CLINICAL GRADE VITRIFICATION OF HUMAN OVARIAN TISSUE FOR FERTILITY PRESERVATION

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“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, autotransplantation 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


IV. Mona Sheikhi, M.Sc., Kjell Hultenby, Ph.D., Boel Niklasson RN.M., Monalill Lundqvist, Ph.D. and Outi Hovatta, M.D., Ph.D. Preservation of human ovarian follicles within tissue frozen by vitrification in a xenofree closed system using only ethylene glycol as a permeating cryoprotectant.
(In press in Fertility and Sterility)
RELATED PUBLICATION NOT INCLUDED IN THIS THESIS

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

Anti- Müllarian hormone (AMH), inhibins, activins, bone morphogenic proteins (BMPs) and growth differentiation factors (GDFs) belong to the Transforming growth factor- beta (TGF-β) superfamily. These growth factors are known to be important in early follicular growth (Massague and Chen 2000) in the ovary. TGF-β 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-β 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 β–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 β–subunits (βA and βB). 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 autotransplantation (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 cyclophosphamid 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; Marhmom 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, autotransplantation 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; Hreinsdson 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.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vitrification</th>
<th>Slow freezing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct contact with liquid nitrogen</td>
<td>Yes (Before our studies)</td>
<td>No</td>
</tr>
<tr>
<td>Ice crystal formation</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Cooling rate (°C/min)</td>
<td>15.000- 30.000</td>
<td>0.15- 0.30</td>
</tr>
<tr>
<td>Exposure to cryoprotectant</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Concentration of cryoprotectant</td>
<td>High (&gt;40%)</td>
<td>Low (10-15%)</td>
</tr>
<tr>
<td>Time consuming (for one run)</td>
<td>Minutes</td>
<td>Hours</td>
</tr>
<tr>
<td>Cost</td>
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<td>Expensive</td>
</tr>
<tr>
<td>Special equipment required</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Freezing methods used</th>
<th>Article I</th>
<th>Article II</th>
<th>Article III</th>
<th>Article IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow freezing using EG</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Slow freezing using PrOH</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Vitrification using DMSO, PrOH, EG</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Vitrification using only EG</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

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- propanedioil (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 second 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

<table>
<thead>
<tr>
<th>Article I</th>
<th>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).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Article II</td>
<td>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 (1 ng/ml) all obtain from Sigma-Aldrich, Sweden (Telfer et al. 2008).</td>
</tr>
<tr>
<td>Article III</td>
<td>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).</td>
</tr>
<tr>
<td>Article IV</td>
<td>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).</td>
</tr>
</tbody>
</table>

**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 $97.14 \pm 7.5 \%$ in fresh, $90.87 \pm 10.74\%$ 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

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

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.
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 larger 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- 1.5mm³) 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).
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.
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