pool after 2 days. One reason is hypothesised to be the absence of medullary factors, suggested to regulate activation and growth *in vivo*, since the medulla is removed from the cortex before cryopreservation. The other hypothetical reason given was that the growth factors supplemented in culture medium caused *in vitro* conditions to be richer than *in vivo*. Culture conditions to date supports only limited follicle growth of ovarian tissue.

Demeestere *et al.* (2006) used *in vitro* culture of 1x1x3mm cryopreserved cortex pieces to assess the follicular viability of thawed tissue. Increased concentrations of estradiol were measured in the culture medium after 15, 25 and 35 days confirmed viability and development of the follicles estimated at a density of 12 follicles per $mm^2$. Actual data was not given.

### 5.7.4. Grafting

Grafting is a more physiological approach to assess follicular competence after cryopreservation but depend on successful grafting techniques (Gook *et al.*, 2004b) which could affect results negatively.

Xenografting of human ovarian pieces under the kidney capsule of immunodeficient female oophorectomized mice (SCID) showed growth of antral follicles on both non-frozen and frozen tissue grafts, irrespective of exogenous administration of gonadotrophins. Preservation of follicular function were also histologically evident as these antral follicles underwent periovulatory changes, i.e. luteinized cells, mucification of cumulus matrix and germinal vesicle breakdown, after administration of human chorionic gonadotropin (hCG). In these studies, xenografting were suggested to be more reliable compared to *in vitro* culture of both tissue and isolated follicles (Gook *et al.*, 2004b, 2004c).

The technology of transplantation after cryopreservation is in its infancy, where follicle numbers in cryopreserved tissue are lower and only a minority of these follicles initiates development (Gook *et al.*, 2000). Newton *et al.* (1996) found a loss of about 26% of primordial follicles due to grafting and the resulting ischeamic insult that may additionally cause oxidative stress. This was supported by Lornage & Salle, (2007) who estimated a loss of 75% or more follicles, based on histological results as well as rapid graft exhaustion studies. The follicles were lost during the post transplantation ischeamia phase since graft recovery is entirely dependent upon neovascularisation.

The longevity of a graft is another area of concern. Mathematical calculations show accelerated follicle loss when primordial follicle numbers reach <25000 and loss of ovarian function at <1000 (Newton, 1998b). This implicated the follicular loss due to cryopreservation and grafting may be to such an extent that ovarian function may only be regained for a short time and that the fertile window of the graft may be short lived (Newton, 1998b; Gook *et al.*, 2000). More than 60% of primordial follicles can be lost during the initial ischeamia before revascularization after transplantation, where the freeze-thaw procedure may result in only a additional 7% primordial follicle loss (Oktay, 2001).

Considering these losses of primordial follicle population by ischeamic damage after grafting, improvement in the numbers of remaining functional transplanted follicles may improve the longetivity of the graft as well as the quality of endocrine profiles and cycles (Oktay & Buyuk, 2004).

One of the main drawbacks of the grafting as evaluation method is that the graft takes weeks to revascularize and then human primordial follicles require several more months of growth to reach maturity (Newton *et al.*, 1996).

Transplantation of tissue allows several optional sites for different grafts and recipients and an optimal site to achieve best results is unknown. Oocyte quality could be altered because of differences in temperature and blood flow in subcutaneous grafts compared to the orthotopic location. Follicles in heterotopic locations of the same phase grow to different sizes compared to orthotopic or pelvis grafts which may affect oocyte outcome. This may imply that the longer follicles are allowed to develop, the lower the quality of the follicle or oocyte. It was seen that a germinal vesicle retrieved from a small antral follicle from the forearm resulted in a superior quality embryo after ICSI compared to the metaphase II oocytes (Oktay *et al.* 2004).

Successful transplantation, therefore, relies on sites with rich blood supply which can provide superior graft reception. Insufficient blood supply can cause insults to follicles and oocytes, as well as possible necrosis of the implant. Fresh and cryopreserved ovarian cortex transplants showed significant reductions in morphologically normal follicles, indicating that transplantation caused morphological changes to some follicles, mainly due to ischeamic damage. There is a direct relationship between developmental stages of follicles and their susceptibility to insufficient blood supply. Larger antral follicles invariably undergo damage while smaller resting follicles survive well. The pool of resting follicles is responsible for keeping the longevity of the graft, which is ultimately the goal of ovarian tissue cryopreservation (Deng *et al.*, 2007).

In light of the abovementioned studies on cryobiology and ovarian cryopreservation, a protocol was designed that combined three protocols from reputable researchers (Oktay *et al*., 2000; Gook *et al*. 1999; Shaw, 2000, 2003a, 2003b; Gosden *et al.*, 1994) (*see Appendix 1*). The customized protocol was developed to accommodate two different cryoprotectant media, which had different cryoprotective agents, equilibration times and temperatures as well as slightly different cooling practices, without compromising efficacy of original protocols.

## AIMS AND OBJECTIVES

Our main aim was to initiate, develop and implement a practical ovarian tissue cryopreservation and re-transplantation protocol that can be used to possibly restore ovarian function, and maybe fertility, in young female cancer patients undergoing sterilizing cancer therapies in South Africa.

The objective of the study was to identify the better cryoprotectant by comparing two well-known cryoprotectants in slow cryopreservation of ovarian tissue by investigating their effects on the ultrastructure of early follicles in cortex tissue before and after freezing.

The ultrastructure was evaluated on transmission electron microscopy micrographs using a novel scoring system to assess the extent of the follicular damage induced by the cryopreservation of ovarian tissue.

# CHAPTER TWO

# MATERIALS AND METHODS

## 1. STUDY DESIGN

This is a single blind, single group experimental study.

The cortex tissue of each patient was divided into each investigated group to give paired results in all groups for comparisons, including own fresh control.

Five outcome groups were investigated, each with a different intervention. Fresh tissue was taken as a control sample with no treatment or intervention. Toxicity testing after completion of equilibration in either DMSO or PROH was the first intervention investigated for comparison. Slow cryopreservation with subsequent rapid thawing in either DMSO or PROH was the second intervention investigated for comparison.

The histological evaluation of all tissue samples was done in a blind fashion.

The study was conducted in parallel with a clinical study by Dr MH Botha from the Department of Gynaecological Oncology, Tygerberg Hospital. Ovarian tissue was obtained through Dr MH Botha for cryopreservation studies.

## 2. STUDY SAMPLE

The sample of convenience was patients receiving all consultations, surgeries and therapies in 2006 and 2007 at Tygerberg Hospital, South Africa.

Entry into the study was on voluntary basis subject to required prescribed informed consent (*see Appendix 4*).

## 3. EXCLUSION CRITERIA

### 3.1. To reduce statistical noise

- Patients younger than 20 years and older than 42 years at time of study.
- Patients diagnosed with any other serious illness or complication other than squamous cervical carcinoma or fallopian tube carcinoma.
- Patients not showing a normal LH, FSH and estradiol profile.
- Patients who previously received any form of cancer treatment before the time

of the study.

- Patients not scheduled for curative chemotherapy and/or radiotherapy.

### 3.2. Inevitable exclusions due to unforeseen circumstances

Eleven patients entered the study as participants but the histological results of three participants were excluded due to unforeseen circumstances.

The fresh control and equilibration tissue samples of one participant were lost and the patient was inevitably excluded.

After histological processing and evaluation of HE slides and TEM grids of several tissue samples of two patients, the tissue seen could not be recognised as cortex tissue and were also excluded from the study.

## 4. SAMPLE SIZE

N = 8

## 5. DIVISION INTO GROUPS

Patients were not divided into different groups.

## 6. THE CRYOPRESERVATION PROCEDURE

*See Appendix 2 for details on all mediums and consumables used.*

### 6.1. Ovarian tissue retrieval and dissection (Dr MH Botha)

Ovarian tissue retrieval was done during the staging evaluation procedure by laparoscopic examination under general anaesthesia.

An unilateral or bilateral oophorectomy was performed.

The ovary or ovaries was collected at room temperature (± 25 degrees Celsius (°C)) in either a standard sterile 90mℓ specimen container containing 40mℓ Quinn's Advantage™ Medium with HEPES pre-heated to ±37ºC or in a sterile endobag with no medium.

The ovarian specimen was transported to the laboratory within 10 minutes after retrieval from the abdomen.

The dissection of ovarian specimen was done at room temperature under laminar flow in a sterile tissue culture dish containing 10mℓ Quinn's Advantage™ Medium with

HEPES pre-heated to ±37ºC. The inner medulla (red soft tissue) was removed and the outer cortex (white, smooth, tough, collagenous surface layer) trimmed to a thickness of 1-2mm. The ovarian cortex was dissected into strips ranging between 2-3mm wide to 3-7mm long.

Strips were mixed and washed in clean Quinn's Advantage™ Medium with HEPES at room temperature.

All unused tissue, consisting of cortical and medullary tissue, was sent in 10% formalin for metastatic analysis.

The dissection was done within 15 to 40 minutes depending on the size of the ovarian specimen(s).

### 6.2. Cryopreservation (CL Els, Dr ML (Windt) De Beer)

*See Appendix 1 for detailed cooling protocols of references used.*

#### 6.2.1. Equilibration

Two to four strips of the fresh cortex tissue were washed in 3mℓ D-PBS and fixed as the control sample.

Remaining ovarian cortical strips were washed in 10mℓ clean Quinn's Advantage™ Medium with HEPES, counted and equally halved into 2 groups. Each group was allocated to a different cryoprotectant group, the DMSO group and the PROH group. The total number of strips was evenly divided to fit into 10 cryo vials at the end of equilibration, with 2-6 strips for each cryo vial (Shaw, 2003b). The colour-coded sterile 2mℓ cryo vials were appropriately labelled with patient details, number of strips in the cryo vial and cryoprotectant used. The vials were then pre-filled with 1-1.2mℓ of the respective cryoprotectant media (Shaw, 2003a, 2003b).

In cases where specimen(s) where larger and tissue strips numbered to more than 70 in total, a second freezing cycle was necessary since the CryoLogic Freeze Control® System is limited to only 10 x 2mℓ cryovials in the cryochamber. In these cases, tissue strips were divided into 4 equal groups of which 2 groups followed the first cryopreservation cycle, while the remaining 2 groups were incubated in 10mℓ clean Quinn's Advantage™ Medium with HEPES at room temperature for ±3 hours awaiting a second cryopreservation. These delayed samples were not investigated, only stored for patient's future use.

The PROH group was equilibrated and cryopreserved in 0.2µm sterile filtered PROH-medium containing 1.5M 1,2-Propandiol + 0.1M sucrose + 10mg/mℓ Quinn's Advantage™ Human Serum Albumin (HSA) in D-PBS (Gook *et al.,* 1999).

The DMSO group was equilibrated and cryopreserved in 0.2µm sterile filtered DMSO-medium containing 1.5M DMSO + 0.1M sucrose in D-PBS pre-cooled to 4ºC (Shaw, 2000, 2003a, 2003b).

The PROH group was equilibrated in ±5mℓ of the PROH-medium for 90 minutes in a single step at room temperature (±25ºC) in a 35x10mm culture dish (Gook *et al.,* 1999).

The DMSO group equilibrated in ±5mℓ of the DMSO-medium, pre-cooled to 4ºC, for 30 minutes in a 35x10mm culture in a fridge set at 4ºC (Oktay, 2000b; Shaw, 2000, 2003a, 2003b).

The culture dishes containing the tissue and cryoprotectant solution were agitated every 5-10 minutes during the equilibration to ensure that all the strips were evenly exposed to the cryoprotectant (Shaw, 2003a).

The equilibration protocols of DMSO and PROH differed in time and temperature. To ensure both sample groups could be cryopreserved in the same slow-freezing cycle, equilibration of both cryoprotectants needed to run in parallel without changing the specified protocol. DMSO tissue samples, therefore, were incubated in a small culture dish in clean D-PBS for 60 minutes at room temperature before starting equilibration.

Tissue samples of each group were placed into their 5 pre-filled cryo vials during the last 10 minutes of their equilibration time. Two to four strips of each group were left in equilibration medium and were fixed at completion of equilibration time.

### 6.2.2. Customized ovarian tissue slow cooling protocol

Slow-freezing was done in the CryoLogic Freeze Control® machine (Marcus Medical) run by the Cryogenesis™ V.5 Software programmed with the following customized cryopreservation protocol to accommodate both cryoprotectant groups as well as shorten total freezing time.

The 5 PROH tissue cryo vials were loaded into the cryochamber at 24°C, 2 minutes before equilibration time was completed. One vial was loaded into each ampoule

chamber (*see Fig. 2.3.*)

The cryochamber temperature was held constant at 24ºC for 2 minutes to ensure that cryo vials and tissue samples are at 24ºC before cooling started.

Cooling rate was started from 24ºC at -2°C/min until 4°C. A bell signalled and the temperature was held constant at 4°C for 2 minutes to load 5 DMSO vials. Again, one vial was loaded into each ampoule chamber.

Cooling was continued from 4ºC at a rate of -2°C/min until -7°C (Oktay *et al.*, 2000).

Temperature was held constant at -7°C for 5 minutes during which time vials were manually seeded.

**Manual seeding protocol** (*see Fig.1.7.*).

- The upper most vial (DMSO) was transferred to a neighbouring ampoule slot with forceps.
- The bottom vial (PROH) was lifted from the ampoule slot with forceps until the meniscus of the medium inside was visible.
- To seed, a cotton swab or the tip of a forcep, pre-cooled in liquid nitrogen, was placed on the meniscus until an ice crystal front was seen (Gook *et al.*, 1999; Oktay *et al.*, 2000; Shaw, 2003b).
- The vial was returned to its original location.
- The moved vial (DMSO) was then also seeded and returned to its original location.
- This sequence was repeated until all vials were seeded.
- The vial that was seeded first was re-examined to ensure the ice crystal front was still present (Shaw, 2003b).

Cooling rate continued at -0.3°C/min until -40°C (Gook *et al.*, 1999; Oktay *et al.*, 2000; Shaw, 2003b).

“Freefall” to below -60°C. (Shaw, 2003b)

Vials were plunged into liquid nitrogen.

Vials were clipped onto canes and placed into cane holder in canister of the dewar.

Samples were stored in liquid nitrogen for at least six weeks to simulate long-term low temperature storage.

### 6.2.3. Rapid thawing

Samples of both groups were thawed at the same time.

The vial at the top of the cane of each group was unclipped and kept at room temperature for a few seconds to remove excess nitrogen (Gook *et al.*, 1999; Oktay *et al.*, 2000; Shaw, 2003b).

Vials were transferred into sterile water bath at ±37°C. The vials were gently swirled while completely immersed for ±5 minutes until medium and the tissue sample were completely thawed (Gook *et al.*, 1999; Oktay *et al.*, 2000; Shaw, 2003b).

Each tissue sample was decanted into a small sterile culture dish and strips were transferred to subsequent wash-out media with sterile forceps (Gook *et al.*, 1999; Oktay *et al.*, 2000; Shaw, 2003b).

The samples were washed step-wise in lower concentrations of cryoprotectant media at room temperature to remove all cryoprotectant (Gook *et al.*, 1999; Shaw, 2003b).

The PROH group was washed for 5 minutes in 10mℓ sterile-filtered medium containing 0.75M PROH + 0.2M + 10mg/ml Quinn's Advantage™ HSA in D-PBS and then in 10mℓ 0.2M sucrose + 10mg/ml Quinn's Advantage™ HSA in D-PBS for 10 minutes (Gook *et al.,* 1999).

The DMSO group was washed for 5 minutes in 10mℓ sterile-filtered medium containing 0.75M DMSO + 0.25M sucrose in D-PBS and then in 10mℓ 0.25M sucrose in D-PBS for 10 minutes (Shaw, 2000, 2003a, 2003b).

All tissue samples were fixed according to standard histological procedures (*see Appendix 3*).

Each container containing the fixed samples was labelled with only a sample number to ensure blind sample processing.

## 6.3. FLOW DIAGRAM OF CRYOPRESERVATION PROTOCOL

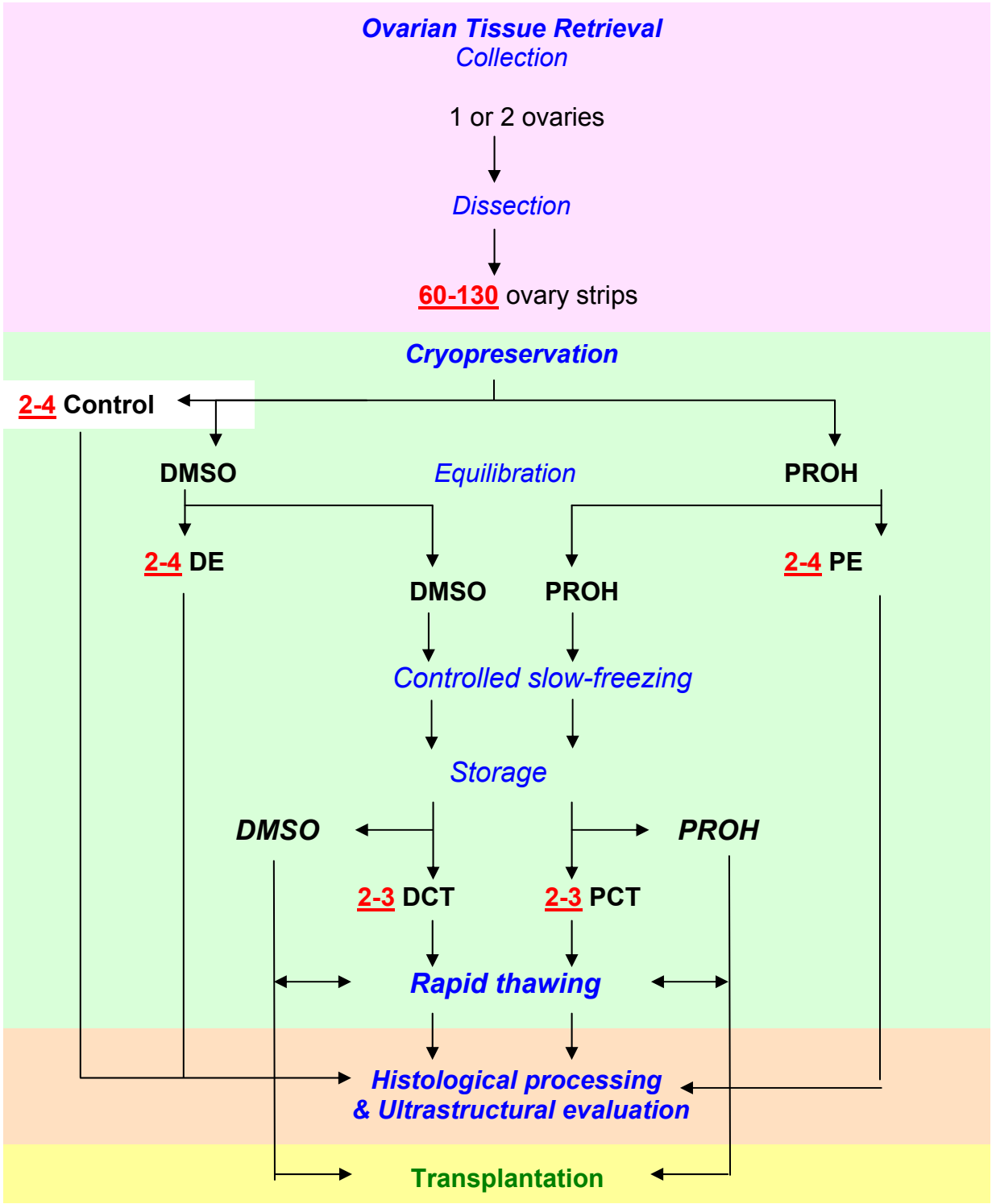



*Figure 2.1.* Flow diagram of the cryopreservation protocol of the study.
(**Key:** n **=** number of ovarian tissue strips; **Bold print** = name of tissue sample group; ***Protocol*** = different protocols; *method* = phase or stage of protocol; **Protocol** = future prospective phase of protocol; Groups: **FC**: fresh control, **DE**: DMSO equilibrated, **PE**: PROH equilibrated, **DCT**: DMSO cryopreserved and thawed, **PCT**: PROH cryopreserved and thawed.)

## 6.4. EQUIPMENT: THE CRYOLOGIC FREEZE CONTROL® SYSTEM

Manufacturer: CryoLogic Pty. Ltd.

Address: 54 Geddes St, Mulgrave, Victoria 3170, Australia

Product type: Freeze Control®

Model: CL-8800 System

The Freeze Control® System is a precise, reliable and versatile controlled rate freezer specifically designed for the cryopreservation of a wide variety of biological specimens.

### 6.4.1. Components

The Freeze Control® System consists of the following main components (*see Fig.2.2*):

- The temperature controller which regulates and monitors the temperature of the cryochamber. The temperature controller also has temperature protocols programmed into memory to specify temperature changes with time for given specimens during freezing operation.
- The cryochamber in which the specimens are placed, keeping the specimens at a uniform temperature and ensuring even heat distribution.
- The cryobath in which the cryochamber is placed and is filled with liquid nitrogen to provide cooling of specimens.
- CryoGenesis™V5 software for managing freezing operations with a computer. It allows for the design of specific freezing protocols, as needed by the user.

This system is reliable and quiet since it has no valves, solenoids or other moving parts. The system has different cryochambers that can be interchanged to be adaptable for different specimen types.


*Figure 2.2.* The main components of the CryoLogic Freeze Control® System, including the CryoGenesis™V5 software.

Inside the cryochamber all specimens are held at a highly uniform temperature which is measured by a precision platinum temperature sensor, read by the controller. The cryochamber standing in the cryobath acts as a heat sink, where the controller regulates the heat flow from the specimens to the liquid nitrogen with a control element tightly coupled to the cryochamber core. It is strictly regulated to follow the selected temperature protocol. The temperature protocol to be followed is selected from either the temperature controller or CryoGenesis™. The temperature controller contains up to 16 fixed internal protocols stored on a program chip by the manufacturer. These programs are always available but cannot be changed or modified. The instruction guide provides the assigned protocol number and corresponding protocol specifications (graphs and appropriate sample type) needed for selection of a required temperature protocol. If the temperature controller is connected to a computer installed with the CryoGenesis™V5 software, the user has access to external protocols which can be fully customised and stored and edited as required. These external protocols have no limit to duration of the protocol or number of steps in the protocol.

The temperature controller can control temperatures between 40°C and -120°C. It can record temperatures between 200°C and -200°C. Temperatures below -120°C can be reached by including a 'Freefall State' in the temperature protocol. In the

'Freefall' state the cryochamber element is turned off allowing the temperature to drop towards that of nitrogen inside the cryobath. The rate of Freefall is governed by the cryochamber model, the initial temperature and the amount and type of samples loaded. The manual provides graphs of maximum cooling rates (°C per minute) at temperatures ranging from 40°C to -120°C of the different unloaded cryochambers for user's reference. The temperature controller uses less than one litre of liquid nitrogen per hour. It can be stored at temperatures between 50°C and -10°C and safely operated at temperatures between 40°C and 0°C.

The cryochamber is the holding compartment for the samples and contains the heating element for cooling or thawing. The cryochamber has a thermally conductive heating core enclosed in a customised insulation layer and a protective thermal conductive shell into which the samples are inserted. The core heating element is coupled to a platinum temperature sensor which is constantly monitored and adjusted by a control loop between the temperature sensor, temperature controller and control element, to maintain the samples at a uniform temperature as specified by the temperature controller. The cylindrical shape of the holding compartment and the tight coupling between the samples and the core optimizes uniform heat transfer from all sides and along the whole length of each straw or ampoule (*see Fig.2.3.*).

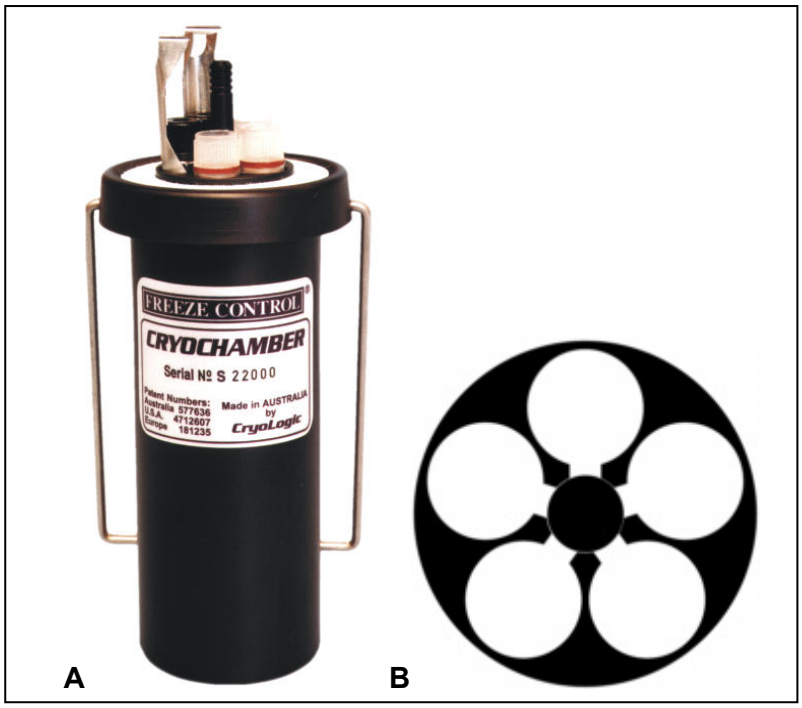



*Figure 2.3.* The 5-slot ampoule cryochamber (A) is designed to hold 3 x 1.0mℓ, 2 x 2.0mℓ or 1 x 5.0mℓ cryo vials in each of the 5 slots (B) [http://www.cryologic.com/cc_amp.htm].

The cryobath is a well insulated 1.5ℓ capacity container designed to hold the cryochamber in liquid nitrogen. The cryobath insulation layer and lid minimises liquid nitrogen consumption by limiting rate of evaporation and provides two hours of operation before refilling is needed. Samples can be plunged directly into the liquid nitrogen from the cryochamber after a cooling protocol has finished prior to transfer to the storage tank.

The CryoGenesis™V5 is proprietary software developed by CryoLogic for cryopreservation management. The CryoGenesis™ software runs under Microsoft Windows when the temperature controller is connected to a computer via either the standard communications port or USB port. CryoGenesis™ can be used to create, edit and save temperature protocols for a specific specimen. CryoGenesis™ collects, displays and stores all logged cryochamber temperatures and freezing operation data in records called 'Jobs' for easy management and future data recollection (*see Figs.2.4., 2.5., 2.6.*).

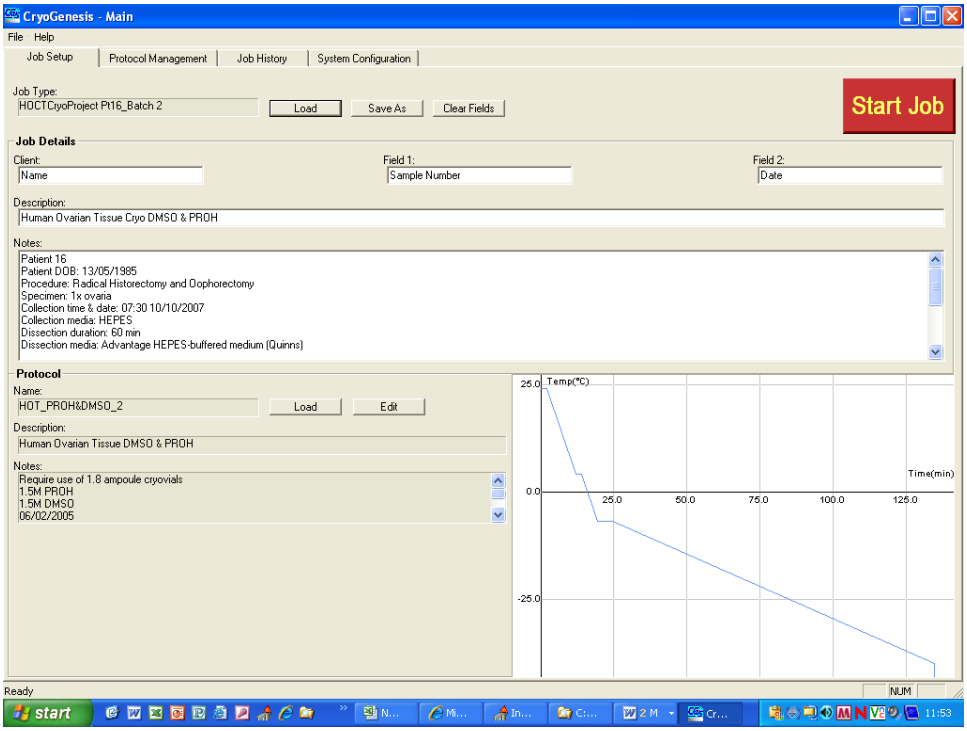



*Figure 2.4.* A screen print of CryoGenesis™ software running in windows. The main page shows a overview of the last 'Job' done. The new 'Job' protocol is loaded and details or notes of the new 'Job' are entered and saved.

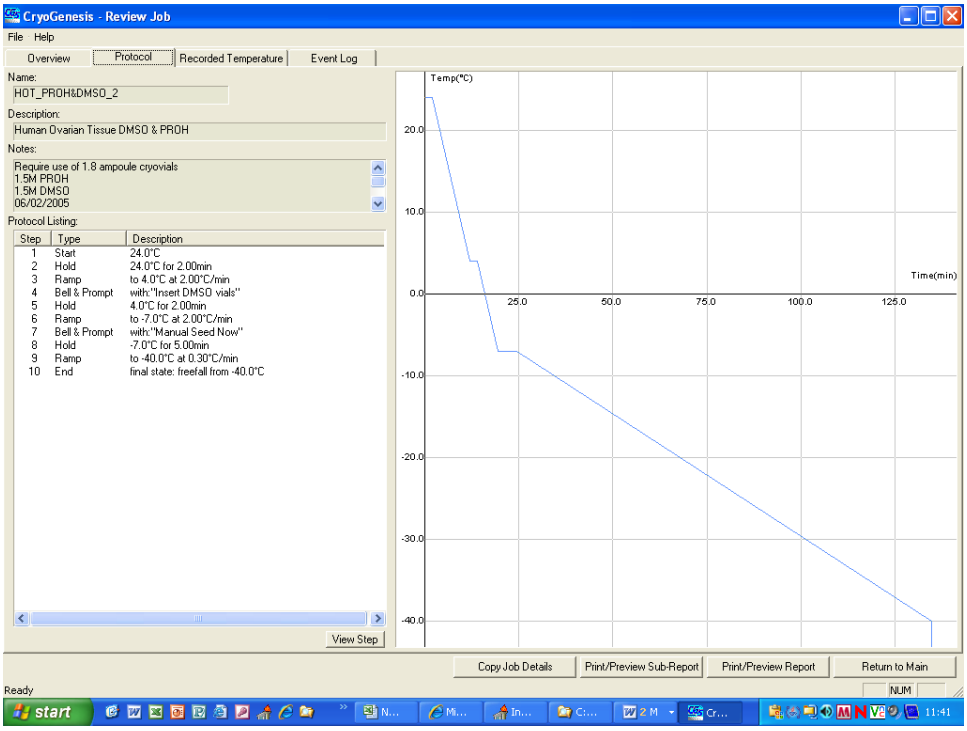



*Figure 2.5.* The customized cryopreservation protocol is programmed and saved in the CryoGenesis™ software program. The protocol is then loaded for each freezing; therefore all samples are cooled with the same protocol.

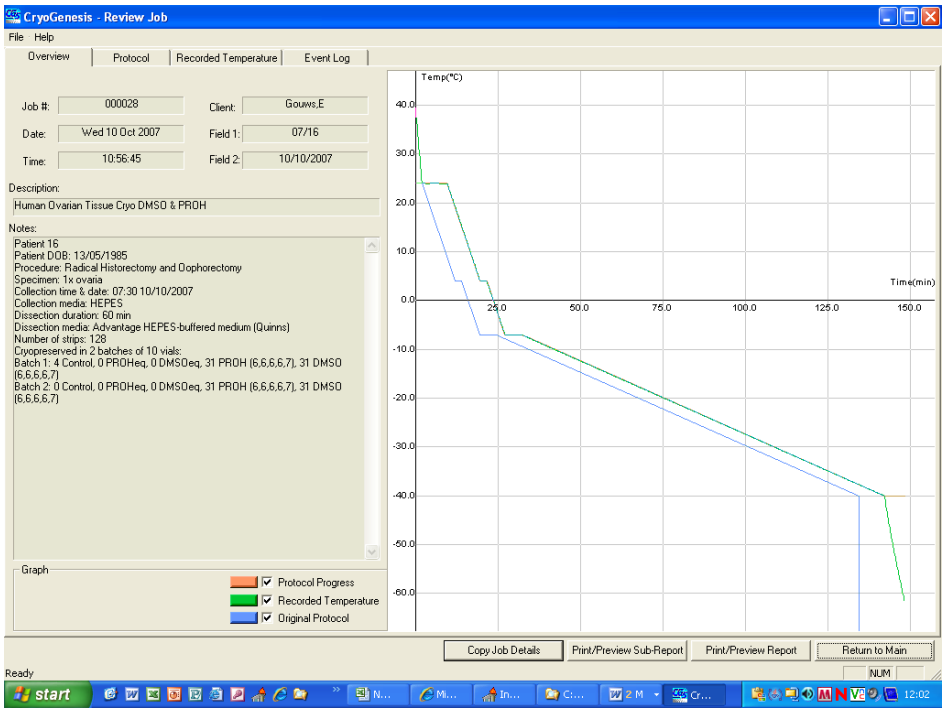



*Figure 2.6.* An 'Overview' page is set up where the data of the protocol (blue line), recorded temperature (green line), progress of protocol (red line) and extra information are stored and could be recalled for future reference.

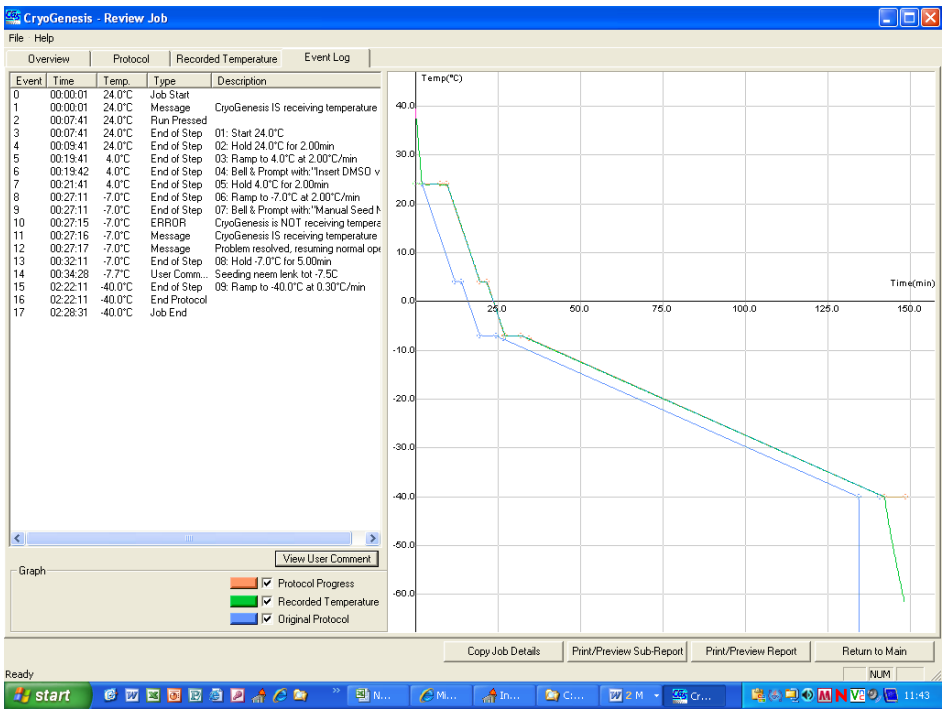



*Figure 2.7.* An 'Event log' is automatically compiled and stored by CryoGenesis™ for each freezing 'Job' and can be recalled at any time.

### 6.4.2. Specifications and recommendations

The CryoLogic Freeze Control™ system had several specifications and handling recommendations for optimal use. The original protocols had to be adapted accordingly in the customized protocol.

It is recommended that after manual seeding, the cryochamber temperature needs to be held for approximately five minutes at the seeding temperature to allow the Heat of Fusion to be absorbed and the first stage of dehydration to reach equilibrium. The ice front should be propagating to the bottom of the sample when checked one minute after seeding before the next cooling step is started.

When the 5-slot cryochamber for ampoule (vial) samples in combination with manual seeding is used, it is recommended that only five ampoules are frozen at a time. This implied that only a few tissue pieces could be cryopreserved at a time and thus, several (more than 4) cycles of the protocol would have to be done to cryopreserve the cortex tissue of one or two ovaries. Since the slow cryopreservation protocol was very time consuming (4 hours per equilibration and cooling cycle), a customized manual seeding protocol was introduced to accommodate 10 x 2mℓ vials (double the

number of vials) per cooling cycle. This allowed two whole ovaries be frozen in two cooling cycles which reduced the time frame (total of 7 hours) for practical reasons and minimizing the incubation period of tissue awaiting cooling, reducing further ischeamic damage to the follicles. This also allowed the cooling of an equal number of vials and tissue strips from the two cryoprotectant groups in one cooling cycle.

The customized manual seeding method entailed the quick shifting of the top vial of a slot to a neighbouring slot in the cryochamber, followed by the manual seeding of the bottom vial and the shifted vial, which is then replaced into their original positions after being seeded. This shifting is repeated until all vials are seeded.

The cryochamber has a specified maximum cooling rate at a specific core temperature. According to Oktay *et al.* (2000) and Gook *et al.*, (1999), a cooling rate of -10ºC is prescribed from -40ºC to -140ºC before plunging samples in liquid nitrogen. This cooling rate, however, is too fast for the cooling chamber at -40ºC and below. A setting called 'Freefall' is optional in the CryoGenesis™ software program, during which time the heating core is switched off and the cryochamber is allowed to cool at its maximum cooling rate (*see Fig.2.8.*). The maximum cooling rate was closest to the prescribed cooling rate between -40ºC and -60ºC (±8°C/min). The maximum cooling rate then slowed to less than 4ºC, becoming even slower. It was decided to plunge the samples at -60ºC because of time constraints. Shaw (2003a) suggested that plunging could be done at -40ºC and further cooling was only optional.

## 7. HISTOLOGICAL PROCESSING (N Muller, Dr CJF Muller)

Standard histological preparation and processing procedures were followed in all cases as required by the National Health Laboratory Service (NHLS) (*see Appendix 3.*).

A total of 55 samples, 11 participants with 5 samples each, were sent for histological processing. Tissue samples were fixed in phosphate-buffered gluteraldehyde (pH 7.4) and kept at 4°C overnight and until processed.

Tissue samples were halved under a dissection microscope. One section was prepared into paraffin blocks and stained with haematoxylin and eosin (HE). HE tissue slides were inspected under light microscopy to identify the presence of primordial follicles (*see Fig.2.8.*).

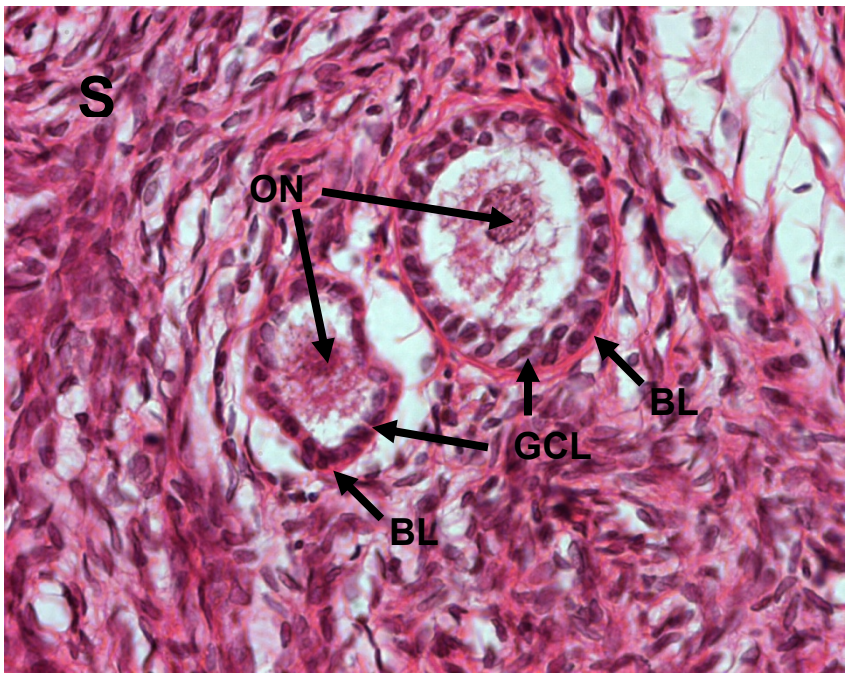



*Figure 2.8.* Example of primordial follicle identification under light microscopy on HE stained slide. Two primordial follicles were identified in a PROH post-equilibration tissue sample processed by HE staining. The surrounding stromal cells (S), basal lamina (BL) and granulosa cell layers (GCL) and oocyte nuclei (ON) of the primordial follicles are visible.x20.

If no follicles could be found on several HE slides of a tissue sample, the corresponding half of the tissue section did not undergo TEM processing, as the chances of capturing a follicle on TEM were too low.

A total of 31 tissue sections contained primordial and/or primary follicles on HE slides (*see Table 2.*).

*Table 2.* The amount of tissue samples in each group which presented primordial follicles.

| Samples per group | N |
|---|---|
| Fresh Control (FC) | 7 |
| Equilibration: | |
| DMSO (DE) | 5 |
| PROH (PE) | 7 |
| Cryopreserved & Thawed: | |
| DMSO (DCT) | 6 |
| PROH (PCT) | 6 |
| Total | 31 |

The other half of the 31 tissue sections still fixed in gluteraldehyde were processed for transmission electron microscopy (TEM).

Each follicle identified on the TEM grid was photographed at several magnifications. A lower magnification (1500-4500x) of the whole follicle with surrounding stroma and several higher magnifications (up to 15000x) of specified areas of the follicle were used for optimal evaluation. Micrographs were developed in a dark room on A4-sized photographic paper.

The micrographs of eight follicles were not optimal for accurate ultrastructural analysis and were excluded (*see Fig.2.9.*).

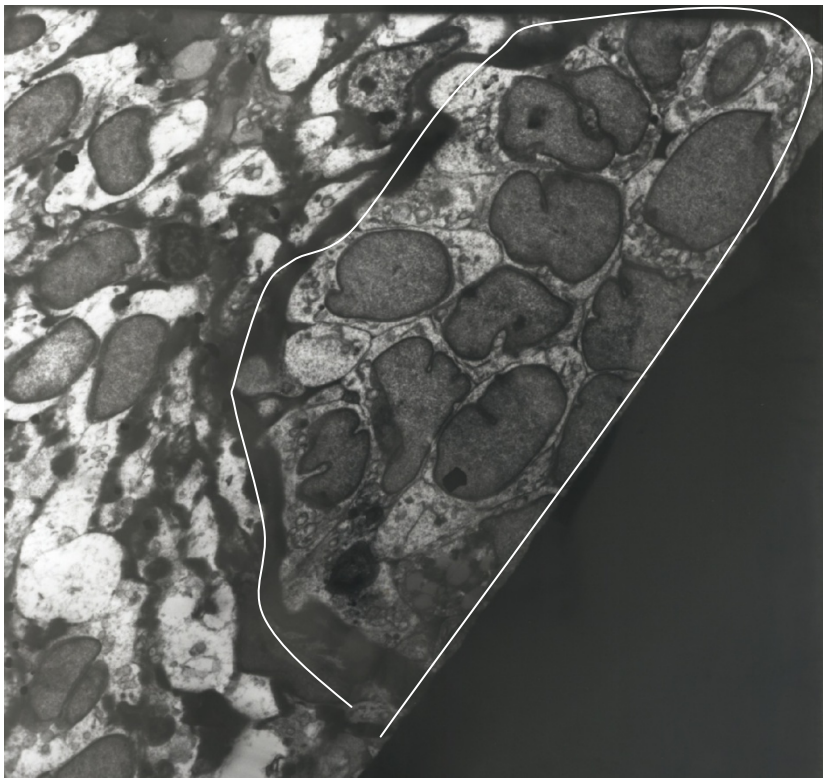


*Figure 2.9*. Example of an excluded electron micrograph of a primordial follicle in the DMSO equilibration sample group. The follicle was halved on the TEM grid (white insert) and too dark for accurate analysis.x1500.

A total of 145 micrographs (±5 per follicle) of 30 follicles were used for ultrastructural evaluation (*see Table 3.*).

*Table 3*. The distribution of evaluated follicles into the groups.

| Sample / Group | Follicles | Micrographs |
|---|---|---|
| Fresh Control (FC) | 10 | 48 |
| Equilibration: | | |
| DMSO (DE) | 5 | 23 |
| PROH (PE) | 5 | 22 |
| Cryopreserved & Thawed: | | |
| DMSO (DCT) | 5 | 26 |
| PROH (PCT) | 5 | 26 |
| Total | 30 | 145 |

## 8. ULTRASTRUCTURAL EVALUATION (Prof M Sousa)

Ultrastructural evaluation was done on original micrographs. Each follicle was individually evaluated on a blind basis. The following ultrastructures of each primordial or primary follicle were evaluated on the micrographs:

- **Stroma**:
  - Stromal cells: membrane, nucleus, cytosol, organelles.
  - Extracellular matrix.
  - Basal lamina.
- **Granulosa cell layer**:
  - Nucleus: nuclear envelope, nuclear pore complexes, nuclear lamina, heterochromatin, euchromatin, nucleolus.
  - Cytoplasm: membrane, interdigitations/microvilli, pynocytosis, receptor mediated endocytosis, exocytosis, desmosomes, gap junctions, mitochondria, peroxisomes, primary lysosomes, refractile bodies, golgi complexes, secretory vesicles, smooth endoplasmic reticulum, rough endoplasmic reticulum, lipid droplets, centrosome, microfilaments, intermediary filaments, microtubules, cytosol.

- **Oocyte**:
    - Nucleus: nuclear envelope, nuclear pore complexes, nuclear lamina, heterochromatin, euchromatin, nucleolus.
    - Cytoplasm: membrane, interdigitations/microvilli, pynocytosis, receptor mediated endocytosis, exocytosis, desmosomes, gap junctions, mitochondria, peroxisomes, primary lysosomes, refractile bodies, golgi complexes, cortical vesicles, smooth endoplasmic reticulum, rough endoplasmic reticulum, annulate lamellae, nuage, lipid droplets, centrosome, microfilaments, intermediary filaments, microtubules, cytosol.
    - Perivitelline space.
    - Zona Pellucida.

Evaluation was done using the following scoring system:

1 = Poor, degenerative;

2 = Fair, severe damage;

3 = Average, recoverable damage;

4 = Good, little damage;

5 = Excellent, normal, no damage.

No scores were awarded in cases where a structure or organelle was absent or could not be seen clearly enough for evaluation on the micrograph.

Descriptive notes were also collected.

## 9. STATISTICAL ANALYSIS (Dr M Kidd, CL Els)

The resulting scores (1-5) of evaluated organellae were recorded (*see Section 8.*)

A statistician (Dr M. Kidd, Centre for Statistical Consultation, Dept of Statistics and Actuarial Sciences, University of Stellenbosch) was consulted for appropriate statistical analysis as well as assisting in the interpretation of the results.

Missing data was not included in statistical analysis. This resulted in the exclusion of many ultrastructure evaluations that were either absent or too infrequent for statistical

analysis.

All actual scores awarded were used in the statistical analysis. In cases where a few scores were absent, average scores of smaller organelles were calculated to award a score to the greater structure for statistical analysis. The scores of the major structures, i.e. stroma, granulosa cell layer and oocyte; were calculated from its main components. The scores of the stroma were the calculated mean scores from the scores of the stromal cells, extracellular matrix and basal lamina. The scores of the granulosa cell layer were calculated from scores of the nucleus and cytoplasm of the granulosa cells. The scores of the oocyte were calculated as the average of scores of the oocyte nucleus, cytoplasm and perivitelline space. Missing scores of the nuclei and cytoplasms of the stromal cells, granulosa cells and oocyte were calculated as the average of the nuclear components and cytoplasmic components, respectively.

Descriptive statistics; i.e. mean, standard error of the mean, number of evaluations and 95% confidence intervals, were calculated for evaluated ultrastructures.

The null hypothesis ($H_0$) was formulated and assumed that the mean score of all groups was similar. A *P*-value of ≤ 0.05 was accepted as rejection of the $H_0$ and indicated a statistical significant trend or difference between groups. Same letters (a vs. a or b vs. b) on graphs were used to indicate no significant difference between sample groups, while opposite letters indicated a significant difference between sample groups (a vs. b).

The mixed model analysis of variance (ANOVA) for repeated measures was used to indicate a treatment effect compared to the control (untreated) sample. Post hoc analysis was done using the Fisher least significant difference (LSD) test to compare the mean scores of all possible sample group pairs.

All statistical analysis was done on Statistica 8.0 software program. The ANOVA used was called the Variance estimation, precision and comparison (VEPAC) fixed effect test and the post hoc test was called the LSD test on the Statistica 8.0 software program.

The Spearman rank test was used for correlation studies between selected structures where $r = 1.00$ indicated an exact positive linear relationship.

# CHAPTER THREE

# RESULTS

## 1. INFORMATION OF PARTICIPANTS AND HANDLING OF OVARIAN TISSUE

The clinical data of participants and the ovarian tissue handling information is presented in Table 4.

The average age of the 11 female participants included in the study was 35 years (range 27-41). The majority of the participants (n=9) presented with Stage IIIB squamous carcinoma of the cervix. Ovarian tissue was removed before cancer therapy commenced. The majority of the participants received both radiation and chemotherapy, after which six patients received heterotopic autotransplantation of cryopreserved ovarian strips. No metastases were found in any of the participants' ovarian tissue. Details of the ovarian tissue strips cryopreserved, evaluated, stored and transplanted are shown in Table 4.

*Table 4*. Summary of clinical characteristics of participants and ovarian tissue handling.

| | N | Mean | Range | Median | Percentage (%) |
|---|---|---|---|---|---|
| Participants (including3 excluded patients) | 11 | | | | |
| Age | | 35 | 27-41 | 36 | |
| Cancer diagnosis: | | | | | |
| Cervix Squamous IIIB | 9 | | | | 81.8 |
| Vaginal IIIB | 1 | | | | 9.1 |
| Fallopian Serous Adeno | 1 | | | | 9.1 |
| 6-Week Cancer treatment: | | | | | |
| Radiation | 2 | | | | 18.2 |
| Chemotherapy | 1 | | | | 9.1 |
| Radiation & Chemotherapy | 8 | | | | 72.7 |
| No. Ovaries removed | 20 | 1.8/patient | 1-2 | 2 | |
| No. Cortex strips | | | | | |
| Total | 900 | 45/ovary | | | |
| Per patient | | 81.5 | 48-129 | 75 | |
| Used for Histology | 159 | 14.5 | 7-20 | 15 | 21.6 |
| Currently in Storage | 670 | 60.9 | 40-108 | 58 | 74.4 |
| Heterotopic autotransplantation: | | | | | |
| Patients | 6 | | | | |
| DMSO tissue | 3 | | | | |
| PROH tissue | 3 | | | | |
| No. Cortex strips | 71 | 11.8 | 10-14 | 12 | 54.5 |
| DMSO | 37 | 12.3 | 11-14 | 12 | |
| PROH | 34 | 11.3 | 10-12 | 12 | |

## 2. TRANSMISSION ELECTRON MICROSCOPY

### 2.1. Evaluated ultrastructural micrographs

Thirty follicles were evaluated and scored (*see Materials and Methods*) using a total of 145 low and high magnification micrographs (±5 micrographs per follicle) (*see Table 3.*). The scoring results of one or two follicles from each sample group will be presented below for illustration of ultrastructural damage since not all TEM micrographs could be included. The type of damage and areas damaged are reported where indicated. The location of all ultrastructures that were evaluated, scored and described are not constantly indicated on every micrograph. Actual scores awarded to ultrastructures are shown in brackets (1-5).

#### 2.1.1. Fresh control samples

The follicle from the fresh control sample group of patient 2 showed the best scores and thus the most normal follicle ultrastructure (*see Fig.3.1.*).

The lower magnification micrograph showed a single layer of granulosa cells indicating a primordial stage follicle. An overview of the stroma (S), granulosa cell layer (GCL) and oocyte (OC + ON) showed excellent normal cell structures (*see Fig.3.1.*).

The stroma showed normal stromal cells with normal membranes (5), cytosol (5) and organelles (5). The nuclei (SC-N) (5) of the stromal cells were elongated and indented with abundant peripheric chromatin in the nucleoli (Nu) (5). The extracellular matrix (ECM) showed minimal damage (4). Lipid droplets (L) of the follicle were normal (5) (*see Fig.3.1.*).

The oocyte nucleus (ON) was round, central and completely euchromatic, since chromosomes were clearly visible (*see Fig.3.1.*).

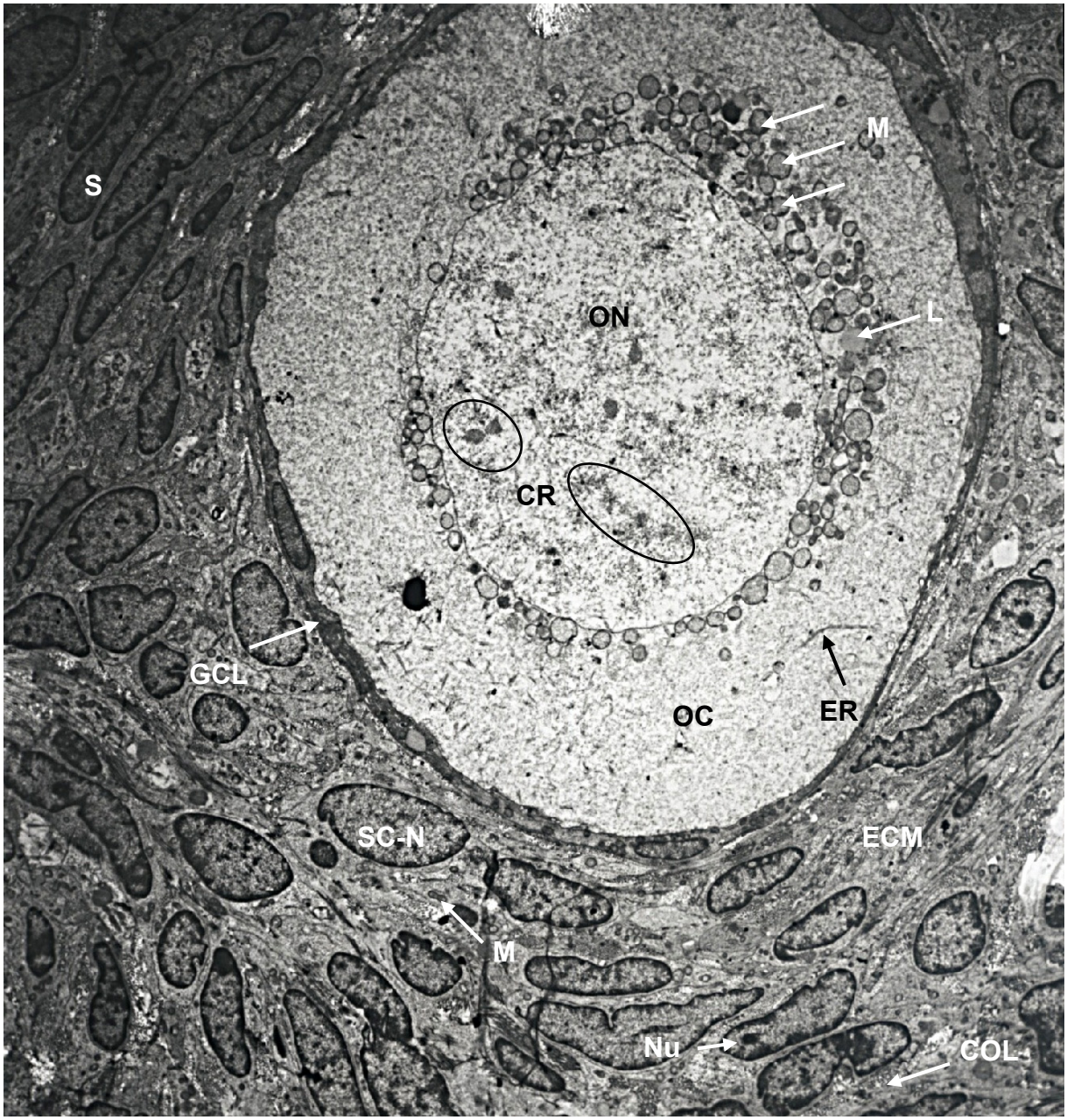



*Figure 3.1*. Electron micrograph of an intact fresh control sample primordial follicle. The stroma (S) consisted of stromal cells with nuclei (SC-N), containing nucleoli (Nu) and the extracellular matrix (ECM), containing mitochondria (M) and collagen bundles (COL). The granulosa cell layer (GCL) surrounds the oocyte cytoplasm (OC) containing the oocyte nucleus (ON) and chromosomes (CR & encircled). Surrounding the oocyte nucleus are abundant mitochondria (M) and lipid droplets (L).x1000-3000.

The higher magnification micrograph of the granulosa cell layer (GCL) showed a normal granulosa cell (5). The nucleus was normal (5) although the nucleolus and nuclear pore complexes were absent. The membrane, mitochondria (M), endoplasmic reticulum (ER) of the granulosa cells were evaluated as normal (5).

The perivitelline space (PVS), microfilaments (MF) and intermediary filaments (IF) were minimally present but did not show signs of damage (5). The basal lamina (BL) was thin and normal (5) (*see Fig.3.2.B*).

The smooth and rough endoplasmic reticulum (ER) (5), pynocytosis (PY) (5) and receptor mediated endocytosis (RME) of the oocyte were normal (*see Figs.3.2.A, 3.2B*). The mitochondria (M) of the oocyte showed the most damage from minimal to severe (2,3,4). The nuclear lamina (ONL), nuclear pore complexes (NPC) and euchromatin were normal (5). The nuages (n) were normal (5) (*see Fig.3.2.A*).

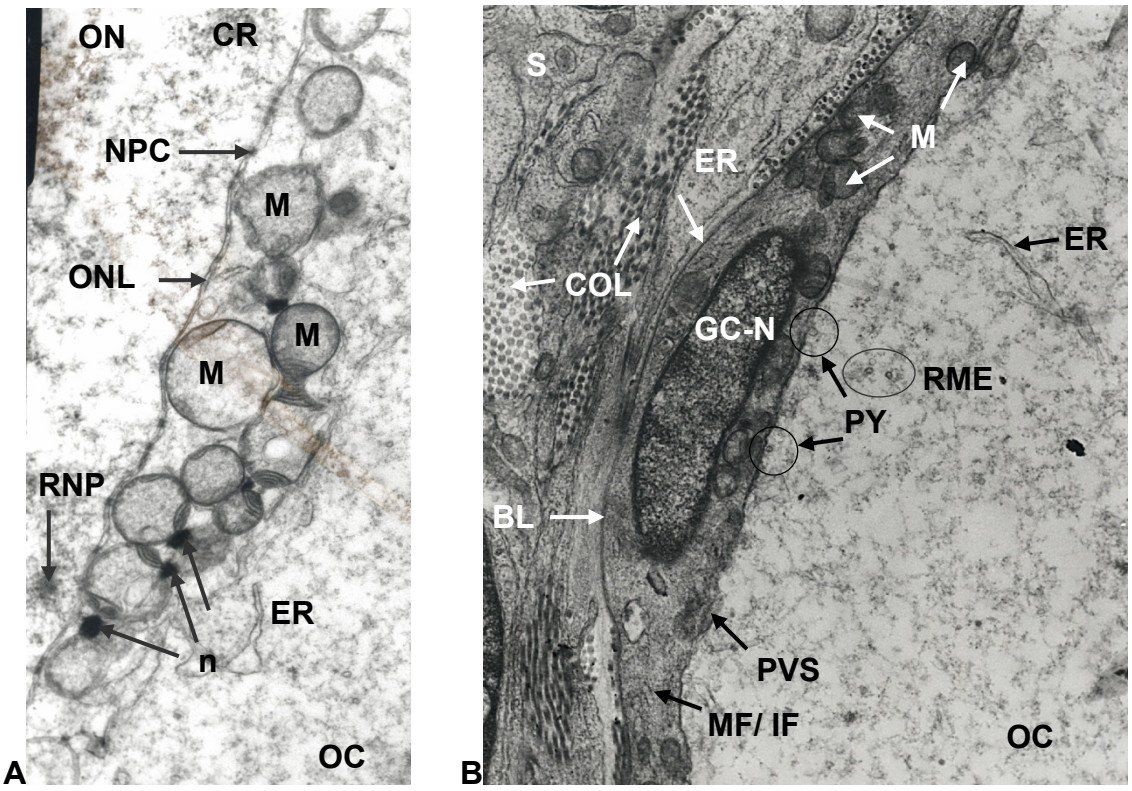



*Figure 3.2*. Two higher magnifications of selected areas of the intact fresh control follicle. **A**) High magnification of the mitochondria (M) in the oocyte cytoplasm (OC) surrounding the oocyte nucleus (ON). The oocyte nuclear lamina (ONL) contained nuclear pore complexes (NPC). Chromosomes (CR) and RNA export proteins (RNP) leading to nuages (n).x6000-18000. **B**) High magnification of a section of the granulosa cell layer with the nucleus of a granulosa cell (GC-N), mitochondria (M), endoplasmic reticulum (ER) and microfilaments and intermediary filaments (MF/IF) in the granulosa cytoplasm. Along the oocyte membrane the perivitelline space (PVS), pynocytosis (PY & encircled) and receptor mediated endocytosis (RME) were present and endoplasmic reticulum (ER) of the oocyte cytoplasm (OC) were also seen. The stroma (S) contained collagen bundles (COL) and a thin basal lamina (BL).x8000-24000.

In contrast, the fresh control follicle of patient 1 presented with various areas of lytic damage (×) in the stroma, granulosa layer (GCL) and oocyte (*see Fig.3.3.*).

The condition of the stroma was poor with many areas where lysis (×) induced severe damage. The stromal cell membranes (1), nuclei (SC-N) (1), cytosol (1) and organelles (1) were in a degenerative state (*see Fig.3.3.*). The extracellular matrix showed severe damage (2) (*see Fig.3.4.*).

The basal lamina was thick in some areas but showed no signs of damage (5) (*see Fig.3.3.*).

The cytoplasm, specifically the cytosol of the oocyte showed degenerative properties (1) although some ultrastructures were normal. The oocyte membrane (5), microvilli (MV) (5), giant refractile bodies (RB) (5), nuages (n) (5) and lipid droplets (L) (5) showed no damage while pynocytosis (PY) (4), the golgi complexes (G) (4), smooth and rough endoplasmic reticulum (ER) (4) and annulate lamellae (AL) (4) showed minimal damage. The mitochondria (M) of the oocyte showed recoverable to severe damage (2,3) (*see Figs.3.3.,3.4.*).

The perivitelline space (PVS) was in a good condition with minimal damage (4).

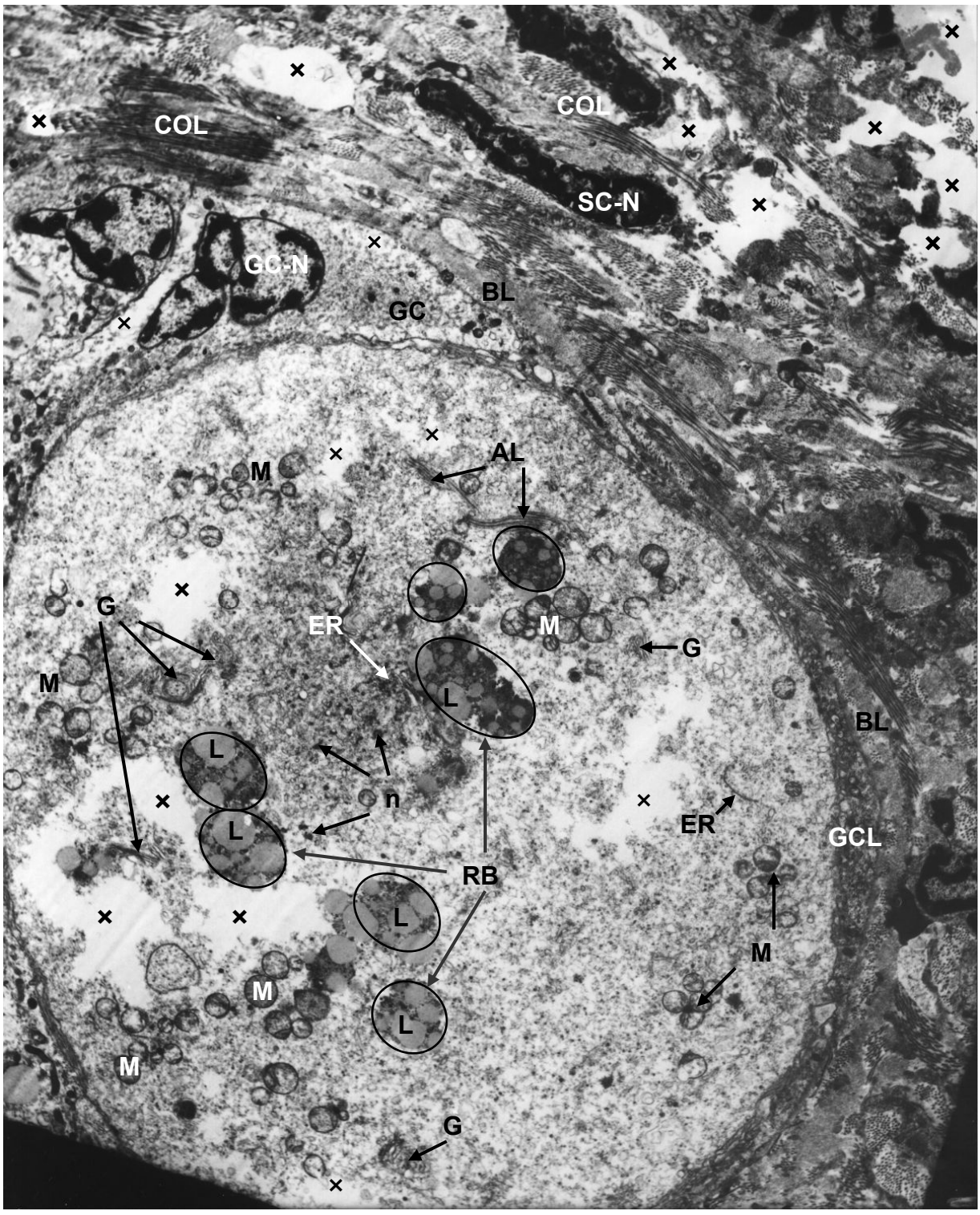



*Figure 3.3*. Electron micrograph of a damaged fresh control sample primordial follicle. The stroma, granulosa layer (GCL) and oocyte were damaged by lysis (**x**). Giant refractile bodies (RB) containing several lipid droplets (L), golgi complexes (G), nuages (n) annulate lamellae (AL), endoplasmic reticulum (ER) and mitochondria (M) were identified in the oocyte. Collagen bundles (COL), the basal lamina (BL), granulosa cell layer (GCL) with granulosa cells (GC) and nuclei (GC-N) are visible. .x2000-6000.

The higher magnification of the granulosa layer of this follicle showed that the granulosa cells (GC) had a normal membrane (5) with no damage to the microvilli (MV) (5), desmosomes D) (5), gap junctions (GJ) (5), pynocytosis (PY) (5), receptor mediated endocytosis (RME) (5), centrosomes (Ce), secretory vesicles (SV) (5), microtubules (MT) (5) and filaments (5). The nuclear lamina (4), golgi complexes (4) and rough endoplasmic reticulum (RER) (4) showed little damage. The heterochromatin of the granulosa nuclei (GC-N), however, showed detachment indicating severe damage (2). The mitochondria (M) of the granulosa layer showed recoverable to severe damage (2,3) (*see Fig.3.4.*).

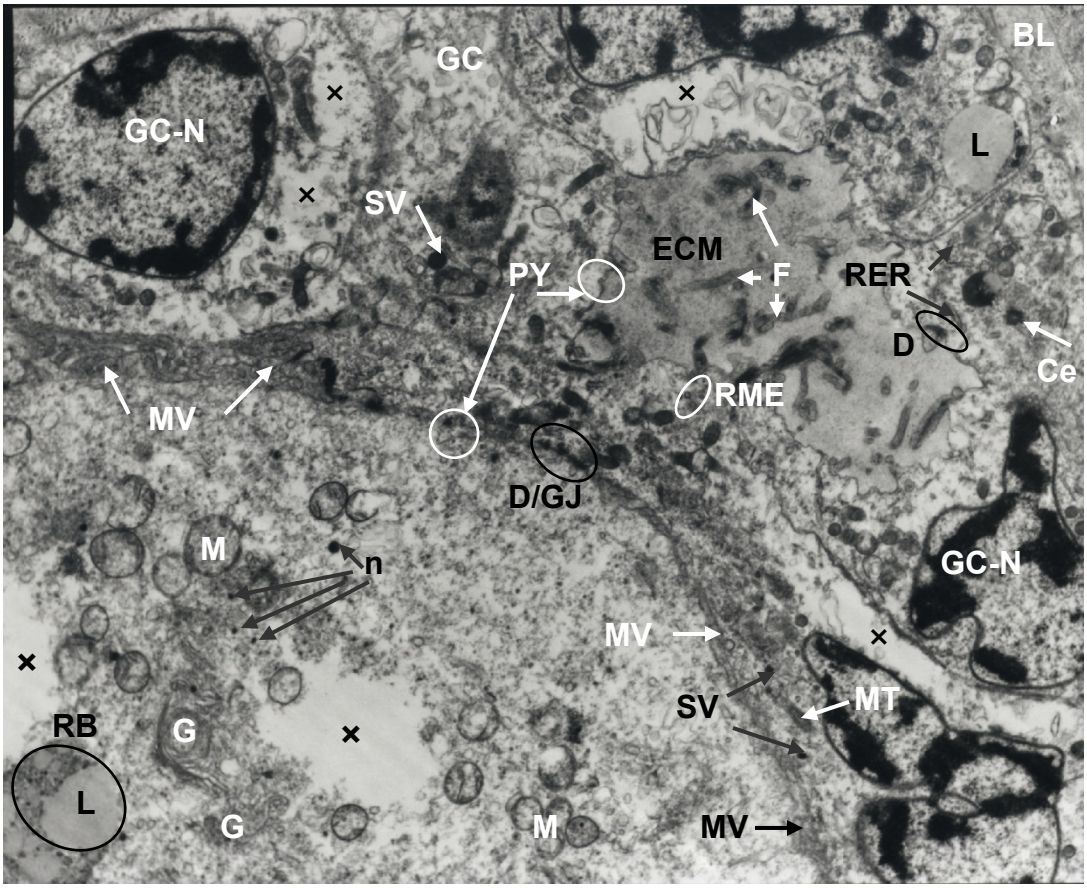



*Figure 3.4*. Higher magnification of the damaged fresh follicle with granulosa layer (GCL) and oocyte (O) with areas of lysis (**x**). The basal lamina (BL), extracellular matrix (ECM), granulosa cells (GC) and nuclei (GC-N), desmosomes (D), gap junctions (GJ), filaments (F), receptor mediated endocytosis (RME), pynocytosis (PY), secretory vesicles (SV), microvilli (MV), microtubules (MT) and centrosomes (Ce) were identified in the granulosa layer. Refractile bodies (RB) containing several lipid droplets (L), golgi complexes (G), nuages (n) annulate lamellae (AL), rough endoplasmic reticulum (RER) and mitochondria (M) were identified in the oocyte.x2000-6000.

### 2.1.2. Equilibration samples

The follicle of patient 2 from the **DMSO** equilibration sample showed areas of severe lytic damage (**x**) in the oocyte but not in the stromal or granulosa cells (*see Fig.3.5.*).

The single layer of squamous granulosa cells indicated a primordial stage follicle (*see Fig.3.5.*).

No large areas of lysis are visible, but the state of the stromal cells was evaluated as degenerative, where the stromal cell membrane (1), nuclei (SC-N) (1), cytosol (1) and organelles (1) were in a poor condition. The nuclei were elongated, indented and condensed (*see Fig.3.5.*). The extracellular matrix (2) and basal lamina (BL) (2) showed severe damage (*see Figs.3.5., 3.6.*).

The oocyte nucleus was absent. The oocyte cytoplasm (OC) showed degeneration with only the membrane (1,2), microvilli (1,2), mitochondria (M) (1), smooth and rough endoplasmic reticulum (ER) (1) and cytosol (1) present and in degenerative or severely damaged condition. The perivitelline space (PVS) (1,2) with microvilli (MV) (1,2) were minimally present and in a poor state with severe damage (*see Figs.3.5., 3.6.*).

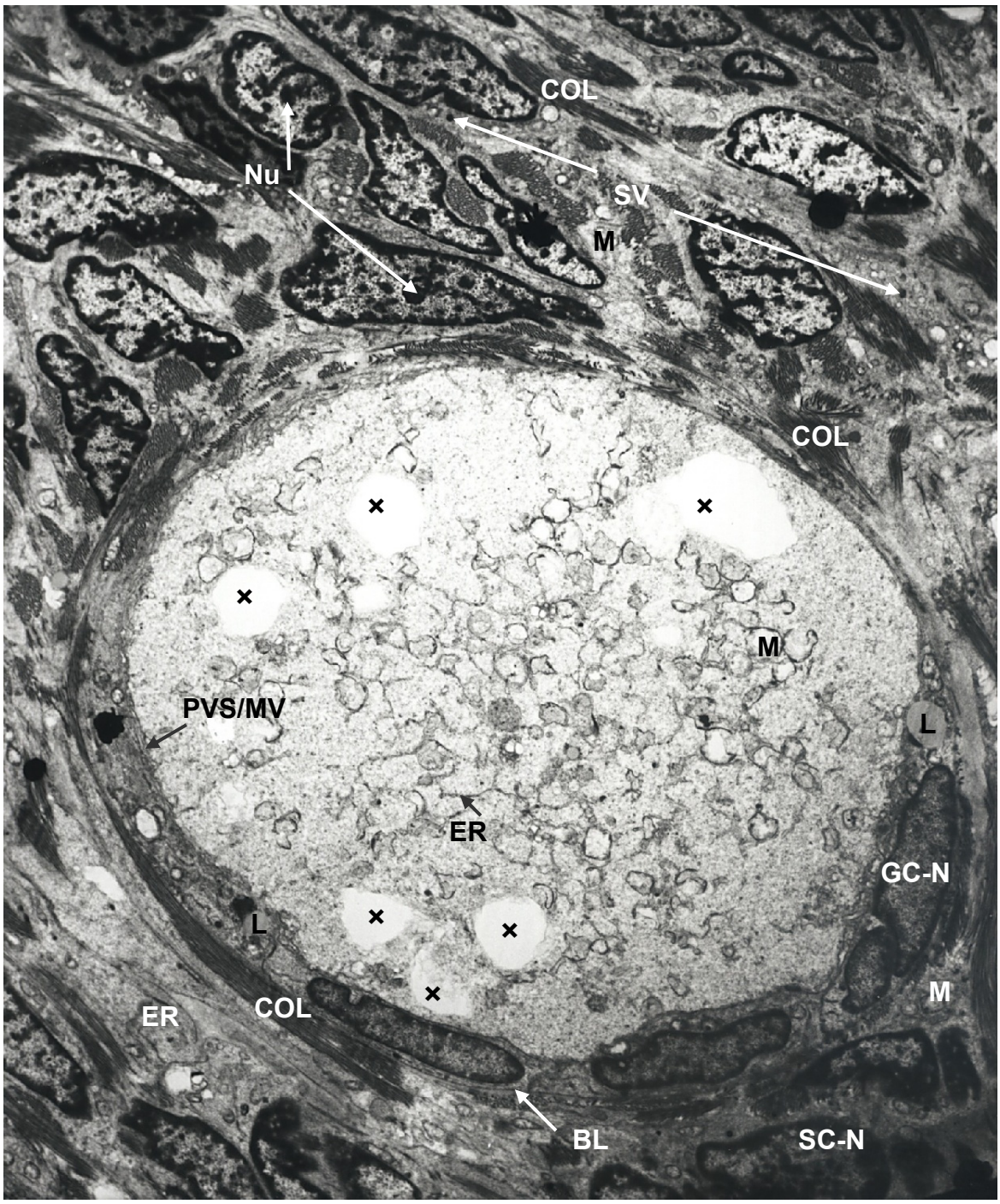



*Figure 3.5.* Micrograph of a DMSO equilibrated primordial follicle with large areas of lysis (×) in the oocyte cytoplasm. Ultrastructures present for evaluation were stromal nuclei (SC-N) with some nucleoli (Nu), secretory vesicles (SV), mitochondria (M) and endoplasmic reticulum (ER). The granulosa layer contained granulosa cell nuclei (GC-N), lipid droplets (L) a thin basal lamina (BL) and minimal microvilli(MV) and perivitelline space (PVS) were visible.

The nuclei of the granulosa cells (GC-N) were elongated and indented with peripheric heterochromatin and in fair condition (2). The nuclear envelope (2), nuclear lamina (2) and chromatin (2) showed severe damage. Nuclear pore complexes were absent.

The granulosa cell cytoplasm showed a poor condition where the membrane (1), mitochondria (M) (1,2), endoplasmic reticulum (ER) (1,2), and cytosol (1,2) were in a degenerative or severely damaged state. Lysis of some mitochondria (M) was observed (low electron density / whiteness). The secretory vesicles (SV) (5) and lipid droplets (L) (5) were normal (*see Fig.3.6.*).

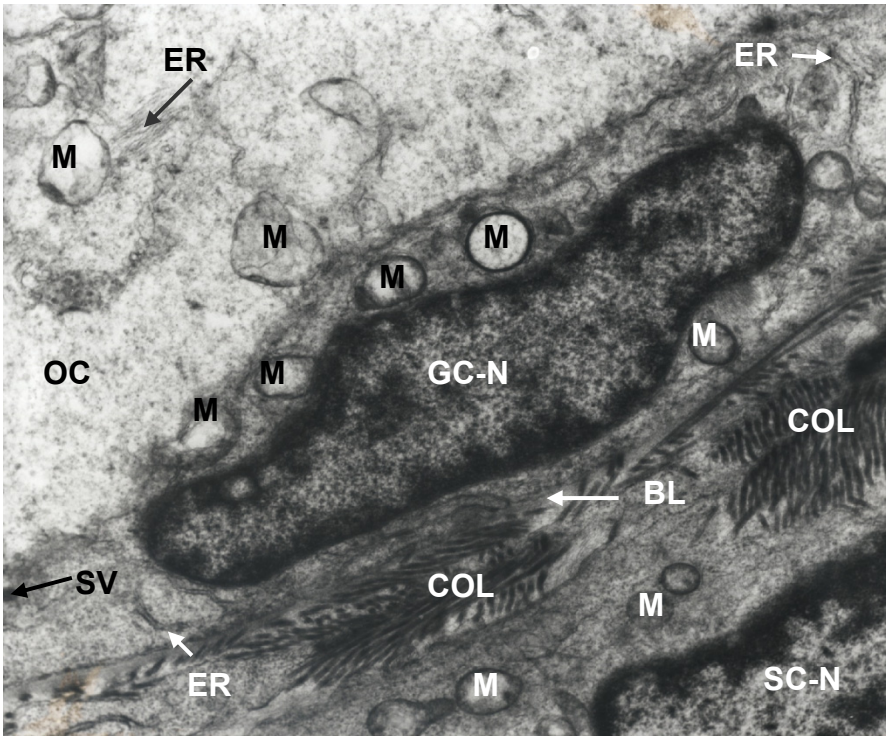



*Figure 3.6.* Higher magnification of the granulosa cell layer of the DMSO equilibrated primordial follicle. Ultrastructures evaluated here were the basal lamina (BL), secretory vesicles (SV), mitochondria (M) and endoplasmic reticulum (ER). stromal nuclei (SC-N) with some nucleoli (Nu), The neighbouring oocyte cytoplasm (OC) and stromal cell nucleus (SC-N) are indicated.x24000.

The follicle of patient 3 in the DMSO equilibration group demonstrated areas of lysis mainly in the surrounding stroma that were not seen in the granulosa cells or the oocyte. The single layer of squamous granulosa cells indicated a primordial stage follicle. TEM processing artefacts were observed (∞) (*see Figs.3.7.,3.8.*).

The stromal cells suffered severe damage, where the stromal membranes (1), cytosol (1) and organelles (1) were of degenerative quality. The nuclei (SC-N) (1) were elongated, indented and condensed with severe damage and degenerative properties. The extracellular matrix (2) showed severe damage and the basal lamina (BL) (2,3) presented with recoverable to severe damage (*see Fig.3.7.*).

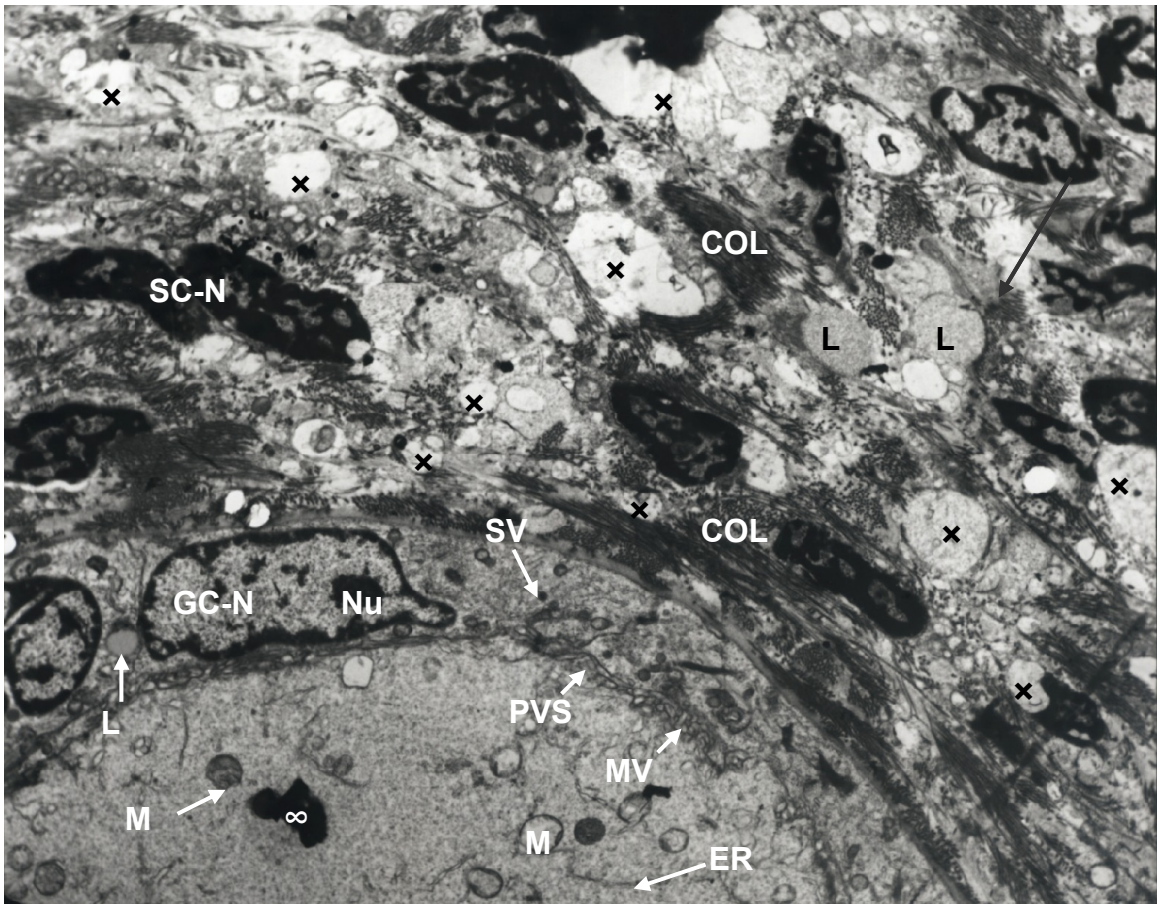



*Figure 3.7.* A primordial follicle in the DMSO equilibration group with lytic areas (**x**) in the stroma. Stromal cell nuclei (SC-N), collagen bundles (COL) and lipid droplets (L) were present in the stroma. Lipid droplets (L), secretory vesicles (SV), perivitelline space (PVS), microvilli (MV), mitochondria (M) and endoplasmic reticulum (ER) were observed in the follicle. TEM artefacts are indicated (∞).x2000-6000.

The granulosa cells were of average quality mostly with recoverable damage. The nuclei of the granulosa cells (GC-N) were elongated and indented (#) with peripheric heterochromatin. The nuclear envelope (3), nuclear lamina (3), heterochromatin (3) and nucleoli (Nu) (3) demonstrated recoverable damage while the euchromatin (2) suffered more severe damage (*see Figs.3.8.A,3.8.B*).

The cytoplasm of the granulosa layer was in a fair state with recoverable to severe damage to the membrane (ICS: intracellular separation) (2,3), and severe damage to the secretory vesicles (SV) (2), mitochondria (M) (2), smooth and rough endoplasmic reticulum (ER) (2), lipid droplets (L) (2), microtubules (2) and cytosol (2) (*see Figs.3.7.,3.8.*).

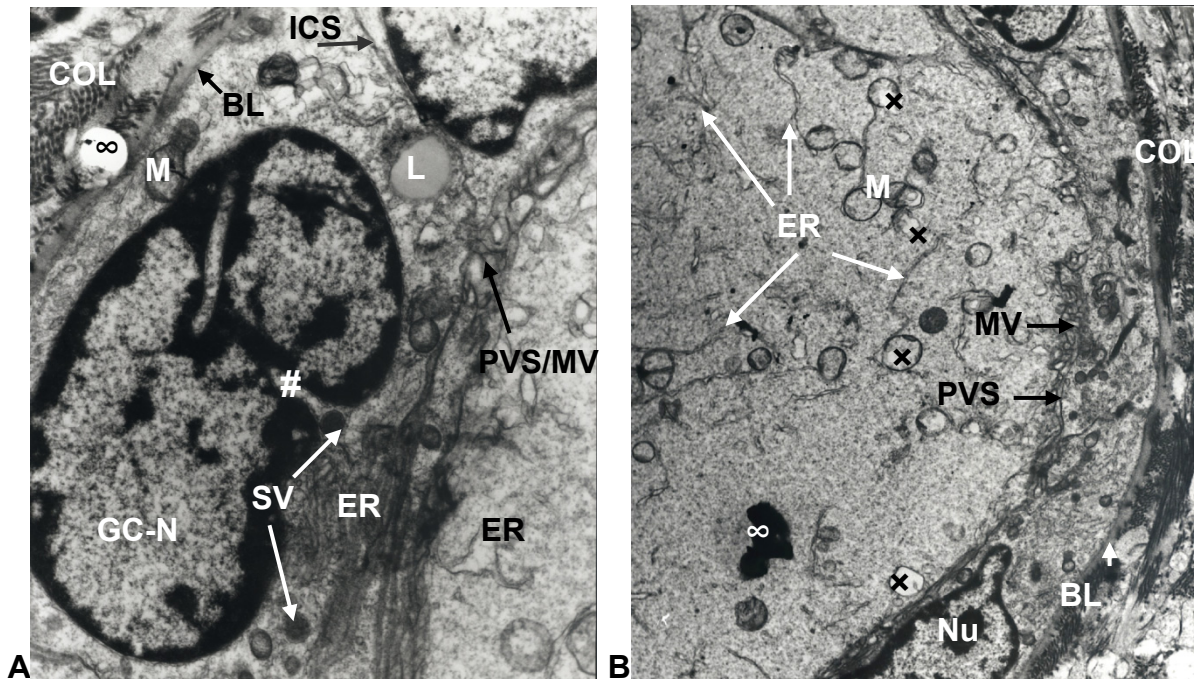



*Figure 3.8.* Higher magnifications of areas in the granulosa layer and oocyte of a primordial follicle in the DMSO equilibration group. TEM artefacts were indicated (∞). A) The granulosa cell nucleus (GC-N) showed indentation (#) and the cytoplasm contained secretory vesicles (SV), endoplasmic reticulum (ER), lipid droplets (L) and mitochondria (M). The intracellular separation (ICS) of the neighbouring granulosa membranes was clearly visible.x24000. B) Several of the mitochondria in the oocyte showed lysis (**x**). The granulosa cell nucleoli (Nu), microvilli (MV), perivitelline space (PVS), and basal lamina (BL) were visible, as well as collagen bundles (COL).x12000.

The oocyte cytoplasm showed severe damage of the mitochondria (2), smooth and rough endoplasmic reticulum (ER) (2) and cytosol (2). The nucleus and other cytoplasmic ultra structures were absent. Lysis (**x**) of several mitochondria could be seen in this follicle (*see Fig.3.8.B.*).

The perivitelline space (PVS) (2) and associated microvilli (MV) (2,3) were minimally present and showed severe damage (*see Fig.3.8.B.*).

A follicle from the **PROH** equilibrated sample of patient 1 showed several areas of lysis (**x**) in the stroma and centre of the follicle. The single layer of cuboidal granulosa cells indicated a primary follicle. The oocyte was absent on the micrograph (*see Fig.3.9.*).

The stromal cells showed signs of degeneration with poor quality membranes (1), cytosol (1), and organelles (1). The nuclei (SC-N) (1) were elongated and indented with peripheric heterochromatin.

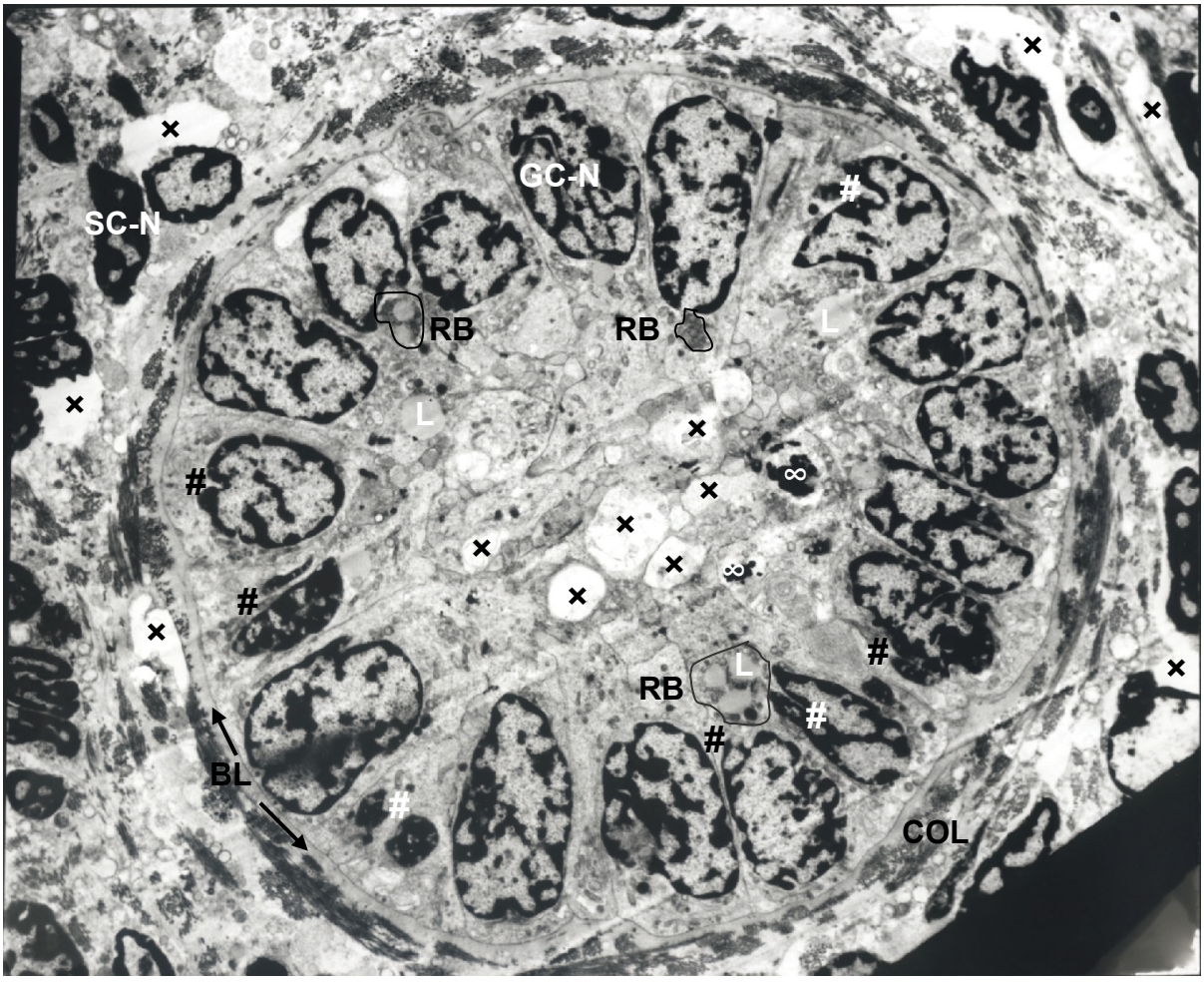



*Figure 3.9.* A primary follicle of a PROH equilibration sample. The oocyte was absent. Lytic damage (**x**), granulosa nuclei indentation (#) and TEM artefacts (∞) were indicated. Several lipid droplets (L), some in refractile bodies (RB), were present in the granulosa cytoplasm. The basal lamina (BL) and collagen bundles (COL) were observed.x1500-4500.

The extracellular matrix (ECM) (5) and basal lamina (BL) (5), however, showed no damage (*see Fig.3.10.*).

The granulosa nuclei (GC-N) were large, oval, indented with peripheric heterochromatin and showed normal morphology, where the nuclear envelope (5), nuclear lamina (5), heterochromatin (5), euchromatin (5) and nucleoli (5) showed no damage. The desmosomes (D) (5), gap junctions (GJ) (5), refractile bodies (RB), golgi complexes (G) (5), secretory vesicles (SV), lipid droplets (L) (5), centrosomes (Ce) (5), intermediary- and microfilaments (IF/MF) (5) showed no damage. The mitochondria (M) (2,3), endoplasmic reticulum (ER & RER) (2,3) and cytosol (2) showed recoverable to severe damage (*see Fig.3.10.*).

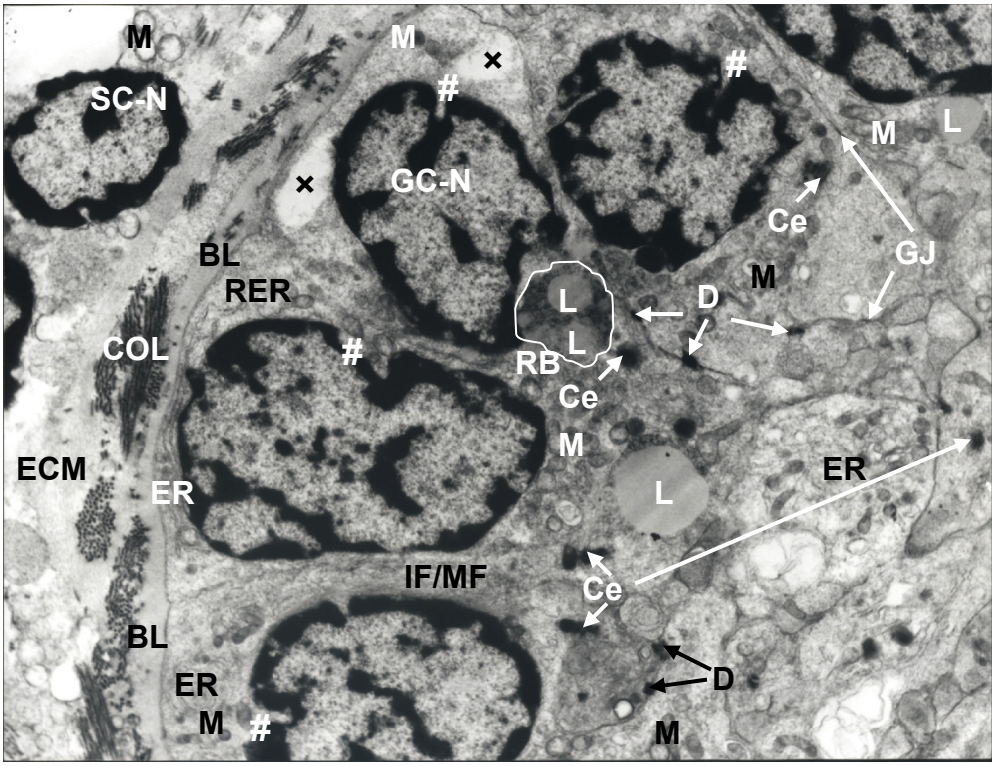



*Figure 3.10.* Higher magnification of a part of the granulosa layer of the PROH equilibrated follicle. Desmosomes (D) and centrosomes (Ce) were especially abundant. Gap junctions (GJ) are also clearly seen. Several lipid droplets (L), some in a refractile body (RB) were observed. Areas of lysis (**x**) and granulosa nuclei indentations (#) are indicated. The basal lamina (BL), mitochondria (M), endoplasmic reticulum (ER & RER), intermediary- and microfilaments (IF/MF) were visible. Stromal nuclei (SC-N), collagen bundles (COL) and extracellular matrix (ECM) were observed.x12000.

### 2.1.3. Cryopreserved and thawed samples

The follicle of patient 6 in the **DMSO** cryopreserved group showed good cell survival [appeared to have good cell integrity with only small areas of lysis visible, but higher magnifications revealed actual severe damage to the ultrastructures.] with small visible areas of damage in the stroma. A single layer of a mixture of squamous and cuboidal granulosa cells surrounding the oocyte indicated a primordial follicle in transition phase towards the primary stage. TEM processing artefacts were visible on the micrograph and are indicated (∞) (*see Fig.3.11.*).

Some areas of the stroma (2) suffered severe lytic damage (**×**) and most stromal cell membranes (1), cytosol (1) and organelles (1) showed degeneration. The stromal cell nuclei (SC-N) were elongated and indented with peripheric heterochromatin (*see Fig.3.11.*).

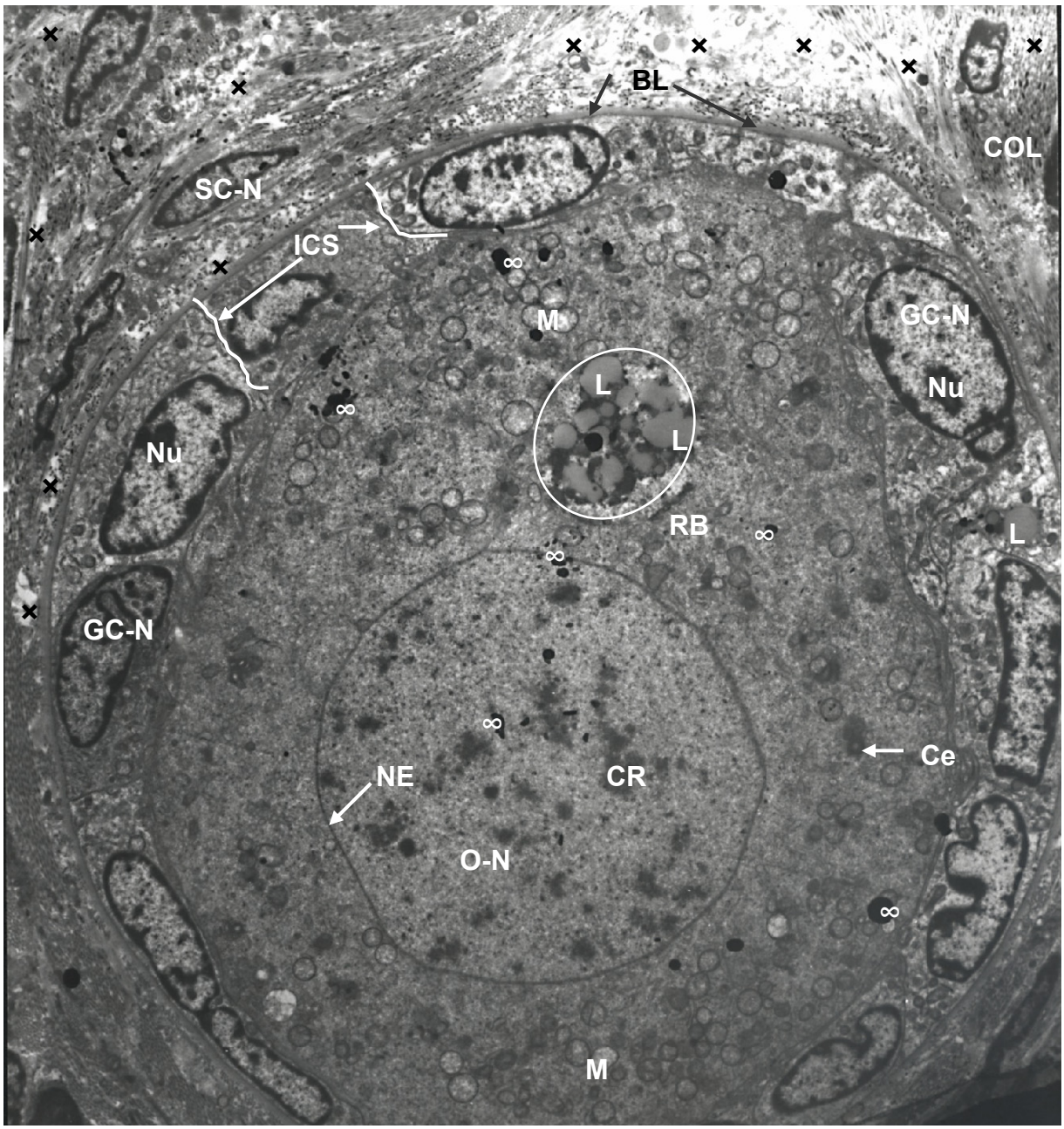



*Figure 3.11.* Micrograph of the DMSO cryopreserved primordial follicle with small areas of lysis (×) in the stroma. TEM artefacts were indicated (∞). The stromal nuclei (SC-N), collagen bundles (COL) and the basal lamina (BL) are visible. The granulosa layer contained granulosa cell nuclei (GC-N), lipid droplets (L). The intracellular separations (ICS) by the granulosa cell membranes were clearly seen in this follicle. Abundant mitochondria (M) and endoplasmic reticulum (ER) were present in the oocyte as well as a centrosome (Ce) and a giant refractile body containing several lipid droplets (L).x1500-4500.

Higher magnification of the granulosa cell layer showed that the nuclei of the granulosa cells were oval and indented (#) with peripheric heterochromatin but showed no signs of damage since the membranes (ICS) (5) nuclear envelope (NE) (5), nuclear lamina (5), chromatin (5) and nucleoli (Nu) (5) were normal. The perivitelline space (PVS) (5) was minimally present but appeared normal with normal associated microvilli (MV) (5). The rough endoplasmic reticulum (RER) (5) showed no signs of damage (*see Fig.3.12., 3.13.*).

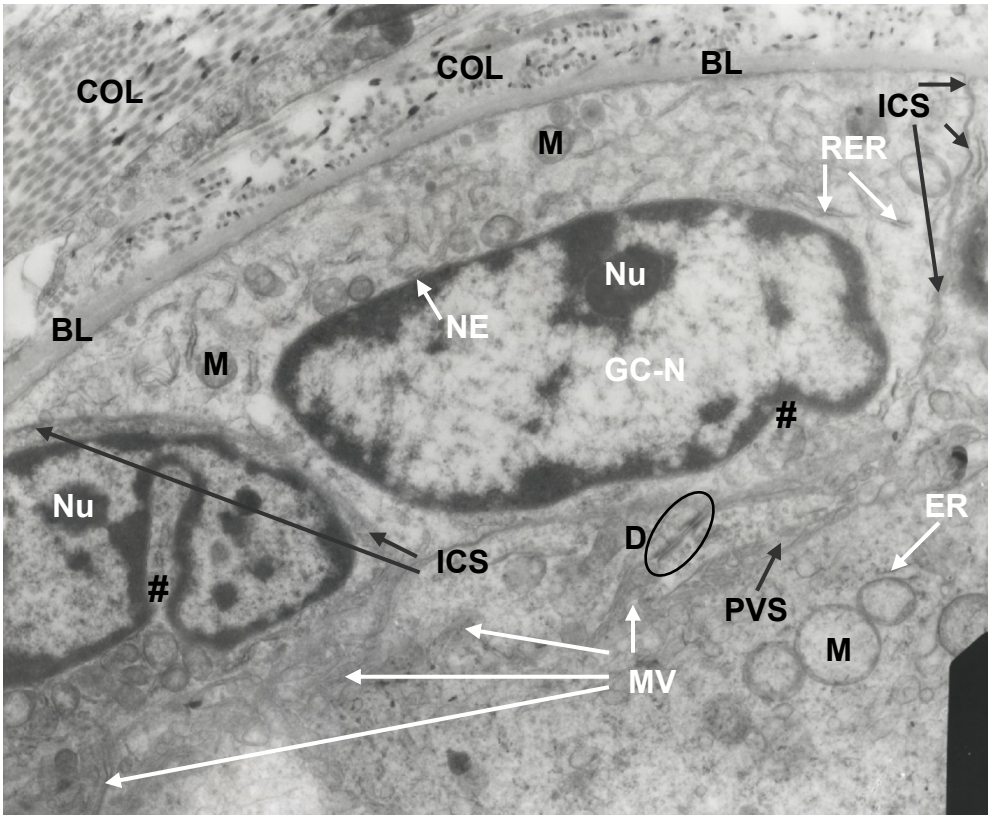



*Figure 3.12.* Higher magnification micrograph of the granulosa cell layer of the DMSO cryopreserved primordial follicle. The collagen bundles (COL) and the basal lamina (BL) was visible. The granulosa layer contained granulosa cell nuclei (GC-N) and the intracellular separations (ICS) by neighbouring granulosa cell membranes were clearly defined in this follicle. Indentations in the granulosa nuclei are indicated (#). Rough endoplasmic reticulum (RER) was shown in the granulosa cytoplasm (RER). Mitochondria (M) and endoplasmic reticulum (ER) were present in the oocyte cytoplasm.x18000.

Another high magnification micrograph of the oocyte showed that organelles were distributed throughout the ooplasm and the nucleus was round, situated centrally and was euchromatic (5). The nuclear envelope (NE) (5) and nuclear pore complexes (NPC) (5) were normal. A giant refractile body (RB) (5) containing multiple lipid droplets (L) (5) showed no signs of damage. The endoplasmic reticulum (ER) (5), nuages (n) (5) and centrosome (Ce) (5) were normal. Damaged ultrastructures were the cytosol (3,4) showing little to recoverable damage, and the mitochondria (M) (2,3) which showed recoverable to severe damage (*see Fig.3.13.*).

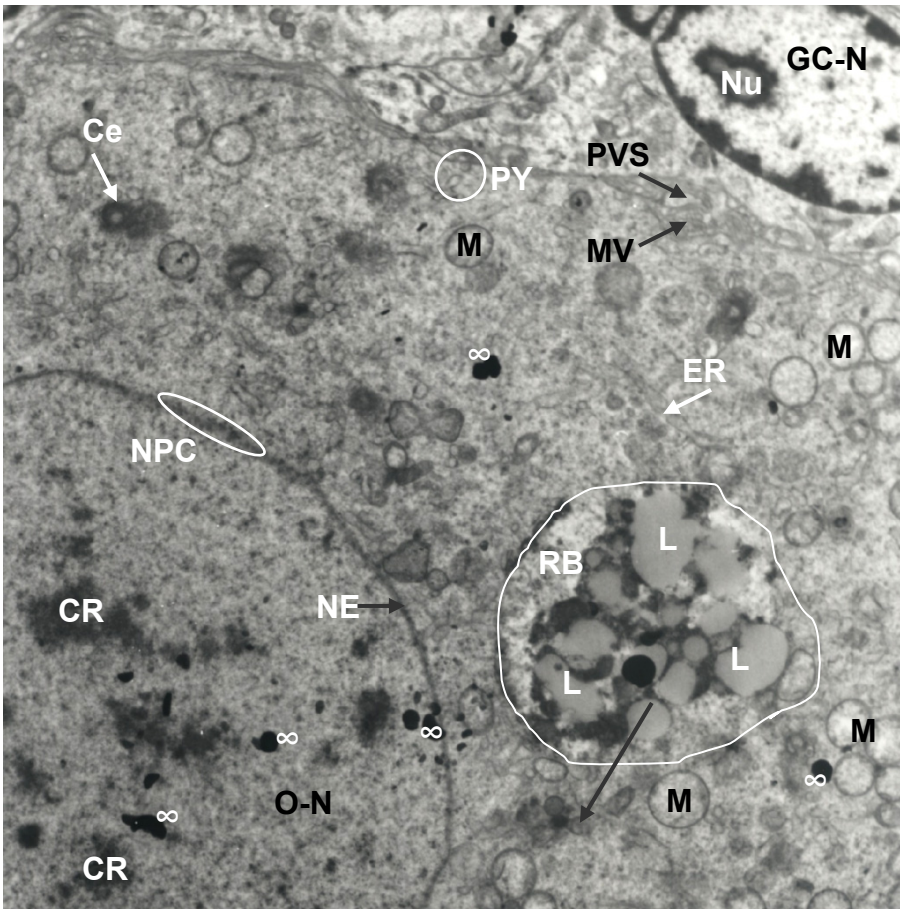



*Figure 3.13.* Higher magnification of the oocyte cytoplasm and oocyte nucleus (O-N) DMSO cryopreserved primordial follicle. Chromosomes (CR) were visible in the oocyte nucleus with nuclear pore complexes (NPC) in the nuclear envelope (NE). A giant refractile body (RB) containing several lipid droplets (L) are shown. A centrosome (Ce), abundant mitochondria (M) and endoplasmic reticulum (ER) were present in the oocyte cytoplasm. Microvilli (MV), pynocytosis (PY) and a perivitelline space (PVS) are seen at the oocyte membrane. A granulosa nucleus (SC-N) and nucleolus (Nu) was also visible. Several sites of TEM artefacts were indicated (∞).x9000.

In the **PROH** cryopreserved sample, the follicle of patient 1 showed a large area of vacuolisation (**v**) and several smaller lytic areas (**x**) in the granulosa cell cytoplasm. One layer of granulosa cells, with a mixture of squamous and cubic forms, showed a primordial follicle in transition to the primary phase (*see Fig.3.14.*).

Stromal cells were too few and incomplete for evaluation and the oocyte was also absent. The extracellular matrix (ECM) (5), however, did not show signs of damage (*see Fig.3.14.*).

Most ultrastructures of the granulosa cytoplasm present were normal. The granulosa cell membranes (5), microvilli (5), desmosomes (D) (5), giant refractile bodies (RB) (5), secretory vesicles (SV) (5), endoplasmic reticulum (ER) (5), lipid droplets (L) (5), centrosomes (Ce) (5), intermediary- and microfilaments (IF/MF) (5) and microtubules (5) showed no signs of damage. The mitochondria (M) (1,2) and cytosol (1,2,3), however, suffered severe damage (*see Fig.3.14.*).

The granulosa cell nuclei (GC-N) were oval, large and indented (#) with abundant peripheric chromatin. The nuclear envelope (5), nuclear lamina (5), euchromatin (5) and nucleoli (5) were normal. Marginalisation (MAR) of heterochromatin was observed in some granulosa cell nuclei indicating possible apoptosis initiation (AP). Apoptotic bodies (APB) were present in the granulosa cytoplasm (*see Fig.3.14.*).

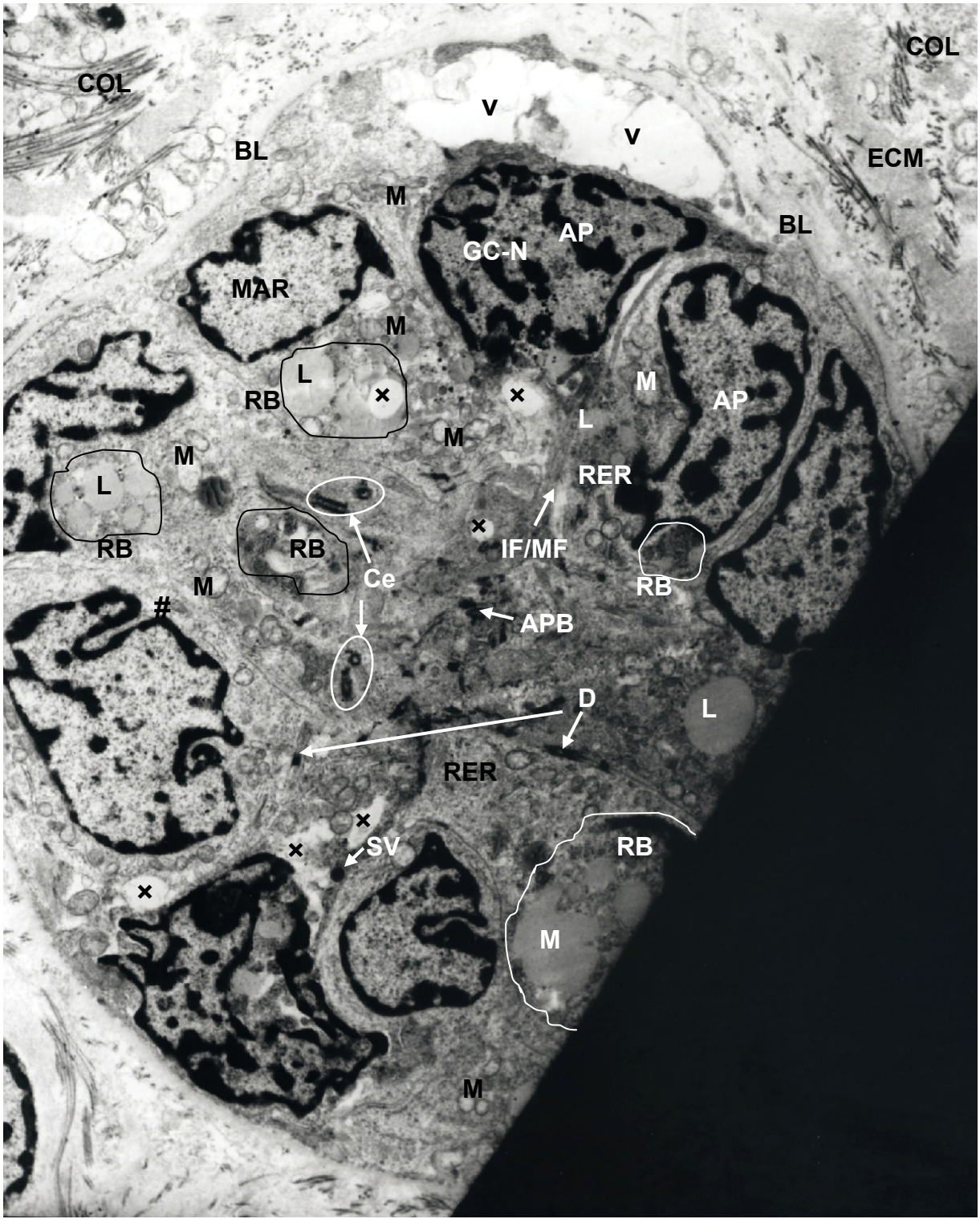



*Figure 3.14.* Electron micrograph of a PROH cryopreserved follicle. Vacuolisation (v) and lysis (**x**) was observed in the granulosa layer. Some granulosa cell nuclei (GC-N) showed marginalisation (MAR) and apoptosis (AP). Apoptotic bodies (APB) were observed. Large refractile bodies (RB) and abundant lipid droplets were seen. Centrosomes (Ce), desmosomes (D), secretory vesicles (SV), mitochondria (M), rough endoplasmic reticulum (RER), intermediary-and microfilaments (IF/MF) were present in the granulosa cells. Indentation of nuclei (#) was indicated. Collagen bundles and the extracellular matrix were present.x3000.

## 2.2. Comparative studies

The scores of the follicle ultrastructures that were most frequently present for evaluation were used in the statistical analysis. The mean scores of the sample groups were then compared to assess the probability of follicle damage caused by equilibration and cryopreservation treatment. The statistical analysis tested the extent of the equilibration and cryopreservation treatment effect (damage) on the follicles compared to the patients' fresh control sample. Existence of possible trends between groups was investigated with post hoc analysis.

*Table 5*. Summary of the overall treatment effect (equilibration and cryopreservation) test results on follicle structures compared to the control sample.

| Evaluated Follicle Structure | *P* | Result |
|---|---|---|
| Stroma | 0.85 | NS |
| Stromal cells | 0.57 | NS |
| Extracellular matrix | 0.15 | Trend? |
| Basal lamina | 0.74 | NS |
| Granulosa cells | 0.34 | Trend? |
| Cytoplasm of granulosa cells | 0.35 | Trend? |
| Mitochondria of granulosa cells | 0.60 | NS |
| Smooth and Rough endoplasmic reticulum of granulosa cells | 0.13 | Trend? |
| Nuclei of granulosa cells | 0.09 | Trend? |
| Oocyte | 0.62 | NS |
| Perivitelline space | 0.32 | Trend? |
| Cytoplasm of oocytes | 0.54 | NS |
| Mitochondria of oocytes | 0.91 | NS |
| Smooth and Rough endoplasmic reticulum of oocytes | 0.69 | NS |
| Nuclei of oocytes | 0.14 | Trend? |

Sample groups were labelled as follows: FC: fresh control sample, DE: DSMO equilibrated, PE: PROH equilibrated, DCT: DSMO cryopreserved and thawed and PCT: PROH cryopreserved and thawed.

Significant differences between groups were indicated by different letters, and no significant differences were indicated by same letters (*see Chapter 2*).

### 2.2.1. Stroma

The stroma showed no treatment effect on the treatment groups compared to the fresh control group and (*P* = 0.85) (*see Fig.3.15.*).

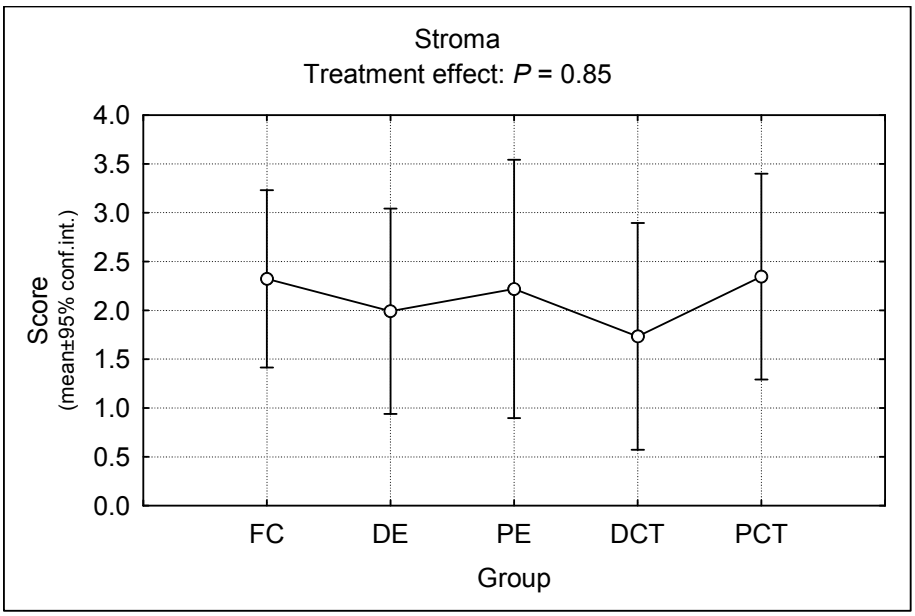



*Figure 3.15*. A graph illustrating the mean scores ±95% confidence intervals of the stroma.

No significant treatment effect on the extracellular matrix was indicated (*P* = 0.15). Post hoc analysis indicated a possible trend of higher scores in PROH cryopreserved and thawed group compared to the fresh control group (FC vs. PCT: *P* = 0.03) (*see Fig.3.16.*).

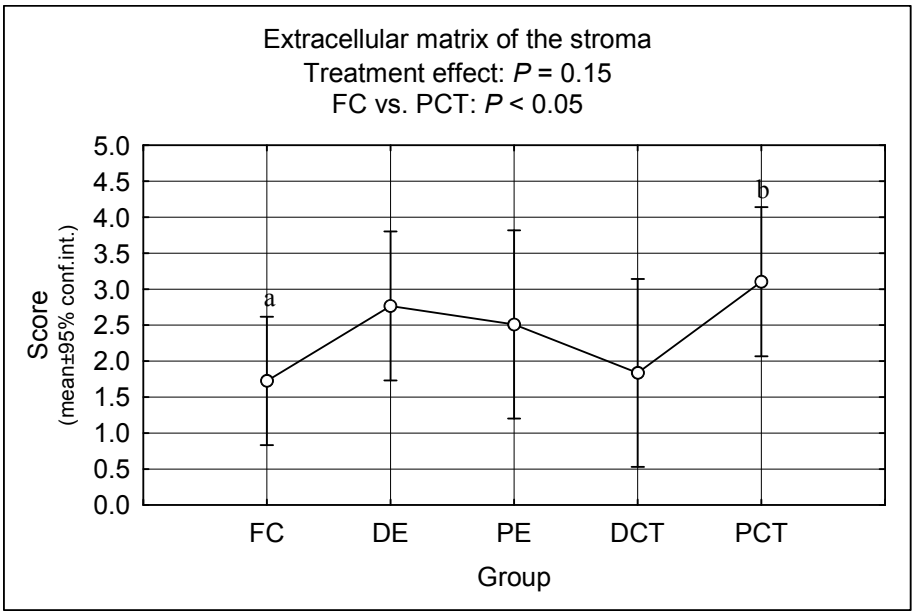



*Figure 3.16*. A graph illustrating the mean scores ±95% confidence intervals of the extracellular matrix.

There was no significant treatment effect on the basal lamina compared to the fresh control group ($P$ = 0.74) (*see Fig.3.17.*).

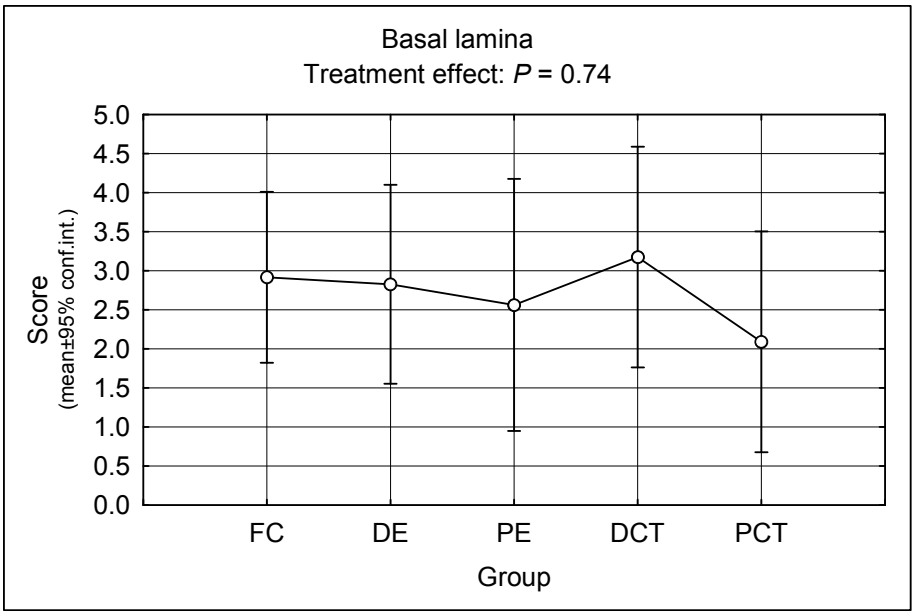



*Figure 3.17*. A graph illustrating the mean scores ±95% confidence intervals of the basal lamina.

### 2.2.2. Granulosa cell layer

No significant treatment effect was shown on the granulosa cells compared to the fresh control group (*P* = 0.34). Post hoc analysis showed a possible trend of severe treatment damage in the PROH cryopreserved and thawed group compared to the fresh control group (FC vs. PCT: *P* = 0.05) (*see Fig3.18.*).

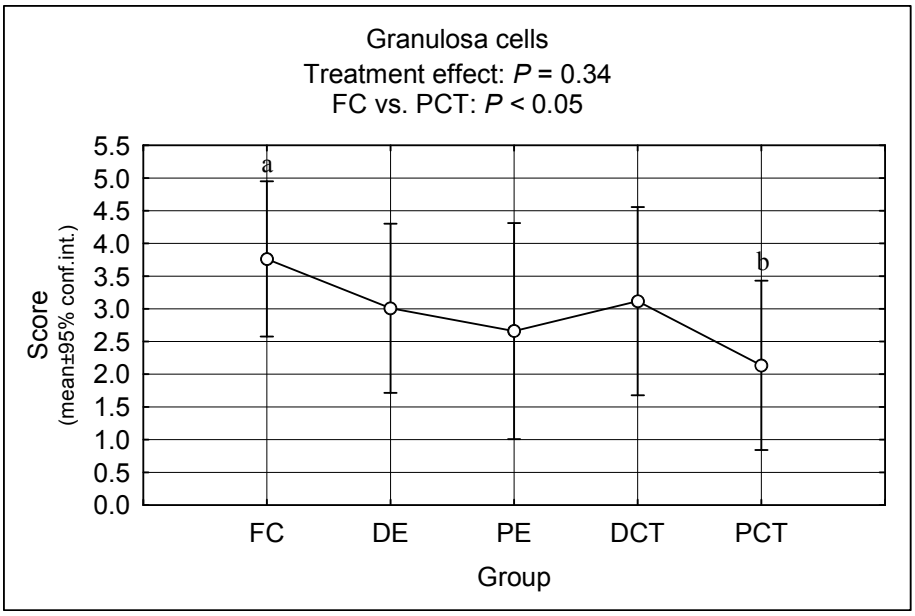



*Figure 3.18*. A graph illustrating the mean scores ±95% confidence intervals of the granulosa cells.

A marginally significant treatment effect was shown in the nuclei of the granulosa cells (*P* = 0.09). Post hoc analysis revealed a possible trend of severe treatment damage in the PROH equilibration group, DMSO cryopreserved and thawed group and the PROH cryopreserved and thawed groups compared to the fresh control (FC vs. PE: *P* = 0.02; FC vs. DCT: *P* = 0.03; FC vs. PCT: *P* = 0.02). The extent of the treatment effect (damage) was similar in the treatment groups since their scores did not differ significantly from each other (*see Fig.3.19.*).

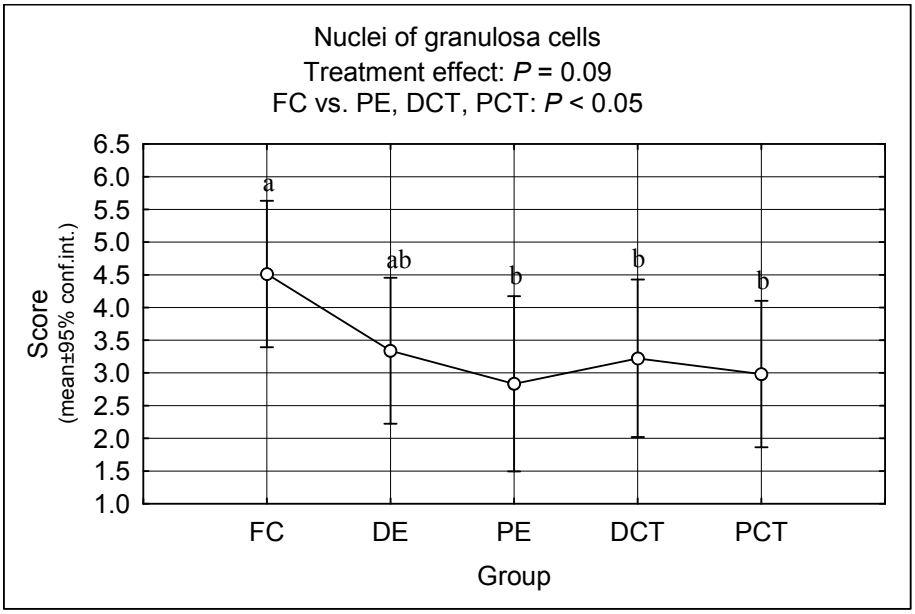



*Figure 3.19*. A graph illustrating the mean scores ±95% confidence intervals of the nuclei of the granulosa cells.

No significant treatment effect was shown in the cytoplasm (*P* = 0.35) or the mitochondria (*P* = 0.60) of the granulosa cells (*see Table 5*.).

The smooth and rough endoplasmic reticulum did not show a treatment effect (*P* = 0.12) but post hoc analysis indicated a possible trend of more severe damage in the PROH equilibration and PROH cryopreserved and thawed groups compared to the fresh control (FC vs. PE: *P* = 0.04; FC vs. PCT: *P* = 0.02) (The smooth and rough ER were reported as one entity here since all of their scores correlated exactly (*see Fig.3.31.*).

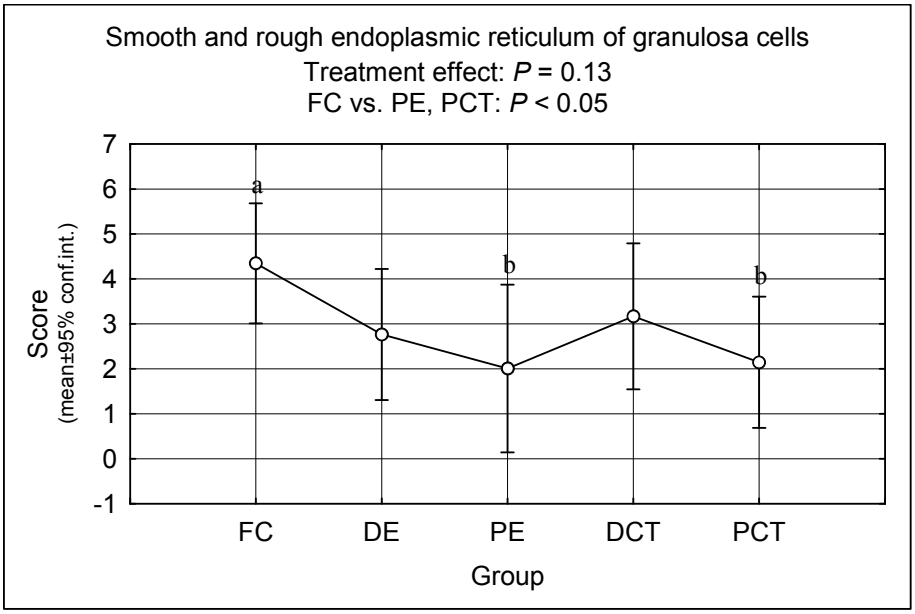



*Figure 3.20*. A graph illustrating the mean scores ±95% confidence intervals of the smooth and rough endoplasmic reticulum of the granulosa cells.

### 2.2.3. Oocyte

No significant treatment effect was shown on the oocyte ($P$ = 0.62) (*see Fig.3.21.*).

The oocyte cytoplasm ($P$ = 0.64), oocyte mitochondria ($P$ = 0.97) and oocyte smooth and rough ER ($P$ = 0.69) did not show any treatment effect between the groups (*see Table 5.*) (The smooth and rough ER are reported as one entity since all of the scores correlated exactly (*see Fig.3.32.*).

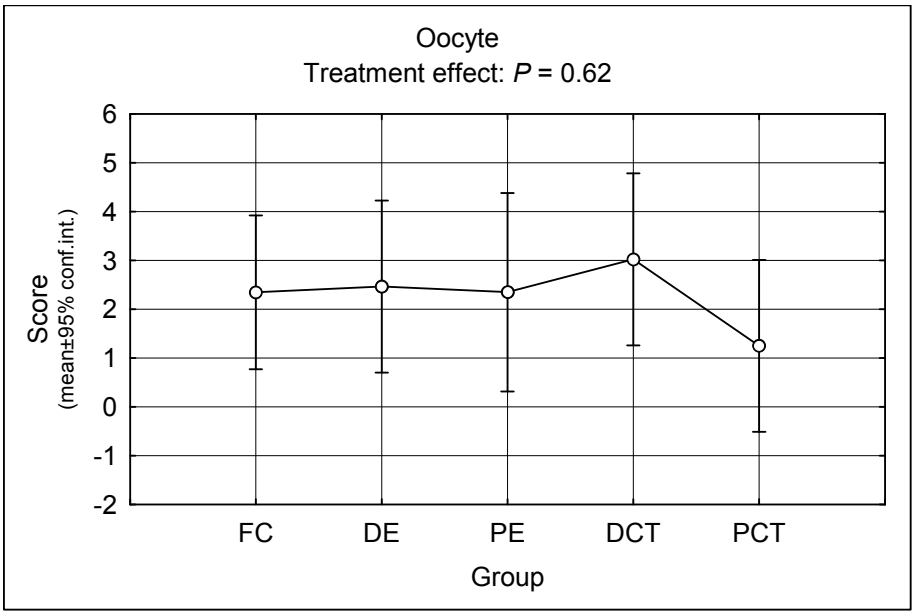



*Figure 3.21*. A graph illustrating the mean scores ±95% confidence intervals of the oocyte.

The perivitelline space showed no treatment effect compared to the fresh control

group (*P* = 0.32) (*see Fig.3.22.*).

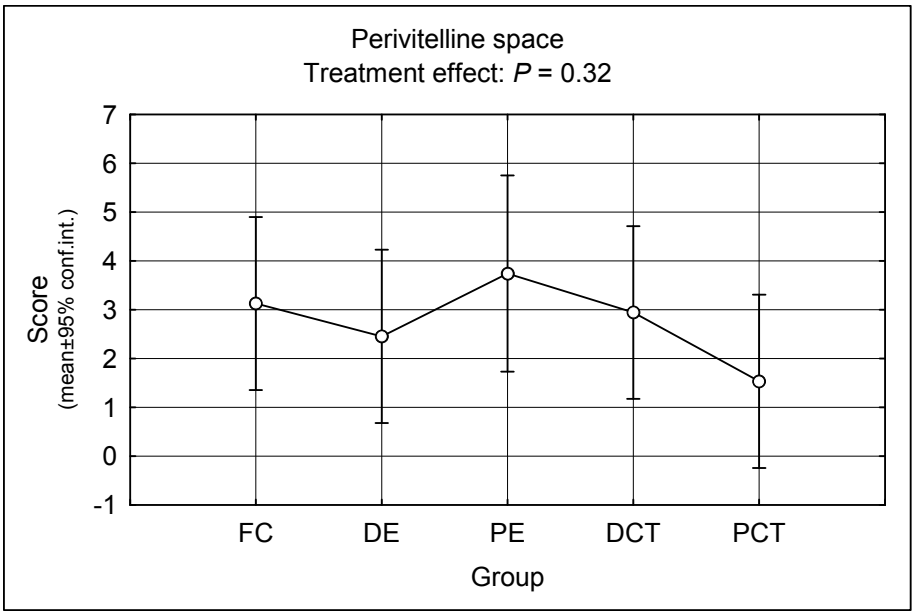



*Figure 3.22.* A graph illustrating the mean scores ±95% confidence intervals of the perivitelline space.

The nuclei of the oocytes did not show a significant overall treatment effect compared to the fresh control group (*P* = 0.14). Post hoc analysis, however, revealed a possible trend of more severe treatment damage in the PROH equilibration and PROH cryopreserved and thawed groups compared to the fresh control group (FC vs. PE: *P* = 0.03; FC vs. PCT: *P* =0.03) (*see Fig.3.23.*).

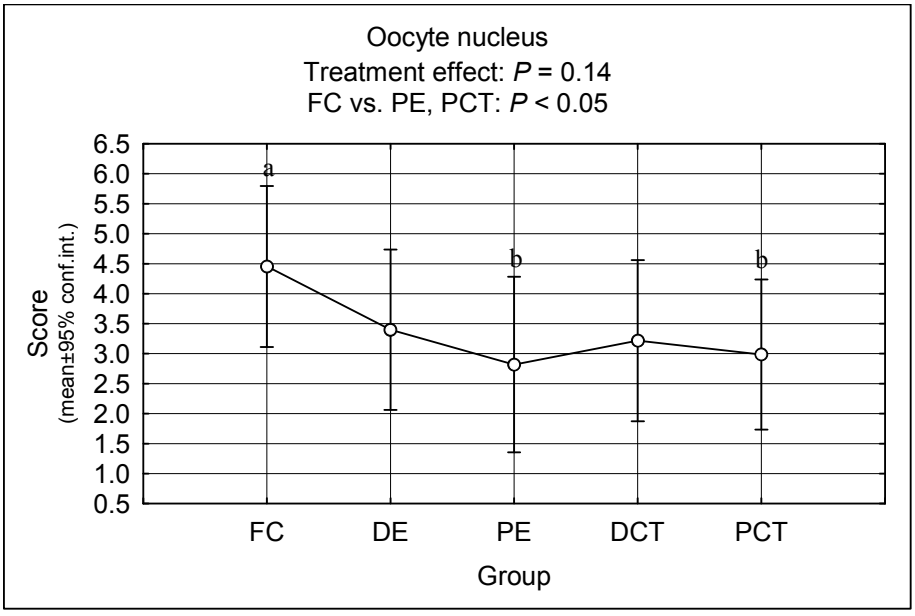



*Figure 3.23.* A graph illustrating the mean scores ±95% confidence intervals of the oocyte nuclei.

## 2.3. Correlation studies

Reviewing of evaluations revealed recurring similarities between scores of some ultrastructures irrespective of the treatment group. Correlation studies were done to show relationships between scores of selected ultrastructures.

Strong correlations were shown between the evaluations of the selected structures (*see Table 6.*).

*Table 6*. Summary of the Spearman rank correlation test results on selected follicle structures.

| Compared Follicle Structures | | *r* | *P* | Relationship |
|---|---|---|---|---|
| Stroma | Granulosa cells | 0.60 | <0.01 | Positive linear |
| Stroma | Oocyte | 0.68 | <0.01 | Positive linear |
| Granulosa cells | Oocyte | 0.77 | <0.01 | Positive linear |
| Nuclei of granulosa cells | Nuclei of oocytes | 0.86 | <0.01 | Positive linear |
| Cytoplasm of granulosa cells | Cytoplasm of oocytes | 0.68 | <0.01 | Positive linear |
| Mitochondria of granulosa cells | Mitochondria of oocytes | 0.62 | <0.01 | Positive linear |
| Smooth and Rough endoplasmic reticulum of granulosa cells | Smooth and Rough endoplasmic reticulum of oocytes | 0.65 | <0.01 | Positive linear |
| Stroma | Extracellular matrix | -0.18 | 0.44 | NS |
| Basal lamina | Granulosa cells | 0.71 | <0.01 | Positive linear |
| Basal lamina | Oocyte | 0.81 | <0.01 | Positive linear |
| Basal lamina | Perivitelline space | 0.85 | <0.01 | Positive linear |
| Basal lamina | Stromal cells | 0.07 | 0.75 | NS |
| Basal lamina | Extracellular matrix | 0.30 | 0.17 | NS |
| Perivitelline space | Granulosa cells | 0.90 | <0.01 | Positive linear |

Significant correlations and therefore, linear relationships were shown between scores of the stroma, granulosa cells and oocyte (Stroma vs. granulosa cells: $r$ = 0.60, $P$ <0.01; Stroma vs. Oocyte: $r$ = 0.68, $P$ < 0.01; Follicular cells vs. Oocyte: $r$ = 0.77, $P$ < 0.01) (*see Figs.3.24., 3.25., 3.26.*).

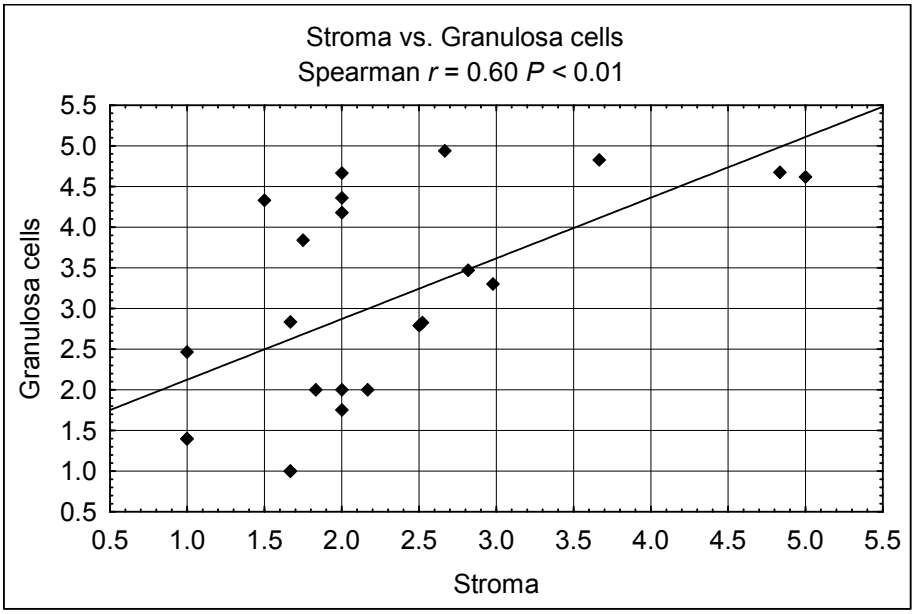



*Figure 3.24*. A correlation graph illustrating the significant positive linear relationship between the scores of the stroma and granulosa cells.

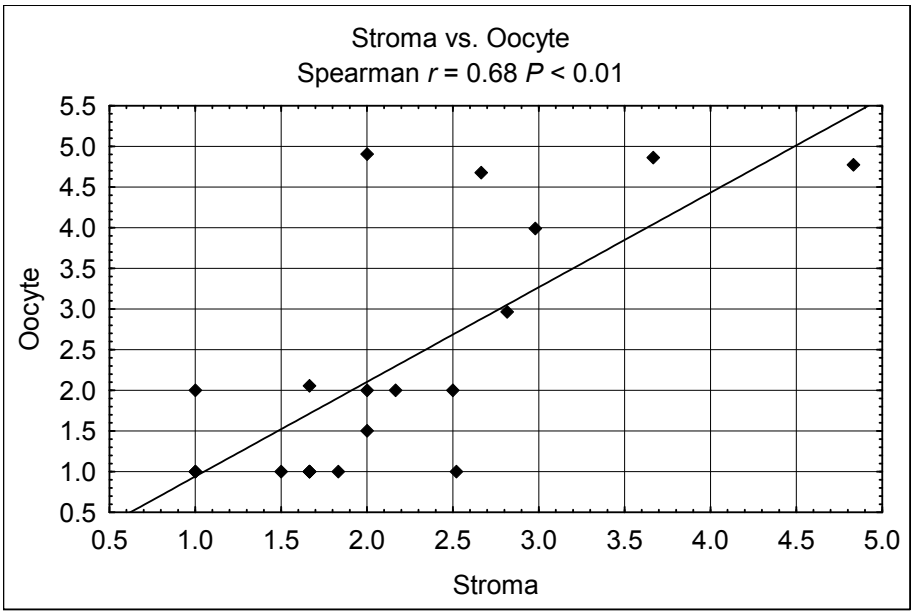



*Figure 3.25*. A correlation graph illustrating the significant positive linear relationship between the scores of the stroma and oocyte.

In agreement with the significant correlation between the granulosa cells with the oocyte (*see Fig.3.26*), significant positive correlations were also shown between the scores of matching organelles of the granulosa cells and the oocyte, such as the nuclei ($r$ = 0.86, $P$ < 0.01), mitochondria ($r$ = 0.62, $P$ < 0.01), cytoplasm ($r$ = 0.68, $P$ < 0.01) and smooth and rough ER ($r$ = 0.65, $P$ < 0.01) (*see Table 6.*).

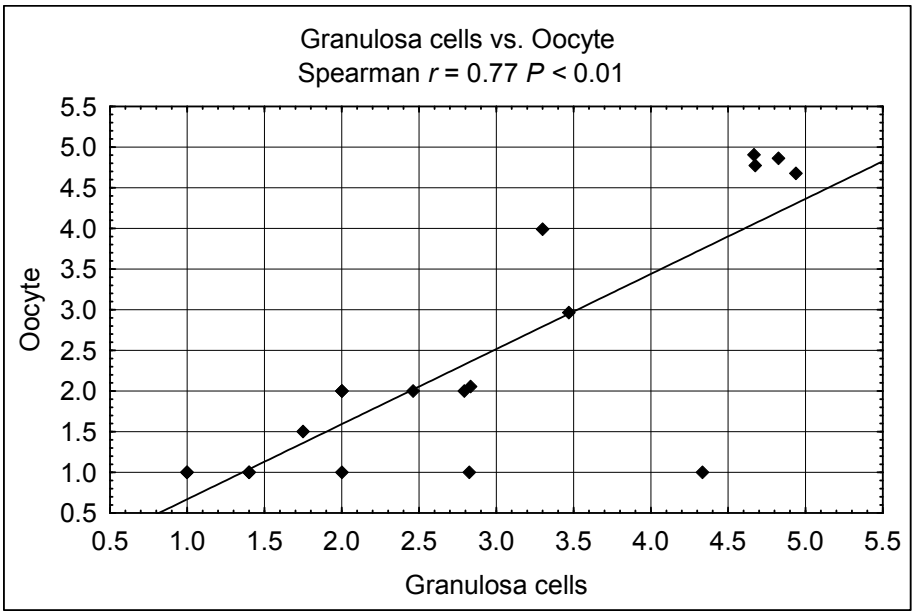



*Figure 3.26*. A correlation graph illustrating the significant positive linear relationship between the scores of the granulosa cells and oocyte.

The scores of the smooth and rough endoplasmic reticulum (ER) of the granulosa cells and of the oocyte, respectively, were exactly the same in all cases and showed an exact linear relationship (Granulosa cell smooth ER vs. Granulosa cell rough ER: $r$ = 1.00; Oocyte smooth ER vs. Oocyte rough ER: $r$ = 1.00) (*see Figs.3.27., 3.28.*).

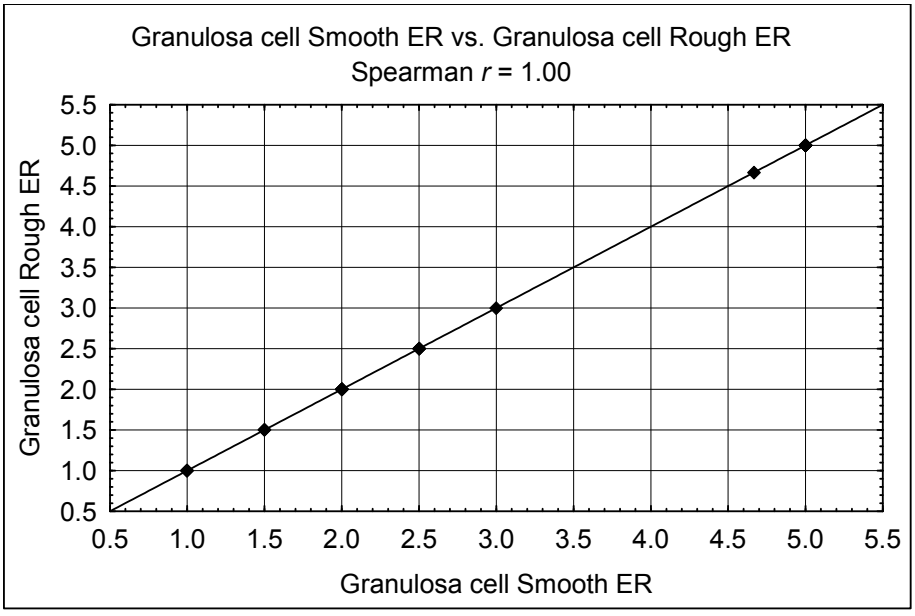



*Figure 3.27*. A correlation graph illustrating the exact positive linear relationship between the scores of the smooth and rough endoplasmic reticulum of the granulosa cells.

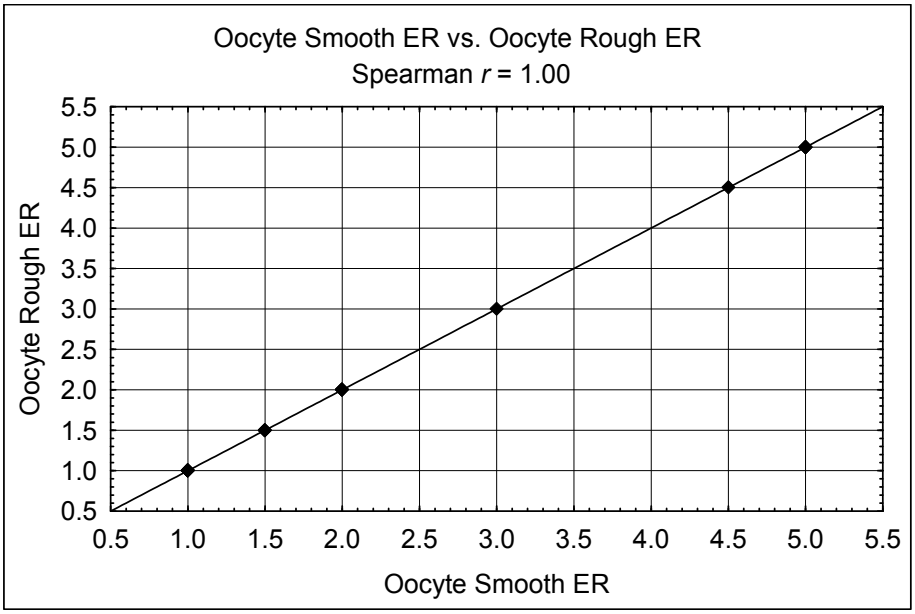



*Figure 3.28*. A correlation graph illustrating the exact positive linear relationship between the scores of the smooth and rough endoplasmic reticulum of the oocyte.

The basal lamina showed strong correlations with the granulosa cells ($r$ = 0.71, $P$ = <0.01) (*see Fig.3.29*.) as well with the organelles of the granulosa cells, i.e. nuclei ($r$ = 0.56, $P$ = 0.01), cytoplasm ($r$ = 0.74, $P$ = <0.01), mitochondria ($r$ = 0.63, $P$ = <0.01) and endoplasmic reticulum ( $r$ = 0.68, $P$ <0.01).

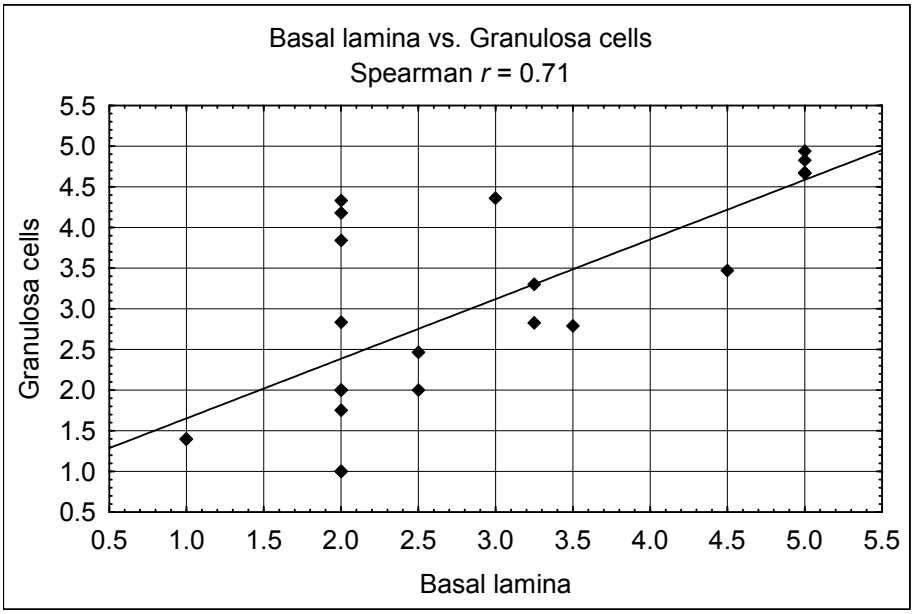



*Figure 3.29*. A correlation graph illustrating the significant positive relationship between the scores of the basal lamina and the granulosa cells.

The basal lamina also showed a significant linear relationship with the oocyte ($r$ = 0.81, $P$ <0.01) (*see Fig.3.30.*) as well as the nucleus ($r$ = 0.82, $P$ = <0.01), cytoplasm ($r$ = 0.79, $P$ = <0.01), mitochondria ($r$ = 0.75, $P$ = <0.01), endoplasmic reticulum ($r$ = 0.85, $P$ <0.0.1) and perivitelline space ($r$ = 0.85, $P$ < 0.0.1) of the oocyte.

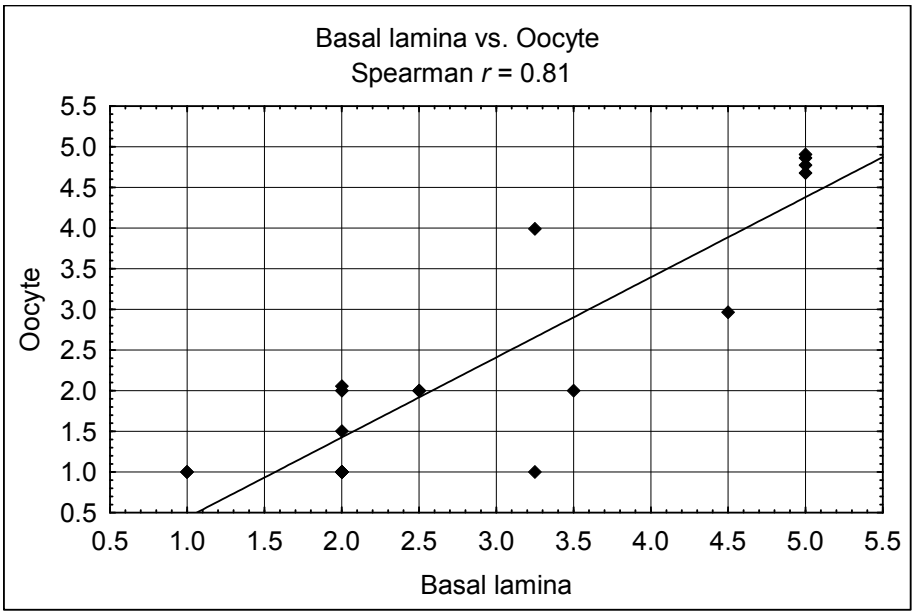



*Figure 3.30*. A correlation graph illustrating the significant positive linear relationship between the scores of the basal lamina and the oocyte.

The basal lamina, however, showed no relationship with the stromal cells or the extracellular matrix (*see Fig.3.31., 3.32.*).

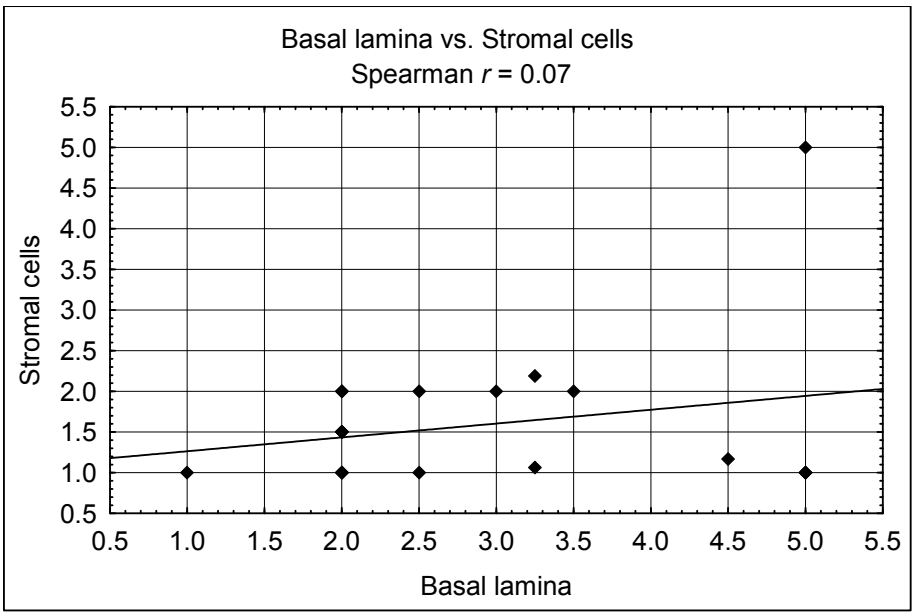



*Figure 3.31*. A correlation graph illustrating no relationship between the scores of the basal lamina and the stromal cells.

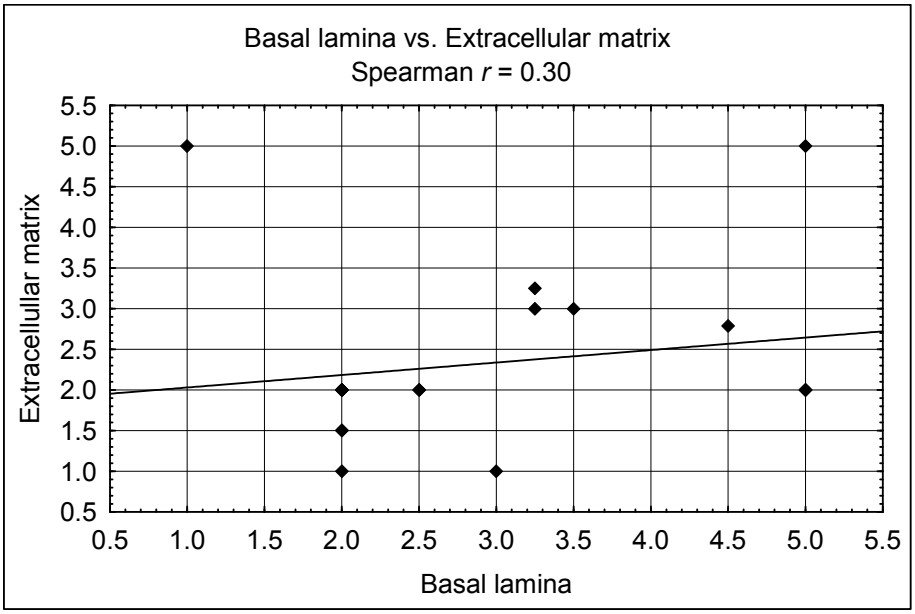



*Figure 3.32*. A correlation graph illustrating no relationship between the scores of the basal lamina and the extracellular matrix.

The perivitelline space showed a strong positive correlation with the granulosa cells ($r$ = 0.90, $P$ <0.01) (*see Fig.3.33*).

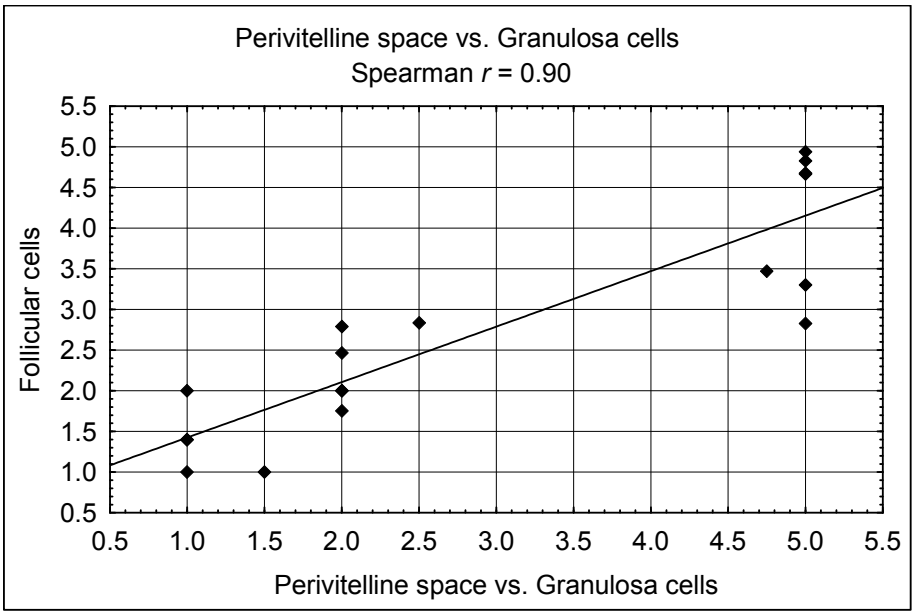



*Figure 3.33*. A correlation graph illustrating the significant positive linear relationship between the scores of the smooth and rough endoplasmic reticulum of the oocyte.

The scores of the extracellular matrix and the stromal cells also showed a negative, but not significant relationship (r = -0.18, P = 0.44) (*see Fig.3.34*.).

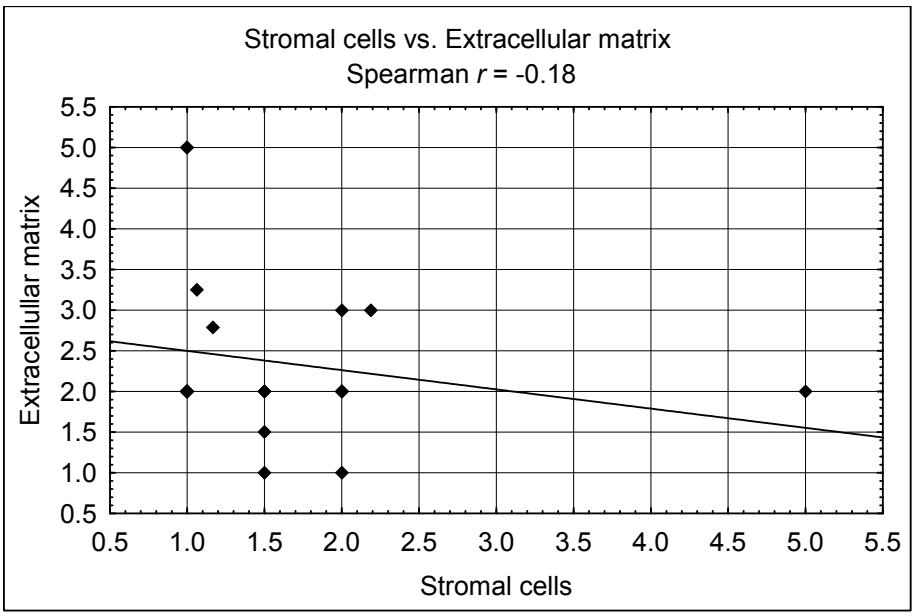



*Figure 3.34*. A correlation graph illustrating the negative, but not significant relationship between the scores of the smooth and rough endoplasmic reticulum of the oocyte.

# CHAPTER FOUR

# DISCUSSION

The transplantation of cryopreserved ovarian tissue has shown successful restoration of endocrine function and possibly fertility in several case studies published on cancer patients sterilized by cancer therapies. The cryopreservation of ovarian tissue has also shown more flexibility as an ovarian function preservation treatment and/or a fertility preservation treatment for cancer patients since the procedure can be used in conjunction with other established ovarian sparing procedures (Martin *et al*., 2007; Demeestere *et al*., 2006). Ovarian tissue cryopreservation also allow the inclusion of very young patients, even pre pubertal girls, and very urgent cancer cases for whom ovarian stimulation cycles to obtain oocytes and embryos may be unethical, contraindicative or inappropriate.

The method of ovarian tissue cryopreservation was initially based on the standard embryo cryopreservation protocol but many variations with different cryoprotectants, cryoprotectant concentrations, supplementations, cooling rates and sample sizes have been described and published. The two cryoprotectants that have dominated the ovarian cryopreservation field are DSMO and PROH showing mostly similar results except in human tissue autotransplantation studies, where DMSO has been shown to be superior (*see Table 1*).

The ovarian tissue grafts, however, have shown endocrine function for only a few months after which the endocrine profile returned to menopausal levels indicating a faster than normal rate of exhaustion of the resting follicle reserve (Oktay, 2001; Gook *et al.*, 2000; Newton, 1998b).

We have aimed in our study to investigate whether the two cryoprotectants (DMSO and PROH) affect ovarian cortex ultrastructures differently and to which extent damage is inflicted to the ultrastructures of the primordial follicles and the surrounding supporting tissue. The outcomes were aimed to identify the better cryoprotectant for implementation in our ovarian cryopreservation protocol.

The ultrastructure (TEM) of thirty follicles, fresh and cryopreserved, from eight participants was evaluated and scored by an expert in the field of electron microscopy. Broad descriptive evaluations were reported and scoring results have been statistically analysed to compare the survival outcomes before and after

cryopreservation and the extent of damage on different ultrastructures (*see Chapter 2*).

Ultrastructural damage of the fresh control follicles was expected to be absent and in overview of the TEM micrographs, most follicles in the fresh control group showed normal morphology. Some follicles, however, presented with small areas of lysis in the stroma (*see Fig.3.11*). This result corresponded to the electron microscopy results of Gook *et al*. (1999, 2000). Higher magnification of these slightly damaged appearing follicles, revealed normal granulosa cells, but severely damaged stroma cells and oocyte mitochondria. Recurrence of this stroma damage in fresh cortex tissue may be a result of the tissue handling during the dissection process. The resulting scores of the fresh control follicles were lower than expected.

The comparative studies did not show any overall significant treatment effect on the ultrastructures evaluated between equilibration and cryopreservation groups compared to the fresh control sample group. This outcome may be because all the groups also showed great variation within the scores of ultrastructures as indicated by the large ranges of the 95% confidence intervals.

Trends of possibly more severe damage as a result of cryopreservation (treatment) with PROH have been shown in some ultrastructures when compared with the fresh control samples. An overview of the TEM micrographs of the follicles in the PROH groups in comparison with the follicles in the DSMO groups suggested that larger and more areas of lytic damage and vacuolation were present in the PROH groups. This was especially apparent in the PROH cryopreservation group. A similar trend to our study was observed in TEM studies of bovine (Lucci *et al*., 2004) and caprine (Rodrigues *et al*., 2004) ovaries cryopreserved in PROH or DSMO as cryoprotectant and both these studies showed that cryopreservation of ovarian tissue in 1.5M DMSO was superior to 1.5M PROH. Gook *et al*. (1999, 2000) also observed large areas of lysis in human primordial follicles of cryopreserved ovarian cortex pieces using 1.5M PROH. In our study, however, the areas of lysis were smaller and not frequently seen in all groups.

In our study, the stroma showed severe or recoverable damage in the stromal cells in all the groups. Damage to the basal lamina was also similar in all groups.

The extracellular matrix, however, showed an unexpected result where a trend of less damage was seen in the PROH cryopreserved group when compared to the fresh control group. The overall treatment effect, however, was not significant.

The granulosa cells showed a trend of more severe damage in the PROH cryopreserved group compared to the fresh sample. The smooth and rough endoplasmic reticulum in the granulosa cytoplasm showed a trend of more damage in the PROH equilibration and PROH cryopreserved groups compared to the control. The nuclei of the granulosa cells showed a marginally significant treatment effect with a trend of extensive damage in both cryopreserved groups (DMSO and PROH) and the PROH equilibration group, but not the DMSO equilibration sample.

Overall, the oocyte showed similar damage in all groups, which was also seen in the oocyte mitochondria and smooth and rough endoplasmic reticulum. The nucleus of the oocyte, however, showed a trend of more severe damage in the two PROH treated groups (equilibration and cryopreserved) compared to the fresh control group.

Even though the perivitelline space was minimally present, which is normal for early follicles, it showed no treatment effect compared to fresh control samples.

The comparative studies revealed a recurring trend where the tissue samples of the two PROH groups, especially the cryopreserved group, have suffered the most damage although the extent of damage did not differ from the DSMO samples. The nuclei of the granulosa and the nuclei of the oocytes seemed to be the most affected by, and may therefore be the most sensitive to equilibration and cryopreservation treatment.

The absence of significant treatment effects in the evaluated ultrastructures may suggest that both cryopreservation protocols were effective. Post hoc analysis of these results, however, revealed less damage and better follicle ultrastructure and stroma survival when ovarian tissue was cryopreserved with DMSO as cryoprotectant. Our results were comparable with and supported by the literature where the use of DMSO as cryoprotectant in ovarian tissue cryopreservation and transplantation has shown superior survival outcomes not yet achieved with any other cryoprotectant (*see Table 1*). In addition, the DMSO equilibration time was an hour shorter than the PROH equilibration time and therefore a practical advantage for the use of DMSO in the cryopreservation protocol.

The correlation studies were done to establish whether all ultrastructures in and around the follicle was similarly affected by cryopreservation. Strong positive linear correlations were shown in several ultrastructures. The stroma, granulosa cell layer and the oocyte showed strong positive linear relationships, suggesting that the probability is high that the extent of the damage seen in one of these structures may be similar in the other structures. The ultrastructures that were found in both the granulosa cells and the oocyte, i.e. nuclei, cytoplasm, mitochondria and endoplasmic reticulum, showed strong positive relationships. This revealed that these ultrastructures were affected to the same extent, irrespective of origin. The smooth and rough endoplasmic reticulum in the granulosa cells and in the oocyte showed exact positive linear correlations, respectively, indicating that the smooth and rough endoplasmic reticulum were affected by equilibration and cryopreservation to the same extent.

The correlation studies also showed that the severity of the damage seen in all areas and structures or organelles around and within the follicles was almost similar. This suggested that the evaluation or assessment outcome (extent of damage) of one area or certain structures of the early follicle or immediate surrounding stoma tissue may be representative of the state of other areas or structures of the same follicle. This may imply that detailed TEM evaluation of all ultrastructures may not be necessary and follicle damage can be assessed under light microscopy (HE slides). The micrographs and the recurring low scores in the fresh control group, however, suggested that even apparently small areas of lytic damage or vacuoles in or around the early follicle resulted in severe damage to the ultrastructures. These small lytic areas or vacuoles may be more easily missed or considered as non-fatal damage to the follicle during light microscopy evaluations.

Some participants of this study who received heterotopic autotransplantation of their cryopreserved ovarian tissue showed restored endocrine function and follicle development for a few months. It can be suggested that either a certain degree of damage to the follicles and surrounding stroma of the cortex tissue was acceptable and could be repaired. Alternatively, a minority of the early follicles could have survived the cryopreservation and transplantation procedures without any damage and resulted in a small viable pool of follicle reserve that were large enough to restore some endocrine function for follicle growth but was quickly exhausted due to the

normal processes of programmed atresia.

The main limiting factor of our study was large areas of missing data as a result of several reasons. The main shortfall was the age range of the participants. Although all of the participants showed normal endocrine function and healthy menstrual cycles, the primordial follicle reserves were very low and the follicles were difficult to target for TEM. In two patients no follicles could be found under light microscopy in any of their tissue samples. Another participants' samples were lost during transport. Many of the ultrastructures were frequently missing on the micrographs and some were completely absent on all micrographs. These scarce ultrastructures were evaluated when present but the resulting sample size was too small to be included in the statistical analysis or to be used to draw conclusions from. Some TEM micrographs were either too light or too dark for accurate evaluation of minute ultrastructures and could not be reassessed because of time and financial constraints.

Ovarian tissue cryopreservation is still an experimental method for the preservation endocrine function and/or fertility in patients with prospects of premature ovarian failure. Transplantation procedures in humans have shown success in only a few case studies. The future of the technology, however, shows an immense potential for improvement and adaptability in the treatment of ovarian failure.

# CHAPTER FIVE

# CONCLUSION

Comparison of the effects of DSMO and PROH on the ultrastructures of primordial follicles during cryopreservation has shown that DMSO appears to cause less damage. The equilibration time of the DMSO protocol is also shorter and therefore more user friendly. DMSO was therefore indicated as the cryoprotectant of choice in our ovarian cryopreservation protocol.

Considering that no significant changes were shown in the ultrastructures of the evaluated follicles when compared to the fresh tissue samples and that endocrine function has been temporarily restored in several participants who received heterotopic autotransplantation, our main aim of initiating, developing and implementing a practical and successful cryopreservation protocol has been achieved.

Younger patients are needed for further improvement and development of our protocol and investigations of other future prospects in the field.

# CHAPTER SIX

# FUTURE PROSPECTS

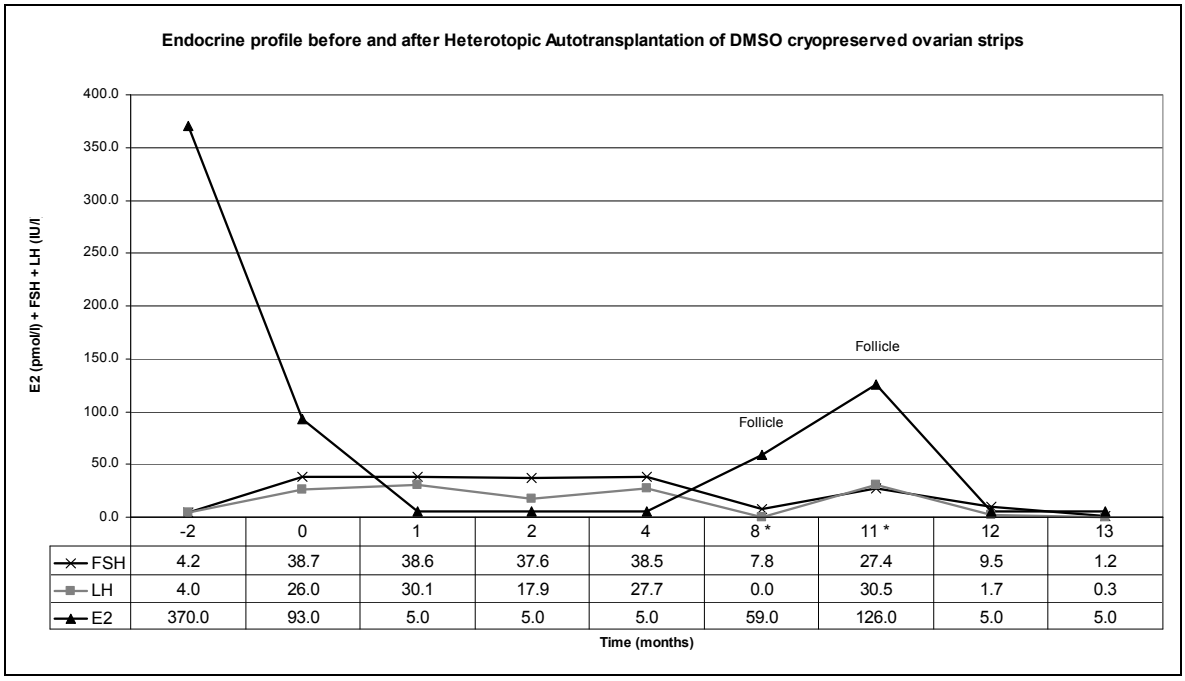
## 1. THE SUCCESS ACHIEVED AFTER HETEROTOPIC TRANSPLANTATION OF CRYOPRESERVED OVARIAN TISSUE IN PARTICIPANTS OF THIS STUDY

A parallel study is currently executed by Dr MH Botha where the cryopreserved ovarian tissue is heterotopically transplanted to evaluate the return of hormonal function in cancer patients that would otherwise be sterilized by cancer therapy and subsequently would have needed on HT.

From a current total of 15 participants so far, nine patients (six from this study) have received heterotopic autotransplantation of their cryopreserved ovarian strips after completion of their 6-week cancer therapy course. About 10-15 cryopreserved strips from one randomly selected cryoprotectant treatment were transplanted subcutaneously ±2cm above the media fossa of either arm. Participants are scheduled to return once a month to assess estradiol, progesterone and FSH blood levels for comparison to blood levels before oophorectomy, cryopreservation and transplantation.

The resulting endocrine profiles showed increased estradiol levels at 6 to 8 months post transplantation (see Appendix 5).

Follicle growth has been evident in two participants so far. The patients observed follicle growth at 8 and 9 months and a ‘bump’ could be seen at the site of transplantation (*see Fig.6.1.)*.




| | -2 | 0 | 1 | 2 | 4 | 8 * | 11 * | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|
| FSH | 4.2 | 38.7 | 38.6 | 37.6 | 38.5 | 7.8 | 27.4 | 9.5 | 1.2 |
| LH | 4.0 | 26.0 | 30.1 | 17.9 | 27.7 | 0.0 | 30.5 | 1.7 | 0.3 |
| E2 | 370.0 | 93.0 | 5.0 | 5.0 | 5.0 | 59.0 | 126.0 | 5.0 | 5.0 |

*Figure 6.1.* A graph illustrating the endocrine profile of a study participant after subcutaneous heterotopic transplantation of cryopreserved ovarian cortex strips compared to blood hormone levels before ovarian cryopreservation (2 months before transplantation procedure: time=-2) and after cancer therapy, at the time of the transplantation procedure (time=0). Follicle development was observed at 8 and 9 months (*).

These results implicated successful cryopreservation of ovarian tissue. The parallel study is still continuing at this time and future results are promising.

## 2. FUTURE DIRECTIONS

### 2.1. Whole ovary cryopreservation

The cryopreservation of whole ovaries with subsequent transplantation by vascular anastomosis is one of the newer prospective procedures currently in an experimental stage. The procedure has the advantage of the immediate return of blood supply with transplantation, reducing ischemic insult to the tissue. The successful freezing and thawing of a whole ovary, however, is a technical challenge. In contrast to the success achieved in animal studies and when compared to freezing of cortex tissue pieces, cryopreservation of whole human ovaries are complicated by its large size and more complex and fibrotic composition. The large organ volume implicates a low surface area to volume ratio, which negatively affects equilibration and cooling rates. The higher complexicity of cell composition further complicates the optimization of protocol conditions (Kim, 2006).

Imhof *et al.* (2004) investigated primordial follicle morphology after slow cryopreservation and vitrification of whole porcine ovaries. Ovaries were perfused with 1.5M DSMO during 30 min equilibration in cryoprotectant on ice. The primordial follicle viability of slow cryopreserved ovaries was 84% compared to fresh control of 98%. Vitrified ovaries resulted in 21% viability.

Bedaiwy *et al.* (2006) applied the same cryoprotectant (1.5M DMSO) perfusion technique to human ovaries removed with its vascular pedicle. After slow cryopreservation of the whole ovary, results showed 75-78% primordial follicle viability compared to tissue strips with 81-83% viability.

## 2.2. Ovarian cryopreservation and transplantation for bone marrow transplantation patients

Stem cell transplantation procedures (previously known as bone marrow transplantation) result in infertility in >95% of patients because of the high dose chemotherapy and/or radiotherapy used for preconditioning (Oktay, 2005; Sanders, 1996).

In 2005, Oktay *et al.* reported a unique case of a live birth in a patient who conceived naturally after heterotopic ovarian transplantation following 2.5 years of POF due to chemotherapy. The 32-year-old female was initially diagnosed with Hodgkin lymphoma at age of 28. Initial radiation therapy did cause ovarian failure, but after a relapse a year later, she underwent gonadotoxic chemotherapy before autologous hematopoietic stem cell transplantation. One ovary was laparoscopically removed for cryopreservation before chemotherapy. The patient showed POF directly after the stem cell transplant procedure which continued for 2.5 years with no HT. Ovarian cortex pieces were transplanted subcutaneously in the suprapubic area. Endocrine function was restored after two months. Ultrasound examination showed no antral follicles in the graft, but vaginal ultrasound revealed an intrauterine pregnancy with a corpus luteum on the native ovary. No foetal heart beat could be detected and the pregnancy was terminated. In the next cycle, a positive LH surge was detected and a positive pregnancy 17 days later. The pregnancy resulted in a birth of a healthy female at 40 weeks of gestation (Oktay & Oktem 2005, Oktay, 2006).

This spontaneous recovery of fertility through an unexplained mechanism indicates the need of extreme caution about the origin of pregnancies following ovarian transplantation (Oktay, 2006).

The likelihood for infertility following autologous stem cell transplantation is around 95% and thus, pregnancies have occurred in adult patients, even in those who appeared menopausal for several years. Schimmer *et al.* (1998) showed that all patients became menopausal immediately after bone marrow transplantation and ovarian function recovered 6 to 48 months later in 30% of the patients (n=17). Sanders *et al.* (1996) showed that out of 708 post pubertal patients, 110 (15%) recovered normal ovarian function of which 32 (4.5%) fell pregnant within a median range of 2-4 years after SCT. Pregnancy rate of patients receiving only chemotherapy was 14.2% (n=176) compared to 1.3% in patients that received only total body irradiation (n=532). Live birth rate was 78.5% and 50%, respectively.

Besides coincidental ovarian function recovery with ovarian transplantation, an alternative hypothesis can be possible. A discrepancy was observed in the rate of apoptosis of primordial follicles and the rate of decline in the ovarian follicle reserve in several mice strains. At the calculated rate of apoptosis, menopause should be reached within weeks of birth; however, fertility prevails for years. The expression of germ line cell markers in bone marrow and peripheral blood have been observed in rodents and women. The ovarian reserve of rodents sterilized by chemotherapy has been replenished with bone marrow and peripheral blood sample injections taken from normal control animals. It was noted that oophorectomy completely abolished the expression of these markers in the bone marrow. Subsequent HT did not restore the marker expression. The results suggested that there is a communication between the ovaries and bone marrow that is not hormonal but cellular, which may be re-instated after heterotopic autologous transplantation of ovarian tissue (Oktay, 2006).

Bath et al. (2004) reported a case of a spontaneous conception and live birth in a 20-year-old woman with documented ovarian failure for 6 years after chemotherapy and radiation therapy for non-metastatic Ewing's sarcoma. Ovarian tissue biopsy and cryopreservation have been done at the age of 14 before cancer treatment, but was not transplanted after cancer treatment.

In a case reported by Meirow *et al.* (2005a) a patient fell pregnant after 33 months of amenorrhea resulting from high-dose chemotherapy for Hodgkin's lymphoma, after receiving autologous stem cell support. She received an orthotopic ovarian graft in both ovaries and menses returned for two months before she fell pregnant with an IVF-ET cycle. Donnez *et al*. (2004) reported a spontaneous pregnancy and live birth after orthotopic transplantation of cryopreserved ovarian tissue. The patient was POF for more than 2 years after chemotherapy. In both these case studies, the ovum concerned, however, could have originated from the native ovarian tissue.

The question arises whether the transplanted ovary provides the signal for the bone marrow to provide new germ cells via the blood stream to the sterilized menopausal ovary for de-novo production of primordial follicles. These results of possible germ cell renewal and migration from bone marrow to the ovary needs further in-depth investigation (Oktay, 2006).

### 2.3. Freezing of oocytes collected during dissection of ovary before cryopreservation

Another possible future strategy is the cryopreservation of immature oocytes released into the collection dish during the dissection of ovarian tissue as supplemental to ovarian tissue cryopreservation. This strategy may be an option when the freezing protocols for immature and *in vitro* matured human oocytes is well established (Shaw, 2003a) and needs future development.

**The cryopreservation of ovarian tissue, whole organ or in cortex pieces, and subsequent transplantation opened a wide field of unexplored possibilities for the treatment of induced premature ovarian failure. Future investigation is needed to improve the restoration of ovarian function.**

## CHAPTER SEVEN

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## CHAPTER EIGHT

## ABBREVIATIONS

| | |
|---|---|
| CPA | Cryoprotectant / cryoprotective agent |
| DMSO | Dimethyl sulfoxide |
| D-PBS | Dulbecco's Phosphate-buffered saline |
| EG | Ethylene glycol |
| ET | Embryo transfer |
| GLY | Glycerol |
| GnRH-a | Gonadotropin-releasing hormone agonist |
| Gy | Gray: Standard International Unit of absorbed radiation |
| HE | Haematoxylin and eosin staining |
| HT | Hormone therapy (previously also referred to hormone replacement therapy (HRT)) |
| HSA | Human serum albumin |
| ICSI | Intra cytoplasmic sperm injection |
| IIF | Intracellular ice formation |
| IVF | *In vitro* fertilization |
| IVM | *In vitro* maturation |
| LDH | Lactate dehydrogenase |
| PBS | Phosphate buffered-saline |
| POF | Premature ovarian failure |
| PROH | Propanediol |
| SCID-mice | Severe combined immunodeficient mice |
| SCT | Stem cell transplant or bone marrow transplant |
| TEM | Transmission electron microscopy |

## APPENDICES

### APPENDIX 1

### OVARIAN CRYOPRESERVATION PROTOCOLS USED TO FORMULATE THE CUSTOMIZED PROTOCOL

**Gook *et al*. 1999**

Dissection & Collection

Solution: A) HEPES-buffered human tubal fluid (HTF) + 4mg/ml human serum albumin (HSA)
B) 3.7% gluteraldehyde + 0.1M cacodylate buffer
C) 0.1M cacodylate buffer

Method: A) At 37°C, "slices" 4x3x1mm at 6%$CO_2$/94% air
B) Fix 1 slice immediately at room temp
C) Storage at 4°C

Time: B) Min 2h

Equilibration

Solution: 1.5M 1,2 propanediol (PROH) + 0.1M sucrose + PBS + 10mg/ml HSA

Vial: Not specified

Prepare: Pre-filled tubes & cryovials; label cryovials
1) unspecified amount in test tube
2) 1ml in cryo vial

Method: 1) Transfer tissue to tubes at 37°C
2) Tip contents into sterile dish
3) Rapidly allocate tissue evenly to cryovials

Time: 1) 90min

Cooling

Solution: 1.5M PROH + 0.1M sucrose + PBS + 10mg/ml HSA

Prepare: Pre-cool tip of large forceps in $N_2$, set automated freezer

Method: 1) Load vials, cool from ~18°C at -2°C/min to -8°C
2) Manual seeding, touch vial with forceps just above meniscus, examine for ice-crystal formation
3) Cool at -0.3°C/min to -30°C, cool at -10°C/min to -150°C}
4) Plunge into $N_2$ for storage

Time: 1) 10min

3) ±2h

Thawing

Solution: A) 0.75M PROH + 0.2M sucrose + PBS + 10mg/ml HSA

B) 0.2M sucrose + PBS + 10mg/ml HSA

C) HEPES-buffered HTF

Prepare: A & B) 37°C; sterile water bath at 37°C, sterile small forceps

Method: 1) Immerse in water bath

2) Note details, sterilise vial with OH, remove lid

3) Transfer tissue with forceps or by decantation of contents

A-C) Wash by agitation

6) Optional: transfer tissue to culture medium

Time: 1) 2-3min

A & B) 5 min each

C) 10min

**Oktay *et al.* 2000**

Dissection & Collection

Solution: Serum supplemented media

Prepare: Ice

Method: “strips” 1-2x5x15mm, transport on ice

Time: Max 4-6h

Equilibration

Solution: Phenol free buffered medium (L-15) + 1.5M DMSO (propanediol / ethylene glycol) + 10% serum + 0.1M sucrose

Vial: Type? Strips per vial?

Prepare: Label cryovials, tissue roller, ice

Method: 1) Allocate tissue to vials

2) Incubate at 4°C

Time: 2) 30min

Cooling

Solution: Phenol free buffered medium (L-15) + 1.5M DMSO (propanediol / ethylene glycol) + 10% serum + 0.1M sucrose

Prepare: Pre-cool tip of large forceps in $N_2$ , set automated freezer

Method: 1) Load vials, start at 0°C, cool at -2°C/min to -7°C

2) Manual seeding, touch vial with forceps just above meniscus, examine for ice-crystal formation

3) Cool at -0.3°C/min to -40°C, cool further at -10°C/min to -140°C}

4) Plunge into $N_2$ for storage

Time: 1) 5min

2) Unknown

3) $\pm$2h

4) Long-term?

Thawing

Solution: A) Phenol free buffered medium (L-15) + 1.5M DMSO + (propanediol / ethylene glycol) + 10% serum + 0.1M sucrose

B) Phenol free buffered medium (L-15) + 1.0M DMSO + (propanediol / ethylene glycol) + 10% serum + 0.1M sucrose

C) Phenol free buffered medium (L-15) + 0.5M DMSO + (propanediol / ethylene glycol) + 10% serum + 0.1M sucrose

D) Phenol free buffered medium (L-15) + 10% serum + 0.1M sucrose

E) Phenol free buffered medium (L-15) + 10% serum

Prepare: Sterile water bath at 37°C, sterile small forceps

Method: 1) Hold each vial at room temp

2) Immerse in water bath

3) Note details, remove lid

4) Transfer tissue

A -E) Wash by gentle agitation

6) Transfer tissue to fresh E) for grafting

Time: 1) 30s

2) 2min

A to E) 5min each

**Shaw *et al*., 2000, 2003a, 2003b**

Dissection & Collection

Solution: Phosphate buffered saline (PBS), sterile, no protein

Prepare: 100ml/whole ovary, transport on ice

Method: At room temp, “sheets” 5x5x1mm ; “strips” 1-2x0.5-1x1mm

Time: 20 – 40min

Equilibration

Solution: A & B) 1.5M DMSO + 0.1M sucrose in PBS, sterile filtered through a 0.22 micro filter, no protein

Vial type: 1.7ml star-foot, conical / round base, external/internal thread

Prepare: Pre-filled & pre-cooled on ice for at least 30min; label cryovials

A) 5-10ml/ovary, in tube

B) 1ml, in cryovial

Method: 1) Transfer all tissue samples to A); agitation every 5-10min

2) Tip contents into sterile dish

3) Rapidly allocate tissue evenly to B), return vials to ice

Time: A) 15-20min

B) 30min

Cooling

Solution: 1.5M DMSO + 0.1M sucrose in PBS, sterile, no protein

Prepare: Pre-cool tip of large forceps in $N_2$, set automated freezer

Method: 1) Load vials, vials to reach -6°C

2) Manual seeding, touch vial with forceps just above meniscus, examine for ice-crystal formation

3) Cool at -0.3°C/min to -40°C,

{optional: further cool at -10°C/min to-140°C}

4) Plunge into $N_2$ for storage

Time: 1) 5min

3) ±2h

4) Long-term?

Thawing

Solution: A) 0.75M DMSO + 0.25M sucrose in PBS, sterile, no protein
B) 0.25M sucrose in PBS, sterile, no protein
C) PBS, sterile, no protein

Prepare: A & B) 3ml/vial at room temp, 20-22°C; sterile water bath at 37°C, sterile small forceps,

Method: 1) Hold each vial just above $N_2$ level to rid of excess $N_2$ in vial
2) Immerse in water bath until thawed
3) Note details, sterilise vial with OH, remove lid
4) Transfer tissue with forceps or by decantation of contents
A & B) Wash without agitation
6) Transfer tissue to C) for grafting / culture

Time: 1) Until no sign of $N_2$
2) Several minutes
A & B) 10 min

**Gosden *et al*., 1994: Sheep**

Dissection & Collection

Solution: Leibovitz L-15 medium

Prepare: Sterile specimen cup with medium

Method: At room temp, 4-5 “sheets” per ovary cortex

Time: ?

Equilibration

Solution: 1.5M DMSO + 10% calf serum, in Leibovitz L-15 medium, no sucrose

Vial type: 2mℓ cryo vials

Prepare: Pre-filled & pre-cooled on ice, 1mℓ, in cryovial

Method: 1) Transfer all tissue samples to cryo vials
2) Return vials to ice

Time: 15min

Cooling

Solution: 1.5M DMSO + 10% calf serum, in Leibovitz L-15 medium, no sucrose

Prepare: Pre-cool tip of large forceps in liquid $N_2$ , set automated freezer

Method: 1) Load vials, cool at 2°C/min to reach -7°C

2) Manual seeding, touch vial with forceps just above meniscus, examine for ice-crystal formation

3) Cool at -0.3°C/min to -40°C,

4) Cool at -10°C/min to-140°C

5) Plunge into $N_2$ for storage

Time: 1) 5min

2) 10min

3 & 4) $\pm$2h

5) 3 weeks

Thawing

Solution: Leibovitz L-15 medium

Prepare: sterile water bath at room temperature

Method: 1) Hold each vial just above $N_2$ level to rid of excess $N_2$ in vial

2) Immerse in water bath until thawed

4) Transfer tissue with forceps or by decantation of contents

A) Wash 3 times in clean Leibovitz L-15 medium

6) Transfer tissue for grafting

Time: 1) Until no sign of $N_2$

2) Several minutes

A) 10 min

## APPENDIX 2

## DETAILS OF CHEMICAL COMPOUNDS, MEDIUMS AND CONSUMABLES USED

### Chemical compounds

Cryoprotectants:

**Dimethyl Sulphoxide** (DMSO) Hybri-Max™ D2650. Sterile filtered. Sigma® Sigma-Aldrich Company Ltd., Irvine, Ayrshire KA128NB, UK.

| | |
|---|---|
| Molecular formula: | $C_2H_6OS$ |
| Molar mass: | 78.13g/mol |
| IUPAC Name: | Dimethyl sulfoxide |
| Other names: | Methyl sulfoxide; Methyl sulfinylmethane; DMSO |
| Melting point: | 16-19°C (292K) |
| Boiling point: | 189°C (462K) |
| Density: | 1.1004g/cm$^3$ |

**1,2-Propandiol** *Art*. 7478 *Ph Eur, B P, USP*. Merck, Darmstadt, Germany

| | |
|---|---|
| Molecular formulae: | $C_3H_8O_2$ |
| IUPAC name: | Propane-1,2-diol |
| Other names: | Propylene glycol; *1,2-Propylenglycol*; 1,2-Propanediol (*1,2-propylene glycol*); Propanediol-1,2 (*propylèneglycol-1,2*); Propandiol-(1,2) (*1,2-propilenglicole; 1,2-propilenoglicol*); 1,2-Propanodiol (*1,2-propilenglicol*) |
| Molar mass: | 76.10g/mol |
| Melting point: | -59°C |
| Boiling point: | 188.2°C |
| Density: | 1.036g/cm$^3$ |

**Sucrose** >99.5% (GC). S1888. Cell culture tested.

Manufacturer: Sigma® Sigma-Aldrich Company Ltd., St. Louis, MO 63103, USA.

**MEDIUMS**

Base solution:

**Dulbecco's Phosphate-Buffered Saline** with Calcium and Magnesium without Phenol Red (D-PBS + $CaC\ell_2$ + $MgC\ell_2$) liquid. Sterile.1178.

Manufacturer: Gibco™Invitrogen™Auckland, N.Z.

Melting point: No data available.

Boiling point: No data available.

Mediums:

**Quinn's Advantage™ Media with HEPES**. Sterile.

Manufacturer: SAGE Assisted Reproduction Products™ A Cooper Surgical Company. In-Vitro Fertilization, Inc., Trumbull, CT 06611, U.S.A.

Quinn's Advantage™ HSA (Human Serum Albumin). Sterile. Manufacturer: SAGE Assisted Reproduction Products™ A Cooper Surgical Company. In-Vitro Fertilization, Inc., Trumbull, CT 06611, U.S.A.

Solution: 100mg/ml total protein (weight/volume) in saline solution.

pH 7.4±0.2

Osmolality: 280±10mOsm/kg water

**Consumables**

Cryovials:

**Cryo.s**™PP, 2mℓ conical round-based internal thread with screw cap

Manufacturer: Greiner Bio-One GmbH, 72636 Frickenhausen, Germany

Petri dishes:

Falcon®3003, 100x20mm, Becton Dickinson Labware, NJ, USA.

Manufacturer: Cellstar®, 35x10mm Greiner Bio-One GmbH, 72636 Fickenhausen, Germany.

Sterilizing micro filters:

Manufacturers: Ministart®, 0.20µm, Sartorius, Vivascience, Hannover, Germany

## APPENDIX 3

## STANDARD HISTOLOGICAL PROCESSING OF TISSUE SAMPLES

### Standard haematoxylin and eosin stain for paraffin sections

1. Dewax sections in xylol, hydrate through graded alcohols to water.
2. Remove fixation pigments if necessary.
3. Stain in an alum haematoxylin of choice for suitable time.
4. Wash well in running tap water until sections 'blue' – 5 minutes or less.
5. Differentiate in 1% acid alcohol (1%HCl in 70% alcohol) – 5-10 seconds.
6. Wash well in tap water until sections are again 'blue' – 5 minutes or less.
7. Stain in 1% eosin Y for 10 minutes.
8. Wash in running tap water for 5 minutes.
9. Dehydrate through alcohols, clear in xylol, mount in DPX.
10. Results in:

| | |
|---|---|
| Nuclei | Blue-black |
| Cytoplasm | varying shades of pink |
| Muscle fibres | deep pinky red |
| Collagen | pale pinky red |
| Red blood cells | orange/red |
| Fibrin | deep pink |

Note that structures and substances other than nuclei may be haematoxyphilic in varying degrees. Examples include fungal hyphae which are faintly haematoxyphilic, and calcium deposits which are often deep blue-black.

Reference:

Bancroft, John D. Theory and Practice of Histological Techniques.

**Standard tissue preparation for transmission electron microscopy**

1. Tissue are kept overnight in phosphate-buffered gluteraldehyde (pH 7.4) at 4ºC.
2. Wash or store in phosphate buffer (pH 7.4).
3. Equilibrate in osmiumtretoxide solution for 1 hour.
4. Wash 2x in distilled water.
5. 30 minutes in 2% uranyl acetate in 70% alcohol.
6. 5 minutes in 70% alcohol.
7. 5 minutes in 96% alcohol.
8. 10 minutes in 2% uranyl nitrate in 96% alcohol.
9. 10 minutes in 100% alcohol in sodium sulphate.
10. 15 minutes in 100% alcohol in sodium sulphate.
11. 20 minutes in 100% alcohol in sodium sulphate.
12. Make resin and keep tightly closed until used.
13. 90 minutes in 50/50 solution of 1ml resin and 1ml alcohol.
14. 1 hour in clean resin in clean container.
15. 1 hour in clean resin in clean container.
16. Imbed in clean resin in capsules.
17. Leave overnight at 60ºC.

Reference:

Operating manual of Department of Anatomical Pathology, Tygerberg Hospital.

## APPENDIX 4

## PATIENT FORMS

### Patient Information and Consent forms

**Hierdie vorm is ook in Afrikaans beskikbaar**

### Information Sheet for Ovarian Biopsy and Cryopreservation

We are providing you with the following information in order for you to be able to make an informed decision about whether you wish to freeze your ovarian tissue. We want to be sure you understand this fully before you formally agree to participate. Be sure to ask any questions you have about the information that follows and we will do our best to explain and to provide any further information you require.

In order to treat the illness from which you are suffering, you will require surgery, chemotherapy and/or radiotherapy which are likely to cause permanent damage to your ovaries such that they will no longer be able to produce eggs. By removing some ovarian tissue before you start this treatment and carefully freezing it, it will be possible to protect some of your eggs from the harmful effects of the chemotherapy or radiotherapy you are shortly to undergo.

Ovarian tissue is collected at a minor operation called a laparoscopy. This is performed under general anaesthesia, when a telescope, about the thickness of a pencil, is placed through your navel and a small piece of ovarian tissue is removed through two smaller incisions, lower down on your abdomen.

There are potential risks involved with this procedure, of which you should be aware:
There is a very small risk of a reaction to the anaesthetic agents; a small chance of infection; damage to the other pelvic organs; and if you have abnormal blood clotting mechanisms, a chance of bleeding after the procedure.

Some women undergoing abdominal surgery for their condition may be able to have ovarian tissue removed at the time of surgery and so avoid the need for a laparoscopy. A piece or pieces of ovarian tissue will be removed. In some cases it may be necessary to remove a whole ovary, in order to maximize your chances of saving a useful number of eggs.

You will not be asked to take any drugs, make any extra visits to the hospital or to have any additional blood tests.

The use to which we can put your ovarian tissue is complicated by the fact that only very immature eggs in the ovary will survive the freezing process. The frozen tissue may, at a later stage, be re-implanted into your body and possibly restore ovarian function. The benefits of normal ovarian function may be hormonal. Hormones secreted by the ovaries protect the body against ageing and in particular the strength of your bones.

Some people have expressed concern that the re-implantation may risk reintroducing some untreated cancer cells back into your body. There may be other risks associated with ovarian re-implantation that we do not know about at present. Research is presently on-going to look at both these risks and ways of reducing the potential harm.

At this stage you are consenting only to collect ovarian tissue for storage. If and when you decide you wish to use the tissue, the details of any procedure will be explained to you and you will be asked to sign a separate consent form. Your participation is entirely voluntary and, whatever your decision, it will not affect your treatment in any way. We understand that this is a difficult time for you and that you may wish to discuss the implications of the storage of your ovarian tissue.

In the event of your death, the stored ovarian tissue will either be destroyed or be used for research purposes. You will be asked to sign a separate consent form instructing us what to do.

If you require any further information regarding the proposed research or have any concerns regarding any potential side-effects, please feel free to discuss them with your doctor.

## Information Sheet for Ovarian Tissue Re-implantation

We are providing you with the following information in order for you to be able to make an informed decision about whether you wish re-implant your stored ovarian tissue. We want to be sure you understand this fully before you formally agree to participate. Be sure to ask any questions you have about the information that follows and we will do our best to explain and to provide any further information you require.

In order to treat the illness from which you are suffering, you required surgery, chemotherapy and/or radiotherapy which could cause permanent damage to your ovaries. Part of your ovarian tissue was stored and can now be re-implanted.

The re-implantation is done under local anaesthesia except when you may require general anaesthesia for another reason. A small cut of less than 1 centimetre is made in your forearm and the piece of tissue is then inserted and sutured with absorbable sutures. The cut is made on the inner aspect of the arm. Sometimes it may be necessary for the tissue to be implanted in the abdominal cavity. This will happen during a laparoscopy. Your doctor will inform you about the most suitable place for your implantation.

Some people have expressed concern that the re-implantation may risk reintroducing some untreated cancer cells back into your body. There may be other risks associated with ovarian re-implantation that we do not know about at present. Research is presently on-going to look at both these risks and ways of reducing the potential harm. There is a very small risk that cancer cells may be re-implanted with the ovarian tissue. The tissue is carefully examined before re-implantation to prevent this problem.

You will not be asked to take any medication after re-implantation.

The benefits of re-implantation may be hormonal. Hormones secreted by the ovaries protect the body against ageing and in particular the strength of your bones.

At this stage you are consenting to re-implantation of ovarian tissue. Your participation is entirely voluntary and, whatever your decision, it will not affect your treatment in any way.

If you require any further information regarding the proposed research or have any concerns regarding any potential side-effects, please feel free to discuss them with your doctor

**Hierdie vorm is ook in Afrikaans beskikbaar**

## PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

## TITLE OF THE RESEARCH PROJECT:

A STUDY TO EVALUATE OVARIAN CRYOPRESERVATION TO REDUCE LONG TERM HORMONAL DYSFUNCTION ASSOCIATED WITH CANCER TREATMENT IN WOMEN

**REFERENCE NUMBER: N05/10/182**

**PRINICIPAL INVESTIGATOR:** Dr MH Botha

**ADDRESS:** Dept. Obstetrics & Gynaecology, Tygerberg Hospital, TYGERBERG

**CONTACT NUMBERS:**

Tel: 021 9385696
Cell: 0844024023
Fax: 021 9384648
Email: mhbotha@sun.ac.za

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you did agree to take part.

This study has been approved by the Committee for Human Research at Stellenbosch University and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

**What is this research study all about?**

- Please read the information sheet "Information Sheet for Ovarian Biopsy and Cryopreservation" that will be provided to you, carefully.

**Why have you been invited to participate?**

- Your participation in this project will result in the broadening of the knowledge of preservation of ovarian function after cancer treatment.

Section 1.01 Will you benefit from taking part in this research?

- By removing some ovarian tissue before you start this treatment and carefully freezing it, it may be possible to protect some of your eggs from the harmful effects of the chemotherapy or radiotherapy you are shortly to undergo. The frozen tissue may, at a later stage, after

thawing be re-implanted into your body and may possibly restore ovarian function. The benefits of normal ovarian function may be hormonal. Hormones secreted by the ovaries protect the body against ageing and in particular the strength of your bones. This procedure may sometimes restore fertility.

**Are there in risks involved in your taking part in this research?**

- The possible risks are clearly described in the information sheet - Please read the information sheet that will be provided to you, carefully.

**If you do not agree to take part, what alternatives do you have?**

- Participation is voluntary, and you may refuse to participate in the project and may at any time withdraw your participation from the project. Refusal or withdrawal from the project will in no way affect your present or future treatment at the clinic. The researcher may also withdraw you from the project if he/she considers it to be in your best interest.

**Who will have access to your medical records?**

- All information collected will be treated confidentially. The results will be used for publication in human fertility related journals, without revealing the identity of any individual. On completion of the project, the final outcomes of the project will be available to you.

**What will happen in the unlikely event of some form of injury occurring as a direct result of your taking part in this research study?**

- At this stage you are consenting only to collect ovarian tissue for storage. If and when you decide you wish to use the tissue, the details of any procedure will be explained to you and you will be asked to sign a separate consent form. The freezing and storage of ovarian tissue will not cause any injury to you.

**Will you be paid to take part in this study and are there any costs involved?**

- No, you will not be paid to take part in the study and there will be no costs involved for you, if you do take part.

**Is there anything else that you should know or do?**

- You should inform your family practitioner or usual doctor that you are taking part in a research study.
- You can contact **Dr MH Botha at Tel 021 9385696** if you have any further queries or encounter any problems.
- You can contact the **Committee for Human Research at 021-938 9207** if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.
- You will sign a consent form to instruct us that in the event of your death, frozen ovarian tissue will either be destroyed or used for research purposes.

By Signing below, I…….. agree to take part in a research study entitled:-

**A STUDY TO EVALUATE OVARIAN CRYOPRESERVATION TO REDUCE LONG TERM HORMONAL DYSFUNCTION ASSOCIATED WITH CANCER TREATMENT IN WOMEN**

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalized or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*)…….on (*date*) …….. 2……

…….. …… ……

Signature of Participant Date of birth Signature of Witness

## Declaration by Investigator

I (name ) ……declare that:-

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a translator. (*If a translator is used then the translator must sign the declaration below.*

Signed at (*place*)……..on (*date*) …….. 2…….

…….. ……

Signature of Investigator Signature of Witness.

## Declaration by Translator

I (*name* ) ……declare that:-

- I assisted the investigator (*name*)……. to explain the information in this document to (*name of participant*)…….. using the language medium of

Afrikaans/Xhosa/Other ……..……...

- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

.

Signed at (*place*)……..on (*date*) …….. 2……

…….. ……

Signature of Translator. Signature of Witness.

## WRITTEN CONSENT FOR HANDLING OF OVARIAN TISSUE

**Ovarian Cryopreservation Project for Oncology Patients**

Name of Patient: .....…… Date:……..
Address: ……
……
……

I have read the attached information on the experimental freezing programme for ovarian tissue. I have been given a copy to keep. I have had the opportunity to discuss the details and ask questions about this information. I understand that I must undergo the operation of laparoscopic ovarian biopsy in order that some of my ovarian tissue may be cryopreserved (frozen) and stored for my possible future use. I understand that this tissue may not survive the freezing and thawing process and that the Hospital cannot be held responsible for this. I understand that this technique is new and experimental and thus I understand that the technique may not work.

However, I am willing to undergo the operation accepting these reservations and I understand that no guarantee can be given to me regarding future success.

I understand that in the event of my death, or should I no longer require my ovarian tissue, it will be:- a) disposed of *
b) made available for research purposes*
(* delete as appropriate)

I have been given the opportunity to take part in counselling regarding the implications of the proposed storage of my ovarian tissue.

**PATIENT'S** NAME *(BLOCK CAPITALS)* . .....……..…..

PATIENT'S SIGNATURE …… …

PATIENT'S DATE OF BIRTH .....……..…..

**PATIENT'S WITNESS'** NAME *(BLOCK CAPITALS)* …….

WITNESS' SIGNATURE ……..

**DOCTOR'S** NAME *(BLOCK CAPITALS)* ……..

DOCTOR'S SIGNATURE ……..

**Cryopreservation forms**

## PATIENT DETAILS FORM

***Project no:N05/10/182***

***Note: 1. To be completed for/by every patient entering this project!***
***2. Attach 2 copies of signed cryopreservation consent forms to this form!***
***3. Place TBH patient stickers on every form and/or page!***

Please complete in block letters, as accurate and thorough as possible.

**Personal Details**

| |
|---|
| Date: |
| Surname: |
| Full names: |
| Date of Birth: Place of Birth: |
| ID no. (Identification book no.): |
| Hospital no.: |
| Local Clinic / Doctor: |
| Occupation: |
| Do you have any children? Ages: |
| Any previous serious illnesses?(specify): |
| Any previous serious operations?(specify): |
| Any prescription medication past 90days?(specify): |
| Tel(h): (w) |
| Cell: |
| Home Address: |
| |
| |
| |
| Postal Address: |
| |
| |
| |
| Next of kin: Relationship: |
| Tel(h): (w) |
| Cell: |
| Home Address: Postal Address: |
| |
| |
| |
| Possible personal reason(s) for entering the study: |
| To have children: To regain hormones: Both: |
| Other (specify): |
| |

**Completed by _____ at _____**

**Patient Signiture _____ Name _____**

**Medical Doctor _____ Name _____**

# PATIENT & PROCEDURE INFORMATION FORM

***Project no:N05/10/182***

***Note: 1. To be completed for every patient entering the project!***
***2. Attach 2 copies of signed cryopreservation consent forms to this form!***
***3. Place TBH patient stickers on every form and/or page!***

**Patient**

| | |
|---|---|
| Surname: | |
| Full names: | |
| DOB: | ID no: |
| Hospital no: | Cell: |

**Diagnosis and Pre-Operation Details**

| | | | |
|---|---|---|---|
| Date diagnosed: | | Treatment: | |
| Cancer type: | | | |
| Location: | | | |
| Stage: | | Treatment duration: | |
| Date: | FSH (IU/ℓ): | LH (IU/ℓ): | $E_2$ (pmol/ℓ): |
| | | | |
| | | | |
| | | | |
| Any other illness? Specify: | | | |
| Additional medication prescribed: | | | |

**Surgical Procedure**

| | |
|---|---|
| Date: | Theatre: |
| Procedure: | Time procedure started: |
| Specimen: | Time procedure finished: |
| Collection holder: | Time tissue retrieved: |

**Cryopreservation**

| | |
|---|---|
| Sample no: | Dissection by: |
| Time tissue recieved: | Cryo by: |
| Time freezing started: | Total strips in storage: |
| Initial total strips: | % Strips for storage: |
| Total vials in storage: DMSO: | PROH: |
| Storage place: DMSO: | PROH: |
| Colour of vials: DMSO: | PROH: |
| Date cryopreservation results report sent: | |

**Pre-Transplantation Details – Dr MH Botha**

| | | | |
|---|---|---|---|
| Date treatment completed: | | | |
| Metastasis present?: Yes / No Date: | | | |
| Date: | FSH (IU/ℓ): | LH (IU/ℓ): | $E_2$ (pmol/ℓ): |
| | | | |
| | | | |
| | | | |
| Any other illness during/after treatment? Specify: | | | |
| | | | |
| If yes, other prescription medication taken: | | | |

# PATIENT & PROCEDURE INFORMATION FORM

***Project no:N05/10/182***

***Note:1. To be completed for every patient recieving transplantation!***
***2. Attach 2 copies of signed transplantation consent forms to this form!***
***3. Place TBH patient stickers on every form and/or page!***

**Thawing Details**

| Date: | Vials: | Strips: | Start: | End: | Media: | Reason: | Done by: |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |

**Histology**

| Date sent: | Tissue sent: | Date results recieved: | Done by: |
|---|---|---|---|
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |

**Transplantation Details – Dr MH Botha**

| Date: | Strips: | Location: | Anaesthetic: | Start: | End: |
|---|---|---|---|---|---|
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |

**Post-Transplantation Details – Dr MH Botha**

| Date: | FSH (IU/ℓ): | LH (IU/ℓ): | $E_2$ (pmol/ℓ): | Follicles? |
|---|---|---|---|---|
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |

**Medical Scientist: _____Name: _____**

**Medical Doctor: _____Name: _____**

**CRYOPRESERVATION DETAILS**

***Project no:N05/10/182***

***Note:1. To be completed for every patient having ovarian tissue cryopreserved.***
***2. Place TBH patient stickers on every form and/or page!***

| | |
|---|---|
| **Date:** | **Sample no:** |
| **Patient:** | **DOB:** **Age:** |
| **Specimen:** | **Est. time retrieved:** **:** |
| **Total strips counted:** | **Time taken to dissect:** |
| **Batches: 1 / 2** | **% Strips for storage:** |

**Batch 1**: n= Time started: _____:_____

| | | | |
|---|---|---|---|
| Control n= BCode | | | |
| DMSO n= | | PROH n= | |
| DMSOeq n= BCode | | PROHeq n= BCode | |
| DMSO freeze n= | | PROH freeze n= | |
| **Vial no.** | Vial colour: | **Vial no.** | Vial colour: |
| **1**. n= | Bcode<br>Tank | **1**. n= | Bcode<br>Tank |
| **2**. n= | Tank | **2**. n= | Tank |
| **3**. n= | Tank | **3**. n= | Tank |
| **4**. n= | Tank | **4**. n= | Tank |
| **5**. n= | Tank | **5**. n= | Tank |

**Batch 2**: n= Time started: _____:_____

| | | | |
|---|---|---|---|
| Control n= BCode | | | |
| DMSO n= | | PROH n= | |
| DMSOeq n= BCode | | PROHeq n= BCode | |
| DMSO freeze n= | | PROH freeze n= | |
| **Vial no.** | Vial colour: | **Vial no.** | Vial colour: |
| **1**. n= | Bcode<br>Tank | **1**. n= | Bcode<br>Tank |
| **2**. n= | Tank | **2**. n= | Tank |
| **3**. n= | Tank | **3**. n= | Tank |
| **4**. n= | Tank | **4**. n= | Tank |
| **5**. n= | Tank | **5**. n= | Tank |

Please mark block with ✓when tissue thawed and/or sent for histology.

**Medical Scientist: _____Name: _____**

## RESULTS REPORT OF FREEZING OF OVARIAN TISSUE

***Project no: N0/10/182***
***Note: 1. To be completed for every patient entering the project!***
***2. Attach 1 copy of consent forms to this form!***

**Date: _____**

**Dear _____**

**Tygerberg Hosiptal number: _____**

**Your ovarian tissue were frozen and stored. Yes / No**

**If yes, please complete:**

| | | |
|---|---|---|
| Date of freezing: | | |
| Ovarian tissue recieved for freezing: | | |
| % Ovarian tissue kept in storage *: | | |
| Vial colour identification: | | |
| Storage place: | | |
| | | |
| | | |
| Referring Dr. | | |

* Amount of ovarian tissue left in storage available for future treatment, for example transplantation, expressed as % of original tissue sample.

**If not, please explain: _____**
**_____**
**_____**
**_____**
**_____**
**_____**

**Signed at _____ on _____ of _____20___.**

**Medical Scientist: _____ Name: _____**

**Senior Medical Scientist: _____ Name: _____**

**Medical Doctor: _____ Name: _____**

## APPENDIX 5

## SPECIFIC DETAILS OF PARTICIPANTS

Age, diagnosis and treatment of each participant.

| | | Diagnosis | | | Treatment | | |
|---|---|---|---|---|---|---|---|
| Participant | Age | Carcinoma | Type | Stage | Chemo | Radio | Duration |
| 1 | 36 | Fallopian | Serous Adeno | III | | | |
| 2 | 33 | Cervix | Squamous | III B | Yes | Yes | 6 weeks |
| 3 | 33 | Cervix | Squamous | II B | Yes | | 6 weeks |
| 4 | 36 | Cervix | Squamous | III B | Yes | Yes | 6 weeks |
| 5 | 34 | Cervix | Squamous | III B | Yes | Yes | 6 weeks |
| 6 | 27 | Cervix | Squamous | III B | Yes | Yes | 6 weeks |
| 7 | 30 | Cervix | Squamous | III B | Yes | | 6 weeks |
| 8 | 41 | Cervix | Squamous | III B | Yes | Yes | 6 weeks |
| 9 | 38 | Cervix | Squamous | III B | Yes | Yes | 6 weeks |
| 10 | 40 | Vaginal | Squamous | III B | Yes | Yes | 6 weeks |
| 11 | 38 | Cervix | Squamous | III B | Yes | Yes | 6 weeks |

Ovarian tissue handling of participants

| Participant | No. of ovaries removed | No. cortex strips | Strips used for histology | Strips transplanted | Total no. of strips left in storage |
|---|---|---|---|---|---|
| 1 | 1 | 75 | 17 | 0 | 58 |
| 2 | 2 | 68 | 18 | 0 | 50 |
| 3 | 2 | 103 | 20 | 11 | 72 |
| 4 | 2 | 116 | 20 | 12 | 84 |
| 5 | 1 | 48 | 8 | 0 | 40 |
| 6 | 2 | 129 | 7 | 14 | 108 |
| 7 | 2 | 87 | 15 | 0 | 72 |
| 8 | 2 | 75 | 11 | 12 | 52 |
| 9 | 2 | 82 | 10 | 12 | 60 |
| 10 | 2 | 63 | 15 | 0 | 48 |
| 11 | 2 | 54 | 18 | 10 | 26 |

Indication in which cortex tissue samples follicles was seen after HE processing and assessment. Samples where no follicles could be identified were not sent for TEM processing

| Participant | Group | PROH | | DMSO | |
|---|---|---|---|---|---|
| Sample | Fresh Control | Equilibration | Cryopreserved | Equilibration | Cryopreserved |
| 1 | Yes | Yes | Yes | Yes | Yes |
| 2 | Yes | Yes | Yes | Yes | Yes |
| 3 | No | No | No | Yes | No |
| 4 | Lost | Lost | Lost | No | No |
| 5 | Yes | Yes | No | No | No |
| 6 | Yes | Yes | Yes | Yes | Yes |
| 7 | Yes | Yes | Yes | No | Yes |
| 8 | Yes | Yes | Yes | No | Yes |
| 9 | No | No | No | No | No |
| 10 | Yes | Yes | Yes | Yes | Yes |
| 11 | No | No | No | No | No |

Amount of follicles identified per tissue sample group for TEM evaluation.

| Participant | Group | PROH | | DMSO | |
|---|---|---|---|---|---|
| Sample | Fresh Control | Equilibration | Cryopreserved | Equilibration | Cryopreserved |
| 1 | 3 | 1 | 1 | 1 | 1 |
| 2 | 2 | 1 | 0 | 1 | 2 |
| 3 | - | - | - | 1 | - |
| 4 | - | - | - | - | - |
| 5 | 1 | 0 | - | - | - |
| 6 | 1 | 2 | 1 | 2 | 1 |
| 7 | 1 | 0 | 1 | 0 | 1 |
| 8 | 1 | 1 | 1 | - | 0 |
| 8 | - | - | - | - | - |
| 10 | 1 | 0 | 1 | 1 | 0 |

| | | | | |
|---|---|---|---|---|
| 11 | - | - | - | - |
| Total | 10 | 5 | 5 | 5 |

Blood LH, FSH and $E_2$ results of participants who received an ovarian transplantation

| Pt no | Date of Cryopre-servation | Date of Trans-plantation | CPA | No. Strips | Date of blood test | E2 | FSH | LH | Time (months) | Follicle develop-ment |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 07/06/06 | - | - | - | - | | | | | - |
| 2 | 24/03/06 | - | - | - | 27/03/06 | <37 | 11.6 | - | - | |
| 3 | 31/03/06 | 05/06/06 | DSMO | 11 | 30/03/06 | 370.0 | 4.2 | 4.0 | -2 | - |
| | | | | | 05/06/06 | 93.0 | 38.7 | 26.0 | 0 | - |
| | | | | | 05/07/06 | <37 | 38.6 | 30.1 | 1 | No |
| | | | | | 02/08/06 | <37 | 37.6 | 17.9 | 2 | No |
| | | | | | 10/10/06 | <37 | 38.5 | 27.7 | 4 | No |
| | | | | | 07/02/07 | 59.0 | 7.8 | - | 8 | Yes |
| | | | | | 08/05/07 | 126.0 | 27.4 | 30.5 | 11 | Yes |
| | | | | | 06/06/07 | <37 | 9.5 | 1.7 | 12 | No |
| | | | | | 04/07/07 | <37 | 1.2 | 0.3 | 13 | No |
| | | | | | 29/08/07 | <37 | - | 54.6 | 15 | ? |
| | | | | | 12/11/07 | <37 | 24.7 | 2.9 | 18 | |
| 4 | 05/05/06 | 24/07/06 | PROH | 12 | 24/07/06 | <37 | 54.0 | 47.8 | 0 | |
| | | | | | 09/10/06 | <37 | 67.7 | 30.8 | 3 | |
| | | | | | 12/04/07 | <37 | 75.9 | 37.6 | 9 | |
| | | | | | 16/05/07 | 300.0 | 69.6 | 47.8 | 10 | ? |
| | | | | | 20/06/07 | <37 | 81.4 | 51.1 | 11 | Yes |
| | | | | | 19/09/07 | <37 | 24.7 | 2.9 | 14 | |
| 5 | 26/05/06 | - | - | - | 25/05/06 | <37 | 6.7 | 0.7 | | |
| 6 | 07/07/06 | 19/09/06 | DSMO | 14 | 06/07/06 | <37 | 5.8 | 3.1 | -2 | |
| | | | | | 19/09/06 | <37 | 33.9 | 21.6 | 0 | |
| | | | | | 11/10/06 | <37 | 54.7 | 42.0 | 1 | |
| | | | | | 01/11/06 | <37 | 59.6 | 73.7 | 2 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 11/12/06 | <37 | 87.0 | 84.8 | 3 | |
| | | | | | 21/02/07 | <37 | 28.9 | 5.0 | 5 | ? |
| | | | | | 05/03/07 | <37 | 1.1 | 0.1 | 6 | ? |
| 7 | 25/08/06 | - | - | - | 25/08/07 | <37 | 3.1 | 6.3 | | |
| 8 | 19/09/06 | 30/10/06 | PROH | 12 | 30/08/06 | 94.0 | 4.4 | 7.8 | -2 | |
| | | | | | 31/10/06 | <37 | 67.0 | 71.1 | 0 | |
| | | | | | 11/12/06 | 19.0 | 97.5 | 67.7 | 2 | |
| 9 | 22/09/06 | 27/11/06 | DMSO | 12 | 22/09/06 | 138.0 | 5.4 | 1.4 | -2 | |
| | | | | | 27/11/06 | 38.0 | 52.2 | 80.5 | 0 | |
| | | | | | 06/02/07 | <37 | 93.7 | 102.5 | 2 | |
| 10 | 03/11/06 | - | - | - | 03/11/06 | 59.0 | 4.9 | 2.6 | | |
| 11 | 08/12/06 | 29/01/07 | PROH | 10 | 08/12/06 | 346.0 | 3.2 | 2.8 | -2 | |
| | | | | | 29/01/07 | <37 | 200.0 | 173.4 | 0 | |
| | | | | | 28/02/07 | <20 | 150.0 | 207.4 | 1 | |
| | | | | | 04/05/07 | 34.0 | 150.0 | 156.2 | 4 | |
| | | | | | 06/07/07 | <20 | 150.0 | 250.0 | 6 | |

# APPENDIX 6

# UNPUBLISHED REFERENCES

## Ovarian tissue cryopreservation

J. Shaw

Monash Institute of Reproduction and Development,
Monash University
Current September 2003

**Contact Information for further details:**
J. Shaw at jill.shaw@med.monash.edu.au

**Publications using this protocol:**
**Book Chapters**

1. Shaw JM & Nakagata. N. (2002) Cryopreservation of transgenic mouse lines. Ch 10 in: *Methods in Molecular biology*, vol 180:207-228 "Transgenesis techniques: Principles and Protocols" 2nd Edn Editor A. Clark Humana press Totowa.
2. Shaw JM. & Trounson AO. (2002) Ovarian tissue transplantation and cryopreservation: its application to the maintenance and recovery of transgenic mouse Ch 11 in: *Methods in Molecular biology*, vol 180:229-251 "Transgenesis techniques: Principles and Protocols" 2nd Edn Editor A. Clark Humana press Totowa

**Papers**

3. Cleary, M. Snow, M. Wolvekamp, M. Shaw, J.M. Cox S-L and Jenkin G. (2001) Cryopreservation of mouse ovarian tissue following prolonged exposure to an ischaemic environment. **Cryobiology**. 42:121-133.
4. Snow, M. Cleary, M. Cox, S-L. Shaw J., Paris M. and G. Jenkin (2001) Comparison of the effects of in vitro and in situ storage on the viability of mouse ovarian tissue collected after death. **Reprod. Fertil. Dev**. 13:389-394.
5. Paris MCJ., Snow, M., Cox, S-L, and Shaw JM. (2003) Xenotransplantation: can this technology provide a tool for reproductive biology and animal conservation ? Theriogenology (In press)

### Application

Ovarian tissue cryopreservation has been applied to a wide range of species including:

- *Eutherians*: Mouse, rat, rabbit, sheep, pig, cow, marmoset, macaque, human, cat, elephant, dog.
- *Marsupials*: Wallabies, Sminthopsis, common wombat.
- Others: e.g. Silkworm.

In all these cases the tissue has been viable after thawing (as assessed by *in vitro* or *in vivo* assays).

Live young can be derived from frozen thawed grafted ovarian tissue, but to date only a small number of studies have proven that this is possible (mainly mouse and sheep). This indicates that ovarian tissue banking should be possible for a wide range of species.

Most studies have used protocols that are identical or very similar to that described in this document.

We recommend that it, or another proven procedure, be followed as closely as possible, because even though some variations to this protocol have relatively little impact on the outcome [e.g. using a different buffer, an equivalent cryoprotectant (ethylene glycol instead of DMSO), or dehydrating agent (e.g. trehalose instead of sucrose) or slightly different equilibration temperatures or times], other changes can be detrimental (e.g. using glycerol rather than DMSO) or lethal (e.g. altered cooling or warming rates).

The protocol provided here is a "slow cooling" procedure because this is the one that has (to date) proven most versatile and reliable.

**Background**

Cryopreservation of ovarian tissue is a simple procedure which can be used to store the germline of valuable female animals. In brief, it involves putting ovarian tissue into antifreeze solution and then cooling it to a low sub zero temperature (liquid nitrogen) for storage. The tissue can subsequently be thawed for use or analysis.

For best results use good quality tissue. Ideally the tissue should be collected from young healthy females and processed for cryopreservation immediately after collection with sterile/ aseptic techniques. The reasons for this are:

A) Age: ovarian tissue of mammals contains the maximum number of germ cells (eggs/ oocytes) at or around the time of birth, after this time there is an exponential loss with age. In some species old females may have no viable oocytes left in their ovaries.

B) Health: Diseased cells present within ovarian tissue (e.g. cancer cells) can survive frozen storage. Other infectious agents can also survive frozen storage.

C) Sterile/aseptic technique: Frozen material may be used to generate offspring by any of a range of techniques including in vitro maturation, in vivo maturation (following grafting to a suitable recipient), or nuclear transfer. The presence of contaminating bacteria, fungi or other infectious agents could prevent or compromise these uses.

D) Rapid processing. Aim to collect ovaries by surgery or immediately after the death of an animal. Delays between the death of the animal and collection have a detrimental effect on the tissue. The viability of follicles declines as the delay between collection and cryopreservation increases. A delay of only a few hours within the body of the dead animal, is highly detrimental and kills most follicles. Ovarian tissue which is removed from an animal immediately after its death (or is removed by surgery) and placed in e.g. Phosphate buffered saline on ice or at room temperature, deteriorates less rapidly than within a dead animal, but even with this treatment few, if any, viable follicles remain 48 h after collection.

Current cryopreservation procedures focus on collecting the outermost ovarian cortex. The reason for this is that the primordial follicles (the oocyte stage which is most abundant in the ovary, and which tolerate cryopreservation very well) are mainly located within the outermost layer (2mm or less from the surface) of the ovary. Unfortunately primordial follicles are very small (<50microns) and very difficult to see except by histology or after enzymatic digestion of the ovary. A freezing protocol which preserves primordial follicles should allow later stage follicles to be generated if required because the primordial follicles grow to form mature oocytes/ follicles. In the mouse it takes approximately 3 weeks for a primordial follicles to grow and mature into a mature oocyte either in vitro or in vivo. In other species this process takes longer (months) and can (currently) only be completed in vivo (by auto, allo or xenografting). During the period of maturation many cytoplasmic and nuclear maturation (including imprinting) steps take place within the oocyte which prepare the egg for fertilization and development to term.

This report details a standard slow cooling cryopreservation protocol for ovarian tissue which is known to be compatible with the survival of primordial follicles within ovarian tissue for a wide range of species. Rapid cooling procedures are available but they have only been tested on a few species.

## Summary: Media/products

A) *For collection*: PBS or other bench type handling media (100 ml per large ovary) (sterile, without protein)

Note: If **oocytes** are to be collected approx. 10 ml of protein containing bench solution for oocyte handling (e.g. 3-8 mg/ml Bovine Serum Albumin or 5-10% serum) should be prepared.

B) *For equilibration and freezing*: Cryoprotectant solution: one 10 ml tube per large ovary (1.1 ml DMSO; 0.32g sucrose made up to 10 ml with buffer. Sterile without protein)

C) *For dilution after thawing*:

First dilution solution: 0.55 ml DMSO and 0.62 g sucrose in a 10 ml tube (3ml per vial). Sterile without protein

Second dilution solution: 0.62 g sucrose in a 10 ml tube (3ml per vial). Sterile without protein

Solutions should be sterile. They can be sterilized by filtration through a sterile 0.22micron filter (e.g. syringe type filter).

**Summary: Large equipment**

- Storage tank (liquid nitrogen or equivalent)
- A vapour shipper may be needed if specimens are to be transported frozen.
- Cooling device
  - Freezing machine (Note most freezing machines require access to electricity)
  - OR Passive cooling container or equivalent (this requires dry ice in an eski or a –80 freezer)\
- Sterile work area (biohazard hood or equivalent is best)
- Microscope if there is a need to look for/ collect eggs.

**Summary Other materials/ equipment**

| Note: If the tissue has to be sterile then it is advisable to pre- sterilize items marked with * | Collection & dissection | For freezing | For thawing |
|---|---|---|---|
| **ITEMS** | | | |
| *Gloves (mask/ cap) | Y | Y | Y |
| Safety goggles | (optional) | Y | Y |
| Betadine/ alcohol or equivalent to sterilize skin prior to dissection | Y | - | - |
| Clippers (to remove/ shave fur /wool/hair) | (optional) | - | - |
| *Dissection equipment (e.g. scalpel, scissors, forceps, retractor) | Y | - | - |
| *Specimen jar/ tube (e.g. 50 ml screwcap) for transport of the ovary/ ovaries | Y | N | N |
| Ice, crushed, in container (e.g. polystyrene eski) | Recommended | Y | N |
| *Tubes containing the required solutions (outlined above) | Y | Y | Y |
| *Petridishes, 10 cm (allow at least 2 per ovary) | Y | (optional) | N |
| *Petridishes, 35 mm | Y (for oocytes) | N | Y |
| *Pipettes or equivalent for measuring solutions (e.g. 1 and 10 ml) | Y | Y | Y |
| Test tube rack (optional) | Y | Y | Y |
| *Scalpel #20 or #22 blade (for ovary dissection) | Y | N | optional |
| *Scissors (small for ovary dissection) | Y | N | N |
| *Forceps: Small, for holding and moving the tissue. | Y | Y | Y |
| *Forceps: large (or equivalent, for handling and seeding) | (optional) | Y | Y |
| *Pipettes or equivalent (e.g. pasteur pipettes with pipette bulb/ plastic transfer pipette) to move small volumes of solution | Y | Y | Y |
| *1 ml syringe with 26 g needles or equivalent (for puncturing follicles) | Y | N | N |
| Tissues | Y | Y | Y |
| Alcohol (in squirter bottle) | Y | Y | Y |
| Pen/ paper for taking notes | Y | Y | Y |
| Indelible pen (for marking dishes and tubes) | Y | Y | Y |
| Garbage bin/ bin liner | Y | Y | Y |
| Sharps container | Y | Y | Y |
| Hand wash soap/ water to wash hands | Y | Y | - |
| Bench cover (e.g. plastic backed paper) | optional | optional | optional |
| Plastic apron/ gown/ labcoat | advisable | advisable | Advisable |
| Liquid nitrogen | N | Y | Y |
| Benchtop container for liquid nitrogen (can be eski) | N | Y | Y |
| *Cryovials 1.7 ml star-foot, external thread, conical or round base (2 per person) | N | Y | N |
| Test tube rack (for Cryo tubes) (optional) | N | Y | N |
| Cryo Canes | N | Y | N |
| Timer | N | Y | Y |
| Insulating Cryo-gloves | N | Y | Y |
| Thermometer (for water temperature) | N | N | Y |
| Waterbath or Beaker (for warm water for thawing) | N | N | Y |

If eggs are to be collected then a pipettor or equivalent that can be used to move eggs between dishes is needed. If eggs are to be stored or used for ART, then additional solutions and materials (not listed here) would be required.

**Steps in preparing specimens for cryopreservation:**

It is advisable to prepare all *equipment* and *solutions* needed for collection, dissection and cryopreservation (listed in the table above) in advance.

***For collection***

Prepare the media, equipment and items listed above:

**Procedure**

If possible remove fur/ hair/ wool and swab the skin over the intended incision site with antiseptic. Use sterilized dissection instruments. Access the ovary via one (or more) incision through the skin and abdominal wall. Excise the ovary(s) /piece of ovary and place it in a sterile pot/ tube/ dish with sterile (protein free) media e.g. saline or PBS as soon as possible after collection. If the specimen cannot be processed immediately, or has to be transported to another location then place the specimen on ice.

***For dissection***

Prepare the media, equipment and items listed above:

**Procedures**

Very small ovaries (less than 5x5x5 mm) do not need to be dissected, as they can be frozen whole (skip to the section on freezing).

If only a part of an ovary has been collected then identify the outer and inner surfaces. If the piece(s) is less than 1 mm thick it can be cryopreserved without further dissection. (Skip to the section on freezing).

For larger ovaries or large ovarian pieces (> 1 mm thick) dissection is recommended as it facilitates the entry of the cryoprotectant into the tissue. The aim of the dissection is to remove the outer cortical tissue (tough, white, fibrous) for freezing.

If a whole (large) ovary has been collected, place it ovary in a dish of PBS at room temperature (condensation makes it difficult to work with ice cold specimens). Use your instruments to separate the white firm (outermost) region from the underlying red tissue. It can be difficult to separate the two layers, but it can usually be achieved using a combination of sharp, scissors (large and small), and scalpel blades (e.g. #22 &/or #20). If the white tissue surrounding the ovary is normal (smooth, elastic, and tough, around 1mm thick) it can be cut into "sheets" (5 x 5 mm x 1 mm) or "strips" (1-2 mm wide x 0.5 to 1 cm long, 1 mm deep) relatively easily.

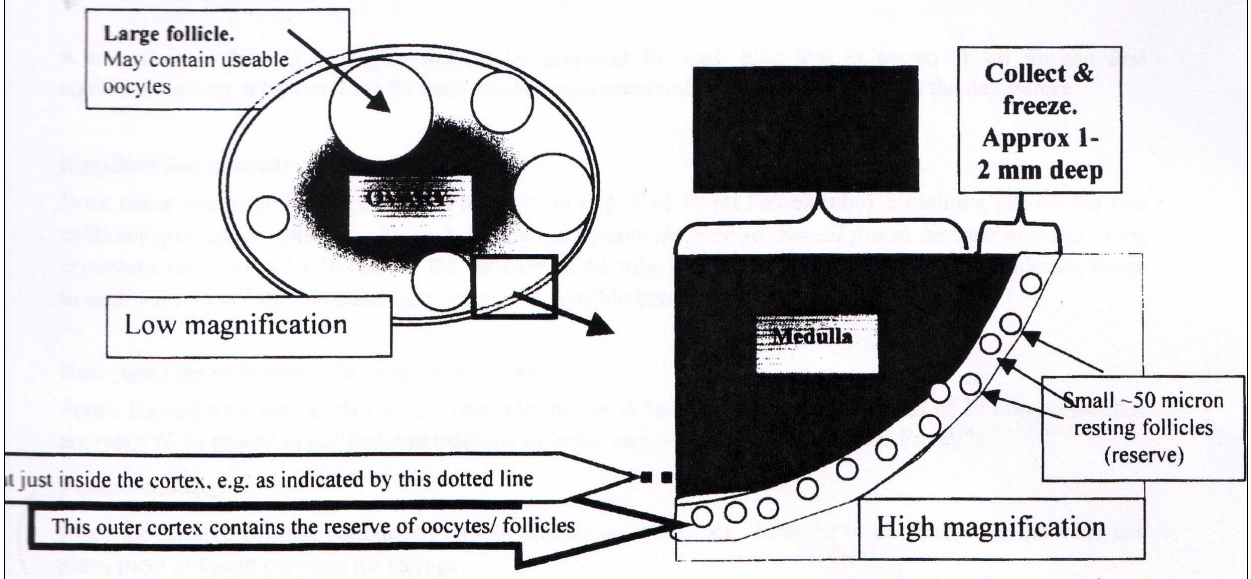


Figure: In larger species the cortex (the outermost region of the ovary) is the region that is collected and frozen because it contains most primordial follicles.

***Cryopreservation***

Have the media, equipment and items listed above prepared in advance:

The protocol outlined here allows ovarian tissue to be cooled to and stored at –196C. Access to a liquid nitrogen storage tank or the equivalent (nitrogen vapour storage tank) in which this tissue can be stored long term is essential.

Preparation of the freezing machine (or equivalent):

- If a programmable freezing machine is available a suitable programme is: hold at -6C until the chamber is loaded and seeded. Once seeding is complete cool the chamber at 0.3°C/ min to -40°C, then rapidly cool to –196C.
- If a programmable freezing machine is **not** available then low cost equipment such as the "Nalgene Mr frosty" (Sigma C1562) can be used. These will, when placed in a –80C freezer, cool ampoules/vials at around 1C/ min to –80C. This is adequate for ovarian tissue freezing. Place the Mr Frosty in the refrigerator or on ice for at least 1 hr before freezing is due to commence. If there is no –80 C freezer available then the Mr Frosty can be placed in a polystyrene box with pellets (or a block) of dry ice (frozen $CO_2$).

*Preparation of the solutions and vials*

Cryoprotectant should be made up, filtered and cooled to 0-1°C (in ice) before the dissection starts.

Ovarian tissue is most commonly frozen in 1.5 M DMSO (i.e. 5.5 ml Dimethyl sulphoxide (e.g. Sigma Hybrimax, D-2650)) and 0.1 M sucrose (i.e. 1.71g sucrose (e.g. Sigma S-7903)) made up to 50 ml with physiological, protein free, buffer (e.g. PBS). If filters which tolerate DMSO are available then sterilize the DMSO+ sucrose solution. If DMSO tolerant filters are not available then filter the sucrose + PBS and then add the DMSO. If DMSO is not available then 1.5 M ethylene glycol or 1.5 M propane diol can be used instead. If a different cryoprotectant is used then the dilution solutions should be made up with the same (corresponding) cryoprotectant.

- Prepare at least 10 ml cryoprotectant per ovary
- Pre-cool cryovials (e.g. 1.7 ml Nunc conical, star based, internal thread, CRYO tubes Cat # 377224) on ice. It is easiest if these are each pre-filled with 1 ml cryoprotectant.

It is best if *at least* 2 ml cryoprotectant be prepared for each tube that is frozen (1 ml for the first equilibration step, a further 1 ml for each freezing ampoule/vial). This can be made up the day before.

**Equilibration procedure:**

Once tissue has been dissected, placed it in a tube (e.g. 5 or 10 ml Falcon tube) containing pre-cooled (ice cold) cryoprotectant solution. *Every 5-10 minutes agitate the tube so that all pieces become exposed to the cryoprotectant.* After 15-20 min tip the contents of the tube out into a sterile dish and rapidly allocate tissue to each vial. Distribute the material as evenly as possible between all vials.

Note: label the vials before freezing commences.

Screw the cap back onto each vial and return to the ice. After the tissue has had a total of 30 min on ice they are ready to be placed in the freezing machine or in the passive cooling device ("Mr Frosty").

**Passive cooling:**

Place the passive cooling container in a –80 freezer or on dry ice. After 12 to 24 h, remove the vials and place them in liquid nitrogen for storage.

**Cryoporeservation in a freezing machine**

Ovarian tissue cryopreserved at 0.3C/ min in a biological freezer is better preserved than material cryopreserved in a passive cooling device (e.g. Mr Frosty). The difference is however not big, and must be counterbalanced against the detrimental effect of deterioration with time after collection. Thus material will be of better quality if it is frozen at 1C/ min (Mr Frosty on dry ice) as soon as possible after collection, than if the cryopreservation is delayed but then cooled at 0.3C/ min (e.g. by being transported to a site with a biological freezer).

After loading the vials containing ovarian tissue into a freezing machine they should be seeded to initiate ice formation (vials placed in a "mr frosty" are not seeded). Seeding is usually performed once the vials have reached -6°C (this can take 5 minutes). Seed each vial by pulling it up and touching its outer surface with a large pair of forceps pre-cooled in liquid nitrogen (place the forceps just above the meniscus of the liquid, taking care to not touch the sample). After all vials have been seeded re-examine each one in turn to establish that the solution still contains ice crystals.

**Cooling programme:**

Once it has been confirmed that ice is present in all tubes the cooling program can start. The cooling rate should be 0.3°C/min to -40°C. On reaching -40°C the vials can be plunged directly into liquid nitrogen, and placed on labelled canes for storage. There may be a benefit from introducing a further rapid cooling ramp (e.g. 10 or 40C/ min) to a temperature of –140°C or less before moving the vials into liquid nitrogen.

**THAWING PROCEDURE:**

Solutions and a water bath should be prepared and sterilized in advance:

(1) The first thawing solution contains 0.75M DMSO and 0.25 M sucrose (i.e. 0.55 ml DMSO and 0.86 g sucrose/10 ml PBS), at room temperature (20-22°C).

(2) The second solution contains only 0.25 M sucrose (i.e. 0.86 g sucrose/10ml PBS) at room temperature (20-22°C).

The water bath should be at 37°C.

After long term storage vials may have filled with liquid nitrogen. Eliminate the risk of explosions by holding the vial **deep** in the nitrogen vapour (just above the surface of the liquid nitrogen). Once there is no sign of liquid nitrogen in the vial immerse the vial into a 37°C waterbath until thawed (this may take several minutes).

Write down the details written of the vial then wipe it over with alcohol before removing the lid.

The tissue is then either grasped with sterile forceps and placed in the first thawing solution (0.75M DMSO+ 0.25 M sucrose), or the whole contents of the vial decanted into it. After 10 min the ovarian tissue is moved to the second solution (0.25 M sucrose), and 10 min later placed in PBS ready for grafting or culture.

Remember sterile technique should be applied throughout, all solutions, equipment, and containers should be sterile.

**OOCYTES.**

Optimized freezing protocols for immature oocytes have yet to be established. Thus it is not currently possible to recommend a strategy by which to preserve the immature oocytes which are released into the collection dish during the dissection of the ovarian tissue. If the embryologist wishes to try to preserve these oocytes for the patient, they could use an existing human oocyte slow cooling (e.g. Fabbri et al 2001; Boldt et al 2003) or rapid cooling protocol (Chen et al 2000; Liebermann and Tucker 2002) either before, or after in vitro maturation.

**OTHER TISSUES**

Note that the protocol given above can be applied to tissues from other organs including skin.
Note that cell suspensions may be better cryopreserved using standard cell freezing protocols.

**Acknowledgements:**

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**Additional References (for oocyte cryopreservation):**

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