Department of Clinical Science, Intervention and Technology
Karolinska Institutet, Stockholm, Sweden

CLINICAL GRADE VITRIFICATION OF HUMAN OVARIAN TISSUE FOR FERTILITY PRESERVATION

Mona Sheikhi

Karolinska Institutet

Stockholm 2013

Published by Karolinska Institutet. Printed by [Larserics Digital Print AB].

ISBN 978-91-7549-149-3

”Try not to become a man of success, but rather a man of value”

Albert Einstein

To my dear children and parents, with love

ABSTRACT

Cryopreservation of human ovarian tissue is one approach to preserve fertility for women who can be predicted to undergo premature ovarian failure as a consequence of chemotherapy, radiotherapy or genetic disorders. It is the most suitable option for pre-pubertal girls and for many young women to store oocytes. To date, auto-transplantation of frozen- thawed cortical tissues has resulted in birth of 24 healthy children, worldwide. Cryopreservation can be performed using slow freezing or vitrification. Vitrification is known as solidification without formation of lethal intracellular ice crystals. The aim of this thesis was to further develop methods for cryopreserving follicles in human ovarian tissue of women who have a risk of losing their fertility.

In the first study, we systematically compared two cryopreservation methods for human ovarian cortical tissue, slow freezing and vitrification. Cryoprotectants we used for slow freezing were either 1,2- propanediol (PrOH)- sucrose or ethylene glycol (EG)-sucrose. For vitrification, we used solutions containing a combination of the cryoprotectants dimethyl sulphoxide (DMSO), PrOH, EG and polyvinylpyrrolidone (PVP). Light microscopy (LM), transmission electron microscopy (TEM) and post-thaw tissue culture were carried out to evaluate the structure and the viability of the follicles. The follicles were well preserved and the ovarian stroma showed better morphological integrity after vitrification. In the second study, we developed a clinical grade vitrification of human ovarian tissue. Ovarian tissue was vitrified in a closed system without any direct contact with liquid nitrogen using a non-toxic and sterile cryotube. Vitrification solutions used contained a combination of cryoprotectants DMSO, PrOH, EG and PVP. The morphology of the follicles in the vitrified tissue, showed well -preserved structures as verified by LM, TEM and also after post -thaw culture. The system used is compatible with the European tissue directive and the Swedish tissue law. In the third article, we studied the occurrence of apoptosis in vitrified tissues. We used Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and DNA fragmentation analysis, which showed no incidence of apoptosis in follicles or stroma after using either vitrification solution (i.e., a combination of DMSO, PrOH, EG and PVP, or EG and bovine serum albumin (BSA). In the fourth one, we performed a study to simplify our earlier described closed vitrification procedure. Permeating cryoprotectants used in vitrification solutions consisted either of a combination of DMSO, PrOH, EG or EG only. Ovarian tissue was vitrified in closed sealed tubes containing either of the vitrification solutions. Morphological analysis (LM and TEM) showed that oocytes, granulosa cells and stroma were equally well preserved when either of the vitrification solutions was used. No apoptosis was observed in primordial and primary follicles using immunohistochemistry for active caspase- 3. Conclusion: Hereby we present new vitrification procedures that can be performed in a clinical setting. The morphology of follicles in the ovarian tissue as evaluated by using LM and TEM proved to be normal after the procedures. Ultra-structural analysis by TEM used in this study is the best-known method to evaluate cryoinjury. We have developed a new effective clinical grade method for cryo-storage of human ovarian tissue.

LIST OF PUBLICATIONS

I. I. Keros V, Xella S, Hultenby K, Pettersson K, **Sheikhi M**, Volpe A, Hreinsson J, Hovatta O. Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue. Hum Reprod. 2009 Jul;24(7):1670-83.

II. **Mona Sheikhi,** Kjell Hultenby, Boel Niklasson, Monalill Lundqvist and Outi Hovatta. Clinical grade vitrification of human ovarian tissue: an ultrastructural analysis of follicles and stroma in vitrified tissue. Human Reproduction, Vol.26, No.3 pp. 594–603, 2011.

III. Mojdeh Salehnia, **Mona Sheikhi**, Shahram Pourbeiranvand, Monalill Lundqvist. Apoptosis of human ovarian tissue is not increased by either vitrification or rapid cooling. Reproductive BioMedicine Online (2012) 25, 492–499.

IV. **Mona Sheikhi,** M.Sc., Kjell Hultenby, Ph.D., Boel Niklasson RNM., Monalill Lundqvist, Ph.D. and Outi Hovatta, M.D., Ph.D. Preservation of human ovarian follicles within tissue frozen by vitrification in a xeno-free closed system using only ethylene glycol as a permeating cryoprotectant.
(In press in Fertility and Sterility)

RELATED PUBLICATION NOT INCLUDED IN THIS THESIS

Borgström B, Hreinsson J, Rasmussen C, **Sheikhi M**, Fried G, Keros V, Fridström M, Hovatta O. Fertility preservation in girls with turner syndrome: prognostic signs of the presence of ovarian follicles. J Clin Endocrinol Metab. 2009 Jan; 94(1):74-80.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AMH	Anti-Müllerian hormone
BSA	Bovine serum albumin
BMP	Bone morphogenetic protein
cAMP	Cyclic adenosine 3, 5- monophosphate
cGMP	Guanosine 3, 5- cyclic monophosphate
CPA	Cryoprotective agent
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
EG	Ethylene glycol
ER	Endoplasmic reticulum
FSH	Follicle-stimulating hormone
GC	Granulosa cell
GDF-9	Growth differentiation factor-9
GnRH	Gonadotrophin releasing hormone
GV	Germinal vesicle
Gy	Gray, absorbed dose of irradiation
HSA	Human serum albumin
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilization
IVM	In vitro maturation
LH	Luteinizing hormone
LM	Light microscopy
PBS	Phosphate- buffered saline
PCOS	Polycystic ovary syndrome
PGC	Primordial germ cell
POF	Premature ovarian failure
PROH	1,2- propanediol
PVP	Polyvinylpyrrolidone
SCF	Stem cell factor
TEM	Transmission electron microscopy
TGF- ß	Transforming growth factor- ß
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

1 INTRODUCTION

Infertility is a significant public health problem that affects about 15% of couples of fertile age worldwide. There are large numbers of factors, which cause infertility. One cause is cancer treatment. Ovarian damage is a common side effect of chemotherapy and radiotherapy for women and girls who are after such treatment at a risk of losing their germ cells (Nicosia *et al.* 1985; Wallace *et al.* 1989; Howell and Shalet 1998; Schmidt *et al.* 2010). There are also genetic causes. Follicles in girls with Turner's syndrome, for instance, disappear prematurely often before 20 years of age (Hreinsson *et al.* 2002; Borgstrom *et al.* 2009).

Sperm cryopreservation has been an option for men to preserve their reproductive capability before starting cancer treatment for over 60 years. The first successful human pregnancy using frozen sperm was obtained in 1953. Ovarian tissue cryopreservation has been a feasible method since 1996 with functioning tissue after thawing. It has been used for young women, including girls with Turner's syndrome (Hovatta *et al.* 1996; Newton *et al.* 1996; Hreinsson *et al.* 2002). The first healthy child has been born after autologous re-transplantation of frozen-thawed tissue in 2004 (Donnez *et al.* 2004), and since then 24 children have been born (Dolmans *et al.* 2013).

Cryopreservation of ovarian tissue is the most suitable technique for pre-pubertal and young women, who do not yet have mature oocytes for in vitro fertilization (IVF). The vast majority of immature ovarian follicles are situated in the ovarian cortex, and cryopreservation of this tissue also enables storing a large number of oocytes (Hovatta *et al.* 1996; Newton *et al.* 1996; Hreinsson *et al.* 2002).
A cryopreservation procedure should ensure the viability of the follicles and minimize changes in morphology and ultra-structure. Cells are exposed to different stresses caused by ice formation, during cooling and warming. These stresses vary due to the cryopreservation techniques used; slow freezing or vitrification (Karlsson and Toner 1996; Mullen and Critser 2007). Until today, slow rate freezing has been mostly employed in clinical programmes. A new promising technique, vitrification of ovarian tissue, has received researchers' considerable interest, first in experimental animals and then also in human (Li *et al.* 2007; Lornage and Salle 2007; Huang *et al.* 2008; Keros *et al.* 2009; Sheikhi *et al.* 2011).

2 REVIEW OF THE LITERATURE

1.1 DEVELOPMENT OF OOCYTES AND OVARIAN FOLLICLES

1.1.1 Ovarian reserve

The pool of female germ cells is formed during foetal period. Female fertility potential can be measured by the ovarian reserve. Sperm production in male is a continuing process, but oocytes in postnatal life are non-renewable (Zuckerman, 1951; (Byskov *et al.* 2011). Even though opposing opinions have recently been presented (Johnson *et al.* 2004; Johnson *et al.* 2005), the results have not been convincing, and they have not been repeatable (Gerner unpublished data). Neither has anybody else managed in culturing up oocytes from stem cells in ovarian stroma (Adhikari and Liu 2009; Byskov *et al.* 2011).

The ovary contains a large number of oocytes surrounded by supporting somatic granulosa and theca cells that form a unit called follicle. The primordial follicles are embedded in stroma, the fibrous tissue in the ovary. Throughout the entire reproductive period of a woman, only 400-500 follicles become ovulated, whilst the rest degenerate (Gougeon 1996). The number of primordial follicles continually decreases by atresia at an exponential rate during reproductive life (Faddy *et al.* 1992; Faddy and Gosden 1995; Faddy 2000). The two main tasks performed by the ovary consist of producing and housing fertilisable oocytes, and secreting of hormones that are responsible for follicle maturation and the appearance of secondary sex characteristics.

1.1.2 Folliculogenesis

Folliculogenesis is a process in which follicles are formed from primordial germ cells in the foetal ovary (Byskov *et al.* 2011), and later on when a resting follicle begins to grow and develop through the primordial, primary, secondary and tertiary (antral) stages into the ovulatory stage. The developing follicles are located within the ovarian cortex. There are four major events involved in the postnatal folliculogenesis, i.e., recruitment of follicles into the growing pool, preantral follicle growth and development, selection of one dominant follicle and maturation of the preovulatory follicle (Gougeon 2010).

In the sexually mature female, primordial follicles, which have remained quiescent for years, are recruited little by little into the growing pool. Each month, a cohort of follicles grows under the influence of gonadotrophins. Normally only one of them ovulates under a complicated hormonally regulated process (Adhikari and Liu 2009). In response to a mid-cycle surge of luteinizing hormone (LH), one dominant follicle releases a mature oocyte ready to become fertilized (Gougeon 1996; McGee and Hsueh 2000). At the antral stage, most follicles die by atresia while a few of them reach the pre-ovulatory stage supported by the pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinising hormone (LH) (Gougeon 2010).

Follicular growth and maturation involve tight regulation of paracrine and endocrine signals. It requires the coordinated communication between the oocyte and its somatic cell companions. Two key somatic cells in the ovary are granulosa cells and theca cells. Granulosa cells support the regulation of the oocyte development by providing nutrients and molecular signals, while the oocyte is needed for granulosa cells to proliferate and differentiate in addition to promoting the organisation of the follicle. The communication between oocytes and granulosa cells occurs via paracrine signalling and bi-directional gap junctions (Reddy *et al.* 2010). Gap junctions, channels through the plasma membrane, allow the exchange of molecules such as amino acids, second messenger cAMP and possibly energy metabolites such as glucose (Eppig 1991, 1992).

1.1.3 **Oogenesis**

Oocytes in the ovarian follicles originate from primordial germ cells (PGC). During early embryonic development, PGCs move a short distance from the dorsal mesentery of the hindgut into the gonadal ridges at the beginning of the fifth week of gestation. They come to rest on either side of the coelomic angle and colonize the gonadal ridges (Mamsen *et al.* 2012). The colonization of the gonadal ridges occurs during the late fifth week or at the beginning of the sixth week of gestation. The PGCs continue to proliferate by mitosis and become rapidly surrounded by cords of somatic cells. During the ninth week, the differentiation of PGCs into oogonia occurs. In the human female, the PGCs enter meiosis at about 11-12 weeks of gestation (Mc *et al.* 1953; Fujimoto *et al.* 1977; Gondos *et al.* 1986; Ding *et al.* 2008; Mollgard *et al.* 2010).
The primordial oocytes enter meiosis and become arrested in the prophase of the first meiotic division, transformed into primary oocytes. At the time of arrest, the primary oocytes become surrounded by a single layer of flat granulosa cells (GCs), forming primordial follicles. The total number of follicles reaches a peak of around seven million at 16-20 weeks of gestation in the female foetus. During follicle formation, many oocytes are lost by apoptosis. The number of follicles subsequently decreases to about one to two million at birth and 3-400.000 at menarche (Faddy *et al.* 1992; Faddy 2000; Martins da Silva *et al.* 2004). The rate of follicular depletion accelerates from the age of 37 years onward, resulting in around 100-1000 follicles per ovary at menopause (Gougeon *et al.* 1994; Broekmans *et al.* 2007).

1.1.4 Growth factors and hormones

Transforming growth factor- beta (TGF-ß) superfamily
Anti- Müllerian hormone (AMH), inhibins, activins, bone morphogenic proteins (BMPs) and growth differentiation factors (GDFs) belong to the **Transforming growth factor- beta** (TGF-ß) **superfamily**. These growth factors are known to be important in early follicular growth (Massague and Chen 2000) in the ovary.
TGF-ß receptors exist in homo- or hetero-dimeric forms. They are single pass serine/ threonine kinase receptors. Receptor types I and II are two subfamilies of TGF-ß receptors distinguished by their structural and functional properties (Massague 1998).

AMH expression in ovarian tissue from healthy women was found to be highest in granulosa cells of secondary, preantral and small antral follicles (≥ 4mm) (Weenen *et al.* 2004). AMH is an important regulator of the initiation of growth of human primordial ovarian follicles (Carlsson *et al.* 2006), in a similar manner as it regulates follicular activation in rodents (Durlinger *et al.* 2002).

The **BMPs** play a significant role during embryogenesis and in the maintenance and repair of bone and other tissue in the adult (Massague and Chen 2000).
BMP receptors are located on the cell surface.

GDF-9 is a paracrine factor essential for mammalian ovarian folliculogenesis and fertility. It is expressed in human oocytes during early development of follicle (Laitinen *et al.* 1998; Hreinsson *et al.* 2002; Mottershead *et al.* 2008). Hreinsson et al. in our group showed that GDF-9 promotes the activation of primordial follicles in human cultured ovarian tissue. A significantly higher proportion of primordial follicles showed growth initiation and reached secondary stage in presence of GDF-9 (Hreinsson *et al.* 2002).

Inhibins are hetero-dimeric glycoproteins consisting of one α -subunit and one ß –subunit (de Kretser *et al.* 2002). They inhibit FSH secretion via a feedback regulatory mechanism. Inhibin B levels are highest in the early follicular phase and decrease in the late follicular phase of a menstrual cycle, whereas inhibin A level is highest in the late follicular and luteal phases (Groome *et al.* 1996). In the ovary, inhibins and activins are produced by granulosa cells of all follicles. In women older than 40 years, a higher level of FSH in the follicular phase was associated with a decrease in total inhibin concentration in both follicular and luteal phases (MacNaughton *et al.* 1992).

Activins are homo- or heterodimers consisting of two different, covalently linked, inhibin ß –subunits (ßA and ßB). There are three forms of activin including active A, activin AB and activin B. Activin is produced in granulosa cells of growing follicles (from primary to tertiary stage). It plays a role in promoting aromatase activity, the formation of antral cavity and proliferation of granulosa cells (Ying 1988; Thompson *et al.* 2005). The activity of activin is regulated by follistatin and inhibin that bind to its receptor and thereby mediate inhibition. Follistatin is a single chain polypeptide that is produced by the granulosa cells (Thompson *et al.* 2005). Activin has been used in promoting ovarian follicular growth in culture (Telfer *et al.* 2008).

Follicle stimulating hormone (FSH)

FSH is a gonadotrophin that stimulates the growth of antral follicles (Oktay *et al.* 1997). The initiation of follicular growth is gonadotrophin-independent, but FSH is still involved in early development of the follicles (Funkenstein *et al.* 1980). Shortly after initiation of growth, the follicles become responsive to FSH (Oktay *et al.* 1997).
FSH receptors (FSHR) are present in granulosa cells of growing follicles from the primary up to the Graafian stage. FSH sensitivity plays an essential role in the selection of dominant follicle for ovulation (Funkenstein *et al.* 1980). Women with an inactivating mutation in the FSHR showed hyper-gonadotrophic ovarian dysgenesis,

amenorrhea and infertility. Follicles do not develop beyond the primary stage in these women (Aittomaki *et al.* 1996).

1.2 OVARIAN TISSUE IN ANIMAL MODELS

Animal models have been used to optimise cryopreservation protocols for human ovarian tissue. These could be applied directly to humans. Studies on mutant mice have given valuable information for understanding molecular mechanisms underlying human follicular activation (Adhikari and Liu 2009; Reddy *et al.* 2010). Long before the human studies, ovarian tissue from several animal species has been cryopreserved. In these experiments glycerol was used as a cryoprotectant and tissues were stored at -79°C. Ovarian tissue from mice, rats and hamsters has been successfully cryopreserved. Transplantation of frozen- thawed mouse ovarian tissue (Deanesly, 1954; Green*, et al.*, 1956; Parkes; 1958) and isolated mouse primordial follicles from post-thawed ovarian tissue has resulted in live offspring (Carroll and Gosden 1993). Live births have been achieved after auto-transplantation of post-thawed ovarian tissues using slow freezing in sheep (Gosden *et al.* 1994). Since then, several successful studies regarding freezing of ovarian tissue from several animal species such as sheep (Almodin *et al.* 2004; Baird *et al.* 2004; Cecconi *et al.* 2004), goat (Rodrigues *et al.* 2004), zebu bovine (Lucci *et al.* 2004) and cat (Bosch *et al.* 2004) have been carried out.
Vitrification of ovarian tissue has been successfully used in mice with much looser ovarian structure than in humans (Salehnia *et al.* 2002; Tokieda *et al.* 2002; Wang *et al.* 2008). In vitro maturation of vitrified mouse ovarian follicles has resulted in live births (Wang *et al.* 2011). Recently, vitrification has been reported as an advanced alternative method for cryopreservation of ovarian tissue with improved viability of vitrified tissue, in various species including; mouse (Wang *et al.* 2009), rat (Deng *et al.* 2009), pig (Gandolfi *et al.* 2006), goat (Santos *et al.* 2007), sheep (Courbiere *et al.* 2006) and monkey (Yeoman *et al.* 2005; Hashimoto *et al.* 2010; Suzuki *et al.* 2012; Ting *et al.* 2013). Non-human primates represent attractive and relevant animal models for preclinical studies of reproductive biology, physiology, ovarian tissue and auto-transplantation (Stevens 1997; Nyachieo *et al.* 2013).

1.3 HUMAN OVARIAN TISSUE

The almond- shaped adult human ovary is 3-5 cm in length, 1.5-3 cm in wide and 0.5-1.5 cm in thickness. A thin layer of dense connective tissue known as the tunica albuginea encapsulates the ovary. Immediately beneath this layer lies the stroma that is composed of special connective tissue formed by spindle-shaped cells and extracellular matrix, supplied by blood vessels. The ovarian follicles are situated at a depth of one to two mm from the surface of the ovary, in a layer of stroma, termed cortex (Figure 1).

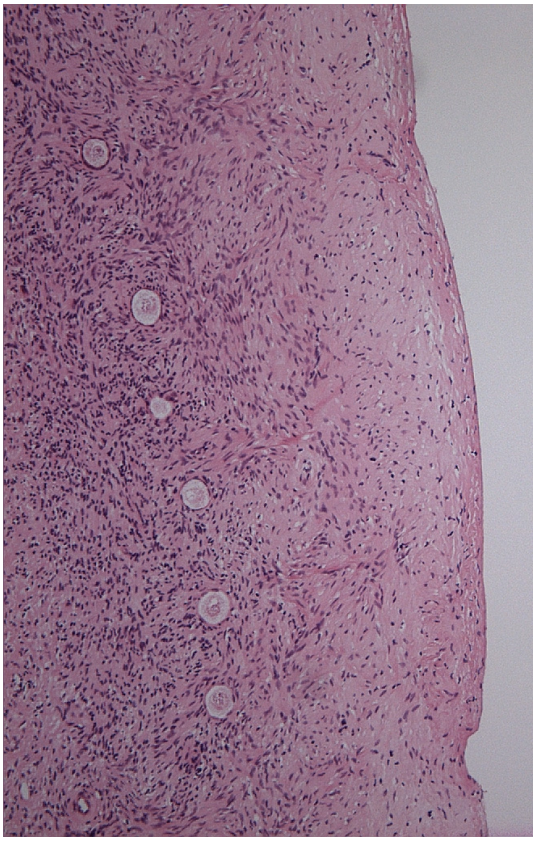

Figure 1. The ovarian follicles are located at a depth of 1-2 mm in the ovarian cortex (magnification: 100X)

The cortex contains highly compact connective tissue. Finally, under the cortical layer lies the ovarian medulla containing loose connective tissue, blood vessels, lymphatic vessels and nerves. The vast majority of the follicles are primordial (88%) whereas primary (8%) and secondary (4%) follicles are less frequent (Faddy *et al.* 1992; Lass *et al.* 1997). Our research group has developed an ovarian tissue culture system in which follicular growth in cryopreserved human ovarian tissue has been successfully achieved. Ovarian tissue was cultured on diluted extracellular matrix (ECM) i.e., Matrigel™ matrix or collagen that maintain the three dimensional organisation of the follicle (Scott *et al.* 2004).

The developmental stages of the human ovarian follicles are classified by the number and shape of granulosa cells around the oocyte. Primordial follicles consist of an oocyte

surrounded by a single layer of flattened granulosa cells. They have a diameter of about 30 μm (Figure 2).

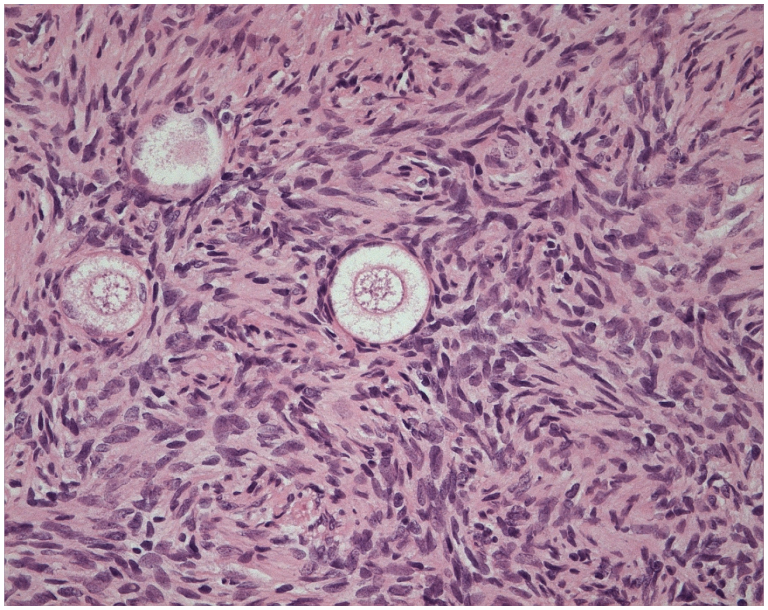

Figure 2. Light micrograph of a primordial follicle (right) and a transitional follicle undergoing formation of a primary from a primordial (left) follicle (magnification: 200X)

Primary follicles are about 30 - 60 μm in diameter and contain a complete single layer of cuboidal granulosa cells. The granulosa cells in the secondary follicles proliferate and form multiple cuboidal layers around the oocyte (Figure 3).

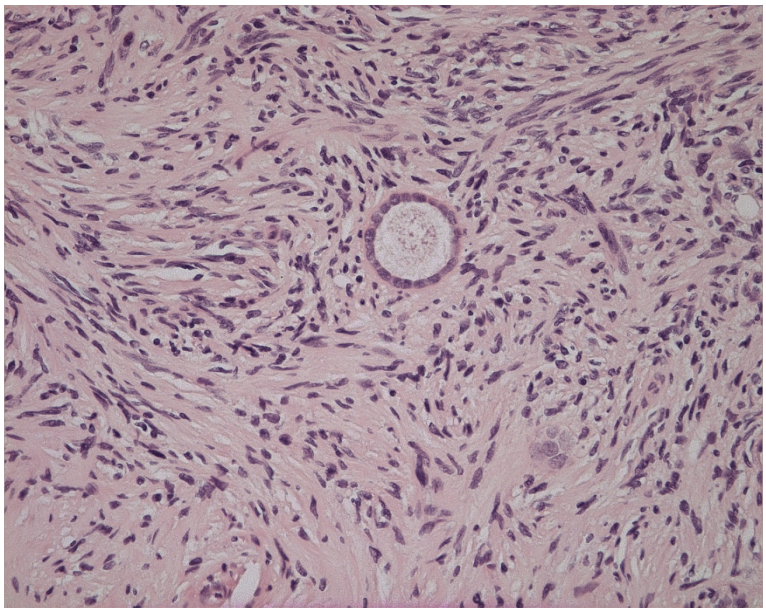

Figure 3. Light micrograph of a primary follicle (magnification: 200X)

At the secondary stage, the granulosa cells grown in several layers, theca interna and theca externa layers have been formed from the outside of the basement membrane (Gougeon 1986) and the diameter of the follicle has increased to about 100-200 μm (Figure 4).

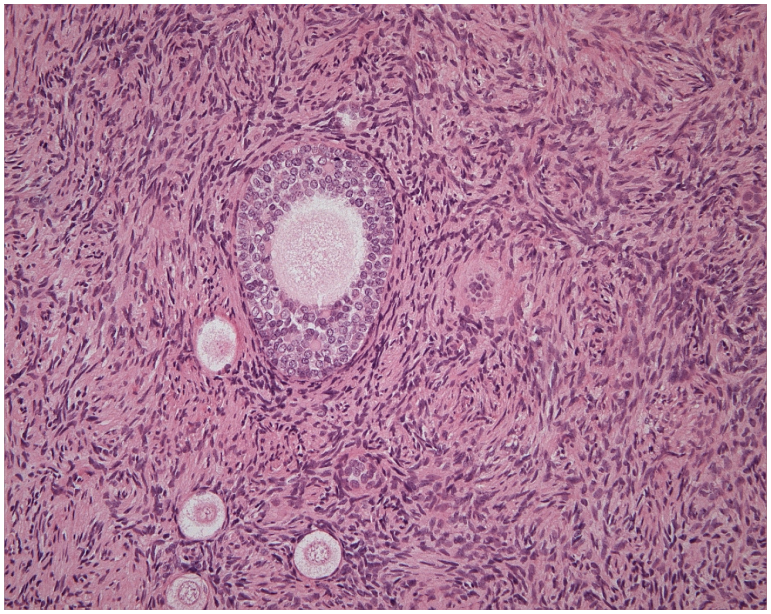

Figure 4. Light micrograph of a secondary (upper) and several primordial follicles (lower) (magnification: 200X)

At the next stage, granulosa cells are growing quickly and start to secrete fluid around themselves. They soon contain a fluid-filled space called antrum. The follicle is called tertiary, or antral and has reached a size of 500 μm in diameter. A fully-grown mature follicle containing a large fluid- filled antrum is known as a Graafian follicle. Two types of granulosa cells are encountered in these follicles; mural granulosa, which is a thin layer around the peripheral of follicle cells, and cumulus cells, surrounding the oocyte. By the time of full maturity, the follicle is about 20 mm in diameter (Figure 5).

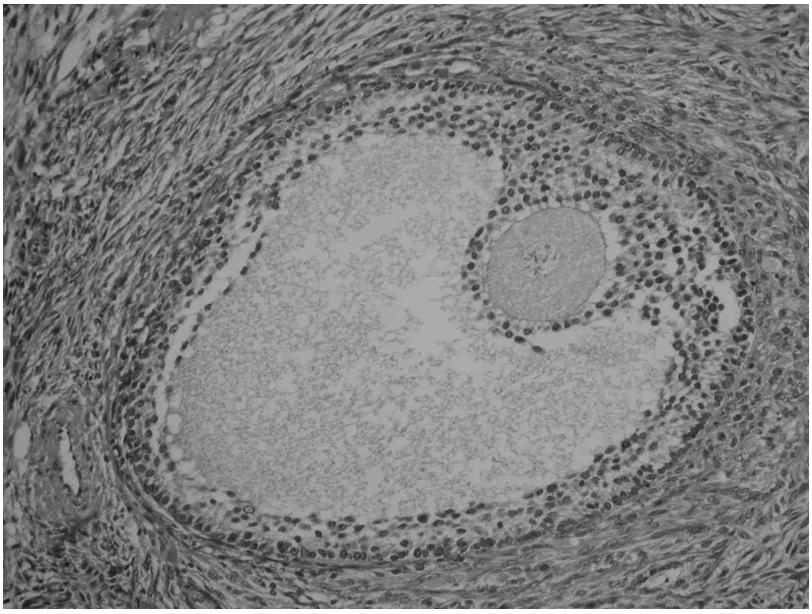

Figure 5. Light micrograph of an antral follicle from a 14-year-old girl with Turner's syndrome, (magnification: 100X). Photo by Julius Hreinsson.

1.4 FERTILITY PRESERVATION

1.4.1 Premature ovarian failure

Female fertility can become negatively affected by a number of conditions, which can deplete follicular reserve in the ovary, resulting in premature ovarian failure (POF) (Peretz *et al.* 2001; Larsen *et al.* 2003; Schmidt *et al.* 2010). POF is a condition in which ovaries stop functioning normally before the age of 40 years and it is estimated to affect about 1-2% of all women. Possible causes of POF include genetic defects such as Turner's syndrome, Fragile X syndrome (Beck-Peccoz and Persani 2006) and FSH receptor mutations (Aittomaki *et al.* 1996). An inactivating mutation in the FSH receptor prevents the maturation of the follicles beyond the primary stage (Aittomaki *et al.* 1996). There are also many other genetic causes.
Turner's syndrome is a chromosomal condition with a total or partial deletion of one of the two X chromosomes (45, X). It is a relatively common genetic disorder that affects about 1 in 2.500 live born females (Singh and Carr 1966). Turner's syndrome can cause cardiac abnormalities, enlargement of blood vessels and ovarian dysgenesis, i.e., streak ovaries with white fibrous stromal tissue. Most girls with this syndrome undergo ovarian failure at an early age. Girls with mosaicism may reach spontaneous menarche and become pregnant (Hovatta 2012). Other genetic disorders that result in premature ovarian failure include fragile X syndrome with a defective gene on the X chromosome (Marozzi *et al.* 2000). POF develops to 13% of the carriers (Cronister *et al.* 1991; Allingham-Hawkins *et al.* 1999; Sullivan *et al.* 2005; Johnston-MacAnanny *et al.* 2011).
Chemotherapy and/or radiotherapy are among the most common causes of premature ovarian failure. Since population of survivors is growing constantly, the development of methods for fertility preservation before starting cancer treatment has become of great importance.

1.4.2 Toxic impact of chemotherapy and radiotherapy

The risk of premature ovarian failure after gonadotoxic anti-cancer drugs depends on the patient's age at treatment, type of malignancy and treatment protocol (Meirow and Nugent 2001; Donnez *et al.* 2006; Gonzalez *et al.* 2012). It has been reported that women up to 40 years treated with chemotherapeutic drugs have a risk of 61% to develop amenorrhea, and the risk increased to 95% when the age of women increased from 40 years (Jensen *et al.* 2011). Meirow also showed that the risk of premature ovarian failure was about 42% in women who received alkylating drugs, while no significant increased risk of POF was observed among those treated with platinum derivatives or plant alkaloids (Meirow 2000). Alkylating agents such as cyclophosphamide are highly gonadotoxic since they are not cell cycle- specific and affect even other cells in the ovary (Jensen *et al.* 2011). Although the gonadotoxic

effects of cancer treatment are known, the effects of such treatments are not predictable since the exact apoptotic pathway involved is not known yet.
Anticancer drugs affect ovarian function by several ways, such as depletion of the primordial follicle pool (burn-out mechanism), ovarian cortical fibrosis and blood vessels damage (Meirow 2000; Meirow *et al.* 2007).
It has been suggested that anticancer drugs cause follicular depletion by inducing apoptosis. Electron microscopic analysis has shown that primordial follicles in human ovarian tissue exposed to chemotherapy become surrounded by basal lamina, which was abnormally thick. Moreover, the incidence of primordial apoptosis has also been demonstrated in human ovarian tissue after transplantation in SCID mice (Oktem and Oktay 2007). However, more research is needed to make sure whether the primordial follicular apoptosis occurs truly in vivo and if the oocyte or granulosa cells are the main targets of apoptosis. Cortical fibrosis and follicular burn-out are other possible alternative mechanisms that have been suggested to explain damage follicular reserve (Meirow *et al.* 2010). It is possible that specific developmental stages of follicles are more sensitive to toxic effects of chemotherapy than others since there are follicles at different developmental stages in the ovary at any time (Morgan *et al.* 2012). There is little data available on this. Decreased numbers of primordial follicles in biopsied ovarian tissue from patients treated with chemotherapy have been reported (Meirow *et al.* 2007; Oktem and Oktay 2007; Meirow *et al.* 2010; Najafi *et al.* 2011; Das *et al.* 2012).

Radiotherapy also causes destruction of the follicular reserve. The extent of harmful effects is related on the treatment dose, irradiation field and patient's age.
Many types of malignancies in central nervous and hematologic systems are treated with high- dose radiation (Meirow *et al.* 2010). Wherever it is possible, gonads are protected from radiation, except if the radiation field overlaps the ovaries, or if total body radiation is required (Sanders *et al.* 1996). In human, the amount of radiation required to destroy half of the total number of resting follicles (LD50) is estimated to 2 Gy (Wallace *et al.* 2003). Radiation to the pelvis results in ovarian damage, and decreases the follicular reserve. It also induces uterine damage that is likely due to disruption of uterine blood vessels.

1.5 METHODS FOR FERTILITY PRESERVATION

Currently, several methods exist for female fertility preservation, including ovarian protection techniques, embryo cryopreservation, oocyte cryopreservation, ovarian tissue banking, followed by auto-transplantation or in vitro culture of follicles within ovarian tissue (Hovatta 2005; Silber *et al.* 2010; Morris and Ryley 2011).
Ovarian suppression using gonadotrophin- releasing hormone (GnRH) agonists prior to and during treatment was claimed to offer some ovarian protection against chemotherapy. However, the results have been contradictory and not repeatable. (Maltaris *et al.* 2006; Blumenfeld 2007).
In 1958, ovarian transposition was described as an ovarian function preserving technique (Mc *et al.* 1958). Surgical ovarian transposition for protecting ovaries from injuries following by ionizing radiation to pelvis or abdomen or total body irradiation has also been used with some success (Bisharah and Tulandi 2003; Han *et al.* 2011).

The methods to preserve fertility in men with cancer are cryopreservation of spermatozoa and cryopreservation of testicular tissue. The latter is the only possible method among pre-pubertal boys (Hovatta 2003; Keros *et al.* 2005). Sperm freezing is now a well-established method that has routinely been applied since the 1970's. Intracytoplasmic sperm injection (ICSI) is used for fertilisation of egg with thawed sperms and good pregnancy rates can be expected (Hovatta 2003).
This thesis focuses on developing improved cryopreservation of ovarian tissue for fertility preservation in clinical practice.

1.5.1 Cryopreservation of embryos

Embryo cryopreservation is a well -established method that is routinely used in all fertility clinics. The first child was born after a pregnancy resulting from a frozen embryo transfer in 1984 (Zeilmaker *et al.* 1984). Children born after frozen embryo transfers have not shown any increased risk of birth defects when compared with children born after naturally conceived pregnancies (Smitz *et al.* 2010; Wikland *et al.* 2010). Embryo cryopreservation before cancer treatment is suitable for women who have partner. However, this procedure needs conventional ovarian stimulation, which may require up to two-three weeks. That is not acceptable for women with highly aggressive malignancies such as some leukaemias, which require immediate cancer treatment (Dolmans *et al.* 2005).
Moreover, many cancer patients respond poorly to conventional ovarian stimulation, which may result in poor quality of collected oocytes during the cycle. An average of about 10 oocytes may be obtained although this amount is variable and age dependent. Ovarian stimulation procedure may not be an optimal option neither for women with hormone-sensitive tumours (e.g. breast and endometrial cancers) (Pena *et al.* 2002; Chen *et al.* 2003) because of increased oestrogen concentrations in serum during the stimulation. In those situations, tamoxifen, a non-steroidal anti-estrogen has been used for ovarian stimulation (Oktay *et al.* 2003). On the other hand, concomitant use of aromatase inhibitors with the ovarian stimulation can be safely used (Azim *et al.* 2007; Reddy and Oktay 2012).
Cryopreservation of embryos is not a feasible option for prepubertal or adolescent girls or single women.

1.5.2 Cryopreservation of oocytes

Cryopreservation of mature oocytes after ovarian hyperstimulation cycle involving administration of GnRH antagonist, or from natural cycles, is an optional fertility preservation method that would abolish the need for a sperm from a partner or donor. The world's first baby using frozen oocytes was born in 1986 (Chen 1986). The live birth rate per frozen oocyte when slow freezing was used, was about 2%, which was much lower than that achieved using fresh oocytes (Gosden 2005). Matured oocytes are more difficult to cryopreserve than embryos. They have a fragile spindle apparatus that makes them more sensitive to cryodamage, and they become easily injured by slow freezing. In contrast to the poor results and pregnancy rates obtained by slow freezing, vitrification of oocytes has resulted in much higher survival rates after warming. It can

be expected to give similar clinical outcomes to those obtained by using fresh oocytes (Cobo *et al.* 2008). Oocyte cryopreservation can be performed for single women, couples without sperm available on the day of oocyte pick up and also for already menstruating adolescent girls. Cryopreservation of immature oocytes after in vitro maturation (IVM) is also another possible option for fertility preservation. Immature oocytes can be obtained from stimulated or non-stimulated cycles (Demirtas *et al.* 2008; Huang *et al.* 2008), and by ovarian tissue biopsy during the ovarian tissue cryopreservation procedure (Revel *et al.* 2003). Maturation of oocytes in vitro followed by vitrification offers also an approach for fertility preservation (Demeestere *et al.* 2009; Anderson and Wallace 2011) because no hormonal stimulation is needed (Baka *et al.* 1995; Cao *et al.* 2009; Wang *et al.* 2012).

1.5.3 Cryopreservation of ovarian cortical tissue

As described above, ovarian tissue cryopreservation would benefit prepubertal girls and young women who need immediate chemotherapy (Hovatta *et al.* 1996). Storing of frozen ovarian tissue may also benefit patients with non-malignant diseases such as recurrent ovarian endometriosis or recurrent mucinous cysts and other patients undergoing prophylactic oophorectomy because of an increased risk of breast cancer and ovarian cancer due to an inherited mutation in BRCA1 or BRCA2 gene. The procedure does not require ovarian hyperstimulation (Oktay *et al.* 1998; Donnez *et al.* 2000; Gosden 2002; Hovatta 2003; Marhhom and Cohen 2007). Cryopreservation of ovarian cortex is the only option for restoring both endocrine function and fertility. To date, re-transplantation of frozen-thawed ovarian tissue has resulted in 24 births of healthy infants (Roux *et al.* 2010; Amorim *et al.* 2012; Greve *et al.* 2012; Dolmans *et al.* 2013).

Ovarian biopsy can be performed via laparoscopy or laparotomy immediately before or after initiation of cancer therapy. A unilateral or bilateral ovariectomy is performed in some rare cases in which no remaining ovarian function can be expected after the treatment (pelvic irradiation). Usually, about 25- 50% of the cortical tissue from one ovary is removed. This varies depending on the expected ovarian follicle injury after exposure to cancer treatment (von Wolff *et al.* 2009). The biopsied tissue should be large enough and representative as follicle density in the ovary is unevenly distributed (Qu *et al.* 2000; Schmidt *et al.* 2003). The vast majority of the follicles are located in the ovarian cortical tissue. Primordial follicles constitute a majority of 70-90% of all follicles in the ovary and a small piece of ovarian tissue may contain hundreds of these small follicles (Gougeon 1986; Lass *et al.* 1997). Hence, they are the main goal of cryopreservation programmes. Using cryopreservation, most primordial follicles are preserved as they resist cryoinjury due to their small size, low metabolic rate, and lack of zona pellucida and cortical granules (Hovatta *et al.* 1996; Shaw *et al.* 2000). In addition, primordial follicles have more time for repairing sub-lethal injury to organelles and other structure during their prolonged growth phase (Picton *et al.* 2000). Considering these advantages discussed above, cryopreservation of ovarian tissue is an attractive method for fertility preservation.

1.6 TRANSPLANTATION OF OVARIAN TISSUE

Cryopreserved ovarian tissue can be used by either transplantation or in vitro culture.
Xenografting is transplantation of ovarian tissue from one species into another. It is an excellent tool for studying the survival and developmental potential of follicles in ovarian tissue after cryopreservation and thawing. However, xenografting is not applicable in clinical practice because of the possible risk for transmission of animal pathogens (Kim *et al.* 2002). Transplantation of ovarian tissue can be done either orthotopically or heterotopically. Heterotopic transplantation refers to transplantation of a tissue from one place in the body to another place in the same body. Orthotopic transplantation refers to re-transplantation of tissue at its origin place. In human, auto-transplantation of cryopreserved ovarian tissue has been described to restore ovarian function and fertility. Heterotopic transplantation of ovarian tissue to the forearm (Oktay *et al.* 2001), or abdomen (Oktay *et al.* 2004) in a woman has also been resulted in regain ovarian function but no pregnancies after embryo transfer have been achieved. Orthotopic transplantation of fresh ovarian tissue resulted in the first live primate birth in 2004 (Lee *et al.* 2004). In the same year, the first childbirth following orthotopic transplantation of human ovarian cortical tissue was reported (Donnez *et al.* 2004).
Despite these achievements, there is a real concern regarding re-transplantation of thawed ovarian tissue because of the possible risk of retransmitting metastatic cells (Shaw *et al.* 1996; Meirow *et al.* 2008). Therefore, it is important to ensure the safety of post-thawed tissue before transplantation by identifying tumour involvement in ovaries and detecting the presence of cancer cells in the preserved tissue.
Before transplantation of ovarian tissue from hematologic cancer patients, minimal residual disease must be identified (Meirow *et al.* 2008). In 2008, chronic myeloid leukemia (CML) cells were detected by real-time quantitative polymerase chain reaction (RT- qPCR) in post-thawed ovarian tissue. That led to cancellation of re-transplanting the post-thawed ovarian tissue. RT- qPCR showed to be positive for BCR-ABL transcripts (Meirow *et al.* 2008).
Recently a Japanese study reported the incidence of ovarian leukemic involvement in 8.4% of leukemia patients. This study was based on retrospective analysis of 5.571 autopsy findings of women younger than 40 years (Kyono *et al.* 2010). Molecular biology has been recently used to detect the presence of leukemic cells in cryopreserved ovarian tissue from patients with CML, acute myeloid (AML) and acute lymphoblastic leukemia (ALL) (Dolmans *et al.* 2010; Rosendahl *et al.* 2010; Dolmans *et al.* 2013; Rosendahl *et al.* 2013).
Taking together, ovarian tissue should actively be cryostored for fertility preservation but much care should be taken for auto-transplantation of cryopreserved tissue because reliable methods to detect the minimal residual disease in grafts are not available yet. Among high-risk patients, culture of the ovarian follicles and oocytes to full maturity in vitro would be a better option.

1.7 CULTURE OF OVARIAN FOLLICLES

In vitro maturation (IVM) of oocytes obtained from maturing follicles would be the safest method for utilizing cryopreserved ovarian tissue from patients with haematological malignancies, which may be transmitted by re-transplantation of the tissue (Shaw *et al.* 1996; Hovatta *et al.* 1997). Maturation of the follicles to antral stage

is a complex procedure that requires understanding of these cells and their requirements at different developmental stages. Primordial follicles in newborn mice ovaries have been successfully cultured in two steps, become matured and ended in one live birth even short-lived (Eppig and O'Brien 1996). In this study, at the first step primordial follicles in ovarian tissue were cultured for 8 days. Thereafter, they were enzymatically isolated and cultured for additional 14 days. Then the oocytes in oocyte-granulosa complexes were removed and become matured by IVM technique. Seven years later, the same researchers reported 59 normal pups after improvement of their previous culture medium (O'Brien *et al.* 2003).

Culture of isolated follicles from the stromal tissue has also been studied. Follicles can be isolated either mechanically e.g., needle or enzymatically e.g., collagenase. However, isolation of human follicles is technically difficult because of the fibrous and high density of ovarian stroma. In human, both mechanical and enzymatic isolation proved to cause irreversible damages in early antral follicles. The oocytes were expelled from the follicles in culture (Hovatta *et al.* 1999). In partially isolated follicles, the oocytes were also extruded after two weeks of culturing. This suggested that the surrounding cells of stroma and theca are important for supporting follicular survival and development.

Immature oocytes can be aspirated from small antral follicles for clinical in vitro maturation (IVM). They can be fertilised, and embryos can be transferred for fertility treatment. Many healthy children have been born after IVM treatment (Cha *et al.* 1991; Barnes *et al.* 1995; Hreinsson *et al.* 2003).

Culture of follicles within the ovarian tissue was shown to be an excellent method in human (Hovatta *et al.* 1997; Hovatta *et al.* 1999). Both fresh and frozen-thawed ovarian tissue was cultured for 21 days (Hovatta *et al.* 1997). The proportion and density of viable follicles in cultured frozen-thawed ovarian tissue was similar to that in fresh tissue. After 10-15 days of culture, two-third of the follicles in the frozen thawed tissues were viable. The availability of human ovarian tissue for research has been a limiting factor in these studies. Human ovarian tissue could be obtained by donation from women undergoing gynaecological surgery or caesarean section (Hovatta *et al.* 1997). The culture of human ovarian follicles is still challenging. Our research group has studied factors that are involved in human folliculogenesis. Adding of GDF-9 and cGMP into the culture increased the viability, recruitment and early growth of ovarian follicles (Hreinsson *et al.* 2002). c-Kit receptor is important in the survival of the follicles in the human ovary during the early development. Kit ligand (KL, also known by name stem cell factor, SCF) mRNA and c-Kit mRNA and protein are expressed in follicles at all developmental stages (Carlsson *et al.* 2006). In addition, antimüllerian hormone (AMH) plays an important role by inhibiting the follicles from entering the growing pool (Carlsson *et al.* 2006).

We now culture human ovarian tissue pieces and isolate follicles mechanically after 4 days. Then the follicles are cultured in a medium comprising Dulbecco's Modified Eagle Medium (DMEM- GlutaMax) supplemented with Antibiotic/ Antimycotic solution, insulin transferrin selenium (ITS), HSA, FSH, a PTEN -inhibitor (bpVHopic) and activin A or GDF-9 for two weeks. The purpose is to isolate cumulus-oocyte complex (COC) and mature the oocyte in vitro in the future (Figure 6).

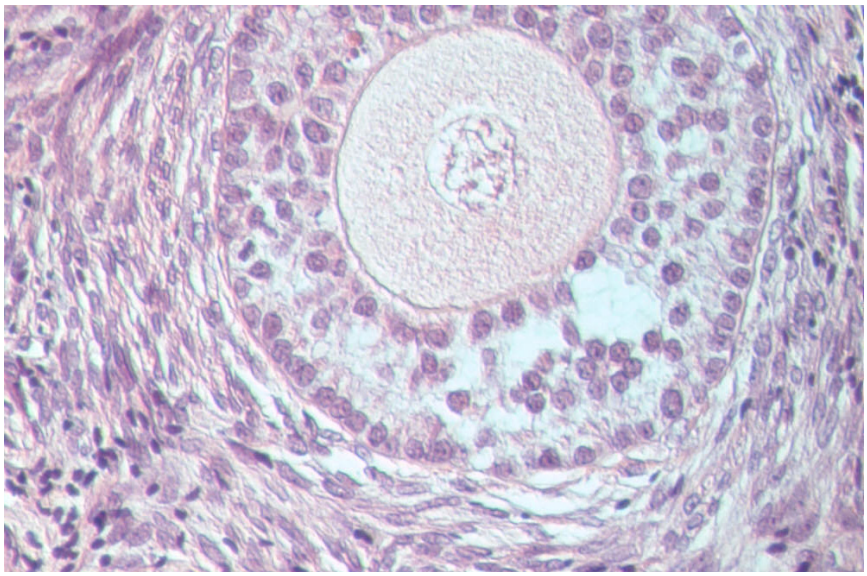

Figure 6. A tertiary follicle in biopsied human ovarian tissue cultured for 4 days, then partially isolated and cultured for further 8 days in activin A and a PTEN inhibitor containing medium.

1.8 CRYOBIOLOGY

To understand the effect of very low temperatures on cells and tissue we have to take into account that many structures and processes are temperature dependent. Hence, cooling has extremely complex effects that produce conditions over the normal physiology. Currently, there are two main methods for cryopreservation of ovarian cortical tissue, slow freezing and vitrification. Both methods permit cells to be stored indefinitely by using extremely cold temperatures to stop metabolic activities. In both procedures, cryoprotective agents are used to prevent cellular damage during the freezing process. Once cells are frozen or vitrified, they can be stored by plunging them into liquid nitrogen.

1.8.1 Freezing injury

At least 80% of the tissue mass consists of water that freezes when cooled below 0°C. Freezing occurs by conversion of water to ice crystals. This results in concentration of the dissolved solutes in the remaining liquid phase, and precipitation of the solutes exceeded their solubility limit.
Following the breakthrough articles by Lovelock (1950), it has been concluded that the freezing damage is caused either by reduction in temperature or changes in the solution composition by freezing, or both (Lovelock 1953; Meryman 1968).
Cooling and warming rates are two important determinants in survival of cells during cryopreservation. In 1963 Mazur discovered the importance of the rate of temperature change that controls the movement of water across the cell membrane and hence, indirectly, the possible intracellular freezing (Mazur 1963).
Crystallisation (nucleation) starts when cooling temperature falls below the freezing point (0°C) and builds crystals. In addition, the growth of ice crystals is a heat generating process, and it tends to increase the temperature of the system to the freezing point. The growth of ice crystals is maximal just below the freezing point, so that by further cooling, it is arrested. At lower temperature (~ -50°C) the concentration of the solution surrounding the cells is changed, which results in increased viscosity of the fluid by osmotic gradient. The cells will dehydrate and shrink at a rate depending on the rate of ice formation. The rate of temperature changes influences the rate at which water moves out of cells (during cooling) or into the cells (during warming). Increased viscosity prevents ice crystal formation. Water leaves the cells quickly across the cell membranes, and the cytoplasm will not cool below its freezing point. This leads to extracellular freezing because all the ice formed is outside the cells. On the other side, if the cooling is too fast, sufficient transport of water out of the cells will not be possible and the cytoplasm of the cells will freeze. Each cell type has maximal survival at a characteristic cooling rate due to the effects of solution and intracellular freezing (Mazur 1963). Ice crystals can also be rebuilt during warming. The rate of warming has an important effect because very small amounts of intracellular ice crystals are capable to grow. Based on the rate of warming, the behaviour of these small ice crystals is different. During slow warming, the ice crystals are recrystallized and grow together

but during rapid warming there is no efficient time to recrystallize and the ice simply will melt (Pegg 2007).

1.8.2 Cryoprotection

Cryoprotection involves addition of cryoprotective agents (CPAs) in solute in order to protect the cells/ tissues from cryoinjury during cryopreservation. Addition of CPAs produces osmotic dehydration and a diffusion force for solutes. Free diffusion of solutes is limited by barriers in the biological system. These barriers can cause transient and equilibrium changes in the volumes of the compartments. These changes can be damaging to cells if they are excessive. Therefore, both processes of diffusion and osmosis are very important in cryopreservation (Kedem and Katchalsky 1958; Lees *et al.* 1989).
CPAs reduce the formation of ice crystals by increasing the total concentration of all solutes in the system. Hence, some of them must be able to penetrate into the cells and have low toxicity. Cryoprotectants must be highly water-soluble, even at low temperatures in order to lower the freezing temperature. Glycerol, dimethyl sulphoxide (DMSO), 1,2 propanediol (PrOH) and ethylene glycol (EG) are some of the CPAs with these properties.

1.8.3 Properties of cryoprotectants

Cells that undergo cryopreservation are exposed to damage caused by intracellular ice crystal formation and build-up extracellular salts in the cells as they dehydrate. Cryoprotective agents protect cellular damage during freezing and thawing. They dehydrate the cells during freezing by creating osmotic gradients (Shaw *et al.* 2000). When CPAs are added in cryopreservation solution, they create an osmotic gradient that causes water moving out of the cells that become shrunken. In addition, CPAs function by lowering the freezing points of the extracellular solution. In this manner, cells have enough time to become dehydrated before the temperature reaches the freezing point of the cytoplasm. Hence, the main function of CPAs is to avoid intracellular ice crystal formation, which is the main cause of damage during freezing and thawing processes. The cryoprotective agents penetrate the cell membrane and stabilise intracellular proteins during freezing (Hovatta *et al.* 1996; Picton *et al.* 2000). Newton et al, (Newton *et al.* 1996) showed that human ovarian tissue frozen without a cryoprotectant was composed of only traces of fibrous tissue, confirming that that CPA is necessary for survival of follicles after freezing and thawing.

Human ovarian tissue has been cryopreserved using several permeating CPAs such as DMSO, PrOH and EG. Two agents, DMSO and PrOH have been compared in solutions for cryopreservation of ovarian tissue. There was no significant difference in any of those cryoprotectants regarding to morphological changes of the follicles after cryopreservation and thawing (Hovatta *et al.* 1996). Follicles in these frozen- thawed tissues were cultured for 24 days and showed a survival rate similar to that in non-frozen tissue (Hovatta *et al.* 1997). Live births have been reported after transplantation of human ovarian tissue cryopreserved using DMSO and EG (Donnez *et al.* 2004; Andersen *et al.* 2008). Follicle growth in frozen-thawed human ovarian tissue was

shown after transplantation into immunodeficient (SCID) mice. Ovarian tissues were cryopreserved in PrOH using of slow freezing (Van den Broecke *et al.* 2001). Both DMSO and EG have been frequently used in ovarian tissue cryopreservation (Kagawa 2009). EG is recently used commonly for rapid cooling (vitrification) of human ovarian tissue (Huang *et al.* 2008; Wang *et al.* 2008; Amorim *et al.* 2011) since it has a lower molecular weight and higher permeability into the cells than DMSO (Bautista and Kanagawa 1998; Kuleshova *et al.* 1999). DMSO is an effective glass former (Fahy *et al.* 2004) that usually is used in combination with another strong glass former such as PrOH or poor glass former such as EG, acetamide and formamide (Fahy *et al.* 2004).
In cryopreservation of ovarian tissue, a permeating CPA is combined with non-permeating CPAs such as polymers, sucrose, albumin and serum proteins. The non-permeating CPAs influence viscosity of freezing solutions and act as osmotic buffers to prevent cells from becoming shrunken and swollen during freezing and thawing (Picton *et al.* 2000). Usually sucrose is used at a concentration of 0.1- 0.2 M whilst the concentration of used serum and albumin is varying. Our group has showed that human serum albumin (HSA) was as efficient as serum when added in respective cryoprotectant solutions (Hreinsson *et al.* 2003). HSA is used in clinical practice because the risk of infection is smaller than that with serum.

1.8.4 Preservation of cells and tissues

The basic knowledge about cryobiology has helped researchers to develop effective cryopreserving methods for a wide range of cells. It is possible to predict the conditions for effective cryopreservation if the characteristics of the cell are known.
The situation is more difficult for a complex system with a heterogeneous collection of cells. Hence, it is necessary to find a technique that can favour the adequate safety of survival of the all cell types that are important for tissue function. Moreover, it is important to protect extracellular structures from damage and maintain the normal interactions between the cells and their attachments to the basement membranes (Pegg 1987). Extracellular ice remaining in the system can destroy the structure of the tissue. (Pegg *et al.* 1987). The amount of the cryodamage is dependent on where the ice is formed. This location is influenced by the cooling rate used (Hunt *et al.* 1982; Pegg 2010). Functional vasculature is very necessary and ruptured vasculature is lethal in tissue and organs. Therefore, it is important to avoid this problem by preventing freezing or at least reducing the amount of ice crystals in the susceptible locations(Pegg 2007).

1.8.5 Slow freezing/ rapid thawing

Slow freezing has been routinely used for cryopreserving human ovarian tissue since 1996 (Hovatta *et al.* 1996). Ovarian tissue is cooled very slowly (2-3 hours) down to
- 150°C using a programmable freezer, followed by storage in liquid nitrogen (- 196°C) and, thawed rapidly in 1 minute from - 196°C to room temperature. Frozen tissue in a cryovial is thawed by first keeping in the air for 30 seconds and then put it in a water bath (30°C) until the ice melts. The starting temperature for the procedure with PrOH-

sucrose is +18°C and for that with DMSO is 0°C. Ovarian tissue is cooled from the starting temperature to -8.0°C (for PrOH-sucrose) -7°C (for DMSO) and at a rate of 2.0 °C/min. Ice crystal induction outside the tissue is performed with pre-cooled forceps, by touching the container after a 10 min holding time. It is then cooled to -30°C at a rate of 0.3°C/min and plunged into liquid nitrogen during a free fall from -35°C (50°C/min to - 150°C for PrOH-sucrose and 10°C/min to - 150°C for DMSO) (Hovatta 2005).
Ovarian tissue is first equilibrated in a cryoprotectant solution to allow the CPA to penetrate into the cells. The optimal penetration rate for DMSO was achieved at 4°C for 30 min (Newton *et al.* 1998). The equilibration time used for PrOH has varied between 15-90 min at room temperature. The longer incubation time (90min) showed the best ultrastructural morphology for both stroma cells and follicles (Hovatta *et al.* 1996; Hovatta *et al.* 1997).

1.8.6 Vitrification/ warming

Vitrification is another cryopreservation technique. It is usually achieved by rapid cooling of a liquid that contains a high concentration of a cryoprotectant. In vitrification, the cooling induces formation of glass-like substance (glass=vitrum) instead of freezing into solid ice with crystals. Successful vitrification requires both high concentrations of cryoprotectants and high cooling rates, which can be achieved by plunging the tissue directly into liquid nitrogen. A combination of cryoprotectants including other permeating cryoprotectants avoids the toxicity of each individual CPA. The permeability of a combination of cryoprotectants is higher than that of each individual one (Vicente and Garcia-Ximenez 1994). Furthermore, a stepwise exposure of cells to precooled concentrated vitrification solution reduces the suspected toxicity of CPAs (Liebermann *et al.* 2002; Hovatta 2005). Moreover, addition of non-permeating cryoprotectants influence the viscosity of the vitrification solution and promote glass formation, which results in reduction of toxicity since they allow addition of a lower concentration of permeating cryoprotectants without compromising vitrification properties (Bautista and Kanagawa 1998; Liebermann *et al.* 2002; Pegg 2005). The toxicity of CPAs can also be reduced by adding a non-permeating polymers such as Ficoll, dextran, polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) (Fahy *et al.* 1984). These polymers reduce the mechanical stress that occurs during cryopreservation and may partially cause the cryoinjury in the cells. Polymers influence the viscosity of the vitrification solution and lower the concentrations of the cryoprotectants, which in turn reduces the toxicity of the cryoprotectant solution (Shaw *et al.* 1997).
Successful results from vitrified human oocytes and blastocysts have been achieved. The complex and dense human ovarian tissue is much more difficult to vitrify. For vitrification of ovarian tissue, several permeating CPAs such as, DMSO, EG, PrOH, glycerol, acetamid and formamide have been used. EG with low toxicity, has been commonly used as a cryoprotectant for vitrification of human ovarian tissue (Huang *et al.* 2008; Wang *et al.* 2008; Amorim *et al.* 2011).

1.8.6.1 Slow freezing and vitrification of human oocytes

Oocytes can be cryopreserved by two methods, slow freezing (Chen 1986)or vitrification (Kuleshova *et al.* 1999). When slow freezing and vitrification were compared, vitrified oocytes showed better survival rates than those frozen by slow freezing (Monzo *et al.* 2012). Several studies have reported low survival and implantation rates with a few pregnancies, following slow freezing of mature (Porcu *et al.* 1997; Fabbri *et al.* 2001; Borini *et al.* 2006; Levi Setti *et al.* 2006) (Konc *et al.* 2008) and immature oocytes (Cha *et al.* 1991; Trounson *et al.* 1994; Tucker *et al.* 1998). Slow freezing can cause modification of the membrane structure and affect oocyte microtubules, cytoskeletal organization and the zona pellucida (Ghetler *et al.* 2005; Bromfield *et al.* 2009). Based on poor survival rates, cryopreservation of oocytes by slow freezing has not been extensively applied.
Vitrification does not affect the physiology of oocytes as extensively as slow freezing. A higher survival rate has been achieved after warming. Hence, it has recently been used as an alternative method for cryopreserving oocyte in several laboratories, (Lucena *et al.* 2006; Antinori *et al.* 2007; Cobo and Diaz 2011). In a study on 936 children born after oocyte cryopreservation, Noyes et al. reported that there was no higher incidence of congenital anomalies when compared with naturally conceived newborn babies. The study included 532 live born children resulting from slowly frozen oocytes, 392 from vitrified oocytes and 12 from a combination of both cryopreservation methods (Noyes *et al.* 2009).

1.8.6.2 Slow freezing and vitrification of human ovarian tissue

Cryopreservation of human ovarian cortical tissue is challenging because it contains many different cell types such as stromal cells, follicles with oocytes that are surrounded by granulosa cells and blood vessels. These cells have different requirements for optimal survival during cryopreserving and thawing processes (Hovatta 2005). Cryopreservation of human ovarian tissue using slow freezing is a method with remaining follicular function after thawing (Hovatta *et al.* 1996; Newton *et al.* 1996). Cryoprotectants such as DMSO and PrOH with sucrose are widely used in slow freezing protocols that result in high survival rates of follicles. Frozen-thawed follicles in cortical slices survived two weeks of culture and remained functional after transplantation to SCID mice (Hovatta *et al.* 1997; Van den Broecke *et al.* 2001). After xeno-grafting, mature oocytes were obtained (Gook *et al.* 2005). Despite these achievements, cryopreservation of ovarian tissue by slow freezing is limited by relatively poor survival of the stroma (Gook *et al.* 2000; Hreinsson *et al.* 2003). The stroma and the blood vessels play a critical role in the development of follicles and restoration of gonadal function after transplantation. Hence, a proper preservation of stroma and blood vessels is basically important (Woodruff and Shea 2007).

Therefore, we have developed vitrification protocols for human ovarian tissue to improve the viability of the stroma in addition to the follicles and oocytes. Vitrification of human ovarian tissue has been investigated during the recent 10 years. Many researchers have studied various vitrification solutions and carrier systems and evaluated the effect of different vitrification protocols on ovarian tissue (Isachenko *et al.* 2007; Huang *et al.* 2008; Wang *et al.* 2008; Isachenko *et al.* 2010; Xiao *et al.* 2010). Many of the conclusions from these early diverse studies have been contradictory.

There are limited data from studies in which vitrification and slow freezing of human ovarian tissue have been compared (Gandolfi *et al.* 2006; Isachenko *et al.* 2007). Vitrification is not one single method, and conditions can vary widely. The different results reported may due to differences in the procedures such as cryoprotectant composition and concentration, exposure times to cryoprotectants, cooling rates, carrier systems and sizes of fragments. Many studies support vitrification of human ovarian tissue by reporting results on well- preserved ultrastructure of follicles and stroma tissue (Wang *et al.* 2008; Kagawa *et al.* 2009; Xiao *et al.* 2010). Therefore, vitrification seems to be preferable for preserving more complex and heterogeneous tissues such as ovarian cortical tissue. The main differences between slow freezing and vitrification are demonstrated in **Table 1**.

Parameters	Vitrification	Slow freezing
Direct contact with liquid nitrogen	Yes (Before our studies)	No
Ice crystal formation	No	Yes
Cooling rate (ºC/min)	15.000- 30.000	0.15- 0.30
Exposure to cryoprotectant	Yes	Yes
Concentration of cryoprotectant	High (>40%)	Low (10-15%)
Time consuming (for one run)	Minutes	Hours
Cost	Inexpensive	Expensive
Special equipment required	No	Yes

Table 1. Comparison of cryopreservation methods for human ovarian tissue

1.9 THE SWEDISH TISSUE LAW AND THE EUROPEAN UNION TISSUES AND CELLS DIRECTIVE

The European Union Tissues and Cells Directive (EUTCD) establishes the regulation for cells and tissues used clinically in Europe. The Directives set a standard for handling, application and traceability of the cells and tissues from a donor to a patient. The EUTCD includes three directives, the directive (2004/23/EC), which contains the framework legislation, and the two technical directives (2006/17/EC and 2006/86/EC). **The Swedish Tissue Law** 2008:286 is built upon the EU Directives and consists of quality and safety standards for processing tissues and cells for clinical use and for manufacture of medical products for humans. (www.vavnad.se).

3 AIMS OF THE STUDIES

General aim:

The overall aim of the thesis was to improve the cryopreservation of human ovarian tissue by developing a clinical grade vitrification.

Specific aims listed according to each article:

1. To improve the method of cryopreservation of ovarian tissue

2. To develop a vitrification method for human ovarian tissue that could be applied in a clinical setting.

3. To evaluate the incidence of apoptosis after vitrification, by using TUNEL assay and DNA laddering.

4. To improve and simplify the cryopreservation procedure by using a solution that only contains one permeating cryoprotectant, EG, in addition to the non-permeable ones, Ficoll and sucrose.

4 MATERIALS AND METHODS

1.10 ETHICS STATEMENT

Ethics approvals were obtained for all the studies presented in this thesis from the Regional Ethics Board in Stockholm, project number 2005/589-31. All of the tissue biopsies used in articles I, II, III and IV were collected at the Karolinska University Hospital Huddinge, while for article III collection of biopsies were performed at the Karolinska University Hospital Huddinge and at the Tarbiat Modares University Hospital, Tehran. The Iranian ethics approval was obtained from the Ethics Committee of Tarbiat Modares University (ref. 5274856; 7 December 2010).

1.11 HUMAN OVARIAN TISSUES FOR RESEARCH

The methods are described in a general manner in this section, for further details please see the individual articles.

Small pieces of ovarian cortical tissue (diameter <5 mm) were obtained by biopsy from 59 women 22-43 (mean 33) years of age. All women underwent elective Caesarean section and had given informed consent for participation in all studies.
Ovarian tissue was collected and transported directly to the laboratory in sterile 50 ml Falcon tubes (Becton Dickinson, Bedford MA, USA) containing 20-50 ml Flushing medium (Medicult Jyllinge, Denmark). The tissue was transferred to a culture dish (Becton Dickinson, Bedford MA, USA) containing flushing medium and most of the medullar tissue was removed. The cortical ovarian tissue was then cut into pieces of about 1-1.5 mm^3 with scalpel, keeping the tissue immersed in collection medium while working under a stereomicroscope.
Within the individual studies, each biopsy sample was equally divided into groups and exposed to the different treatments.

Article I
Cortical ovarian tissue was cut into small pieces of approximately 1-2 x 5-8 mm^3. Two small tissue pieces were taken as non-vitrified controls and fixed for light microscopy (LM) and transmission electron microscopic (TEM) evaluation. The remaining pieces of the tissue were frozen either by using slow freezing or vitrification. After warming, two pieces were fixed for LM and TEM analyses. The cortical tissue was stored in liquid nitrogen for at least one week before thawing/warming and culturing.

Article II
Small cortical ovarian tissue pieces 1-1.5 mm^3 were taken for both non-vitrified controls and for vitrification. Two control pieces, and pieces from the vitrified tissue were taken for LM and TEM analysis. Tissue was cultured for 24h either before or after thawing.

Article III and IV

Ovarian tissue 1-1.5 mm^3 was vitrified using two vitrification solutions. Samples from non-vitrified and warmed/ vitrified tissue were fixed for LM and TEM evaluation,

before and after culture. **Table 2** summarize the cryopreservation methods used in the four articles presented in the thesis.

Freezing methods used	Article I	Article II	Article III	Article IV
Slow freezing using EG	Yes	No	No	No
Slow freezing using PrOH	Yes	No	No	No
Vitrification using DMSO, PrOH, EG	Yes	Yes	Yes	Yes
Vitrification using only EG	No	No	Yes	Yes

Table 2. Freezing methods used in the four studies

1.12 CRYOPRESERVATION AND THAWING OF OVARIAN TISSUE (ARTICLE I)

After preparation of ovarian tissue, the tissue pieces were equilibrated with cryoprotectants and cryopreserved according to either the method using PrOH and sucrose as cryoprotectants for cryopreservation of ovarian tissue described by Hovatta et al. (Hovatta *et al.* 1996) or using EG and sucrose as cryoprotectants.

Slow freezing (PrOH and sucrose)
The cryopreservation medium was commercially obtained (Freezing Kit I, Vitrolife, Gothenburg, Sweden). It consisted of three solutions. The basic solution was Phosphate- buffered Saline (PBS) supplemented with human serum albumin (HSA). The first cryo- solution contained 1.5M PrOH as a permeating cryoprotectant and the second one contained 1.5M PrOH and 0.1M sucrose as a non-permeating cryoprotectant. The incubation times were 5, 10 and 15 min in each cryo-solution. All steps were carried out at room temperature. The tissue was transferred into a 1.8 ml Nunc cryovial (Nunclon, Roskilde, Denmark) containing 1ml of the third cryo-solution and placed in a programmable freezer (CryoLogic, Australia). Then samples were cooled from room temperature to -6.5 °C at a rate of -2.0 °C/min. Seeding was performed by using a forceps, precooled in liquid nitrogen during 10 min of holding. Then samples were cooled to -35 °C at a rate of 0.3°C/min and plunged in liquid nitrogen during a free fall from -35°C to -196°C.

Slow freezing (EG and sucrose)
The tissue was rinsed several times in an isotonic saline solution, and then it was transferred to 1 ml of PBS (Invitrogen, Scotland) containing 1.5 M EG and 0.1 M sucrose (Sigma-Aldrich, Sweden) and HSA (10mg/ml) (Vitrolife, Gothenburg,

Sweden) for 30 min at 4 °C. Then the piece was placed into 1.8 ml cryovials. The samples were cryopreserved at similar rates as described above but the starting temperature was 4 °C.

Thawing after slow freezing

For PrOH and EG, thawing was performed at room temperature. The cryovials were removed from the liquid nitrogen, air-thawed for 30s and plunged into a water bath (37°C) until the ice was melted.

PrOH thawing procedure

The tissues were thawed using thawing solutions, Cryo-PBS, containing HSA (Thaw Kit 1, Vitrolife). The tissue piece was immediately transferred into thawing solutions with 1.0M PrOH and 0.2M sucrose. After five min they were transferred into medium with 0.5 M PrOH and 0.2M sucrose and after another five min into medium with 0.2M sucrose for 10 min.

EG thawing procedure

After thawing, tissues were rinsed in a medium containing PBS, 0.75 M EG and 0.25 M sucrose for 10 min. Then they were transferred to a medium containing PBS and 0.25 M sucrose and incubated for 10 min, and transferred to another medium containing PBS and again incubated for 10 min.

1.13 VITRIFICATION AND WARMING OF OVARIAN TISSUE (ARTICLES I, II, III & IV)

Vitrification procedure: Solution containing a combination of DMSO, EG, PrOH and PVP (Articles I, II, III, IV)

In our vitrification procedure, for ovarian tissue samples we used solutions with increasing concentrations of dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Sweden), 1,2- propanediol (PrOH) and ethylene glycol (EG) (Invitrogen, Corporation, Scotland, UK) supplemented with 10 mg/ml HSA (Vitrolife). After washing for 5 min in Hank's balanced salt solution (HBSS) with 10 mg/ml HSA, cortical tissue pieces were incubated sequentially in vitrification solutions (VS) including VS1 (0.35 M DMSO, 0.38 M PrOH, 0.38 M EG), VS2 (0.70 M DMSO, 0.75 M PrOH, 0.75 M EG) and VS3 (1.4M DMSO, 1.5 M PrOH, 1.5 M EG), at 2.5, 5 and 10% of each cryoprotectant, respectively. The third solution was also supplemented with (10%w/v) polyvinyl pyrrolidon (PVP, Sigma- Aldrich, Sweden). The first and second incubation steps were performed at room temperature. The third one contained higher concentration of cryoprotectants at 4°C.
In article I, two incubation times of 5 min (Vit5) and 10 min (Vit 10) were tested in each solution. In articles II, II and IV the stepwise incubation times were 5 min in VS1 and 10 min in VS2, VS3 and VS4, respectively.

In article I, an open system was used to vitrify cortical tissue. Pieces of ovarian tissue were put in a 0.5 ml insemination cryostraws (IMV- Technologies, L ´Aigle, France), which were hand-cut by scalpel. Afterwards, the cryostraw with tissue was plunged

into liquid nitrogen and placed in a pre-cooled 5.0 ml Nunc cryovial (Nunclon, Roskilde, Denmark), the cap lid closed and stored in liquid nitrogen.

In articles II and IV, a closed system was utilised to vitrify cortical tissue. In article III, an open vitrification system was used. The tissue piece was transferred into a pre-cooled 1.8 ml NUNC cryotube (Nunclon, Roskilde, Denmark), with a minimum of the vitrification medium using a small spoon for not to squeeze the tissue. The cryotube was tightly closed and plunged directly into liquid nitrogen. Tissue preparation and banking was carried out in a closed sterile, system with traceable components, in a controlled and defined sterile environment.

Warming

For warming, the open straw (Article I) was removed from the cryotube and directly plunged into the first pre-warmed, 37°C, solution, which consisted of HBSS and 10 mg/ml HSA supplemented with 0.5 M sucrose, until the tissue sample was rolled out from the straw into a dish. In articles II, III and IV, the cryovial was removed from liquid nitrogen, kept at room temperature for 30 sec and then immersed in a 37°C water bath until the ice melted. After warming, the cortical tissue was transferred into the first pre-warmed, 37°C, solution for about two min. Then the tissue piece (Articles I, II, III and IV) was incubated in the warming solutions that consisted of HBSS/ HSA supplemented with 0.25 and 0.125 M sucrose, respectively. The fourth solution consisted of HBSS supplemented with (10 mg/ml) HSA. The incubation time for the last three solutions was five min in each. All steps were performed at room temperature.

Vitrification procedure: A solution containing 40% EG and Ficoll (Article III)

Ovarian cortical tissue was equilibrated in one incubation step in solution containing 40% EG (v/v), 30% Ficoll 70 (w/v) and 1 M sucrose supplemented with bovine serum albumin (BSA) for 5 minutes at room temperature. The tissue pieces were transferred to a cryotube with a minimal volume of the vitrification medium and plunged into liquid nitrogen. The vitrified tissues were stored in a liquid nitrogen storage tank until further experiment.

Vitrification procedure: A solution containing 40% EG and Ficoll (Article IV)

The vitrification procedure was as described previously in article III, but in this study (Article IV) we used human serum albumin (HSA) instead of BSA. The aim of this study was to perform a vitrification procedure for human ovarian tissue that could be carried out in a clinical setting. Hence, HSA instead of BSA was used in vitrification solution. Moreover, after vitrification, the tissues were stored in the vapour-phase nitrogen storage freezer (Air liquid- DMC, Espace, France) to avoid possible microbial contamination by liquid nitrogen. The tissue preparation, the vitrification, banking and warming procedures were carried out in a closed sterile and traceable system, in a controlled and defined sterile environment. Hence, the system is compatible with European tissue directive and the Swedish Tissue Law.

To carry out the vitrification itself, tissue pieces were transferred with a minimum volume of the vitrification medium into pre-cooled NUNC cryotube containing an internal thread cap, to make it leak proof. The cryotube was immersed into liquid nitrogen leaving the screw cap above the surface to avoid leaking.

After vitrification and culture, the morphology of the follicles was analysed by using light (LM) and electron microscopy (TEM). The ultrastructural changes of the oocytes, granulosa cells and stromal cells after vitrification and culture were analysed by using a scoring system. Two different investigators judged the morphology blind from each other's results. In the oocyte, the appearance of the nuclear membrane integrity, the contents and density of the cytoplasm, microvilli and attachment to the granulosa cells were evaluated. In the granulosa cells the chromatin, the density and appearance of the cytoplasm, mitochondria and other cellular components in the cytoplasm, and the contact to the basement membrane were evaluated. In the stroma, morphology of the fibroblasts, the cytoplasm, nuclear contents and collagen matrix were evaluated. The maximum scores for the oocytes were three, granulosa cells two and stroma, one respectively. A highest score of 1.0 (100%) was given to perfectly preserved cells. The final score reflected the preservation of the tissue after using different vitrification methods.

Warming (Articles III and IV)
The warming procedure was similar to that in the second vitrification article. After warming, the vitrified tissue pieces were transferred into two warming solutions with decreasing concentrations of sucrose (1 and 0,5M) for 5 min in each and at room temperature.

1.14 TOXICITY TESTING (ARTICLE III)

The toxicity of vitrification solutions was tested by subjecting pieces of ovarian tissue to the solutions for the two vitrification procedures. All dehydrating and rehydrating steps were carried out except plunging into liquid nitrogen. The ovarian tissues were fixed in Bouin's solution for light microscopic analysis.

1.15 TISSUE CULTURE (ARTICLES I, II, III & IV)

We evaluated the viability of follicles in the tissue after 24 h culture.
After thawing all samples (non-vitrified and vitrified) were cultured on 24- well plates in humidified air with 5% CO_2 at 37°C for 24 hours.
In article I, the wells contained MilliCell culture inserts (MilliCell, Sigma-Aldrich, Stockholm, Sweden) which were coated with 100 µl pre-diluted Matrigel extracellular matrix (Becton Dickinson, Stockholm, Sweden) to support tissue growth (Hovatta *et al.* 1997). The contents of culture media in all four studies are summarized in the **Table 3**. The details are described in the individual articles.

	Culture medium
Article I	α – minimum essential medium (α – MEM), Antibiotic/ Antimycotic solution (50 IU/ml, Invitrogen Inc), insulin transferrin selenium (ITS, 10 µg/ml) (Gibco Invitrogen, Sweden), 10% HSA (Vitrolife,Göteborg, Sweden),0.5 IU/ml FSH (Gonal-F; Serono Nordic, Sweden) and 8-bromo-cGMP (Sigma- Aldrich, 1.1 mg/ml(Scott *et al.* 2004).
Article II	McCoy's 5a medium with bicarbonate supplemented with HEPES (20 mM), L-glutamine (3mM), penicillin (100 IU/ml), streptomycin (0.1 mg/ml), transferring (2.5 µg/ml), selenium (4 ng/ml), insulin (10 ng/ml), 0.1% HSA and FSH (1ng/ml) all obtain from Sigma - Aldrich, Sweden (Telfer et al 2008).
Article III	α – MEM, 100 µg/ml penicillin and 50 µg/ml streptomycin, 1 % ITS, 5% fetal bovine serum, 100 ml/m recombinant FSH (Gonal-F; Serono), 20 ng/ml murine recombinant epidermal growth factor (Sigma- Aldrich, Germany).
Article IV	McCoy's 5a medium with bicarbonate supplemented with HEPES (20 mM), L-glutamine (3mM), 0.5% antibiotic/antimycotic solution (50 IU/ml), insulin transferrin selenium (ITS, 10 µg/ml), 10% HSA and 0.5 IU/ml FSH (Gonal F, Serono, Sweden).

Table 3: A summary of the contents of culture media in all four studies

1.16 LIGHT MICROSCOPY (ARTICLES I, II, III & IV)

Ovarian tissue pieces were fixed in Bouin's solution for 24 hrs at 2-8°C and then dehydrated starting in 70% ethanol. The fixed tissues were embedded in paraffin and cut in serial sections of 4µm thickness. Every 11th section of each tissue piece was mounted on glass slides and stained with haematoxylin- eosin. To prevent double counting, each follicle was followed through neighbouring sections and counted only once. Analysing adjacent sections confirmed the status of the follicle. Follicles were counted and their developmental stages were classified as defined by Gougeon (Gougeon 1986) Briefly, follicles were defined as primordial if they contained only a single layer of flat granulosa cells and those containing a complete single layer of cuboidal granulosa cells were classified as primary follicles and follicles surrounded by two complete layers of cuboidal granulosa cells identified as secondary follicles. Atretic follicles were defined as those containing an oocyte with eosinophilic cytoplasm, contraction and clumping of the chromatin material and pyknotic granulosa cells. A digital image analysis system (Infinity Analyze Imaging Software from Lumenera Corporation) connected to an inverted microscope (Nikon, Tekno Optik, Stockholm, Sweden) was used to measure the area of the pieces. The volume was calculated by multiplying the area of the tissue piece by the known thickness of 4µm.

1.17 TRANSMISSION ELECTRON MICROSCOPY (ARTICLES I, II, III & IV)

Tissue specimens taken for TEM were fixed in 2% glutaraldehyde and 0.5% paraformaldehyde in 0.1%mol/ml sodium cacodylate buffer and 0.1 mol/ml sucrose pH 7.4 at room temperature for 2 h and post- fixed in 1% osmium tetroxide. After dehydration, the specimens were embedded in LX-112 (Ladd Research Industries Inc., Burlington, VT, USA). Thereafter, the samples were cut into about 0.5 µm sections, stained with toluidine blue and observed in light microscopy for selecting sections with follicles for further examination. Sections were cut into about 50 nm and stained with alcoholic uranyl acetate. The ultra-thin sections were examined using a transmission electron microscope (Tecnai, Fei, Eindhoven, and The Netherlands). Digital images were taken at magnifications of x 1250 to x 30 000 using Megaview III camera.

In all studies, the ultra structural changes in both fresh and cryopreserved ovarian tissue were analysed. The structures of oocytes, granulosa cells and stromal cells were evaluated separately. The investigators were blinded to the experimental background of the specimens. In the oocytes, the integrity of nuclear organelles, the membranes, cristae of the mitochondria and their density, the density of the cytoplasm, the content of membrane vesicles and attachment to the granulosa cells were evaluated. The same parameters were judged in granulosa cells. The attachment between granulosa cells and the attachment to the basement membrane were also evaluated.

Follicles without any damaged structures in the oocytes and granulosa cells were classified as intact whereas those with slight but non-lethal changes were considered as influenced. A cell with collapsed and/ or nuclear or plasma membrane disrupted was regarded as degenerated.

In study IV, a scoring system was performed. The maximum scores for the oocytes were three, granulosa cells two and stroma, one respectively. Each individual cell type

was given a final score (ratio) by dividing the total number of score gained by evaluation to the maximum possible score. A highest score of 1.0 (100%) was given to perfectly preserved cells. The final score reflected the preservation of the tissue after using different vitrification methods.

1.18 IMMUNOHISTOCHEMISTRY (IHC) (ARTICLE IV)

The occurrence of apoptosis in follicles and stroma in fresh and vitrified tissue was assessed by immunohistochemistry for active caspase-3.
The presence of the active caspase-3 was evaluated by immunohistochemistry using a monoclonal antibody against human caspase-3 (Cleaved Caspase-3 (Asp175) (5A1) Rabbit mAb, Cell signaling technology). Primary antibody, Cleaved Caspase-3 (Asp175) (5A1) Rabbit mAb) (Catalogue no. 9664, Cell Signaling Technology Inc, Danvers, Massachusetts, USA) and Polyclonal Goat Anti-Rabbit Immunoglobulins/ Biotinylated (Code No. E 0432, DakoCytomation, Denmark) were used. For negative controls we used tris-buffered saline instead of primary antibody. For positive and negative control tissue, mouse ovarian tissue was used.
The immunostaining was performed on two slides, from the three different groups, before culture (fresh, vitrified using DMSO, PrOH and EG, and vitrified using only EG) (three patients from each group).

1.19 TUNEL ASSAY (ARTICLE III)

After deparaffinization and rehydration, sections were stained to detect DNA fragments from apoptotic cells by terminal deoxynucleotidyl transferase- mediated dUTP nick-end labelling (TUNEL kit; In situ Cell Death Detection, Roche, Germany). The apoptotic signal was recorded as positive when green staining of the nucleus was apparent or negative when the nucleus stained red. The test was performed according to the manufacturer's instructions. Ten sections per sample were randomly selected and examined by using a fluorescence microscope (Axiophot; Zeiss, Germany). For a positive control, dexamethasone- treated mouse thymus was used (Mazoochi *et al.* 2008).

1.20 DNA LADDERING (ARTICLE III)

Detection of DNA fragments in apoptotic cells was evaluated using DNA laddering kit purchased commercially (Apoptotic DNA Ladder Kit 1835246; Roche, Germany). The test was performed according to the instruction from the manufacture. The DNA content was determined spectrophotometrically loaded in 1% agar gel. For a positive control, dexamethasone- treated mouse thymus was used (Mazoochi *et al.* 2008).

1.21 STATISTICAL ANALYSIS

Article I
Chi-square test for independence with exact p- values was used to assess if the degree of follicle viability in LM evaluation was associated with different cryopreservation

protocols or fresh control. Pairwise comparisons between protocols were performed by Fisher's exact test. Similar statistical analysis was performed for TEM evaluation of oocytes, granulosa cells and stroma. Kruskal- Wallis analysis of variance was used for difference in viability of stroma between the cryopreservation protocols. Pairwise comparisons between protocols were also performed. P- value was adjusted according to the Bonferroni procedure for multiple tests of significance. $P<0.05$ was considered statistically significant.

Article III

The Mann- Whitney U-test was used to test differences between groups regarding proportions of the developmental stage of the follicles. $P<0.05$ was considered statistically significant.

Article IV

The Friedman ANOVA test was used to test the rate of follicular viability in LM evaluation when tissues were vitrified or not. The Friedman ANOVA test was also performed for TEM evaluation of oocytes, granulosa cells and stroma. Pairwise comparisons between protocols were performed in both LM and TEM evaluations. $P=0.03$ in LM evaluation and $p=0.02$ in TEM evaluation were considered statistically significant.

5 RESULTS

1.22 ARTICLE I

In article I, we carried out the first systematic comparison of slow freezing and vitrification of human ovarian cortical tissue. The cryopreservation solutions used in slow freezing were PrOH- sucrose and EG- sucrose, and for vitrification it was a combination of DMSO, PrOH, EG and PVP with two different incubation times of 5 min (Vit5) or 10 min (Vit10). The results showed similar and well-preserved morphology in follicles within tissues cryopreserved either by slow freezing or vitrification. Electron microscopic analysis revealed that the ovarian stroma was better preserved in vitrified tissue than that frozen by slow freezing.

A total of 354 follicles was analysed by **LM** in fresh, slowly frozen and vitrified tissue. Of these follicles, 110 (31%) were primordial, 214 (60%) primary, 28 (8%) secondary and 2 (1%) atretic follicles. Most follicles were at primordial or primary stage and in all samples. Microscopic analysis of hematoxylin-eosin stained sections did not show any differences in the structures of follicles frozen after using different protocols. The structure of ovarian stroma was impaired in most samples that had been frozen slowly. When the stromal structure in slowly frozen tissue was compared to that in fresh tissue, there were higher numbers of necrotic stromal cells with pyknotic nuclei and empty spaces in the extracellular matrix with disorganized collagen fibres. The quality of the stroma in vitrified tissues was as good as that in fresh tissue. The cryo-changes in stroma in all cryopreserved ovarian tissue were evaluated by using high power field (n= 18/ block and treatment). Most stromal cells (98%) in the fresh tissue had intact nuclei. There was significantly higher proportion of intact stromal cells in all vitrified tissue samples than was found in the slowly frozen samples using either PrOH or EG.

A total of 62 follicles and 633 granulosa cells was analysed by **TEM**. Fifty-five oocytes in these follicles were analysed. Of these oocytes, 14 were in fresh, ten in PrOH, six in EG, 19 in Vit5 and six in Vit10 groups. In those sections in which the oocyte was not visible, only granulosa cells were analysed.
The ultrastructure of the **oocytes** did not vary between groups in any of the tissues.
The nuclei in almost all samples showed a prominent nucleolus with normally distributed euchromatin and heterochromatin. In the majority of the follicles subjected to the different cryopreservation methods, the inner and outer nuclear membranes were clearly seen. The nuclear membrane of some oocytes in tissue samples cryopreserved using slow freezing with EG was not well preserved. The nuclei and the cytoplasm of the oocytes in the cryopreserved tissue that had been frozen slowly using EG were blurred after obvious cryoinjury. In tissue samples slowly frozen with EG, the main sign of cryoinjury was the increased vacuolization in the cytoplasm and disruption of the contact between the oocytes and the granulosa cells. The mitochondria were not well preserved in any of the tissue samples cryopreserved using slow freezing.
In the vitrified tissue samples, the mitochondria with clearly visible cristae showed highly organized structure and well preserved membranes. There were no significant differences in the viability of the oocytes between any of the studied groups.

The ultrastructure of the **granulosa cells** in all the studied groups was well preserved. Their contact with basement membranes, neighbouring granulosa cells and oocytes looked similar to that in fresh tissue. They contained an intact nucleus with nucleolus, homogeneous euchromatin and peripheral heterochromatin. The cytoplasm of the granulosa cells contained undamaged organelles and the endoplasmic reticulum (ER) was clearly seen.
There were clearly visible differences in the preservation of ovarian **stromal tissue**. The stroma was better preserved in vitrified tissue than it was in the slowly frozen tissue. Vitrified tissues after both 5 and 10 min of exposure to vitrification solutions had uniform and intact collagen fibres. Vitrified tissue samples after 10 min of exposure to the vitrification solutions (Vit10) showed the best morphology of stroma. It was composed of collagen fibres and spindles-shaped fibroblast- like cells. The longer incubation time (10 min) was probably more optimal than the short time incubation time (5 min). The nuclei had normal morphology with intact nuclear membranes. Most stromal cells contained intact mitochondria and other organelles. The centrioles were usually close to the nucleus or the Golgi apparatus.
After slow freezing with PrOH and EG, the stroma showed cryodamage indicated by shrunken stromal cells containing pyknotic nuclei, ruptured cell membranes and vacuolization in the ER as a sign of early necrosis. Most of the stromal cells were at necrosis and indicated by disruption of nuclear and cellular membranes, organelle breakdown and cell lysis. Collagen fibres and extracellular matrix were disrupted.
Poor preservation of ovarian stroma was clearly found in the slowly frozen tissue with PrOH or EG. The viability of the stroma in the vitrified samples was significantly better than that in slowly frozen samples. There were no significant differences in the morphology of the ovarian stroma in tissue samples vitrified with different incubation times.

1.23 ARTICLE II

In article II, we carried out vitrification of human ovarian tissue in a closed system in which the tissue was never in direct contact with liquid nitrogen (LN2), neither during vitrification nor during storage in a vapour-phase nitrogen storage freezer. We transferred the tissue pieces in a sterile and leak proof cryotube with a minimum volume of vitrification medium. The vitrification medium consisted of a mixture or cryoprotectants DMSO, PrOH, EG and PVP. The results did not show any differences in the light and electron microscopic ultrastructure of oocytes between non-vitrified and vitrified tissues. There were no subcellular alterations in the vitrified tissue samples.

Histological analysis on 100 follicles in both non-vitrified (control) and vitrified tissues (before culture) was analysed by **LM**. Of these follicles, 37 were primordial, 42 primary, 13 secondary and 8 atretic. The morphology of a total of 136 follicles in both non-vitrified and vitrified tissues after 24 h culture was also evaluated. Of these follicles, 15 were primordial, 66 primary, 12 secondary and 43 atretic. We did not found any clear differences in the morphology of follicles and stroma after closed vitrification.

Transmission electron microscopy (**TEM)** was used to analyse the ultrastructural changes in ovarian tissue caused by cryoinjury, during vitrification and warming.

Ultrastructural alterations of ovarian tissues after vitrification and culture were analysed by evaluating the structures of oocytes, granulosa cells and stroma, separately. Forty-one follicles in both vitrified and non-vitrified tissues were analysed. Of those follicles, ten were in tissues from non-vitrified, six from vitrified, 16 from non- vitrified cultured and nine from vitrified cultured tissue.
The ultrastructure of the **oocytes** in vitrified tissue was similar to that in the fresh tissue. The nuclei were dominated by euchromatin. The inner and outer nuclear membranes containing distinct nuclear pores were clearly seen. The cytoplasm was well preserved showing a homogenous structure without obvious increased vacuolization. The round or ovoid shaped mitochondria had highly organised structure with condensed matrix and intact cristae. Normal looking endoplasmic reticulum (ER) and mitochondria were seen in the oocytes. Golgi complexes were well preserved. The contacts between the oocytes and granulosa cells were sharp, and they contained gap junctions and microvilli.
The **granulosa cells (GCs)** in vitrified tissues had a normal morphology and organelle distribution. The nuclei had peripheral aggregates of chromatin. The cytoplasm contained mitochondria with cristae, and ER. Microvilli were present and Gap junctions connecting neighbouring cells were seen. The GCs showed uniform contact without any obvious increased in vacuolization.
In vitrified tissues, the **stroma** was well preserved. The viability of the follicles was evaluated by culture for 24 hours. The morphology and ultrastructure of the follicles in the ovarian tissue after warming and culture was normal. The ultrastructure of the follicles in both non-vitrified and vitrified tissues was similar after culture. There were neither obvious morphological abnormalities nor necroses in the oocytes after culture. Well-defined nuclear pores and well-preserved round-shaped mitochondria and ER were seen in the oocytes. The GCs had nuclei with peripheral heterochromatin and a large number of rod-shaped mitochondria in their cytoplasm. The stromal cells contained hetero- and euchromatic nuclei. After culture, the vitrified tissues had elongated stromal cells and large numbers of collagen bundles.

Summarising the results in the article II, we have shown an intact structure of vitrified ovarian tissue. Our vitrification method is clinically applicable, because the tissue was never in direct contact with liquid nitrogen. The tissue preparation, vitrification, banking, warming procedures have been carried out in a closed sterile, traceable system, in a controlled and defined sterile environment. This system fulfils the quality requirements of the European directives 2004/23, 2006/17/EG and 2006/86/EG and Swedish tissue law 2008:286.

1.24 ARTICLE III

In article III, we studied the occurrence of possible apoptosis in the tissue after vitrification. We evaluated the light and electron microscopic morphology of the follicles in the vitrified tissues. Human ovarian tissue was vitrified either with a solution consisting of a combination of cryoprotectants, DMSO, PrOH, EG and PVP or EG and bovine serum albumin (BSA). The results showed that neither of the vitrification methods caused apoptosis in primordial nor primary follicles.
Light microscopic (**LM)** analysis on 264 follicles showed that the proportion of viable follicles per donor before culture was 97.14 ± 7.5 % in fresh, 90.87 ± 10.74% in tissue

vitrified with DMSO, PrOH, EG and PVP, and 92.16 ± 8.6% in tissue vitrified with EG and BSA. The proportion of viable follicles after equilibration and removal of vitrification solution (toxicity test) was 97.43 ± 4.4% in tissue vitrified with DMSO, PrOH, EG and PVP, and 95.54 ± 5.98% in tissue vitrified with EG and BSA respectively.

Ultrastructural evaluation of the follicles by **TEM** showed well -preserved morphology in both tissues vitrified using DMSO, PrOH, EG and PVP, and EG and BSA. In tissues vitrified using any of the methods, the **oocytes** contained euchromatic nuclei. The mitochondria of the oocytes were well preserved.
The **granulosa cells** and **stromal cells** had heterochromatic nuclei. The mitochondria of oocytes were well preserved although a few alterations were seen. Fresh and vitrified tissues after warming and 24h culture were used for TUNEL assay. There were no signs of apoptosis in primordial or primary follicles in either fresh or vitrified groups. The fresh and the vitrified tissue after 24h culture did not show any TUNEL-positive cells in the primordial or primary follicles. DNA laddering was used to evaluate the incidence of apoptosis in ovarian tissue from both fresh and vitrified groups. There was no DNA laddering on gel electrophoresis from genomic DNA isolated from fresh or vitrified tissues, neither after warming or 24h culture. Taking all the data together, vitrification of human ovarian tissue using solutions DMSO, PrOH, EG and PVP, or EG and BSA does not cause apoptosis in primordial or primary follicles.

1.25 ARTICLE IV

In article IV, we evaluated the use of only one permeating protectant, EG, instead of a combination of three cryoprotectants, DMSO, PrOH and EG in a closed vitrification system, presented earlier by us. Human ovarian tissue was vitrified in closed sealed cryotubes using either a combination of cryoprotectants DMSO, PrOH, EG and PVP or only EG and human serum albumin (HSA).
A total of 988 follicles was analyzed by **LM**. Of these, 584 follicles were evaluated before culture and 404 follicles after culture. **Table 4** shows the proportion of viable follicles from nine donors in ovarian tissue analyzed by LM. There were no clear differences in the morphology of the follicles in the tissue vitrified with any of the different solutions. There were no significant differences between the fresh and vitrified tissue after culture.

Light microscopy	Fresh	Vitrified using DMSO, PrOH, EG	Vitrified using only EG	Fresh, Cultured	Vitrified using DMSO, PrOH, EG-Cultured	Vitrified using only EG-Cultured
Primordial	97 (51.1)	113 (57.1)	108 (55.1)	47 (24.1)	35 (27.3)	13 (16.0)
Primary	76 (40)	52 (26.3)	62 (31.6)	112 (57.4)	49 (38.3)	41 (50.6)
Secondary	12 (6.3)	11 (5.6)	8 (4.1)	16 (8.2)	7 (5.5)	8 (9.9)
Viable follicles	185 (97)	176 (89)	178 (91)	175 (90)	91 (71)	62 (77)
Atretic	5 (2.6)	22 (11.0)	18 (9.2)	20 (10.3)	37 (28.9)	19 (23.5)
Total no. Follicles	190	198	196	195	128	81
Total F. density (mm3)	1838	1149	1511	1957	874	362

Table 4. Numbers of viable follicles from nine donors, within ovarian tissue analysed by LM. Values are presented in n (%).

A total number of 77 follicles was evaluated by **TEM**. The number of follicles found in the tissue before culture was nine in the fresh tissue, 17 in the tissue vitrified with DMSO, PrOH, EG and 13 in the tissue vitrified with only EG. Of the follicles found in the tissue after culture, were ten in the fresh tissue, 14 in the tissue vitrified with DMSO, PrOH, EG and 14 in the tissue vitrified with only EG.

The results from the evaluation of the ultrastructure of **oocytes** in vitrified tissues did not show any variation between the vitrified or control tissue. There were no cellular differences between the tissue vitrified using either of the two solutions, as regards to the ultrastructure of mitochondria, microvilli or endoplasmic reticulum (ER) of the oocytes. The ER and Golgi apparatus were well defined. The outer and inner nuclear membranes with distinct nuclear pores were clearly seen.
The **granulosa cells** in tissue vitrified using either DMSO, PrOH, EG or only EG were morphologically normal. The nuclei with peripheral aggregates of chromatin, the cytoplasm contained organelles such as mitochondria with cristae and ER showed normal ultrastructure. The contact between the granulosa cells and the basement membrane was well preserved.
Stromal tissue contained abundant collagen fibres and spindle- shaped fibroblast like cells and it appeared to be well preserved in both vitrified groups.
Follicular viability after warming and 24 h culture was evaluated. There were no severe morphological changes or necroses in the oocytes after culture. The granulosa

cells and stroma appeared to be normal. We used immunostaining for active caspase-3 for detection of apoptosis in tissue vitrified with either DMSO, PrOH, EG or only EG. There was no apoptosis in primordial or primary follicles or stroma cells in either of vitrification methods.

There were no significant differences, in the electron microscopic scores of oocytes, granulosa cells or stromal cells, between the two vitrification solutions. According to the results, the oocytes, granulosa cells and stromal cells were equally well preserved in tissue vitrified with either vitrification solution.

6 GENERAL DISCUSSION

Fertility preservation by cryopreservation of ovarian cortical tissue is an excellent option for pre-pubertal girls and young women at risk of premature ovarian failure because of cancer therapy or genetic disorders. There are two methods for cryopreserving ovarian tissue, slow freezing and vitrification. Our group has earlier developed a slow freezing method for cryopreserving human ovarian tissue using PrOH and sucrose as cryoprotectants (Hovatta *et al.* 1996; Hreinsson *et al.* 2003). Other permeating cryoprotectants such as DMSO and EG have been also used in slow freezing of human ovarian tissue (Hovatta *et al.* 1996; Newton *et al.* 1996; Oktay *et al.* 1997; Schmidt *et al.* 2003). Auto-transplantation of post-thawed ovarian tissue has resulted in live births worldwide (Dolmans *et al.* 2013). In spite of successful preservation of oocytes and granulosa cells, poor survival of stromal tissue after slow freezing is a limitation.
We carried out the first systematic comparison between cryopreservation of human ovarian tissue by slow freezing and vitrification.

Vitrification is a method for cryopreservation of ovarian tissue without ice crystal formation. It results in good morphology. In our comparative study (Article I) we showed that ovarian stroma was better preserved by using vitrification than it was after slow freezing. There has been limited data regarding comparison of vitrification and slow freezing of human ovarian tissue, and contradictory conclusions have drawn by researchers. Some results have suggested that cryopreservation of human ovarian tissue by using slow freezing is more efficient than vitrification (Gandolfi *et al.* 2006; Isachenko *et al.* 2007). Some other authors concluded that modified vitrification is an effective method for freezing human ovarian tissue because it showed a high rate of normal follicles after warming (Li *et al.* 2007). These different results may be due to differences in the procedure such as concentrations and composition of cryoprotectants, exposure time to the cryoprotectants and the rate of cooling.
Our vitrification procedure was improved at many points compared to those used earlier. The vitrification solution we used in the comparative study consisted of a combination of DMSO, PrOH and EG diluted in serum-free medium (Article I). The concentration of each cryoprotectant was gradually increased from 2.5% (total concentration of cryoprotectant 7.5%) at the first incubation step to 15% at the second and 10% (total 30%) at the final step. At this step non-permeating cryoprotectant, PVP was also added. Hence, the final overall concentration of cryoprotectants in the vitrification solution was 40%. The third step was carried out in 4°C to reduce the toxicity of cryoprotectants at high concentrations. To increase the rate of vitrification in the first study, an open system was used. Insemination cryostraws cut by scalpel were utilised. In every study in this thesis, we used tissue culture to evaluate the function and the viability of the follicles after thawing/warming because the initiation of follicular growth is a fast and safe event. It can be used as a method to evaluate the viability of follicles. (Hovatta *et al.* 1997). The cryoinjury in the tissue was evaluated by ultrastructural analysis, which gives more detailed information about ultrastructural

changes in the cell than LM (Hreinsson *et al.* 2003; Martinez-Madrid *et al.* 2007). Preservation of ovarian tissue including oocytes, granulosa cells and stroma depends on the composition and exposure time to cryopreservation media and the rate of cooling and thawing. Ovarian stromal tissue frozen by slow freezing was poorly preserved showing loss of plasma membrane, increased chromatin condensation, lysis of stromal cells, vacuolization and disintegration of ovarian stroma (Hreinsson *et al.* 2003; Eyden *et al.* 2004). Our observations were in line with these results. There are many variables involved in vitrification processes that affect the effectiveness and the potential of survival rates of the cells/ tissues. The type and concentration of CPA, sample size, carrier system, the temperature of exposure to vitrification solution and the exposure time to the final solution are such factors (Liebermann *et al.* 2002). The size of ovarian tissue pieces plays an important role in a successful vitrification outcome. It would take a longer time for CPAs to penetrate to a lager ovarian fragment and reach to the inner part of the tissue. It would result in overexposure of the cells on the surface by CPAs and cause damages due to the toxicity of high CPA concentration. The use of smaller ovarian fragments allows faster cooling and warming rates, hence avoiding ice crystal formation and decreasing the cryoinjury. In all four studies, we used small size of ovarian tissue fragments (about 1- 1.5mm^3) in order to increase the cooling rate.

However, direct contact of the tissue with liquid nitrogen in an open system is not optimal for freezing human ovarian tissue because of the possible risk of contamination. In the next study, we performed vitrification in a clinical grade closed system in which human ovarian tissue is never in direct contact with liquid nitrogen, neither during the vitrification procedure nor during storage (Article II). The device that was used in this study was a cryotube, containing internal thread with silicon gasket to provide the best possible seal. The long exposure time of the cryoprotectants was applied because our earlier study (Article I) showed a good- quality of stroma with collagen fibres and stromal cells after 10 min of incubation. Stromal cells probably play an essential role in proliferation and differentiation of granulosa cells (Hovatta *et al.* 1999; Liu *et al.* 2000). Interaction between granulosa cells, stromal cells and oocytes is important for ovarian function (Hovatta 2005). Hence, preservation of the integrity of these components is necessary (Gook *et al.* 2004). Our observations from LM showed similar morphology of follicles in both vitrified and fresh tissue. This observation was confirmed by TEM analysis, which revealed that the structure of organelles of granulosa cells, and oocytes were well preserved in vitrified tissue. The results in this study suggested that human ovarian tissue can be well preserved in a closed system using a cryotube.

Our next aim was to develop an even simpler and more reliable clinical grade vitrification procedure for human ovarian tissue. Hence, we decided to study the possible use of only one permeating cryoprotectant, EG, instead of a combination of three permeating cryoprotectants DMSO, PrOH and EG in clinical human ovarian vitrification. We chose EG because it has been shown to give good morphology in vitrification of mouse ovarian follicles (Salehnia 2002). In order to apply a clinical grade vitrification in study IV, we used HSA instead of BSA that was used in study III. Bovine serum albumin as an animal protein is not optimal when used clinically in human, since there is risk of transmitting pathogens such as prions. Moreover, the cells take non-human proteins from the culture medium. Such proteins may make the cells immunogenic and cause immunoreactions (Martin *et al.* 2005; Unger *et al.* 2008). An

open system was used in study III, since after vitrification the vitrified tissues were stored in liquid nitrogen but in study IV, the system was a closed one.
The morphology of the oocytes, granulosa cells and stromal cells in tissue after both vitrification methods were analysed. The LM and TEM showed that the morphology after both vitrification methods in both cultured and non-cultured tissue was similar. No signs of apoptosis were observed when using the TUNEL assay and DNA laddering (Article III). In addition, immunostaining for active caspase-3 did not show any apoptosis in neither primordial nor primary follicles nor stromal cells (Article IV). This was expected, because the cryoinjury is caused by small ice crystals that damage the membrane in the cells and cellular organelles, and not clearly by apoptosis. Our results suggested that vitrification solution with only one permeating cryoprotectant, EG in diluted human serum albumin, instead of three cryoprotectants, can be recommended in vitrification of human ovarian tissue. It is a fast, simple and safe procedure that can be carried out at clinical setting.

7 CONCLUSIONS

- Vitrification of human ovarian tissue is more efficient than slow freezing in maintaining the cellular morphology. The stroma of vitrified ovarian tissue is better preserved than that cryo-stored by slow freezing.

- Closed vitrification of human ovarian tissue without direct contact with liquid nitrogen is as good as open vitrification used earlier. It can be performed in a clinical setting.

- The clinically feasible vitrification method can be further simplified by using only one permeating cryoprotectant, EG instead of a combination of DMSO, PrOH and EG.

8 ACKNOWLEDGEMENTS

Finally!!! It has been a long journey with many ups and downs the hill to accomplish this thesis. Therefore, I wish to express my sincere gratitude to everyone who helped me to get here.

First of all I would like to express my sincere gratitude to the women that donated their ovarian biopsies and made these studies possible.
I am grateful to all the doctors and nurses at the Obstetrics and Gynaecology department at Karolinska University Hospital, Huddinge, who kindly helped with ovarian biopsies.

Professor **Outi Hovatta,** my main supervisor, for your constant enthusiasm, excellent guidance, great support, scientific discussions and comments during these years. Thank you, for accepting me as your student and teaching me about research, for never getting tired of editing my manuscripts.

Docent **Monalill Lundqvist,** my co-supervisor, for your never-ending support, encouragement, concern and trust. Thank you, for constructive and supportive scientific discussions. You encouraged me when it was tough to be a PhD student.

Virpi Töhönen, my mentor, for your support, encouragement and sound advice. For always having time for me and being helpful during my time as a PhD student.

I deeply thank my co-authors, **Hultenby K, Niklasson B, Hreinsson J, Volpe A, Salehnia M, Pourbeiranvand S, Xella S, Keros V** and **Pettersson K.** This could not exist without you.

My other co-authors in the various projects, whether included in my thesis or not: **Borgström B, Rasmussen C, Fridström M and Fried G (R.I.P).** Thank you!
Margareta Fridström, our chats have meant a lot to me. Thank you for being friendly and a warm and fantastic person.

Galina Drozdova, you are fantastic, helpful and friendly. Thank you for all help!

I am deeply grateful to **Liselotte, Sonya** and **Shahla**, for being the most warm and supportive friends in the lab. **Mohammed,** I warmly thank you for helping me with various difficulties with computer work, and kindly providing me with some good advices when I was preparing for my halftime seminar and now for my dissertation. (I am one step after you ☺). Many thanks to my wonderful colleagues at the Hovatta lab for being friendly and supportive: **Sarita, Riina, Fredrik, Jonathan** and **Eva-Britt**. **Pauliina,** thank you for good advices and funny messages when I was feeling down, for helping me with some nice micrographs for my thesis. Skål!

I am grateful to my "biopsy" friend, **Boel Niklasson**, I could never accomplish this work without your enthusiastic help. Good luck with your PhD studies (you are next ☺).

Many thanks to, **Romana Gerner,** for being the best research-colleague one could have. It was a pleasure to work with you here. Thank you for being a wonderful and supportive friend. I miss you with a smile saying, "coooool"! Good luck with your PhD studies in Austria!

Special thanks to my friends and fantastic past members of our research team:
Susanne Ström, Rós Kjartansdottir, Frida Holm, Ami Strömberg and **Suvi Asikainen**.

Special thanks to **Julius Hreinsson,** for your excellent advices and constructive comments when I was writing my study plan and teaching me all about "ovary" for the first year at the beginning of my projects. Thank you for introducing me to the wonderful world of human female fertility and taking your time to come from Uppsala when I needed to discuss science.

José Inzunza, thank you for being friendly and always having time to help, for your encouragement and good advices.

I am grateful to our Finnish and Norwegian collaborators, **Kirsi Jahunkainen, Irma Oskam, Babak Asadi Azarbaijani** and **Mirja Nurmio** for good advices and excellent collaboration in our ongoing project. We are going to publish this together! Babak! I wish that it would soon be your time to write your thesis.

Special thanks to all of you my colleagues in the IVF lab: **Lina Barbunopulos, Sanna Friberg, Rebecka Holmberg, Jessica Lundmark, Lena Möller, Mirja Tolvanen, Kristina Magnusson, Eija Matilainen, Elsa Mesfin, Kristina Miras Wardell, Linda Nordén, Jeanette Gudmundsson, Linda Bouhafs, Lars Pettersson** (my savior whenever I was in trouble with Photoshop)**, Ingalill Persson, Jens Winerdal, Nashwan Jalal Markus and Victoria Keros.** Words cannot express my gratitude to you all, Tusen tack!

I am lucky to have extremely understanding, wonderful and supporting colleagues at the Fertility Unit. Many thanks to: the doctors: **Jan Olofsson, Annika Bladh Blomquist, Per-Olof Karlström, György Csemiczky, Kenny Rodriguez- Wallberg, Gerasimos Tzortzatos, and Karin Rova**, midwives: **Marie Klinta Svensson, Eva Andersson, Malin Broberg, Maria Dahlgren, Susanna Jangklev, Anneth Johansson, Ing-Marie Jonsson, Karin Persdotter Eberg, Eva Persson, Ingegard Lundqvist, Christina Scherman Pukk, Margareta Stefenson, Kerstin Warolin, Lena Ydenius**, the nursing staff and everyone in administration: **Christina Vuorisalo, Annika Abu Esba, Lena Hyberg, Carina Karlsson, Yvonne Kaselli, Kerstin Wahlman, Pamela Bravo Söderhäll, Christina Karlsson,** also our psychologist **Lovisa Linell.** Thank you for being fantastic and supportive. This work would not be done without your generous help.

I would like to thank **Raoul Kuiper** and his group for fruitful scientific discussions and for helping with immnnohistochemistry staining. Special thanks to **Carin Lundmark** and **Tarja Schröder,** Tack så mycket!

I wish to thank **Kjell Hultenby** and his wonderful group members, **Ingrid Lindell** and **Eva Blomén** for helping with histological preparations and electron microscopic analysis. Tusen tack! Thank you **Kjell,** for sharing your scientific and technical skills, and having many valuable scientific discussions.

I would like to thank professor **Kui Liu** and his group, specially **Deepak Adhikari** for an excellent collaboration in our follicle culture project.

I would give my deepest cordial and heartfelt thanks to my dear **parents**. Your unconditional love, support and encouragement have always helped me out through many tough periods of my life. You have always been my inspiration and the reason to get finish with this journey. Many thanks to my dear **brothers,** for truly believing in me and for providing the love and support that one could ever have. You guys have always time for me. I love our philosophic discussions. I love you all!

Most importantly, I have to thank my wonderful children, **Sara** and **Sam** for making my life always sunny. Your love is the source of my life. Dear Sam! I own you a special thank because you was extremely understanding and patient with me when I was writing my thesis. Dear Sara! I promise to come to Göteborg and visit you more often than before ☺. You both have been extremely supportive and understanding during these four years of my PhD study. I love you my dears!

Special thank to my dear **Hans** for your true love, understanding and support. I am looking forward to experience many adventures together. Älskar dig!

Last but not least, my deepest thanks to my special friends outside the lab, **Simin, Shole** and **Elsa** for your phone calls, support and encouragement concerning research and family life. The world needs more people like you!

THANK YOU ALL!

This work was supported by grants from the Swedish Research Council (OH) and by the R and D funds of Karolinska University Hospital and Karolinska Institutet (ALF) (OH).

9 REFERENCES

Adhikari D and Liu K. Molecular mechanisms underlying the activation of mammalian primordial follicles. *Endocr Rev* 2009; **30**:438-464.

Aittomaki K, Herva R, Stenman UH, Juntunen K, Ylostalo P, Hovatta O and de la Chapelle A. Clinical features of primary ovarian failure caused by a point mutation in the follicle-stimulating hormone receptor gene. *J Clin Endocrinol Metab* 1996; **81**:3722-3726.

Allingham-Hawkins DJ, Babul-Hirji R, Chitayat D, Holden JJ, Yang KT, Lee C, Hudson R, Gorwill H, Nolin SL, Glicksman A et al. Fragile X premutation is a significant risk factor for premature ovarian failure: the International Collaborative POF in Fragile X study--preliminary data. *Am J Med Genet* 1999; **83**:322-325.

Almodin CG, Minguetti-Camara VC, Meister H, Ferreira JO, Franco RL, Cavalcante AA, Radaelli MR, Bahls AS, Moron AF and Murta CG. Recovery of fertility after grafting of cryopreserved germinative tissue in female rabbits following radiotherapy. *Hum Reprod* 2004; **19**:1287-1293.

Amorim CA, David A, Van Langendonckt A, Dolmans MM and Donnez J. Vitrification of human ovarian tissue: effect of different solutions and procedures. *Fertil Steril* 2011; **95**:1094-1097.

Amorim CA, Dolmans MM, David A, Jaeger J, Vanacker J, Camboni A, Donnez J and Van Langendonckt A. Vitrification and xenografting of human ovarian tissue. *Fertil Steril* 2012; **98**:1291-1298 e1291-1292.

Andersen CY, Rosendahl M, Byskov AG, Loft A, Ottosen C, Dueholm M, Schmidt KL, Andersen AN and Ernst E. Two successful pregnancies following autotransplantation of frozen/thawed ovarian tissue. *Hum Reprod* 2008; **23**:2266-2272.

Anderson RA and Wallace WH. Fertility preservation in girls and young women. *Clin Endocrinol (Oxf)* 2011; **75**:409-419.

Antinori M, Licata E, Dani G, Cerusico F, Versaci C and Antinori S. Cryotop vitrification of human oocytes results in high survival rate and healthy deliveries. *Reprod Biomed Online* 2007; **14**:72-79.

Azim AA, Costantini-Ferrando M, Lostritto K and Oktay K. Relative potencies of anastrozole and letrozole to suppress estradiol in breast cancer patients undergoing ovarian stimulation before in vitro fertilization. *J Clin Endocrinol Metab* 2007; **92**:2197-2200.

Baird DT, Campbell B, de Souza C and Telfer E. Long-term ovarian function in sheep after ovariectomy and autotransplantation of cryopreserved cortical strips. *Eur J Obstet Gynecol Reprod Biol* 2004; **113 Suppl 1**:S55-59.

Baka SG, Toth TL, Veeck LL, Jones HW, Jr., Muasher SJ and Lanzendorf SE. Evaluation of the spindle apparatus of in-vitro matured human oocytes following cryopreservation. *Hum Reprod* 1995; **10**:1816-1820.

Barnes FL, Crombie A, Gardner DK, Kausche A, Lacham-Kaplan O, Suikkari AM, Tiglias J, Wood C and Trounson AO. Blastocyst development and birth after in-vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. *Hum Reprod* 1995; **10**:3243-3247.

Bautista JA and Kanagawa H. Current status of vitrification of embryos and oocytes in domestic animals: ethylene glycol as an emerging cryoprotectant of choice. *Jpn J Vet Res* 1998; **45**:183-191.

Beck-Peccoz P and Persani L. Premature ovarian failure. *Orphanet J Rare Dis* 2006; **1**:9.

Bisharah M and Tulandi T. Laparoscopic preservation of ovarian function: an underused procedure. *Am J Obstet Gynecol* 2003; **188**:367-370.

Blumenfeld Z. How to preserve fertility in young women exposed to chemotherapy? The role of GnRH agonist cotreatment in addition to cryopreservation of embrya, oocytes, or ovaries. *Oncologist* 2007; **12**:1044-1054.

Borgstrom B, Hreinsson J, Rasmussen C, Sheikhi M, Fried G, Keros V, Fridstrom M and Hovatta O. Fertility preservation in girls with turner syndrome: prognostic signs of the presence of ovarian follicles. *J Clin Endocrinol Metab* 2009; **94**:74-80.

Borini A, Sciajno R, Bianchi V, Sereni E, Flamigni C and Coticchio G. Clinical outcome of oocyte cryopreservation after slow cooling with a protocol utilizing a high sucrose concentration. *Hum Reprod* 2006; **21**:512-517.

Bosch P, Hernandez-Fonseca HJ, Miller DM, Wininger JD, Massey JB, Lamb SV and Brackett BG. Development of antral follicles in cryopreserved cat ovarian tissue transplanted to immunodeficient mice. *Theriogenology* 2004; **61**:581-594.

Broekmans FJ, Knauff EA, te Velde ER, Macklon NS and Fauser BC. Female reproductive ageing: current knowledge and future trends. *Trends Endocrinol Metab* 2007; **18**:58-65.

Bromfield JJ, Coticchio G, Hutt K, Sciajno R, Borini A and Albertini DF. Meiotic spindle dynamics in human oocytes following slow-cooling cryopreservation. *Hum Reprod* 2009; **24**:2114-2123.

Byskov AG, Hoyer PE, Yding Andersen C, Kristensen SG, Jespersen A and Mollgard K. No evidence for the presence of oogonia in the human ovary after their final clearance during the first two years of life Hum Reprod. 2011, pp. 2129-2139.

Cao Y, Xing Q, Zhang ZG, Wei ZL, Zhou P and Cong L. Cryopreservation of immature and in-vitro matured human oocytes by vitrification. *Reprod Biomed Online* 2009; **19**:369-373.

Carlsson IB, Laitinen MP, Scott JE, Louhio H, Velentzis L, Tuuri T, Aaltonen J, Ritvos O, Winston RM and Hovatta O. Kit ligand and c-Kit are expressed during early human ovarian follicular development and their interaction is required for the survival of follicles in long-term culture Reproduction. 2006, pp. 641-649.

Carlsson IB, Scott JE, Visser JA, Ritvos O, Themmen AP and Hovatta O. Anti-Mullerian hormone inhibits initiation of growth of human primordial ovarian follicles in vitro. *Hum Reprod* 2006; **21**:2223-2227.

Carroll J and Gosden RG. Transplantation of frozen-thawed mouse primordial follicles. *Hum Reprod* 1993; **8**:1163-1167.

Cecconi S, Capacchietti G, Russo V, Berardinelli P, Mattioli M and Barboni B. In vitro growth of preantral follicles isolated from cryopreserved ovine ovarian tissue. *Biol Reprod* 2004; **70**:12-17.

Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY and Yoon TK. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program Fertil Steril. 1991, pp. 109-113.

Chen C. Pregnancy after human oocyte cryopreservation. *Lancet* 1986; **1**:884-886.

Chen CH, Zhang X, Barnes R, Confino E, Milad M, Puscheck E and Kazer RR. Relationship between peak serum estradiol levels and treatment outcome in in vitro fertilization cycles after embryo transfer on day 3 or day 5. *Fertil Steril* 2003; **80**:75-79.

Cobo A and Diaz C. Clinical application of oocyte vitrification: a systematic review and meta-analysis of randomized controlled trials. *Fertil Steril* 2011; **96**:277-285.

Cobo A, Domingo J, Perez S, Crespo J, Remohi J and Pellicer A. Vitrification: an effective new approach to oocyte banking and preserving fertility in cancer patients. *Clin Transl Oncol* 2008; **10**:268-273.

Courbiere B, Odagescu V, Baudot A, Massardier J, Mazoyer C, Salle B and Lornage J. Cryopreservation of the ovary by vitrification as an alternative to slow-cooling protocols. *Fertil Steril* 2006; **86**:1243-1251.

Cronister A, Schreiner R, Wittenberger M, Amiri K, Harris K and Hagerman RJ. Heterozygous fragile X female: historical, physical, cognitive, and cytogenetic features. *Am J Med Genet* 1991; **38**:269-274.

Das M, Shehata F, Son WY, Tulandi T and Holzer H. Ovarian reserve and response to IVF and in vitro maturation treatment following chemotherapy. *Hum Reprod* 2012; **27**:2509-2514.

de Kretser DM, Hedger MP, Loveland KL and Phillips DJ. Inhibins, activins and follistatin in reproduction. *Hum Reprod Update* 2002; **8**:529-541.

Demeestere I, Simon P, Emiliani S, Delbaere A and Englert Y. Orthotopic and heterotopic ovarian tissue transplantation. *Hum Reprod Update* 2009; **15**:649-665.

Demirtas E, Elizur SE, Holzer H, Gidoni Y, Son WY, Chian RC and Tan SL. Immature oocyte retrieval in the luteal phase to preserve fertility in cancer patients. *Reprod Biomed Online* 2008; **17**:520-523.

Deng X, Zheng H, Yu X, Yu H, Zhang C, Chao L, Li R and Liu W. Cryopreserved ovarian tissues can maintain a long-term function after heterotopic autotransplantation in rat. *Reproduction* 2009; **138**:519-525.

Ding J, Jiang D, Kurczy M, Nalepka J, Dudley B, Merkel EI, Porter FD, Ewing AG, Winograd N, Burgess J et al. Inhibition of HMG CoA reductase reveals an unexpected role for cholesterol during PGC migration in the mouse BMC Dev Biol. 2008, pp. 120.

Dolmans MM, Demylle D, Martinez-Madrid B and Donnez J. Efficacy of in vitro fertilization after chemotherapy. *Fertil Steril* 2005; **83**:897-901.

Dolmans MM, Jadoul P, Gilliaux S, Amorim CA, Luyckx V, Squifflet J, Donnez J and Van Langendonckt A. A review of 15 years of ovarian tissue bank activities. *J Assist Reprod Genet* 2013; **30**:305-314.

Dolmans MM, Luyckx V, Donnez J, Andersen CY and Greve T. Risk of transferring malignant cells with transplanted frozen-thawed ovarian tissue. *Fertil Steril* 2013.

Dolmans MM, Marinescu C, Saussoy P, Van Langendonckt A, Amorim C and Donnez J. Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe. *Blood* 2010; **116**:2908-2914.

Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, Martinez-Madrid B and van Langendonckt A. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet* 2004; **364**:1405-1410.

Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, Martinez-Madrid B and van Langendonckt A. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue Lancet. 2004, pp. 1405-1410.

Donnez J, Godin PA, Qu J and Nisolle M. Gonadal cryopreservation in the young patient with gynaecological malignancy. *Curr Opin Obstet Gynecol* 2000; **12**:1-9.

Donnez J, Martinez-Madrid B, Jadoul P, Van Langendonckt A, Demylle D and Dolmans MM. Ovarian tissue cryopreservation and transplantation: a review. *Hum Reprod Update* 2006; **12**:519-535.

Durlinger AL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA and Themmen AP. Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary Endocrinology. 2002, pp. 1076-1084.

Eppig JJ. Intercommunication between mammalian oocytes and companion somatic cells Bioessays. 1991, pp. 569-574.

Eppig JJ. Growth and development of mammalian oocytes in vitro Arch Pathol Lab Med. 1992, pp. 379-382.

Eppig JJ and O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod* 1996; **54**:197-207.

Eyden B, Radford J, Shalet SM, Thomas N, Brison DR and Lieberman BA. Ultrastructural preservation of ovarian cortical tissue cryopreserved in dimethylsulfoxide for subsequent transplantation into young female cancer patients. *Ultrastruct Pathol* 2004; **28**:239-245.

Fabbri R, Porcu E, Marsella T, Rocchetta G, Venturoli S and Flamigni C. Human oocyte cryopreservation: new perspectives regarding oocyte survival. *Hum Reprod* 2001; **16**:411-416.

Faddy MJ. Follicle dynamics during ovarian ageing Mol Cell Endocrinol. 2000, pp. 43-48.

Faddy MJ and Gosden RG. A mathematical model of follicle dynamics in the human ovary Hum Reprod. 1995, pp. 770-775.

Faddy MJ, Gosden RG, Gougeon A, Richardson SJ and Nelson JF. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. *Hum Reprod* 1992; **7**:1342-1346.

Fahy GM, MacFarlane DR, Angell CA and Meryman HT. Vitrification as an approach to cryopreservation. *Cryobiology* 1984; **21**:407-426.

Fahy GM, Wowk B, Wu J and Paynter S. Improved vitrification solutions based on the predictability of vitrification solution toxicity. *Cryobiology* 2004; **48**:22-35.

Fujimoto T, Miyayama Y and Fuyuta M. The origin, migration and fine morphology of human primordial germ cells. *Anat Rec* 1977; **188**:315-330.

Funkenstein B, Nimrod A and Lindner HR. The development of steroidogenic capability and responsiveness to gonadotropins in cultured neonatal rat ovaries. *Endocrinology* 1980; **106**:98-106.

Gandolfi F, Paffoni A, Papasso Brambilla E, Bonetti S, Brevini TA and Ragni G. Efficiency of equilibrium cooling and vitrification procedures for the cryopreservation of ovarian tissue: comparative analysis between human and animal models. *Fertil Steril* 2006; **85 Suppl 1**:1150-1156.

Ghetler Y, Yavin S, Shalgi R and Arav A. The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. *Hum Reprod* 2005; **20**:3385-3389.

Gondos B, Westergaard L and Byskov AG. Initiation of oogenesis in the human fetal ovary: ultrastructural and squash preparation study. *Am J Obstet Gynecol* 1986; **155**:189-195.

Gonzalez C, Boada M, Devesa M and Veiga A. Concise review: fertility preservation: an update. *Stem Cells Transl Med* 2012; **1**:668-672.

Gook DA, Edgar DH, Borg J, Archer J and McBain JC. Diagnostic assessment of the developmental potential of human cryopreserved ovarian tissue from multiple patients using xenografting. *Hum Reprod* 2005; **20**:72-78.

Gook DA, Edgar DH and Stern C. The effects of cryopreservation regimens on the morphology of human ovarian tissue. *Mol Cell Endocrinol* 2000; **169**:99-103.

Gook DA, Edgar DH and Stern C. Cryopreservation of human ovarian tissue. *Eur J Obstet Gynecol Reprod Biol* 2004; **113 Suppl 1**:S41-44.

Gosden RG. Gonadal tissue cryopreservation and transplantation. *Reprod Biomed Online* 2002; **4 Suppl 1**:64-67.

Gosden RG. Prospects for oocyte banking and in vitro maturation. *J Natl Cancer Inst Monogr* 2005:60-63.

Gosden RG, Baird DT, Wade JC and Webb R. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at -196 degrees C. *Hum Reprod* 1994; **9**:597-603.

Gougeon A. Dynamics of follicular growth in the human: a model from preliminary results. *Hum Reprod* 1986; **1**:81-87.

Gougeon A. Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr Rev* 1996; **17**:121-155.

Gougeon A. Human ovarian follicular development: from activation of resting follicles to preovulatory maturation. *Ann Endocrinol (Paris)* 2010; **71**:132-143.

Gougeon A, Ecochard R and Thalabard JC. Age-related changes of the population of human ovarian follicles: increase in the disappearance rate of non-growing and early-growing follicles in aging women. *Biol Reprod* 1994; **50**:653-663.

Greve T, Clasen-Linde E, Andersen MT, Andersen MK, Sorensen SD, Rosendahl M, Ralfkiaer E and Andersen CY. Cryopreserved ovarian cortex from patients with leukemia in complete remission contains no apparent viable malignant cells. *Blood* 2012; **120**:4311-4316.

Groome NP, Illingworth PJ, O'Brien M, Pai R, Rodger FE, Mather JP and McNeilly AS. Measurement of dimeric inhibin B throughout the human menstrual cycle. *J Clin Endocrinol Metab* 1996; **81**:1401-1405.

Han SS, Kim YH, Lee SH, Kim GJ, Kim HJ, Kim JW, Park NH, Song YS and Kang SB. Underuse of ovarian transposition in reproductive-aged cancer patients treated by primary or adjuvant pelvic irradiation. *J Obstet Gynaecol Res* 2011; **37**:825-829.

Hashimoto S, Suzuki N, Yamanaka M, Hosoi Y, Ishizuka B and Morimoto Y. Effects of vitrification solutions and equilibration times on the morphology of cynomolgus ovarian tissues. *Reprod Biomed Online* 2010; **21**:501-509.

Hovatta O. Cryobiology of ovarian and testicular tissue. *Best Pract Res Clin Obstet Gynaecol* 2003; **17**:331-342.

Hovatta O. Methods for cryopreservation of human ovarian tissue. *Reprod Biomed Online* 2005; **10**:729-734.

Hovatta O. Ovarian function and in vitro fertilization (IVF) in Turner syndrome. *Pediatr Endocrinol Rev* 2012; **9 Suppl 2**:713-717.

Hovatta O, Silye R, Abir R, Krausz T and Winston RM. Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture. *Hum Reprod* 1997; **12**:1032-1036.

Hovatta O, Silye R, Krausz T, Abir R, Margara R, Trew G, Lass A and Winston RM. Cryopreservation of human ovarian tissue using dimethylsulphoxide and propanediol-sucrose as cryoprotectants. *Hum Reprod* 1996; **11**:1268-1272.

Hovatta O, Wright C, Krausz T, Hardy K and Winston RM. Human primordial, primary and secondary ovarian follicles in long-term culture: effect of partial isolation Hum Reprod. 1999, pp. 2519-2524.

Howell S and Shalet S. Gonadal damage from chemotherapy and radiotherapy. *Endocrinol Metab Clin North Am* 1998; **27**:927-943.

Hreinsson J, Rosenlund B, Friden B, Levkov L, Ek I, Suikkari AM, Hovatta O and Fridstrom M. Recombinant LH is equally effective as recombinant hCG in promoting

oocyte maturation in a clinical in-vitro maturation programme: a randomized study. *Hum Reprod* 2003; **18**:2131-2136.

Hreinsson J, Zhang P, Swahn ML, Hultenby K and Hovatta O. Cryopreservation of follicles in human ovarian cortical tissue. Comparison of serum and human serum albumin in the cryoprotectant solutions. *Hum Reprod* 2003; **18**:2420-2428.

Hreinsson JG, Otala M, Fridstrom M, Borgstrom B, Rasmussen C, Lundqvist M, Tuuri T, Simberg N, Mikkola M, Dunkel L et al. Follicles are found in the ovaries of adolescent girls with Turner's syndrome J Clin Endocrinol Metab. 2002, pp. 3618-3623.

Hreinsson JG, Otala M, Fridstrom M, Borgstrom B, Rasmussen C, Lundqvist M, Tuuri T, Simberg N, Mikkola M, Dunkel L et al. Follicles are found in the ovaries of adolescent girls with Turner's syndrome. *J Clin Endocrinol Metab* 2002; **87**:3618-3623.

Hreinsson JG, Scott JE, Rasmussen C, Swahn ML, Hsueh AJ and Hovatta O. Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture. *J Clin Endocrinol Metab* 2002; **87**:316-321.

Huang JY, Tulandi T, Holzer H, Tan SL and Chian RC. Combining ovarian tissue cryobanking with retrieval of immature oocytes followed by in vitro maturation and vitrification: an additional strategy of fertility preservation. *Fertil Steril* 2008; **89**:567-572.

Huang L, Mo Y, Wang W, Li Y, Zhang Q and Yang D. Cryopreservation of human ovarian tissue by solid-surface vitrification. *Eur J Obstet Gynecol Reprod Biol* 2008; **139**:193-198.

Hunt CJ, Taylor MJ and Pegg DE. Freeze-substitution and isothermal freeze-fixation studies to elucidate the pattern of ice formation in smooth muscle at 252 K (-21 degrees C). *J Microsc* 1982; **125**:177-186.

Isachenko V, Isachenko E, Kreienberg R, Woriedh M and Weiss J. Human ovarian tissue cryopreservation: quality of follicles as a criteria of effectiveness. *Reprod Biomed Online* 2010; **20**:441-442.

Isachenko V, Isachenko E, Reinsberg J, Montag M, van der Ven K, Dorn C, Roesing B and van der Ven H. Cryopreservation of human ovarian tissue: comparison of rapid and conventional freezing. *Cryobiology* 2007; **55**:261-268.

Jensen JR, Morbeck DE and Coddington CC, 3rd. Fertility preservation. *Mayo Clin Proc* 2011; **86**:45-49.

Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R et al. Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell* 2005; **122**:303-315.

Johnson J, Canning J, Kaneko T, Pru JK and Tilly JL. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature* 2004; **428**:145-150.

Johnston-MacAnanny EB, Koty P, Pettenati M, Brady M, Yalcinkaya TM and Schmidt DW. The first case described: monozygotic twin sisters with the fragile X premutation but with a different phenotype for premature ovarian failure. *Fertil Steril* 2011; **95**:2431 e2413-2435.

Kagawa N, Silber S and Kuwayama M. Successful vitrification of bovine and human ovarian tissue. *Reprod Biomed Online* 2009; **18**:568-577.

Karlsson JO and Toner M. Long-term storage of tissues by cryopreservation: critical issues. *Biomaterials* 1996; **17**:243-256.

Kedem O and Katchalsky A. Thermodynamic analysis of the permeability of biological membranes to non-electrolytes. *Biochim Biophys Acta* 1958; **27**:229-246.

Keros V, Rosenlund B, Hultenby K, Aghajanova L, Levkov L and Hovatta O. Optimizing cryopreservation of human testicular tissue: comparison of protocols with glycerol, propanediol and dimethylsulphoxide as cryoprotectants. *Hum Reprod* 2005; **20**:1676-1687.

Keros V, Xella S, Hultenby K, Pettersson K, Sheikhi M, Volpe A, Hreinsson J and Hovatta O. Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue. *Hum Reprod* 2009; **24**:1670-1683.

Kim SS, Soules MR and Battaglia DE. Follicular development, ovulation, and corpus luteum formation in cryopreserved human ovarian tissue after xenotransplantation. *Fertil Steril* 2002; **78**:77-82.

Konc J, Kanyo K, Varga E, Kriston R and Cseh S. Births resulting from oocyte cryopreservation using a slow freezing protocol with propanediol and sucrose. *Syst Biol Reprod Med* 2008; **54**:205-210.

Kuleshova L, Gianaroli L, Magli C, Ferraretti A and Trounson A. Birth following vitrification of a small number of human oocytes: case report. *Hum Reprod* 1999; **14**:3077-3079.

Kuleshova LL, MacFarlane DR, Trounson AO and Shaw JM. Sugars exert a major influence on the vitrification properties of ethylene glycol-based solutions and have low toxicity to embryos and oocytes. *Cryobiology* 1999; **38**:119-130.

Kyono K, Doshida M, Toya M, Sato Y, Akahira J and Sasano H. Potential indications for ovarian autotransplantation based on the analysis of 5,571 autopsy findings of females under the age of 40 in Japan. *Fertil Steril* 2010; **93**:2429-2430.

Laitinen M, Vuojolainen K, Jaatinen R, Ketola I, Aaltonen J, Lehtonen E, Heikinheimo M and Ritvos O. A novel growth differentiation factor-9 (GDF-9) related factor is co-

expressed with GDF-9 in mouse oocytes during folliculogenesis. *Mech Dev* 1998; **78**:135-140.

Larsen EC, Muller J, Schmiegelow K, Rechnitzer C and Andersen AN. Reduced ovarian function in long-term survivors of radiation- and chemotherapy-treated childhood cancer. *J Clin Endocrinol Metab* 2003; **88**:5307-5314.

Lass A, Silye R, Abrams DC, Krausz T, Hovatta O, Margara R and Winston RM. Follicular density in ovarian biopsy of infertile women: a novel method to assess ovarian reserve. *Hum Reprod* 1997; **12**:1028-1031.

Lee DM, Yeoman RR, Battaglia DE, Stouffer RL, Zelinski-Wooten MB, Fanton JW and Wolf DP. Live birth after ovarian tissue transplant. *Nature* 2004; **428**:137-138.

Lees ND, Kleinhans FW, Broughton MC, Pennington DE, Ricker VA and Bard M. Membrane fluidity alterations in a cytochrome P-450-deficient mutant of Candida albicans. *Steroids* 1989; **53**:567-578.

Levi Setti PE, Albani E, Novara PV, Cesana A and Morreale G. Cryopreservation of supernumerary oocytes in IVF/ICSI cycles. *Hum Reprod* 2006; **21**:370-375.

Li YB, Zhou CQ, Yang GF, Wang Q and Dong Y. Modified vitrification method for cryopreservation of human ovarian tissues. *Chin Med J (Engl)* 2007; **120**:110-114.

Liebermann J, Nawroth F, Isachenko V, Isachenko E, Rahimi G and Tucker MJ. Potential importance of vitrification in reproductive medicine. *Biol Reprod* 2002; **67**:1671-1680.

Liu J, Van Der Elst J, Van Den Broecke R, Dumortier F and Dhont M. Maturation of mouse primordial follicles by combination of grafting and in vitro culture. *Biol Reprod* 2000; **62**:1218-1223.

Lornage J and Salle B. Ovarian and oocyte cryopreservation. *Curr Opin Obstet Gynecol* 2007; **19**:390-394.

Lovelock JE. The haemolysis of human red blood-cells by freezing and thawing. *Biochim Biophys Acta* 1953; **10**:414-426.

Lucci CM, Kacinskis MA, Lopes LH, Rumpf R and Bao SN. Effect of different cryoprotectants on the structural preservation of follicles in frozen zebu bovine (Bos indicus) ovarian tissue. *Theriogenology* 2004; **61**:1101-1114.

Lucena E, Bernal DP, Lucena C, Rojas A, Moran A and Lucena A. Successful ongoing pregnancies after vitrification of oocytes. *Fertil Steril* 2006; **85**:108-111.

MacNaughton J, Banah M, McCloud P, Hee J and Burger H. Age related changes in follicle stimulating hormone, luteinizing hormone, oestradiol and immunoreactive inhibin in women of reproductive age. *Clin Endocrinol (Oxf)* 1992; **36**:339-345.

Maltaris T, Koelbl H, Seufert R, Kiesewetter F, Beckmann MW, Mueller A and Dittrich R. Gonadal damage and options for fertility preservation in female and male cancer survivors. *Asian J Androl* 2006; **8**:515-533.

Mamsen LS, Brochner CB, Byskov AG and Mollgard K. The migration and loss of human primordial germ stem cells from the hind gut epithelium towards the gonadal ridge. *Int J Dev Biol* 2012; **56**:771-778.

Marhhom E and Cohen I. Fertility preservation options for women with malignancies. *Obstet Gynecol Surv* 2007; **62**:58-72.

Marozzi A, Manfredini E, Tibiletti MG, Furlan D, Villa N, Vegetti W, Crosignani PG, Ginelli E, Meneveri R and Dalpra L. Molecular definition of Xq common-deleted region in patients affected by premature ovarian failure. *Hum Genet* 2000; **107**:304-311.

Martin MJ, Muotri A, Gage F and Varki A. Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nat Med* 2005; **11**:228-232.

Martinez-Madrid B, Camboni A, Dolmans MM, Nottola S, Van Langendonckt A and Donnez J. Apoptosis and ultrastructural assessment after cryopreservation of whole human ovaries with their vascular pedicle. *Fertil Steril* 2007; **87**:1153-1165.

Martins da Silva SJ, Bayne RA, Cambray N, Hartley PS, McNeilly AS and Anderson RA. Expression of activin subunits and receptors in the developing human ovary: activin A promotes germ cell survival and proliferation before primordial follicle formation. *Dev Biol* 2004; **266**:334-345.

Massague J. TGF-beta signal transduction. *Annu Rev Biochem* 1998; **67**:753-791.

Massague J and Chen YG. Controlling TGF-beta signaling. *Genes Dev* 2000; **14**:627-644.

Mazoochi T, Salehnia M, Valojerdi MR and Mowla SJ. Morphologic, ultrastructural, and biochemical identification of apoptosis in vitrified-warmed mouse ovarian tissue. *Fertil Steril* 2008; **90**:1480-1486.

Mazur P. Kinetics of Water Loss from Cells at Subzero Temperatures and the Likelihood of Intracellular Freezing. *J Gen Physiol* 1963; **47**:347-369.

Mc CM, Keaty EC and Thompson JD. Conservation of ovarian tissue in the treatment of carcinoma of the cervix with radical surgery. *Am J Obstet Gynecol* 1958; **75**:590-600; discussion 600-595.

Mc KD, Hertig AT, Adams EC and Danziger S. Histochemical observations on the germ cells of human embryos. *Anat Rec* 1953; **117**:201-219.

McGee EA and Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev* 2000; **21**:200-214.

Meirow D. Reproduction post-chemotherapy in young cancer patients. *Mol Cell Endocrinol* 2000; **169**:123-131.

Meirow D, Biederman H, Anderson RA and Wallace WH. Toxicity of chemotherapy and radiation on female reproduction. *Clin Obstet Gynecol* 2010; **53**:727-739.

Meirow D, Dor J, Kaufman B, Shrim A, Rabinovici J, Schiff E, Raanani H, Levron J and Fridman E. Cortical fibrosis and blood-vessels damage in human ovaries exposed to chemotherapy. Potential mechanisms of ovarian injury. *Hum Reprod* 2007; **22**:1626-1633.

Meirow D, Hardan I, Dor J, Fridman E, Elizur S, Ra'anani H, Slyusarevsky E, Amariglio N, Schiff E, Rechavi G et al. Searching for evidence of disease and malignant cell contamination in ovarian tissue stored from hematologic cancer patients. *Hum Reprod* 2008; **23**:1007-1013.

Meirow D and Nugent D. The effects of radiotherapy and chemotherapy on female reproduction. *Hum Reprod Update* 2001; **7**:535-543.

Meryman HT. Modified model for the mechanism of freezing injury in erythrocytes. *Nature* 1968; **218**:333-336.

Mollgard K, Jespersen A, Lutterodt MC, Yding Andersen C, Hoyer PE and Byskov AG. Human primordial germ cells migrate along nerve fibers and Schwann cells from the dorsal hind gut mesentery to the gonadal ridge. *Mol Hum Reprod* 2010; **16**:621-631.

Monzo C, Haouzi D, Roman K, Assou S, Dechaud H and Hamamah S. Slow freezing and vitrification differentially modify the gene expression profile of human metaphase II oocytes. *Hum Reprod* 2012; **27**:2160-2168.

Morgan S, Anderson RA, Gourley C, Wallace WH and Spears N. How do chemotherapeutic agents damage the ovary? *Hum Reprod Update* 2012; **18**:525-535.

Morris SN and Ryley D. Fertility preservation: nonsurgical and surgical options. *Semin Reprod Med* 2011; **29**:147-154.

Mottershead DG, Pulkki MM, Muggalla P, Pasternack A, Tolonen M, Myllymaa S, Korchynskyi O, Nishi Y, Yanase T, Lun S et al. Characterization of recombinant human growth differentiation factor-9 signaling in ovarian granulosa cells. *Mol Cell Endocrinol* 2008; **283**:58-67.

Mullen SF and Critser JK. The science of cryobiology. *Cancer Treat Res* 2007; **138**:83-109.

Najafi S, Djavid GE, Mehrdad N, Rajaii E, Alavi N, Olfatbakhsh A, Najafi M, Bahrami A and Heidari K. Taxane-based regimens as a risk factor for chemotherapy-induced amenorrhea. *Menopause* 2011; **18**:208-212.

Newton H, Aubard Y, Rutherford A, Sharma V and Gosden R. Low temperature storage and grafting of human ovarian tissue. *Hum Reprod* 1996; **11**:1487-1491.

Newton H, Fisher J, Arnold JR, Pegg DE, Faddy MJ and Gosden RG. Permeation of human ovarian tissue with cryoprotective agents in preparation for cryopreservation. *Hum Reprod* 1998; **13**:376-380.

Nicosia SV, Matus-Ridley M and Meadows AT. Gonadal effects of cancer therapy in girls. *Cancer* 1985; **55**:2364-2372.

Noyes N, Porcu E and Borini A. Over 900 oocyte cryopreservation babies born with no apparent increase in congenital anomalies. *Reprod Biomed Online* 2009; **18**:769-776.

Nyachieo A, Spiessens C, Chai DC, Kiulia NM, Willemen D, Mwenda JM, Bourgain C and D'Hooghe TM. Ovarian Tissue Cryopreservation by Vitrification in Olive Baboons (Papio Anubis) : A Pilot Study. *Gynecol Obstet Invest* 2013.

O'Brien MJ, Pendola JK and Eppig JJ. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence Biol Reprod. 2003, pp. 1682-1686.

Oktay K, Briggs D and Gosden RG. Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. *J Clin Endocrinol Metab* 1997; **82**:3748-3751.

Oktay K, Buyuk E, Davis O, Yermakova I, Veeck L and Rosenwaks Z. Fertility preservation in breast cancer patients: IVF and embryo cryopreservation after ovarian stimulation with tamoxifen. *Hum Reprod* 2003; **18**:90-95.

Oktay K, Buyuk E, Veeck L, Zaninovic N, Xu K, Takeuchi T, Opsahl M and Rosenwaks Z. Embryo development after heterotopic transplantation of cryopreserved ovarian tissue. *Lancet* 2004; **363**:837-840.

Oktay K, Economos K, Kan M, Rucinski J, Veeck L and Rosenwaks Z. Endocrine function and oocyte retrieval after autologous transplantation of ovarian cortical strips to the forearm. *JAMA* 2001; **286**:1490-1493.

Oktay K, Newton H, Aubard Y, Salha O and Gosden RG. Cryopreservation of immature human oocytes and ovarian tissue: an emerging technology? *Fertil Steril* 1998; **69**:1-7.

Oktay K, Nugent D, Newton H, Salha O, Chatterjee P and Gosden RG. Isolation and characterization of primordial follicles from fresh and cryopreserved human ovarian tissue. *Fertil Steril* 1997; **67**:481-486.

Oktem O and Oktay K. A novel ovarian xenografting model to characterize the impact of chemotherapy agents on human primordial follicle reserve. *Cancer Res* 2007; **67**:10159-10162.

Oktem O and Oktay K. Quantitative assessment of the impact of chemotherapy on ovarian follicle reserve and stromal function. *Cancer* 2007; **110**:2222-2229.

Pegg DE. The role of vitrification techniques of cryopreservation in reproductive medicine. *Hum Fertil (Camb)* 2005; **8**:231-239.

Pegg DE. Principles of cryopreservation. *Methods Mol Biol* 2007; **368**:39-57.

Pegg DE. The relevance of ice crystal formation for the cryopreservation of tissues and organs. *Cryobiology* 2010; **60**:S36-44.

Pegg DE, Hunt CJ and Fong LP. Osmotic properties of the rabbit corneal endothelium and their relevance to cryopreservation. *Cell Biophys* 1987; **10**:169-189.

Pena JE, Chang PL, Chan LK, Zeitoun K, Thornton MH, 2nd and Sauer MV. Supraphysiological estradiol levels do not affect oocyte and embryo quality in oocyte donation cycles. *Hum Reprod* 2002; **17**:83-87.

Peretz NM, Goldberg H, Kuten A, Meller I, Krivoi E, Lorber A, Bentur L, Lightman A, Gorenberg V and Ben Arush-Weyl M. [Long-term sequelae of malignant tumors in childhood: consequences of late side-effects]. *Harefuah* 2001; **140**:95-100, 192, 191.

Picton HM, Kim SS and Gosden RG. Cryopreservation of gonadal tissue and cells. *Br Med Bull* 2000; **56**:603-615.

Porcu E, Fabbri R, Seracchioli R, Ciotti PM, Magrini O and Flamigni C. Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. *Fertil Steril* 1997; **68**:724-726.

Qu J, Godin PA, Nisolle M and Donnez J. Distribution and epidermal growth factor receptor expression of primordial follicles in human ovarian tissue before and after cryopreservation. *Hum Reprod* 2000; **15**:302-310.

Reddy J and Oktay K. Ovarian stimulation and fertility preservation with the use of aromatase inhibitors in women with breast cancer. *Fertil Steril* 2012; **98**:1363-1369.

Reddy P, Zheng W and Liu K. Mechanisms maintaining the dormancy and survival of mammalian primordial follicles. *Trends Endocrinol Metab* 2010; **21**:96-103.

Revel A, Koler M, Simon A, Lewin A, Laufer N and Safran A. Oocyte collection during cryopreservation of the ovarian cortex. *Fertil Steril* 2003; **79**:1237-1239.

Rodrigues AP, Amorim CA, Costa SH, Matos MH, Santos RR, Lucci CM, Bao SN, Ohashi OM and Figueiredo JR. Cryopreservation of caprine ovarian tissue using dimethylsulphoxide and propanediol. *Anim Reprod Sci* 2004; **84**:211-227.

Rosendahl M, Andersen MT, Ralfkiaer E, Kjeldsen L, Andersen MK and Andersen CY. Evidence of residual disease in cryopreserved ovarian cortex from female patients with leukemia. *Fertil Steril* 2010; **94**:2186-2190.

Rosendahl M, Greve T and Andersen CY. The safety of transplanting cryopreserved ovarian tissue in cancer patients: a review of the literature. *J Assist Reprod Genet* 2013; **30**:11-24.

Roux C, Amiot C, Agnani G, Aubard Y, Rohrlich PS and Piver P. Live birth after ovarian tissue autograft in a patient with sickle cell disease treated by allogeneic bone marrow transplantation. *Fertil Steril* 2010; **93**:2413 e2415-2419.

Salehnia M. Autograft of vitrified mouse ovaries using ethylene glycol as cryoprotectant. *Exp Anim* 2002; **51**:509-512.

Salehnia M, Abbasian Moghadam E and Rezazadeh Velojerdi M. Ultrastructure of follicles after vitrification of mouse ovarian tissue. *Fertil Steril* 2002; **78**:644-645.

Sanders JE, Hawley J, Levy W, Gooley T, Buckner CD, Deeg HJ, Doney K, Storb R, Sullivan K, Witherspoon R et al. Pregnancies following high-dose cyclophosphamide with or without high-dose busulfan or total-body irradiation and bone marrow transplantation. *Blood* 1996; **87**:3045-3052.

Santos RR, Tharasanit T, Van Haeften T, Figueiredo JR, Silva JR and Van den Hurk R. Vitrification of goat preantral follicles enclosed in ovarian tissue by using conventional and solid-surface vitrification methods. *Cell Tissue Res* 2007; **327**:167-176.

Schmidt KL, Byskov AG, Nyboe Andersen A, Muller J and Yding Andersen C. Density and distribution of primordial follicles in single pieces of cortex from 21 patients and in individual pieces of cortex from three entire human ovaries. *Hum Reprod* 2003; **18**:1158-1164.

Schmidt KT, Larsen EC, Andersen CY and Andersen AN. Risk of ovarian failure and fertility preserving methods in girls and adolescents with a malignant disease. *BJOG* 2010; **117**:163-174.

Scott JE, Carlsson IB, Bavister BD and Hovatta O. Human ovarian tissue cultures: extracellular matrix composition, coating density and tissue dimensions. *Reprod Biomed Online* 2004; **9**:287-293.

Scott JE, Zhang P and Hovatta O. Benefits of 8-bromo-guanosine 3',5'-cyclic monophosphate (8-br-cGMP) in human ovarian cortical tissue culture. *Reprod Biomed Online* 2004; **8**:319-324.

Shaw JM, Bowles J, Koopman P, Wood EC and Trounson AO. Fresh and cryopreserved ovarian tissue samples from donors with lymphoma transmit the cancer to graft recipients. *Hum Reprod* 1996; **11**:1668-1673.

Shaw JM, Kuleshova LL, MacFarlane DR and Trounson AO. Vitrification properties of solutions of ethylene glycol in saline containing PVP, Ficoll, or dextran. *Cryobiology* 1997; **35**:219-229.

Shaw JM, Oranratnachai A and Trounson AO. Fundamental cryobiology of mammalian oocytes and ovarian tissue. *Theriogenology* 2000; **53**:59-72.

Sheikhi M, Hultenby K, Niklasson B, Lundqvist M and Hovatta O. Clinical grade vitrification of human ovarian tissue: an ultrastructural analysis of follicles and stroma in vitrified tissue Hum Reprod. 2011, pp. 594-603.

Silber SJ, Woodruff TK and Shea LD. To transplant or not to transplant - that is the question. *Cancer Treat Res* 2010; **156**:41-54.

Singh RP and Carr DH. The anatomy and histology of XO human embryos and fetuses. *Anat Rec* 1966; **155**:369-383.

Smitz J, Dolmans MM, Donnez J, Fortune JE, Hovatta O, Jewgenow K, Picton HM, Plancha C, Shea LD, Stouffer RL et al. Current achievements and future research directions in ovarian tissue culture, in vitro follicle development and transplantation: implications for fertility preservation. *Hum Reprod Update* 2010; **16**:395-414.

Stevens VC. Some reproductive studies in the baboon. *Hum Reprod Update* 1997; **3**:533-540.

Sullivan AK, Marcus M, Epstein MP, Allen EG, Anido AE, Paquin JJ, Yadav-Shah M and Sherman SL. Association of FMR1 repeat size with ovarian dysfunction. *Hum Reprod* 2005; **20**:402-412.

Suzuki N, Hashimoto S, Igarashi S, Takae S, Yamanaka M, Yamochi T, Takenoshita M, Hosoi Y, Morimoto Y and Ishizuka B. Assessment of long-term function of heterotopic transplants of vitrified ovarian tissue in cynomolgus monkeys. *Hum Reprod* 2012; **27**:2420-2429.

Telfer EE, McLaughlin M, Ding C and Thong KJ. A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. *Hum Reprod* 2008; **23**:1151-1158.

Thompson TB, Lerch TF, Cook RW, Woodruff TK and Jardetzky TS. The structure of the follistatin:activin complex reveals antagonism of both type I and type II receptor binding. *Dev Cell* 2005; **9**:535-543.

Ting AY, Yeoman RR, Campos JR, Lawson MS, Mullen SF, Fahy GM and Zelinski MB. Morphological and functional preservation of pre-antral follicles after vitrification of macaque ovarian tissue in a closed system. *Hum Reprod* 2013.

Tokieda Y, Ishiwata I, Segino M, Ishikawa H and Sato K. Establishment of a novel method for cryopreservation and thawing of the mouse ovary. *Hum Cell* 2002; **15**:230-237.

Trounson A, Wood C and Kausche A. In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. *Fertil Steril* 1994; **62**:353-362.

Tucker MJ, Wright G, Morton PC and Massey JB. Birth after cryopreservation of immature oocytes with subsequent in vitro maturation. *Fertil Steril* 1998; **70**:578-579.

Unger C, Skottman H, Blomberg P, Dilber MS and Hovatta O. Good manufacturing practice and clinical-grade human embryonic stem cell lines. *Hum Mol Genet* 2008; **17**:R48-53.

Van den Broecke R, Liu J, Handyside A, Van der Elst JC, Krausz T, Dhont M, Winston RM and Hovatta O. Follicular growth in fresh and cryopreserved human ovarian cortical grafts transplanted to immunodeficient mice. *Eur J Obstet Gynecol Reprod Biol* 2001; **97**:193-201.

Vicente JS and Garcia-Ximenez F. Osmotic and cryoprotective effects of a mixture of DMSO and ethylene glycol on rabbit morulae. *Theriogenology* 1994; **42**:1205-1215.

von Wolff M, Donnez J, Hovatta O, Keros V, Maltaris T, Montag M, Salle B, Sonmezer M and Andersen CY. Cryopreservation and autotransplantation of human ovarian tissue prior to cytotoxic therapy--a technique in its infancy but already successful in fertility preservation. *Eur J Cancer* 2009; **45**:1547-1553.

Wallace WH, Shalet SM, Hendry JH, Morris-Jones PH and Gattamaneni HR. Ovarian failure following abdominal irradiation in childhood: the radiosensitivity of the human oocyte. *Br J Radiol* 1989; **62**:995-998.

Wallace WH, Thomson AB and Kelsey TW. The radiosensitivity of the human oocyte. *Hum Reprod* 2003; **18**:117-121.

Wang H, Racowsky C and Combelles CM. Is it best to cryopreserve human cumulus-free immature oocytes before or after in vitro maturation? *Cryobiology* 2012; **65**:79-87.

Wang X, Catt S, Pangestu M and Temple-Smith P. Live offspring from vitrified blastocysts derived from fresh and cryopreserved ovarian tissue grafts of adult mice. *Reproduction* 2009; **138**:527-535.

Wang X, Catt S, Pangestu M and Temple-Smith P. Successful in vitro culture of pre-antral follicles derived from vitrified murine ovarian tissue: oocyte maturation, fertilization, and live births. *Reproduction* 2011; **141**:183-191.

Wang Y, Xiao Z, Li L, Fan W and Li SW. Novel needle immersed vitrification: a practical and convenient method with potential advantages in mouse and human ovarian tissue cryopreservation. *Hum Reprod* 2008; **23**:2256-2265.

Weenen C, Laven JS, Von Bergh AR, Cranfield M, Groome NP, Visser JA, Kramer P, Fauser BC and Themmen AP. Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. *Mol Hum Reprod* 2004; **10**:77-83.

Wikland M, Hardarson T, Hillensjo T, Westin C, Westlander G, Wood M and Wennerholm UB. Obstetric outcomes after transfer of vitrified blastocysts. *Hum Reprod* 2010; **25**:1699-1707.

Woodruff TK and Shea LD. The role of the extracellular matrix in ovarian follicle development. *Reprod Sci* 2007; **14**:6-10.

Xiao Z, Wang Y, Li L, Luo S and Li SW. Needle immersed vitrification can lower the concentration of cryoprotectant in human ovarian tissue cryopreservation. *Fertil Steril* 2010; **94**:2323-2328.

Yeoman RR, Wolf DP and Lee DM. Coculture of monkey ovarian tissue increases survival after vitrification and slow-rate freezing. *Fertil Steril* 2005; **83 Suppl 1**:1248-1254.

Ying SY. Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocr Rev* 1988; **9**:267-293.

Zeilmaker GH, Alberda AT, van Gent I, Rijkmans CM and Drogendijk AC. Two pregnancies following transfer of intact frozen-thawed embryos. *Fertil Steril* 1984; **42**:293-296.