Experimental Studies on Ovarian Cryopreservation and Transplantation

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Have a heart that never hardens, and a temper that never tires, and a touch that never hurts. Charles Dickens

This book is dedicated to my children Nikola and Filip

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ABSTRACT

Around 8% of all cancer victims are below 40 years of age and the survival after cancer treatment during childhood and reproductive years has increased considerably to be around 80% today. The clinical field of fertility preservation has emerged to enable cancer patients that are treated with potentially gonadotoxic chemotherapy-radiotherapy during childhood or reproductive ages, to preserve their fertility. In prepubertal girls and women of reproductive age, where immediate IVF is not an option, ovarian cryopreservation and later re-transplantation is today the only fertility option. Today 13 live births have been reported worldwide after ovarian cortex cryopreservation and avascular re-transplantation some years after the woman has been declared disease-free. However, the effectiveness of the method of ovarian cryopreservation is low. This thesis investigates several models to be used in improvement of ovarian cryopreservation protocols, including whole ovary cryopreservation, and in addition studies different transplantation sites for avascular cortex transplantation in a non human primate species.

The ovine ovarian ovary was used to examine a slow freezing method with the cryoprotectant dimethylsulphoxide (DMSO). Sheep ovaries were cryopreserved in liquid nitrogen and after thawing several viability tests were used. It was shown that the presence of DMSO was advantages for steroid and cyclic AMP output during in vitro perfusion and in cultured ovarian cells. Light microscopy showed well preserved tissue in the DMSO group after perfusion and a higher density of small follicles as compared to ovaries cryopreserved without of CPA. This study shows that the sheep ovary is a suitable method for further studies on whole ovary cryopreservation, including comparisons of different cryopreservation protocols.

The human postmenopausal ovary was evaluated as a tool for further cryopreservation research in the human. Naturally cryopreservation of human ovaries is aiming at preserving premenopausal ovarian ovaries or ovarian tissue. However, this study on post menopausal ovary shows that the aged ovary can be used as a valuable tool for the research, with special emphasises on the function of the stroma and the vascularity. The study showed that human post menopausal ovaries could be effectively cryopreserved in DMSO and that the stroma secreted androgens during in vitro perfusion. Electron microscopy showed a well-preserved morphology in these human ovaries.

The rodents are commonly used in reproductive physiology research and there is a large knowledge about the ovarian function and folliculogenesis in these species. The present study developed a technique for cannulation of the vasculature to the rat ovary and cryopreservation

of the rat ovary by either vitrifiction or slow freezing. The cryoprotectant used was DMSO in high and low concentration. The result of the study indicated that a whole rat ovary can successfully be cryopreserved and that the DMSO concentration of 1.5 M is optimal when evaluating a secretion of steroids and viability of primordial follicles after cryopreservation.

Cryopreserved ovarian cortex tissue can either be transplanted back to an orthotopic or a heterotopic site. The live births reported in the human have all been from the orthotopic site but there are no comparative studies of different transplantation site in primate species. This study used the baboon as a model to compare different heterotopic intraabdominal transplantation sites. It was found that transplantation of the omentum was of advantage compared to transplantation to the pelvic wall or the pouch of Douglas. After a lag phase of 2-3 months the freshly transplanted ovarian tissue showed signs of growth of a large follicle and cyclicity of the animals.

In summary, the study presents several useable models for viability tests after whole ovary cryopreservation. These models can be explored in further research in the area. In a primate species, the omentum has been found a suitable heterotopic ovarian site. This finding can be used also in a human situation where orthotopic ovarian cortex transplantation is impossible because of anatomical or pathophysiological reasons.

LIST OF PUBLICATIONS

I. **Whole sheep ovary cryopreservation: evaluation of a slow freezing protocol with dimethylsulphoxide**

Milenkovic M., Wallin A., Ghahremani M., Brännström M.

J Assist Reprod Genet. 2011;28:7-14.

II. The human postmenopausal ovary as a tool for evaluation of cryopreservation protocols towards whole ovary cryopreservation

Milenkovic M., Ghahremani M., Bergh A., Wallin A., Mölne J., Fazlagic E., Eliassen E., Kahn J., Brännström M.

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III. Viability and function of the cryopreserved whole rat ovary: comparison between different cryoprotectant concentrations and protocols

Milenkovic M., Díaz-García C., Wallin A., Brännström M.

In manuscript

IV. Ovarian cortex transplantation in the baboon: comparison of four different intra-abdominal transplantation sites

Díaz-García C., Milenkovic M., Groth K., Dahm-Kähler P., Olausson M., Brännström M.

Submitted, Hum Reprod

CONTENTS

1 ABBREVIATION

AFC	antral follicle count
AMH	anti-Müllerian hormone
BMT	bone marrow transplantation
BN	Brown-Norway
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CPA	cryoprotectant
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
E2	estradiol
EG	ethylene-glycol
FCS	fetal calf serum
FSH	follicle-stimulating hormone
GLY	glycerol
GnRH	gonadotropin releasing hormone
GV	germinal vesicle
hCG	human chorionic gonadotropin
HAS	human serum albumin
ICSI	intracytoplasmic sperm injection
IHC	immunohistochemistry
IVF	in vitro fertilisation
IVM	in vitro maturation
LH	luteinizing hormone
LM	light microscopy
LN	liquid nitrogen
LR	Lewis
RA	ringer acetate
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
PBS	phosphate buffered saline
PCR	polymerase-chain reaction
PEG	polyethylene-glycol
PI3K	phosphoinositide 3 kinase
PMSG	pregnant mare's serum gonadotropin
PROH	propanediol/propylene-glycol
pSmad2	phosphorylated form of Smad2
PTEN	phosphatase and tensin homolog
PVP	polyvinylpyrrolidone
S-1-P	sphingosine-1-phosphate
SMCA	smooth muscle cell actin
TEM	transmission electron microscopy
TGF	transforming growth factor
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
UV	ultraviolet light
UW	University of Wisconsin
VEGF	vascular endothelial growth factor

2 INTRODUCTION AND LITERATURE REVIEW

2.1 CRYOBIOLOGY

2.1.1 Historical background

Cryopreservation is derived from the Greek word “kryos”, which means “cold or frost”. The term cryopreservation indicates storage of cells or tissue, usually in liquid nitrogen (LN) at temperatures below -130°C. The main goal of the cryopreservation procedure is to minimize tissue injury from low, subzero temperatures (Shaw and Jones 2003). The storage at a low temperature can continue for decades and the only theoretical limitation of storage time is influence by cosmic radiation, which over several thousand years would degrade the genome of the cryopreserved cells. This factor can be neglected in any practical work of cryopreservation in our society. In other words, by usage of cryopreservation the biological clock can be halted for an unlimited time (Kuwayama 2007). The lowest natural temperature on earth is -80°C. Under normal pressure, the inert gas nitrogen which is commonly used in cryopreservation becomes a liquid at -196°C.

A small number of species like various fish, frogs and insects can survive at low temperature using two different biological principles or a combination of these. The first one is the process of cell dehydration, which reduces the freezing point secondary to a concentration of solutes. The other mechanism is by endogenous production of special antifreeze molecules, which prevent formation of large ice crystals. However, these inherent biological principles should not be referred to as cryopreservation, which should be considered an invention of man.

The term cryobiology refers to the knowledge and understanding of the effects of low temperature on cellular systems and utilization of this knowledge to develop improved cryopreservation protocols. The science of cryobiology can be considered to have its starting point about 70 years ago. At that time, Luyet tried to achieve cryopreservation by cooling epidermal plant cells quickly and published a monograph about his pioneer work in 1940 (Luyet, 1940). Three years later a scientist from England succeeded in cryopreservation of human and fowl spermatozoa, using glycerol (GLY) as the agent that would protect against freezing injury (Polge et al. 1949). The term cryoprotective agent/cryoprotectant (CPA) was then launched as the name of any type of substance that would protect biological tissue from low temperature damage. One commonly used CPA, dimethylsulphoxide (DMSO) was introduced in 1959 (Lovelock and Bishop 1959). The authors of that article, using bovine and human red blood cells, showed better cell penetration of DMSO as compared to GLY. In later

experiments on whole mammalian organs, hamster hearts were perfused with 15% GLY as CPA and exposed to a temperature of -20°C. After thawing, the hearts resumed rhythmic beats but complete recovery was not obtained (Smith 1965). Subsequently in 1968, erythrocytes were cooled (-196°C) in GLY by liquid nitrogen (LN) and showed no signs of ice crystal formation (Rapatz and Luyet 1968).

The studies of cells and organ freezing have increased considerably since then and in particular in the clinical area of reproductive medicine (Fahy et al. 2004). The cryopreservation technique may also expand into transplantation surgery. The possibility of organ storage and future transplantation was early also recognized by the pioneering liver transplantation surgeon Thomas Starzl (Starzl 1970) and it may well be that the technique of cryoperservation may be routinely used in the future to store organs that are retrieved and where a suitable recipient can not be found at that moment.

2.1.2 Basic cryobiology

The main constituent of any cell is water, which makes up 60-85% of the cell volume (Mazur, 2004). In addition to free water, biological systems also contain “bound “water (Figure 1). The “bound” water molecules, which are hydrogen-bonded to proteins, nucleic acids and head groups of membrane phospholipids are essential to maintain cells structure and function (Shaw and Jones 2003). These water molecules are incapable of freezing (Sun 1999). Thus, water plays an important role in cryobiology since at cooling to low subzero temperature, 90% of water in a cell will convert into ice (Mazur, 2004) and the 10% of bound water will not freeze.

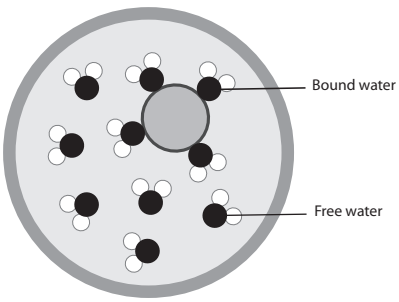

Figure 1 The figure illustrates how water molecules can be either free or bound to larger molecules (proteins, DNA, RNA) of the cell

Cell is in osmotic equilibrium, which means that concentration of any solution inside and outside the cell are the same, since the cell membrane is semipermeable. In the other words, water moves in or out of the cell depending on changes in solution concentration outside the cell and permeability of the cell membrane (Figure 2), which vary for each specific cell type. In addition to passive diffusion through the membrane lipid bilayer (Solomon 1968), water moves through water transport pores known as aquaporins (Verkman et al. 1996). These active transport proteins can transport water up to 100 fold more efficient than passive diffusion. Also, permeability is temperature dependant (Elmoazzen et al. 2002), so that permebility is higher with increasing temperature. Osmotic stress is defined as shrinkage and swelling of a cell due to osmotic differences between the inside and outside of the cell. When these changes are large it may lead to major damage of the cell and in the worst scenario to cell death.

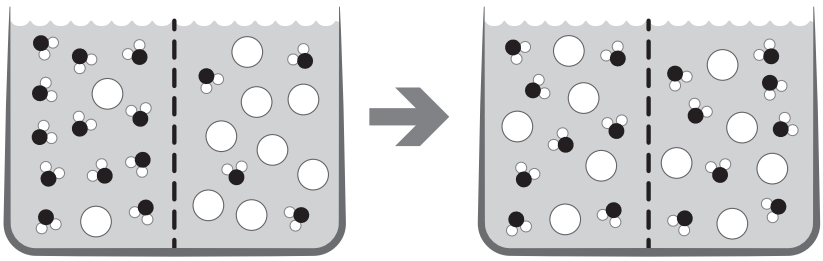

Figure 2 The figure illustrates the principles of osmotic equilibration. Water molecules move from a compartment of lower concentration to a compartment of higher concentration, to equalize the concentrations

The highest temperature when ice can form at normal pressure is 0°C. However, ice in general forms at temperatures between -5 and -15°C; through spontaneous or induced (by seeding) ice nucleation (nucleus for formation of ice crystal). For example, ice nucleation is seen in oocytes at temperature of -5°C (Toner et al. 1991). During the formation of ice crystals releases heat (Shaw and Jones 2003) and consequently the thawing process requires energy input. The solution which remains free of ice in a temperature below freezing point is in a supercooled state (Shaw and Jones 2003). The ice formation can be suppressed by added CPA (Rall et al. 1983; Toner et al. 1991).

It is well established that cell survival rate during cryopreservation depends mostly on the cooling rate (Mazur 1970). In extensive studies of survival of yeast cells and human red blood cells, as a function of cooling rate, it was showed that a curve of survival versus cooling rate

exhibited the shape of an inverted U (Figure 3). This curve can be interpreted as the resultant of two different mechanisms of which one damages living cells at high cooling rates and the other damages at low cooling rates. The highest survival rate is present at an intermediate rate (Mazur 1970).

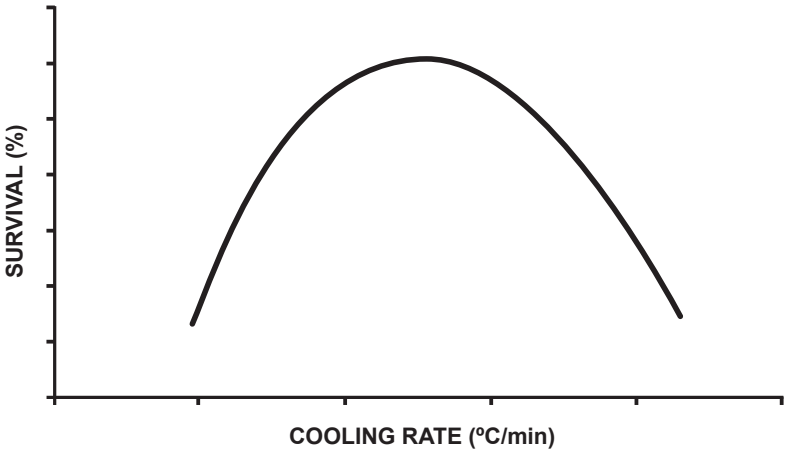

Figure 3 The inverse U-theory. The survival of frozen cells is a function of cooling rate (modified from Mazur, 1970)

2.1.3 Cryoprotectants

Many chemicals have been recognized as having a cryoprotective function and these are collectively referred to as CPAs. Their function is to modify the formation and growth of ice. The CPAs can be divided into two categories, permeable and non-permeable. Permeable CPAs can diffuse through cell membrane and non-permeable CPAs do not enter the cytoplasm.

Permeable CPAs are small, non-ionic molecules with low toxicity and high solubility in water. The rate of CPA permeation and dilution is determined by the species, cell type and stage of development, solution composition, temperature and hydrostatic pressure (Liebermann et al. 2002; Shaw and Jones 2003)**.** The most commonly used permeable CPAs are DMSO, propylene glycol (PROH) and ethylene glycol (EG). The exact mechanism by which these permeable CPAs protect living cells from cryoinjury is not completely understood. However, their general mechanisms seem to be by lowering the freezing point by replacement of some of the bound water molecules in and around proteins, deoxyribonucleic

acid and head groups of phospholipids (Shaw and Jones 2003). Moreover, the permeable CPAs stabilize cellular proteins in the cytoplasm as well as in the cell membrane (Karlsson and Toner 1996). At entrance into the cell, they also reduce the concentration of electrolytes by lowering the amount of ice formed at a given temperature (Pegg 1984).

Non-permeable CPAs are usually long-chain polymers that are soluble in water and increase the osmolality of the solution. The most frequently used non-permeable CPAs are disaccharides (sucrose, glucose, fructose, sorbitol, saccharose, trehalose), some macromolecules (polyvinilpyrrolidone (PVP), polyvinyl alcohol, Ficoll) and proteins (bovine serum albumin; BSA). They contribute to cell dehydration, counteract osmotic stress and reduce the toxicity of permeable CPAs (Muldrew, 2004).

2.1.4 Cryopreservation

There are two principally different cryopreservation procedures, slow freezing and vitrification. Both procedures include four common steps: exposure of the samples to CPA, freezing/cooling to the storage temperature (-196°C), thawing/warming and CPA removal. The terms “freezing and thawing” relate to the slow cooling procedure whereas “cooling and warming” are more correct in relation to the vitrification procedure (Shaw and Jones 2003).

2.1.4.1 Slow freezing

Slow freezing is a method which aims at achieving the optimal balance (equilibrium) between the rates of cell dehydration and extracellular ice formation (Mazur 1990). In this procedure, the CPA is added to the solution around the cells/tissue, and the cooling rate is dependent on the size and permeability of the specific cells/tissue to be cryopreserved. The CPA for whole organ freezing should be delivered by perfusion of the organ through arterial vessels to ensure distribution of the CPA throughout the whole organ. The slow freezing method reduces the likelihood of intracellular ice formation by initiation of extracellular ice crystal formation (seeding) at a high subzero temperature (e.g.-6 to -9°C). The freezing rate is then responsible for how fast the extracellular ice crystals will grow. The extracellular ice draws water out of the cell until little amount of free water remains and only small (non-lethal) ice crystal has been formed (Shaw et al. 2000). The best outcome of this cryopreservation method is obtained when the rate of freezing allows equilibrium between the rate of water loss from the cell (dehydration) and the rate at which water is integrated into extracellular ice crystals. It should be pointed out that formation of ice crystals is an integral part of the slow freezing procedure.

The rate of dehydration, which is the movement of water outward across the cell membrane, depends on the cooling rate that is determined by temperature drop in relation to time. Thus, the movement of water through the cell membrane decreases as a consequence of lower temperature (Mazur 1984). The freezing rate differs between various cell types, by factors such as cell size, cell membrane permeability and diffusion characteristics. The optimal cooling rate vary between 0.3 and 1°C/min for oocytes and embryos (Whittingham et al. 1972) up to >1000°C/min for red blood cells (Mazur 1970). The slow cooling procedure is usually ended when the temperature lies between -30°C and -80°C and after the sample can be plunged into LN (Shaw and Jones 2003). Recently, a new directional freezing technology was introduced (Revel et al. 2004; Arav and Natan 2009) which provides identical cooling rates through the specimen being frozen and it is aimed to cryopreserve whole organs. The method of slow freezing, without the use of programmable temperature decrease, is referred to as uncontrolled-rate freezing. At this procedure, the cell/tissue sample which is exposed to CPA, is simply placed inside a -80°C freezer (Stiff et al. 1987; Almici et al. 2003; Martinez-Madrid et al. 2004) and it is estimated that the cooling rate is about 1°C/min, but the exact cooling rate of the procedure is naturally dependent on several factors such as tissue volume and cellular composition. After 24 h at -80°C, the sample is transferred into LN.

2.1.4.2 Vitrification

Vitri is the Greek word for glass. Vitrification is a procedure when a solution/sample solidifies in a glass-like or vitreous state without any formation of ice crystals during cooling and remains in this state throughout the warming (Shaw and Jones 2003). The terms “ultrarapid freezing” and “non-equilibration freezing” are also used as synonyms to vitrification. The primary idea of ultra rapid cooling is to pass rapidly through the critical temperature zone (for example for the oocyte 15°C to -5°C) where the cells are most sensitive for chilling injury (Liebermann et al. 2002).

The aim of vitrification is to reduce or eliminate both intra- and extra-cellular ice formation by the presence of highly concentrated CPA that interact strongly with water, preventing water molecules from interaction to form ice. Other major differences from slow freezing protocols are that dehydration and CPA penetration occur before cooling and that cooling is usually performed in a single quick step. Typically, the temperature is reduced directly from > 0°C to < 130°C by plunging the sample into LN. Cooling rate at vitrification varies and can be up to > 20000°C /min when samples are positioned directly in LN (Shaw and Jones 2003).

Also, the natural state of liquid water inside living cells is retained during vitrification (Wowk 2010), leading to minimal disturbance within the cryopreserved sample.

A sample that will be vitrified is exposed to CPA at the same manner as during slow freezing, but the vitrification procedure requires higher concentration of CPAs to achieve high cooling rate. Several modifications to find methods to obtain higher cooling rates and to reduce concentration of CPA have been developed such as the cryotop method (Cobo et al. 2008), the open pulled straw (Vajta et al. 1998) and the novel needle immersed vitrification (Wang et al. 2008).

Warming after vitrification is usually also fast. Thus, the warming rate for a small sample like an ovine germinal vesicle (GV)-oocyte is calculated to be from 2000 to 4460°C /min by directly plunging the sample into warming solution (Isachenko et al. 2001).

Vitrification has in addition been recommended as the method of choice for whole organ cryopreservation (Fahy et al. 2006), although this will be associated with great difficulties due to the heat transfer throughout the entire organ. Taken together, vitrification is inexpensive, easy to perform and applicable to many biological systems.

2.1.4.3 Thawing

The thawing and CPA removal are also important steps for a successful procedure. Cells cooled by slow freezing always contain some water and proper thawing is essential to avoid ice damage (Shaw et al. 2000). There is an opinion that the ice formed during thawing is less dangerous (Shaw et al. 1991), although some authors brought up the subject that slow thawing may be detrimental to the cells due to ice formation (Arav and Natan 2009). According to the inverted U curve (Figure 3), the cells which have been cooled at supraoptimal cooling rates have higher survivals rates when they are thawed rapidly (Mazur 1970).

2.1.5 Cryoinjury

All tissues/cells that are subjected to freezing may be damaged during the freezing procedure or during thawing. This is referred to as cryoinjury and includes several types and causes of injuries. It may be a result of excessive cell dehydration (Mazur et al. 1972), intracellular ice formation (Acker et al. 2001), CPA toxicity (Fahy 2010) or a combination of these.

It is well known that formation of ice inside the cell is lethal (Muldrew and McGann 1990). This event occurs when a cell is unable to maintain equilibrium with the extracellular space during slow freezing or when the critical cooling rate is not obtained by vitrification methods.

A recently introduced factor is the possible protective effect of so-called “innocuous” intracellular ice formation (Acker and McGann 2003), which may reduce osmotic stress.

The survival of a cell at freezing can also be affected by the specific forms of ice crystals that are present and mechanical deformation of cells caused by large extracellular ice crystals have been reported (Beckmann et al. 1990; Hubel et al. 1992). Moreover, a phenomenon known as devitrification results in re-crystalisation (Rall et al. 1984), that is a formation of ice crystals during warming. This is usually related to vitrification procedures (Shaw and Jones 2003).

Despite the intended protective effect of CPAs, they may also be toxic and their presence may contribute to osmotic stress (Pedro et al. 1997). However, decreasing the time and temperature of cell exposure to CPAs can reduce the toxicity while stepwise addition and removal of CPAs decreases osmotic stress and excessive volume change of the cells (Wowk 2010). It should be pointed out that the exact mechanism of cell damage during cryopreservation and CPA toxicity has not yet been elucidated (Karlsson and Toner 1996; Fahy 2010).

2.2 OVARY

The main ovarian functions are differentiation and release of mature oocyte competent for fertilisation and production of steroid hormones necessary for secondary sexual characteristics and subsequent achievement of pregnancy. Human ovaries are oval-shaped female gonads that are approximately 3 cm in length, 1.5 cm width and 1 cm in thickness. They are located in the lateral wall of the female pelvis beneath the external iliac artery and in front of the ureter and the internal iliac artery (Figure 4). The lateral pole of the ovary is attached to the pelvic wall by the infundibulopelvic ligament which contains the ovarian artery and veins. Furthermore the ovary is connected to the uterus by the ovarium proprium ligament (Figure 5).

The ovarian arteries arise from the anterior surface of the aorta, just beneath the level of the renal arteries and supply both the ovaries and the Fallopian tubes (Figures 4, 6). The bilateral ovarian veins drain into the vena cava on the right side and into the renal vein on the left side. Both ovarian arteries and veins have long retroperitoneal courses before reaching the cephalic end of the ovary.

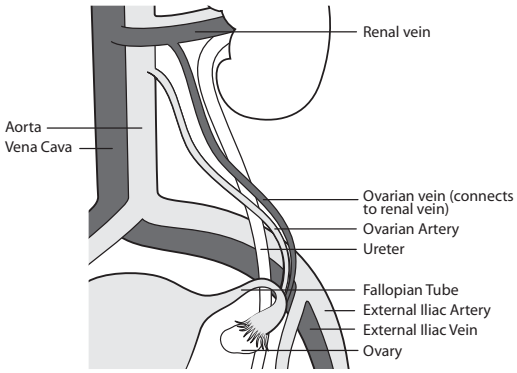

Figure 4 The position of the human ovary in relation to pelvic and abdominal blood vessels

The ovary consists of a medulla and cortex (Figure 5). The medulla is the central part, which contains loose connective tissue, nerves and blood and lymphatic vessels. The ovarian cortex is the outer part of the ovary surrounding the medulla and is composed of the ovarian follicles that embedded in specialized stroma. The small ovarian follicles lie at a depth of approximately 1-2 mm in the ovarian cortex.

The follicles are the functional units of the ovary and are composed each of an oocyte and surrounding somatic granulosa cells (Figure 5) with or without theca cells, depending on the developmental stage of the follicle. A basal membrane, called the lamina propria, separates the granulosa cells from the stromal/thecal tissue and the granulosa cells are devoid of any vascular supply. The intercellular contacts within the granulosa cells are provided by gap junctions, to form a compact functional syncytium of cells and to allow metabolic exchange and transportation of molecules.

From birth the human ovaries contain a pool of about 1 million resting follicles and this number declines during the age (Faddy 2000). The follicles are classified as: primordial follicles (the earliest stage) containing an oocyte surrounded by a single layer of flattened granulosa cells; primary follicles surrounded by at least one cuboidal layer of granulosa cells; secondary follicles with more than one layer of cuboidal granulosa cells and theca externa and interna cells which originate from ovarian stroma; tertiary follicles when a fluid filled antrum inside the granulosa cells (Gougeon 1996).

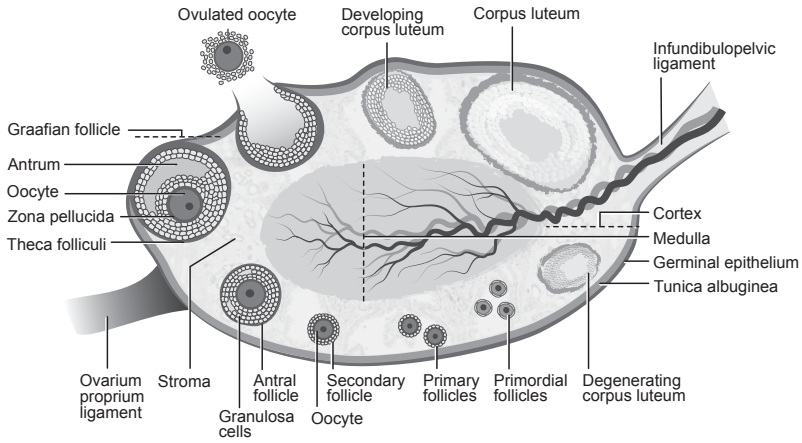

Figure 5 Schematic drawing of the cyclic changes of the ovary. Follicles and corpora lutea of various stages are shown

During follicular development also the oocyte increases in diameter and becomes surrounded by a zona pellucida (Figure 5), which is a thick extracellular coat of glycoproteins. The zona pellucida plays an important role during oogenesis, fertilization and preimplantation development.

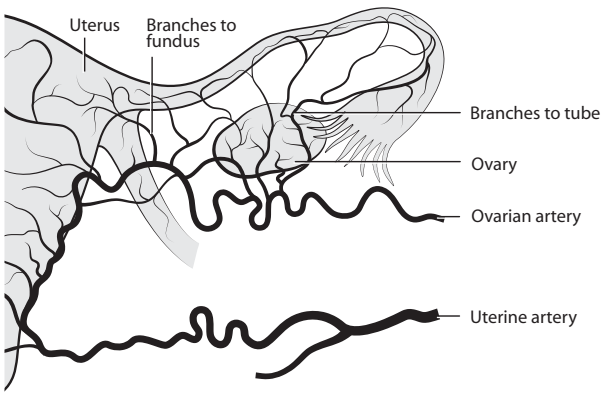

Figure 6 The drawing illustrates the arterial supply to the ovary, oviduct and the uterus

During follicular development, the follicle grows from an initial size of 40 μm to up 15-22 mm (Figure 7) in diameter (Smitz et al. 2010). This whole process is highly complex in its regulation by intra- and extra-ovarian factors. It is suggested that the oocyte is a key factor in the early follicular development, although bidirectional communication between the oocyte

and surrounding follicular cells also seems important (Eppig 2001). The duration of the transition from a primordial into preovulatory follicles in humans is more than 200 days (Gougeon 1996) (Figure 7). The folliculogenesis proceeds at a near constant rate until menopause with less than 0.1% of the primordial follicles growing all the way into mature antral follicle that can ovulate, with the rest degenerating by a process named atresia at any stage of their development. Importantly, about 90% of the follicles in an adult ovary are at the primordial stage (Gook et al. 1999; Schmidt et al. 2003).

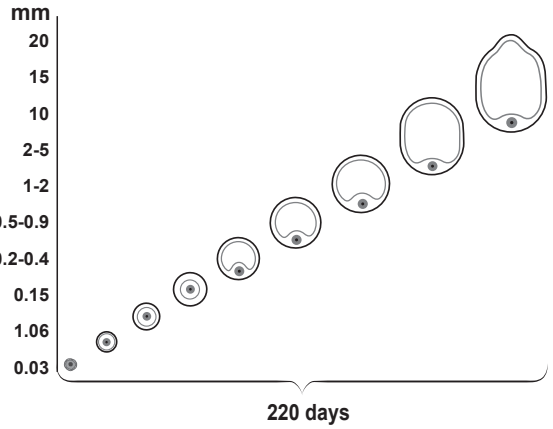

Figure 7 Schematic diagram of follicle growth in the human from the primordial stage to the preovulatory stage

2.3 FERTILITY PRESERVATION

During the last decades there have been dramatic improvements in cancer treatment with increasing survival rates for most types of cancer. The significant increase in survival after cancer treatment is certainly true for the types of cancer that girls and young females may acquire. Leukaemia (particularly acute lymphoblastic leukaemia) is the most common cancer type in children followed by cancer of the central nervous system, neuroblastoma, Hodgkin's, non Hodgkin's lymphoma and Wilms' tumor (Linabery and Ross 2008). The 5-year-relative-survival-rate for children with cancer, including all types of cancer, have improved from around 63% for patients diagnosed during the years 1975-1979 to 79% for those diagnosed 1995-1999 (Linabery and Ross 2008). The most frequent type of malignancy among females during the reproductive age is breast cancer (Jemal et al. 2008) and more than 6% of newly diagnosed breast cancer patients are younger than 40 years old (Gnerlich et al. 2009). Nevertheless, the 5-year-survival-rate for breast cancer patients increased from 75% in the mid-1970s to 88% in the late 1990s (Jemal et al. 2004)**.** In summary, approximately 8% of all cancer victims are younger than 40 years of age (Marhhom and Cohen 2007) and around 0.4 % of all young women are cancer survivors with this proportion increasing in the future (Bleyer 1990).

In view of the fact that the likelihood of survival after cancer treatment among young female cancer victims has increased considerably during the last decades more attention has been drawn to the important quality-of-life aspect of being able to achieve pregnancy and to have your own biological child. Furthermore, the age for attaining the first pregnancy is increasing in many countries, due to social and economic reasons and thereby a larger proportion of women will in the future by choice be nulliparous when cancer is discovered.

In addition to cancer, recurrent ovarian cysts and ovarian torsion as well as endocrine and genetic diseases like Turner syndrome, galactosemia and family history of premature menopause may be indications for fertility preservation (Jadoul et al. 2010). The different methods of fertility preservation will be discussed further below. Ovarian cryopreservation is one such fertility preservation technique. This new and emerging technology, which has all ready resulted in live births (Donnez et al. 2011), still needs improvement and this is the topic of this thesis.

2.3.1 Cancer treatment and ovarian toxicity

Surgery, chemotherapy and/or radiotherapy are the main treatment modalities for malignant tumours. There is always a risk of infertility when surgery is involved in any part of the female genital tract. As a result, surgical management of borderline ovarian tumours/early stage epithelial ovarian cancer, early stage cervical cancer and uterine cancer/sarcoma may lead to infertility although ovarian tissue can be preserved.

Multi-agent chemotherapy is the base for treatment of leukaemia and lymphoma (Linch et al. 2000) and is also used as adjuvant treatment after primary surgery for breast cancer (Bergh et al. 2001). Radiotherapy is the most commonly used primary treatment for advanced cervical (Grigsby and Herzog 2001) and rectal cancer (Gill et al. 2007). Total-body irradiation, in preparation for allogenic hematopoetic stem cell transplantation, is in general use (Tauchmanova et al. 2002). Radiotherapy is also co-treatment together with chemotherapy for lymphoma (Linch et al. 2000), Ewing's sarcoma (Burdach et al. 2000), Wilm's tumor (Wu et al. 2005; Spreafico and Bellani 2006) and after surgery for early stage cervical cancer when lymph node metastasis have been discovered or the surgical margins are inadequate. Furthermore, some precancerous or benign diseases, such as myelodysplasia, aplastic anemia, thalassemia and systemic lupus erythematosus are treated with chemotherapy with or without hematopoietic stem cell transplantation (Oktay and Oktem 2009).

2.3.1.1 Chemotherapy

Numerous chemotherapeutic drugs that are used in cancer treatment are more or less gonadotoxic. The level of the ovarian toxicity is generally divided into high, moderate and low risk where cyclophosphamide, chlorambucil, melphalan, busulfan, nitrogen mustard and procarabazine belong to the high gonadotoxic risk group (Sonmezer and Oktay 2006)**.** The mechanisms by which the chemotherapeutic agents cause ovarian toxicity vary depending on the specific actions of the drug. The toxic effect by cyclophosphamide seems to be mainly on the granulosa cells and the surrounding basement membrane (Raz et al. 2002), with the sensitivity being related to the size of the follicle as shown in a study in the mouse model (Meirow et al. 2001). A decrease in overall ovarian size, as a consequence of a loss of large number of follicles of various stages, including the pool of primordial follicles, is seen in the ovary after chemotherapy (Familiari et al. 1993). Chemotherapy can also provoke injury to the ovarian blood vessels and focal fibrosis of the ovarian cortex (Meirow et al. 2007). A histological study on human ovarian biopsies from women that had received chemotherapy demonstrated that the number of primordial follicle count was decreased in the group of

chemotherapy-treated patients, with the lowest follicle count found in patients that had been treated with alkylating agents, such as cyclophosphamide (Oktem and Oktay 2007). It was also shown that there was a considerable fall in anti-Müllerian hormone (AMH) concentrations as well as partial decrease of inhibin B during the anti-cancer treatment, indicating damage of small and preantral follicles (Anderson et al. 2006). Moreover, combinations of chemotherapeutic drugs, regardless of whether they include alkylating agent, can also have detrimental effects of the ovary and its follicles.

The ovarian injury after treatment with chemotherapeutic drugs is in addition dependent on the patient's age, the cumulative dose of the agent and previous cancer treatment (Meirow et al. 2007). Supporting the role of age-dependency, one study demonstrated that the mean age of women that maintained menstruation after chemotherapy for Hodgkin´s lymphoma was lower than the mean age of those becoming oligomenorrhoeic or amenorrhoeic (Moore 2000). However, it should be accentuated that resumption of menstruation does not directly correlate to restored fertility. Therefore, even if female childhood cancer survivors have regular menstrual periods and normal basal follicle-stimulating hormone (FSH) levels when they are postpubertal, they have lower ovarian volumes and antral follicle counts (AFCs) as compared with age-matched controls (Larsen et al. 2003). Furthermore, it was previously demonstrated a fall in AMH concentration despite maintenance of regular menstrual period in young cancer survivors treated by chemotherapy (Bath et al. 2003). There exists one study indicating that the type of treatment is the most important predictive factor for ovarian function after chemotherapy, rather than age at treatment (van Beek et al. 2007). Moreover it was found in a study of women that had one ovary removed for cryopreservation before cancer treatment, that the presence of regular menstruation 18 months after cryopreservation, was an excellent indicator of good residual ovarian function (Rosendahl et al. 2008) as assessed by the ovarian reserve markers AMH, FSH, inhibin B and AFCs. The dynamics of changes in ovarian reserve markers after chemotherapy are that of an AMH decline after the first treatment cycle of chemotherapy and a decrease of inhibin B and AFCs after three treatment cycles with FSH reaching postmenopausal levels after four chemotherapy cycles (Rosendahl et al. 2010).

A recent large study of post-treatment parenthood in patients that had been treated for Hodgkin's lymphoma (Kiserud et al. 2007) demonstrated that the likelihood of parenthood 15 years after treatment was almost similar in women younger than 20 years of age at diagnosis (85%) as those 20-30 years at diagnosis (89%). Nevertheless, only 25% of the patients older than 30 years of age at diagnosis later achieved parenthood. In the same study the effect on fertility of different treatments protocols were assessed. It was demonstrated that the 10-year

probabilities of achieving parenthood in Hodgkin's lymphoma patients were 55% after treatment with chemotherapy of low gonadotoxicity, 51% after use of chemotherapy considered to be of medium gonadotoxicity and 27% after use of chemotherapy regarded as high gonadotoxic treatment (Kiserud et al. 2007). The endpoint in that study was parenthood, which is the most important measure to study considering that the miscarriage rate may be higher and that there are methodological biases in correctly assessing presence of early pregnancy.

It has been demonstrated in studies in female mice exposed to cyclophosphamide that even if pregnancy is accomplished, the rate of spontaneous abortion and fetal malformations may be substantially increased (Meirow et al. 2001). In contrast, available human large registry studies have revealed that there is no higher risk of genetic abnormality, birth defects, or cancer (aside from hereditary syndromes) in the children of cancer survivors that had been treated with chemotherapy (Lee et al. 2006). One retrospective study conducted on childhood cancer survivors treated by chemotherapy showed no significant differences in neonatal outcome between cancer survivors and the control group (Lie Fong et al. 2010).

2.3.1.2 Radiotherapy

Regarding the ovarian toxicity after radiotherapy the damage to the ovaries is dependent on the dose of radiation to the ovaries (Chiarelli et al. 1999) and fractionation of radiation doses makes it less harmful than a single dose. The oocyte is very sensitive to radiation and it has been estimated that a low dose of 2-4 Gray to the ovaries destroys about half of the oocyte pool (Wallace et al. 2003). Most of the radiation protocols, that are currently used, include radiation doses up to 40 and 50 Gray. Thus, when the radiation fields cover the pelvic area an irreversible and total ovarian damage can be expected.

2.3.2 Categories of subfertility risks

As discussed above the diverse characters of ovarian toxicity after different cancer treatments makes it complicated to predict the outcome for each individual. An evaluation of the overall risks for subfertility after different malignancies/cancer treatments that are frequent in childhood and adolescence has been presented with division into low risk (<20%), medium risk and high risk (>80%) (Wallace et al. 2005). Those that fall into the high subfertility risk group are total body irradiation, localised pelvic radiation, chemotherapy conditioning for bone-marrow transplantation, Hodgkin's disease treated with alkylating agents, and metastatic sarcoma including Ewing's sarcoma (Table 1).

Low risk (<20%)	Medium risk (20-80%)	High risk (>80%)
- Acute lymphoblastic leukemia - Wilms' tumor - Soft-tissue sarcoma (stage 1) - Germ-cell tumors - Retinoblastoma - Brain tumor: cranial irradiation <24Gy	- Acute myeloblastic leukemia - Hepatoblastoma - Osteosarcoma - Ewing' sarcoma (non-metastatic) - Soft-tissue sarcoma (stage 2, 3) - Neuroblastoma - Non-Hodgkin´s lymphoma - Hodgkin´s lymphoma - Brain tumor: craniospinal radiotherapy, cranial irradiation >24Gy	- Total body irradiation - Localized radiotherapy: pelvis - Chemotherapy condit. for BMT - Hodgkin´s lymphoma treated by alkylating agents - Soft tissue sarcoma (stage 4) - Ewing' sarcoma (metastatic)

Table 1 The risk of subfertility after treatment of common cancer in childhood and adolescence. Modified from Wallace et al, 2005. BMT = bone marrow transplantation

2.3.3 Current fertility preservation options

The only clinically established method of fertility preservation in young females that are planned to undergo potentially gonadotoxic cancer treatment is cryopreservation of embryos (Lee et al. 2006) after in vitro fertilisation (IVF). By the use of gonadotropin releasing hormone (GnRH) antagonists mature oocytes for IVF can be collected within about 10-12 days after the decision to use this method, irrespective of the stage of the menstrual cycle (von Wolff et al. 2009). However, IVF cannot be used in prepubertal females and it also requires the presence of a male partner who is present and committed. In addition, stimulation with gonadotropins that cause high estradiol (E2) levels may have negative impact on certain primary cancer diseases (Oktay et al. 2006).

In postpubertal female patients, who need fertility preservation, cryopreservation of mature oocytes may be an option, with no requirements of a male partner at the time of fertility preservation. Nevertheless, cryopreservation of unfertilized oocytes using slow freezing technique is not yet developed to a satisfactory level and the current live birth rate with this method is reported to be only around 2% per thawed oocyte (Oktay, Cil, Bang, 2006). On the other hand, technical developments in the field, including vitrification technique, may in the future substantially improve the success rate (Cobo et al. 2008; Noyes et al. 2010). Methods to cryopreserve immature occytes, followed by in vitro maturation (IVM) after thawing or that IVM is followed by cryopreservation, have practical and theoretical advantages, but these techniques also need improvement (Gosden 2005; Huang et al. 2010). The clinical result of any oocyte cryopreservation procedure depends on numerous factors such as cryopreservation technique, the original quality of the obtained oocyte, condition of the patients, protocol of

hormonal stimulation and each stage of handling of the oocyte (Rodriguez-Macias Wallberg et al. 2009). When pelvic radiation is indicated, ovarian transposition and ovariopexy outside of the pelvis can be performed and may protect the ovarian functions (Cowles et al. 2007). GnRH agonist treatment was proposed as a fertility preservation option (Blumenfeld and von Wolff 2008), but the results are unclear and there is a lack of randomized controlled studies to properly assess this method (Bromer and Patrizio 2008).

2.4 OVARIAN CRYOPRESERVATION AND TRANSPLANTATION IN ANIMALS AND HUMAN

The idea of transplanting ovarian tissue, either fresh or after cryopreservation has been tested in several animal models. The currently performed studies including both ovarian pieces and whole ovary cryopreservation and transplantation in each species are summarized below.

2.4.1 Mouse

2.4.1.1 Avascular fresh ovary transplantation

Experiments on avascular ovarian transplantation were first carried out in the mouse. In an elegant study carried out 50 years ago, both whole and half ovaries were grafted fresh into the orthotopic site of X-ray sterilized animals (Mussett and Parrott 1961). The donors and the recipients were of the same strain. It was calculated that about 65% of oocytes were lost during the post-transplantation period and this was proposed to be due to surgical trauma and ischemia. However, the normal oocyte loss after this post grafting period was compared to that of non-grafted native ovaries of other animals. The conclusion was that the reproductive life span would not be greatly shortened. Fertility was also proved, but with smaller litter size in animals that had received ovarian grafts than in controls. The possible explanation of this subfertility was adhesions and obstructions of the periovarian space which collects the ovulated oocytes sed to be transported into the oviduct for fertilization. Taken together, this study performed at the very beginning of the ovarian transplantation research area, should be regarded as pioneer work and a great step forward the concept of gonadal tissue transplantation for fertility preservation.

In the mouse model, heterotopic ovarian transplantation sites such as beneath the kidney capsule and inside the back muscle have also been explored. Higher survival rate of primordial follicles and better vascularisation after transplantation of fresh hemiovaries into the back muscle was seen as compared to under the kidney (Soleimani et al. 2008). In that study, neoangiogenesis was quantified by the use of immunohistochemical staining with anti

CD31, to visualize this protein that is expressed on the surface of all endothelial cells. In the latter article, live births after IVF and intracytoplasmic sperm injection (ICSI) were reported when mouse oocytes were collected from heterotopic grafts on the back muscle. In another study, mouse ovaries were allografted under the kidney capsule of either male and female recipients (Waterhouse et al. 2004). The male gonads were not removed at the time of grafting and the recipients were killed 3 weeks after grafting and oocytes were collected. Oocytes originating from both female and male recipients were used for IVF and offspring was obtained from both. In addition to survival of primordial follicles after grafting, this study demonstrated that developmentally competent oocytes can be obtained from male recipients, regardless of the male hormonal environment. The authors do not state the possible clinical application of transplantation of female gonadal tissue into males. One study was designed to evaluate possible beneficial effects of antioxidant (vitamin E) to graft survival (Nugent et al. 1998). The mouse ovary was autografted under the kidney capsule and histological examination 7 days after surgery showed about 25% higher follicular survival in animals treated by vitamin E. Also, products of lipid peroxidation, that form after ischemia-reperfusion injury were lower in the vitamin E treated group as compared to controls indicating protective effect of vitamin E to prevent ischemic injury.

2.4.1.2 Avascular frozen ovary transplantation and comparison to fresh ovary transplantation

The initial work on ovarian cryopreservation, during the 1950s, made use of the mouse model. In this species viability of frozen-thawed ovarian tissue was shown for the first time in any species (Parkes 1956). In a later study, fertility after ovarian cryopreservation was demonstrated for the first time with the cryopreservation system being ovarian pieces cryopreserved in GLY and engraftment into X-ray sterilized recipients (Parrott 1960).

The research on ovarian cryopreservation took off during the early 1990s. Mouse ovaries were cryopreserved using 1.4M DMSO as CPA and function of orthotopically grafted fresh and cryopreserved mouse ovaries were compared (Harp et al. 1994). Estrous cyclicity was reestablished in approximately 80% of mice in the non-frozen group and 75% in the cryopreserved group. Post-mortem histology revealed primordial and primary follicles in both the frozen and non-frozen group with slightly better follicular survival in the fresh group, although detailed quantification of primordial and primary follicles was not done. In a slightly different mouse model, avascular orthotopic or heterotopic transplantation of frozen fetal mouse ovaries was performed in oophorectomized adult recipients (Cox et al. 1996) where the ovaries were cryopreserved by slow freezing method using 1.5M DMSO as CPA. Histological examination of the grafts 2 to 8 weeks after transplantation showed follicles of all

developmental stages and cyclic activity was restored both after orthotopic and heterotopic transplantation. Further, fertility was proven in the orthotopic group with a difference depending on the thawing process. The best results were seen after rapid thawing, which was achieved by holding the frozen specimen in -120ºC in the vapour phase of LN for 2 min, followed by placement in 37ºC water bath for 5-10 s.

In another study, the live birth rate was compared after autotransplantation of whole cryopreserved (DMSO and cooling rate of 0.5ºC/min) or fresh adult mouse ovaries and sham-operated animals in a third group (Gunasena et al. 1997). Fresh and frozen-thawed whole ovaries were autotransplanted into the ovarian bursae. Live births were seen from all fresh transplants and from 73% of the cryopreserved grafts. The litter size of the fresh and cryopreserved ovaries was around 1/3 of the sham operated. The authors extended the study to the next generation. No offspring difference was seen in the litter of the second generation neither between the three groups nor between male and female pups. However, the possibility that living pups in this study originated from the residual host ovarian tissue cannot be completely excluded. In a follow up study, the same authors performed allotransplantation of fresh and frozen rat ovarian tissue into the ovarian bursae of nude mice (Gunasena et al. 1997). Live births were reported from 2 out of 3 fresh transplants and 1 out of 4 frozen transplants. Since the recipients were immunodeficient mice, it was not surprising that the allotransplantation was effective.

In these types of experiments, where the native ovary is removed and replaced by a transplanted ovarian piece, one can always argue that there is a possibility that any pregnancy may arise from a remnant of the native ovary. To control this factor one study made use of donors and recipients that were genetically identical apart from different gene for the coat color (Sztein et al. 1998). Donor-strain males were used for mating. The mean number of pups in the frozen group was 3.2 compared to 11.2 in the fresh group which imply reduced fertility in the cryopreserved group. Furthermore, 23 out of 64 animals born in this experiment had the same color coat as the recipient, which demonstrated the origin of these from the recipient's ovary. Thus, 1/3 of the oophorectomized recipients had native follicles left in the ovarian bursae.

Long term ovarian function and reproductive life span after avascular transplantation of both cryopreserved and fresh entire mouse ovaries was evaluated in a study, where the ovary recipient and donor were homozygous for two different isoforms of glucose phosphate isomerase (Candy et al. 2000). The study was designed similarly to the study of Sztein and

coworkers (Sztein et al. 1998), to identify the ovary (native or transplanted) from which pups were derived. It was concluded that more than 80 % of the recipients of fresh and frozen ovarian transplants were fertile and that around 90% of the recipients had litters (normal litter size) obtained from the grafted ovary. Importantly, both the fresh and cryopreserved ovaries continued to function for up to 11 months which is the normal reproductive lifespan of the mouse. The researcher in this experiment used 10-day-old donors, which are ovaries that contain more follicles in the early developmental stages and that can explain high the fertility rate. In addition, based on this and previous studies (Sztein et al. 1998), it is important to emphasize that any experiments on this topic should include excision of the ovary with a large margin, including the tissue surrounding the proximal parts of the ovarian artery, since it is likely that ovarian activity persists in the residual native ovarian tissue. In all the studies on mouse ovary cryopreservation it is important that the results are interpreted in the light of that the mouse ovary is only around $2mm^3$ (Candy et al. 2000). In addition, the ovarian bursae can be regarded as favorable place for neovascularisation as compared with species where the ovary is not positioned inside a bursae sac.

In another study, fresh and cryopreserved newborn mouse ovaries were allografted under the kidney in bilaterally ovariectomized recipients (Liu et al. 2002). The ovaries were frozen using controlled-rate slow freezing technique and 1.5M DMSO as CPA. The follicular loss of frozen grafted ovaries was only 9% higher compared to fresh grafted group. It seems that follicular loss is a result of ischemia rather than the cryopreservation process. Furthermore in this study, apoptosis in grafted tissue was analyzed and it was concluded that apoptosis most commonly occurred 2-12 h after transplantation. Also, granulosa and stroma cells were affected by apoptosis more than oocytes in primordial follicles, which may be explained by a higher metabolic rate of the somatic cells.

In a recently published study, fresh and ovaries frozen in 1.5M DMSO were transplanted in bilaterally and unilaterally ovariectomized recipient mice (Liu et al. 2008). Post grafting ovarian size, follicle survival and litter size were significantly reduced in both fresh and frozen unilateral group. Further, ovarian post transplant size was dramatically reduced in both fresh and frozen groups, confirming other observations that there is a great loss of the follicle pool after grafting rather than after the cryoprocedure.

Cryopreservation by vitrification has also been studied using a mouse model with intraperitoneal (Salehnia 2002) and orthotopic transplantation (Migishima et al. 2003). Follicular survival was seen in both reports and in the report with orthotopic transplantation

(Migishima et al. 2003) live births were seen although fewer pups were born in the vitrified group compared to fresh control group. The same group also reported live birth after orthotopic allotransplantation of fresh and vitrified mouse ovaries into irradiated mice (Migishima et al. 2006).

There exists one report with comparison of different cryopreservation methods for the mouse ovary (Chen et al. 2006). A novel method of direct cover vitrification was compared to conventional vitrification and slow freezing. Direct cover vitrification is a method when LN is applied directly on the sample which has to be cooled. Superior fecundity and higher follicle viability were seen with the novel direct cover vitrification group.

As mentioned before, antifreeze proteins may be beneficial also in ovarian cryopreservation. Antifreeze proteins, produced by some cold-tolerant animals and plants, lower the freezing point of the solution and inhibit growth of ice crystals. In a study with transgenic mice carrying antifreeze protein type III gene (Bagis et al. 2008) the ovaries, both from transgenic and non-transgenic animals, were vitrified and grafted orthotopically into ovariectomized recipients. Fresh and non-transgenic transplants were used as control. The animals that had received transgenic ovaries obtained similar litters as fresh control group and had significantly higher litter size than those that received non-transgenic grafts.

2.4.1.3 Vascular ovary transplantation

Vascular transplantation of whole ovaries in the mouse is a difficult procedure due to the small size of the ovarian vessels in this species. There are no reports on whole mouse ovary transplantations. Nevertheless, it is possible to isolate and perfuse the mouse ovary (Brannstrom and Flaherty 1995) and vascular anastomosis on the aorta and vena cava would be feasible, as demonstrated for uterus transplantation (Racho El-Akouri et al. 2002). Therefore, it would be technically realistic to achieve vascular transplantation of a fresh or frozen whole mouse ovary.

2.4.2 RAT

2.4.2.1 Avascular fresh ovary transplantation

The rat was used fairly early in experiments involving transplantation of ovarian tissue. In pioneering work carried out in the 1940s (Harris and Eakin 1949), ovaries of rats were evaluated concerning function inside subcutaneous tissue. In that study around 90% of all castrated female rats that received subcutaneous ovarian autografts resumed cyclicity and

follicles and corpora lutea were demonstrated within the tissue, supporting the finding of resumed ovarian cyclicity. In the 1950s, further studies showed that oophorectomized rats that had received ovarian grafts showed mating behaviour and developed pseudopregnancy (Deanesly 1956).

Around three decades later, the research around avascular ovarian transplantation in the rat was reinitiated with a study that compared tissue structure of the corpus luteum in the autografted rat ovary as compared to the native ovary (Bagwell et al. 1976). In this study, the rats were oophorectomized at day 44 and each ovary was divided into 4 quarters and autografted into a single subcutaneous pouch created in the flank. In order to obtain ovaries with corpora lutea the rats were euthanized 2 weeks after grafting when vaginal smear showed metestrous, which corresponds to the luteal phase. In transmission electron microscopy (TEM) luteal cells were seen to be viable, but the luteal cells of the grafted ovarian tissue were smaller as compared to those within non-grafted ovaries. A slight disorganizing of cell organelles suggested that structural luteolysis may occur before functional luteolysis in this autografted model, as compared to the inverse in the normal setting. However, it should be pointed out that another study from the same group showed that the ovarian autografts produce ordinary amounts of steroids, resulting in normal peripheral blood levels of estrogens and progesterone during at least a period of 30 day after ovarian transplantation (Chihal et al. 1976).

After these early studies in the rat, which showed that cyclicity was resumed after around 2 weeks post transplantation, it was of particular interest to know exactly when the revascularisation of an ovarian graft occurs and what mechanisms are involved in revascularisation. In a detailed study (Dissen et al. 1994), using 23 days old rats, the ovaries were removed and freshly transplanted subcutaneously at a site of the neck, near the jugular veins. In this study, neovascularisation was studied after the ovaries had been placed within cages prepared of gold wire and analysis was performed with corrosion cast techniques utilizing perfusion of methacrylate. This substance will polymerise and after proper digestion the vessels is the only compartment that is visible within the tissues. In all the rats, evaluation 2 days after transplantation showed that capillary beds and small arterial type vessels had formed in and around the tissue with extravasation of methacryalte at the end of small vessels, suggesting active neovascularisation. It also showed continued increase in capillary diameter over the study period and at the end of the observation period (day 7) new veins were seen. In this article (Dissen et al. 1994) the contributing mechanisms for this fast neovascularisation was investigated by examining expression of presumed angiogenic factors. The two angiogenic factors vascular endothelial growth factor (VEGF) and transforming growth factor beta1 (TGF-

beta 1) showed a marked increase (40-60 fold) in expression already within 24 h after transplantation suggesting that they are important in this angiogenic process. In conclusion this detailed study shows that there is early neovascularisation into an ovarian graft and that this is due to high ovarian expression of the angiogenic factors VEGF and TGF-beta1. The consequence of these findings is probably that local administration of VEGF/TGF-beta and other angiogenic factors can possibly be used to promote neovascularisation after vascular transplantation of ovary or of any other organ.

Several studies exist which have investigated the steroidogenic output and histology of avascular ovarian transplants in the rat. There are some variations in observation times and in what particular transplantation sites that have been analysed. In a study utilizing rats of about 2 months of age, the results after avascular transplantation of the whole ovary or the ovary divided in 4 slices were compared (von Eye Corleta et al. 1998). Ovaries/ovarian fragments were placed in a subcutaneous pocket on the dorsal aspect of the rat and 8 out of 9 rats in both groups showed E2 secretion, but slightly better morphologic characteristics were seen in the sliced-ovary group. This would indicate more efficient neovascularisation and rescue of the tissue after ovarian transplantation of small pieces in comparison to the entire ovary.

The longevity of avascular ovarian transplant in the rat was examined in a study where bilateral oophorectomy was performed with the ovaries then being divided in halves and transplanted either intraperitoneally at an orthotopic position or subcutaneously in the in inguinal region. The levels of follicle FSH and E2 turned back to normal within 7-10 days and grafts stayed viable at least during the observation period of 6 months (Callejo et al. 1999). In the follow up study, the examination period was extended to 12 months with the same procedures. In this analysis, histological evaluation was also performed after 30 days and it was shown that the follicle numbers were decreased when ovarian grafts were placed at the heterotopic intraperitoneal site. One year after transplantation the grafts were non-functional. Thus, this study points out that the longevity of ovarian transplants is restricted in comparison to the ovary in situ. One possible mechanism, which may lay behind this early follicular atresia and the follicular loss, could be that FSH levels are increased in the autotransplanted groups due to decreased local inhibin production in the granulosa cells of the transplanted ovary (Callejo et al. 2003). High FSH may accelerate folliculogenesis and thereby empty the follicular pool at an earlier stage.

The above mentioned study indicates that the functionality of an ovarian graft is not fully normal and this can be due to either damage during the initial ischemic period or that the blood

vessels and their locations after transplantation are suboptimal. In one study, the rat ovary was transplanted to a pelvic intraperitoneal site and two agents that inhibit production of oxygen radicals were and tested (Sapmaz et al. 2003). Oxytetracyclin inhibits leukocytes activity and has an inhibitory action on production of reactive oxygen species. The other tested substance was melatonin which has similar activity. The animals were treated with either melatonin or oxytetracycline intraperitoneally. It was found that melatonin was more effective than oxytetracycline and it partly counteracted ovarian necrosis and the rise in tissue levels of malondialadehyde, an blood indicator of tissue levels of reactive oxygen species (Sapmaz et al. 2003).

It may also be that the precise anatomical location of the ovarian transplant may affect the health of the transplant. In a study of transplanted rat ovaries with transplantation of intact whole ovaries or sliced ovaries, the transplantation site of the greater omentum and the site of the retroperitoneum were compared (Petroianu et al. 2005). It was speculated that there may be differences since the blood drainage from the omentum is by the portal vein and that from the retroperitoneum is through the cava. The results after a follow up period of 3-6 months showed that E2 levels were closer to the normal in rats transplanted with sliced ovaries as compared to intact ovaries and also that the function of those transplanted into the omentum, with portal drainage, was more effective than after transplantation into the retroperitoneum. No further speculation was done in regards to why venous drainage by the porta would be better than venous drainage by the cava. Other alternative transplantation sites are the capsule of the kidney (Candy et al. 1997; Sugimoto et al. 2000) or inside the uterine cavity (Kagabu and Umezu 2000) or a subcutaneous place (Callejo et al. 1999).

When fertility after natural mating is the endpoint, orthotopic transplantation is essential. Transplantation of fresh ovaries within the ovarian bursae has also been demonstrated in the rat (Dorsch et al. 2004). However, evaluation of the results of the study after 3 months showed that ovaries contained cysts and moreover adhesions were found around the ovary. Another site of transplantation, which has recently been evaluated in the rat, is to transplant ovaries to the peritoneal surface located near the epigastric vessels (Barros et al. 2008). In that study hemi-ovaries were transplanted and histology confirmed the usefulness of this transplantation site.

2.4.2.2 Vascular fresh ovary transplantation

Successful syngenic and allogenic tubo-ovarian transplantation were presented more than 20 years ago (Scott et al. 1987). The surgery on Lewis (LR) or Brown-Norway (BN) female rats included isolation of an aortic-vena cava segment that included the origins of the ovarian

vessels on the right side with the ipsilateral oviduct, ovary and distal part of the uterine horn. The specimen was not flushed and the vessel segment was anastomosed end-to-side to the aorta and vena cava of the recipient with also anastomosis of the uterine part to the proximal portion of the recipient uterus. Syngenic transplants (BN-BN or LR-LR) were technically successful in 6 out of 8 rats, and four delivered healthy offspring. Around 50% of the allografts were viable and a low number of pregnancies were reported, with miscarriage when a high dose cyclosporine was used. This is the first report in any species of pregnancies after allogenic ovarian transplantation, with the recipient under immunosuppression.

A similar technique as that described above was used in fresh controls, when the technique of whole ovary cryopreservation of the rat ovary was evaluated (Wang et al. 2002). All transplants showed immediately vascular patency. 9 out of 10 studied rats demonstrated estrous cyclicity, assessed by vaginal cytology, whereas 6out of those 9 rats achieved pregnancy and delivered healthy offspring. At euthanasia 3 months after surgery, thrombosis was demonstrated in the ovarian vessels in the remaining rat without reestablished estrous cyclicity.

2.4.2.3 Avascular frozen ovary transplantation

There exist some papers that have evaluated different cryopreservation procedures on rat ovaries, or pieces of ovaries, before avascular transplantation. In one comparative study (Maltaris et al. 2006) small (1 x 1 x 1 mm) ovarian pieces were subjected to slow freezing, either using a slightly modified protocol described by Newton (Newton et al. 1996) with 1.5M EG or an automatic open-vessel freezing system with equimolar concentrations of DMSO and PROH up to 1.5M. The later method utilizes temperature distribution above the surface of LN. The freezing container moves up and down through the nitrogen vapor to attain temperature changes which are measured during the whole process. Another difference between those two protocols is the cooling rate. The frozen-thawed ovarian pieces were grafted into neck muscle. Survival of the follicles just after thawing, assessed by live/dead viability assay, was similar in both groups with around 40% dead follicles and a further 35% of impaired viability. These were test of the tissues after thawing, and before the warm ischemic period after transplantation. Analysis of the effects of the warm ischemia was performed by morphometric analysis of the samples 3 weeks after transplantation. It showed a significant follicle loss in the frozen groups compared to fresh, but no difference was seen in this regard when the two cryopreservation protocols were compared. Thus, this study indicates that the two cryopreservation procedures are equally effective.

The effectiveness of DMSO, as a CPA for rat ovarian pieces was confirmed in a recent study (Dorsch et al. 2007), aiming at finding a simple protocol for gamete banking of the increasing number of transgenic and coisogenic rat strains, which are used for research purposes. The standard protocol of slow freezing using 1.5M DMSO was used and after thawing, orthotopic transplantation of whole ovaries was performed into the ovarian bursae, after removal of the native ovary. Around 25% of the recipients with cryopreserved ovaries became pregnant and gave birth, which was somewhat lower than the rate (33%) after fresh transplantation. The average litter size was similar between unfrozen and frozen grafts, but significantly lower compared to non- grafted controls. Collectively, the results show that the cryopreservation method works but that there is room for improvement. One way of improving the post transplantation of a frozen thawed graft could be to increase local angiogenesis around the graft in order to shorten the post-transplantation ischemic period.

The use of gonadotropins to possibly promote angiogenesis was tested in the rat model (Yang et al. 2008), where cryopreservation with a slightly modified Gosden protocol (Gosden et al. 1994) was used. The study group was divided further into one group of rats treated by 10IU of pregnant mare's serum gonadotropin (PMSG; mostly FSH activity but also some LH activity) every second day and another group treated only by saline. The grafted glands were recovered on day 2, 7 and 30 and evaluated by histology, VEGF expression and apoptosis. Organized blood vessels were observed at day 7. No major differences regarding apoptosis and folliculogenesis were observed between groups but a transient (day 2) increase in VEGF expression in the gonadotropin group may indicate a positive impact of PMSG.

It is also important to look at the long-term function of an ovarian graft after cryopreservation and a study along this line has been performed with the rat as the animal model (Deng et al. 2009). In this particular study a vitrification protocol (initial equilibration in 7.5% DMSO plus 7.5% EG; second equilibration and vitrification in 15% DMSO, 15% EG and 0.5M sucrose) was used on small ovarian pieces. Transplantation was performed and the ovarian pieces were put under the kidney capsule with animals followed for 5, 8 or 10 months after grafting. The major finding in this study was that the primordial follicle count was equally decreased (around 40% compared to sham operated) in the transplantation groups of fresh and cryopreserved ovaries when evaluated 5 months after surgery and that steroid levels remained normal in these groups during the entire 10 month period. Considering that the oestrus cycle is 4 or 5 days, a great number of cycles have taken place during the 10 months study period.

Vitrification applied on rat ovaries, as discussed above, has as well been evaluated on small postnatal ovaries (Sugimoto et al. 2000), which would mostly contain small follicles and would be smaller in size than the adult ovary. In that study vitrification of whole ovaries was performed with VS1 solution containing 20.5% DMSO, 15.5% acetamide, 10% PROH and 6% EG. The ovaries were exposed to gradually increasing concentration of VS1 from 12.5% to 100% before plunging into LN. At thawing the samples were exposed to decreasing concentrations of VS1 and they were then autotransplanted under the kidney capsule. Rats with fresh and vitrified autotransplanted ovaries reached puberty at a similar time. However, fewer antral follicles were seen in the vitrified group at day 84 post transplantation. This could be due to a follicular loss during vitrification or that the later stages of follicular development are impaired by the treatment.

2.4.2.4 Vascular frozen ovary transplantation

As a consequence of the reassuring results of rat ovary cryopreservation and avascular transplantation as well as due to the presence of techniques for vascular anastomosis of the rat ovary, experiments with whole rat ovary cryopreservation and functional tests regarding fertility have been carried out (Wang et al. 2002). The surgical technique has previously been reported (Wang et al. 2002) for vascular fresh ovarian transplantation. The whole right ovary plus adnexae with its vascular pedicle were cryopreserved essentially by the traditional Gosden protocol (Gosden et al. 1994). In this study (Wang et al. 2002) 4 out of 7 recipients of frozen grafts had follicular activity and visible corpora lutea after transplantation. In addition, higher FSH and lower E2 level than controls were recorded. Pregnancy with two fetuses was seen in one out of those four animals but live birth was not demonstrated. However, regardless of the low pregnancy rate and small size of the rat ovary, this proof-of principle report was a major advance in the area of organ cryopreservation and vascular whole ovary transplantation.

A more detailed description of the previously experiments was later published (Yin, Wang et al. 2003). Apart from vascular transplantation of the whole ovary between syngenic rats of which one resulted in pregnancy, the authors extended the study to allogenic transplantation with and without immunosuppression. Before transplantation, the unfrozen control group was divided into two subgroups: cold ischemia at +4°C <1 h and cold ischemia for 24 h at the same temperature. At autopsy, approximately 70 days after grafting, the female glands that had been exposed to cold ischemia for 24 h showed significantly decreased number of follicles compared to fresh non-grafted ovaries and transplants after cold ischemia of <1 h, but still

higher than in frozen group. In contrast to fresh and syngenic transplants, all allotransplants were completely rejected in spite of treatment by a fairly low dose (4 mg/kg) of cyclosporine. These results should be compared to those of Scott and co-workers (Scott et al. 1987), where pregnancies were also reported after allogenic transplantation. The same rat strains were utilized but the later study used higher doses of cyclosporine.

The concept of successful whole ovary cryopreservation in the rat has also been confirmed in a third study from the authors that published the original study (Qi et al. 2008). In that study the most interesting results were those that apoptosis in the cryopreserved organ was storage time-dependent so that around 75% of cells of ovaries that had been stored for 30 days showed apoptosis, in comparison to 1 day and 7 day storage with apoptosis around 5 and 15%, respectively. This finding is surprising and against the concept, that organ/cells that are stored in LN do not undergo any major changes since all the cellular processes are arrested.

2.4.3 Rabbit

The rabbit is a traditional experimental model in reproductive physiology research. The rabbit is a reflex ovulator with a preovulatory follicle always present to respond to a gonadotropin surge. The rabbit model has been used extensively in research to study ovulation (Dahm-Kahler et al. 2006).

2.4.3.1 Avascular fresh ovary transplantation

There exist only a small number of studies on avascular ovarian transplantation in the rabbit, either as fresh transplants or after cryopreservation of ovarian tissue. Small pieces of rabbit ovary tissue were freshly transplanted, after oophorectomy, into the mesometrium of the uterus or into the ovarian bursae (Deng et al. 2007). Normal histology and follicular development were found in both groups and these results demonstrated that both these sites are feasible transplantation sites in the rabbit. However, the exact transplantation sites are imprecise and the existence of complete ovarian bursae in the rabbit is not as well-described as for the rat and mouse, where intrabursal injection is a used method to experimentally administer drugs that could influence ovarian function (Matousek et al. 2001)

Orthotopic transplantation is possible in cases of an existing ovarian remnant, such as after full gonadotoxic treatment by radiotherapy or chemotherapy, or in cases that an ovarian site is clearly noticeable soon after oophorectomy. Orthotopic allogenic transplantations of fresh intact ovaries or sliced ovaries were tested between New Zealand White and California female rabbits (Petroianu et al. 2007). Interestingly, pregnancy was demonstrated after allogenic

transplantation with only cyclosporine as immunosuppression, with a pregnancy rate in the transplantation groups of between 40 and 60%, as compared to 85% in the sham-operated group. The lower pregnancy rate in the transplanted group may be due to effects by cyclosporine on implantation (Groth et al. 2009) or by local factors such as post-transplantation adhesions. Since the histology and FSH levels were normal it is not likely that follicle loss was a factor behind the lower pregnancy rate (Petroianu et al. 2007).

2.4.3.2 Vascular fresh ovary transplantation

Transplantation of a whole ovary by vascular anastomosis in the rabbit is a possible technique for a skilled microsurgeon. In one experimental study, rabbit ovaries with their vascular pedicle (ovarian artery and vein) were either transplanted orthopically to the ovarian vessels or heterotopically to the inferior epigastric vessels (Denjean et al. 1982). End-to-end techniques with 11-0 sutures were used and ovulation rates after orthotopic or heterotopic transplantation were high and comparable.

Allogenic vascular transplantation of the rabbit ovary, together with the Fallopian tube and the distal uterus, was also tested at an early stage, with both recipients and donors being of the same New Zealand white rabbit strain (Carmona et al. 1993). Half of the animals received cyclosporine and histological analysis showed that >70% of the grafts were well preserved when the animals were treated by cyclosporine. On the contrary the non-treated animals showed graft rejection in 95%. The ultimate test of function of the graft in this experimental series was pregnancy, which was demonstrated in 6 out of 16 transplanted animals. It should be mentioned that the allogenicity between the animals of the same strain was not assessed in this study.

Allogenic transplantation of ovaries was also performed with New Zealand White rabbits as donors and Californian rabbits as recipients (Meraz et al. 2006) and with autotransplantation as a control group. In this study an end-to-end microvascular anastomosis was achieved between the ovarian and the inferior epigastric vessels. Due to the heterotopic and extraperitoneal transplantation sites, all transplants in this study were performed together with a peritoneal pouch in an effort to prevent formation of adhesions. Cyclosporine and prednisone were used as immunosuppressants and one group did not receive any of these drugs. At day 28 after grafting, around 60% viable grafts were found in the autotransplantation group and in the immunosuppressed allotransplantation group. No viable grafts were seen in the allograft group without immunosuppression and furthermore lower blood steroid levels were seen in the same group. In a follow up study to analyse long-term

function including pregnancy, the same research group performed allogenic orthotopic tubal-ovarian transplant using end-to-end micro vascular anastomosis (Meraz et al. 2008). The study had the similar design as the previous study and immunosuppression was obtained by a high dose of cyclosporine (10 mg/kg/day) during the first 21 days, followed by 50% of that dose during the following 7 weeks. Mating was performed around day 30 postoperatively. After allotransplantation and no immunosuppression the pregnancy rate was 0%. Fourteen out of 15 rabbits in the control groups achieved pregnancy and in the allotransplanted group only 1 rabbit out of 8 became pregnant. This is a proof of concept for allogenic whole ovary transplantation, but it also indicates the low effectiveness of the method used. It remains to elucidate whether the low pregnancy rate is due to influences of cyclosporine, low grade rejection or other factors. It would also be good to include a longer observation time to get closer to a possible human situation.

2.4.3.3 Avascular/vascular frozen ovary transplantation

A small number of studies, all published during the last decade, have addressed cryopreservation of rabbit ovarian tissue and in some cases also with evaluation after avascular/vascular transplantation. Orthopic transplantation of small frozen-thawed pieces of rabbit ovaries by subcapsular insertion into radiotherapy-treated and thereby sterilized ovaries was tested (Almodin et al. 2004). The ovarian pieces were cryopreserved by slow-freezing technique and with the use of 1.5M DMSO. All the animals that had undergone this fertility-preservation procedure became pregnant. Heterotopic transplantation, to the mesometrium, of slow-frozen and cryopreserved (1.5M DMSO) ovarian tissue has also been reported to be effective, at least by morphological evaluation (Deng et al. 2007). The optimal rate of slow-freezing for rabbit ovarian tissue seems to be 0.3°C/min and in that study (Neto et al. 2008) PROH showed a somewhat better preservation rate than DMSO, when the preservation was assessed by live-dead viability assay and histology after rapid thawing. Also the non-permeable CPA trehalose showed an acceptable preservation rate of the tissue.

One study of heterotopic autotransplantation by vascular anastomosis of whole cryopreserved rabbit ovaries exists (Chen et al. 2006). In this study the ovarian artery (diameter 0.4-0.7 mm) and vein were carefully dissected free for a length of about 2.5 cm. The experimental ovaries were perfused with medium 199 followed by 1.5M DMSO, 0.1M sucrose, 10 % fetal bovine albumin and 500 IU of heparin sodium. Slow cooling was accomplished 0ºC to -7ºC at the rate of 2ºC /min. Seeding was performed at -7ºC and then temperature was decreased at a rate of 0.3ºC /min to -40ºC followed by 10ºC /min to -180ºC. Thereafter thawing was performed

by plunging the ovaries into a water bath at 40ºC with duration of 10 min. The frozen/thawed ovary was grafted into the groin pocket with an end-to-end vascular anastomosis to the inferior epigastric artery and vein. The outcome was that 10 of 12 rabbits with cryopreserved transplants showed restored ovarian function one week after transplantation, judged by vaginal cytology and hormone secretion. Hormone production remained during the complete observation period of 6 months, although FSH increased 3-fold from 1 week after grafting to the end of the study period. This finding is in line with that follicle density decreased by about 25% as compared to the control ovaries that were neither cryopreserved nor transplanted. The follicle loss of only 25% compared to fresh non-grafted ovaries should be regarded as encouraging results towards whole ovary cryopreservation and transplantation in humans.

2.4.4 Cat

The animal model of the cat has not specially been used in research on ovarian cryopreservation and transplantation. However, it has to be mentioned that the first ovarian transplantation by vascular anastomosis was performed in the cat in 1906 (Carrel and Guthrie 1906). It was an allogenic transplantation, where the ovarian vessels with aortic and caval patch were anastomozed to the aorta and vena cava of the recipient. No more data are available about this particular case.

2.4.5 Cow

Cow ovaries may be suitable for ovarian cryopreservation due to similar tissue architecture, although they are considerable larger (14.3 (+/- 5.7) cm^3 (Gerritse et al. 2008). The function (Yang and Fortune 2006) and the structure (Rodgers and Irving-Rodgers 2010) of the bovine ovary has been extensively studied. In particular, the granulosa cells and the extracellular matrix of bovine follicles of various developmental stages are well described (Lavranos et al. 1994; Irving-Rodgers et al. 2006).

2.4.5.1 Avascular frozen ovary transplantation

In a thorough Pub-Med search I was able to find only one study on transplantation after bovine ovarian cryopreservation. This study examined the effectiveness of avascular ovarian transplantation in the cow after cryopreservation by vitrification using a solution containing 20% EG and 20% DMSO in TCM-199 medium (Kagawa et al. 2009). Vitrification was done by the Cryotissue method (Kagawa et al. 2007) where the ovarian tissue was placed on a thin metal strip, plunged into LN followed by insertion of the metal strip into a protective container for storage in LN. Post thaw warming was rapid with immersion of the metal strip

into TCM-199 supplemented with sucrose at 40ºC and then washing steps of the same solution with decreasing sucrose concentration. Grafting was performed subcutaneously in the neck or orthotopically to oophorectomized cattles, with recovery of cyclicity in both experimental situations within 2 months. Histology of these grafts revealed normal morphological appearance and fluorescent staining showed preantral follicular viability of 95% as compared to fresh control. These results are very encouraging regarding the vitrification method for ovarian cortex cryopreservation.

2.4.5.2 Cryopreservation research on bovine ovary

In research aimed towards in vitro follicle maturation of bovine follicles, slow-frozen bovine ovarian cortical strips were incubated after thawing for time intervals between 1 and 48 h (Paynter et al. 1999). The main finding was that the cryopreserved tissue that had been thawed had a capacity to recover from damage during the subsequent period. This idea was further investigated in later research, where bovine ovarian cortical strips were cultured for 6 days following by isolation of secondary follicles and further culturing in the presence of inhibin (McLaughlin and Telfer 2010). Significant E2 secretion and oocyte growing up to > 100 μm was demonstrated in inhibin group as compared to controls without inhibin.

Bovine ovarian cortex was also used in a study that investigated whether antioxidants may enhance survival of frozen-thawed tissue (Kim et al. 2004). Bovine cortical strips were cryopreserved by slow freezing with 1.5M DMSO and allocated to in vitro culture for periods up to 48 h in MEM medium with or without ascorbic acid. Interestingly, there was no difference in apoptosis rate, assessed by terminal deoxynucleotidyl transferase dUTP nick end labelling test (TUNEL) and DNA laddering, of primordial follicles incubated with or without ascorbic acid. However, signs of protection by ascorbic acid were observed in stromal cells that were cultured for 24 h. In line with another study on cryopreservation of human ovarian cortex it was also observed that stromal cells are more susceptible to damaging mechanisms than primordial follicles (Hreinsson et al. 2003). The concept of supplementation of antioxidant agents to CPA needs further investigation.

Toxicity of different concentrations and types of CPAs that are commonly used for slow freezing was compared using bovine ovarian pieces (Lucci et al. 2004; Celestino et al. 2008). Among several other CPAs, DMSO at concentrations of 1.5M and 3M were evaluated in both studies. While one study (Celestino et al. 2008) demonstrated increased toxicity with increasing concentration of DMSO, the other report (Lucci et al. 2004) showed slightly higher percentage of normal follicles, assessed by histology, in the 3M DMSO group. Nevertheless,

TEM demonstrated some irregularities in the cytoplasm of granulosa cells when 3M DMSO was used (Lucci et al. 2004).

One paper reported the use of bovine ovaries to evaluate the feasibility of whole ovary transplantation (Gerritse et al. 2008). The study evaluated distribution of heparinized solution containing Indian ink particles that were flushed (2.5 ml/min for 5 min) through the ovarian artery. The lumen of the bovine ovarian artery was found to be between 0.8 and 1.2 mm, which is somewhat larger as compared to the human ovarian artery (Silber et al. 2008). Interestingly, the proportion of perfused capillaries was only about 15% but more than 60% of larger vessels contained ink particles as a proof of perfusion, with no augmentation of perfused vessels during perfusion times above 5 min.

It has been debated whether slow freezing or vitrification is the optimal method for ovarian cryopreservation and various results exist, with one study pointing towards higher efficiency of slow-freezing, when bovine cortical strips are evaluated (Gandolfi et al. 2006). Moreover, another report has been published pointing towards the superiority of the vitrification procedure, when whole ovaries with vasculature were used as a model system, but the same study found superiority of slow-freezing with cortical strips as the experimental model (Zhang et al. 2011). In the latter study, the efficiency of these techniques was assessed by Trypan blue test, histology as well as E2 and progesterone levels obtained from supernatant after in vitro culture of the tissue.

Additionally, one study was designed to evaluate the effect thickness of the size of the ovarian cortex strip on follicular morphology after incubation for 20 min and slow freezing with 1.5M PROH (Ferreira et al. 2010). Fragments of 10 x 3 mm, with a thickness of either 2 or 4 mm, were compared and significantly higher percentage of normal follicles were found in the 2 mm group as compared to the 4 mm both fresh and frozen/thawed tissue. This finding may be explained by better tissue impregnation with CPA in 2 mm group, but it needs further investigation.

2.4.6 Pig

The pig is a species that has been used extensively in biomedical research, especially concerning development and training of surgical methods for later use in the human. Concerning the reproductive cycle of the pig large amount of data is available. The cycle of the pig last for 19-23 days and generally 8-12 oocytes are released from each ovary at ovulation (Wolfensholm and Lloyd, 2003). The size of the pig ovary is about 7.3 (+/- 2.2) cm^3

(Gerritse et al. 2008), which is similar to the human ovarian size of 6.5 (+/- 2.9) cm^3 (Munn et al. 1986). The pig ovary has been used extensively for studies of oocyte function (Degenstein et al. 2008), but elaborate studies on the extracellular matrix (Hasegawa et al. 2006), stroma and follicular fluid (Hunter et al. 2000) of the pig ovary also exist. These facts, combined with that fresh ovaries from slaugtherhouses are readily available, would make the pig a good choice as an experimental animal for ovarian cryopreservation research. However, a fairly low number of publications in this area with the pig ovary as an experimental model are available, as shown below.

2.4.6.1 Vascular fresh ovary transplantation

Transplantation of the whole ovary by vascular anastomosis in the pig was first reported in the late 1960s (Binns et al. 1969) and this was later followed by a study in the early 1980s (Harrison 1982), where they, due to the small size of the ovarian artery and the associated complicated microsurgery, used a technique, previously described in the sheep model (Goding et al. 1967). The method used the ovarian artery being retrieved all the way to its origin on the aorta and including an aortic patch. The experiments involved autotransplantation (Harrison 1982) to either an orthotopic site by anastomosis of the patch to the aorta and the utero-ovarian vein to the vena cava or to the heterotopic site of the neck with vascular connections to the carotid artery and jugular veins. Orthotopic transplantation resulted in pregnancy and 8 out of 9 animals delivered offspring. The two pigs that received heterotopic transplants restored normal cyclic activity, which was a proof of that luteolytic signals were transported from the uterus by the blood circulation, in contrast to the local transport of luteolytic prostaglandins from the uterus to the ovary in the sheep (McCracken et al. 1972)**.**

2.4.6.2 Cryopreservation research on porcine ovary

In one detailed study, the goal was to evaluate whether the size of an ovarian cortex graft from the pig ovary is of importance for the cryopreservation outcome (Jeremias et al. 2003). Cortical pieces were all of 1 mm depth but either 1x1 mm in or 5x1 mm in area. The pieces were cryopreserved by slow freezing in 1.5M DMSO and compared after rapid thawing to the fresh tissue (1x1 mm) regarding the number of primordial follicles (Jeremias et al. 2003). The slow freezing was uncontrolled since the cryovials with the ovarian tissue were held in a freezer at -20ºC for 30 min followed by positioning in LN vapor for 30 min and then storage in LN. Interestingly, the frozen/thawed cortical pieces of 5 x 1 x 1 mm showed a similar density of primordial follicles as compared to the fresh tissue, while fewer follicles were seen

in the small frozen/thawed pieces. It was not further speculated on the mechanisms of that the larger pieces were more resistant to cryoinjury. However, it should be mentioned that comparably large, but thin, ovarian cortex grafts are used in human clinical fertility preservation procedures and a Danish group has accomplished most live-births after ovarian cryopreservation using a dimension of the grafts of 5 x 5 x 1 mm (Andersen et al. 2008).

One general view in ovarian cryopreservation research is that only the very small follicles (primordial and primary) survive the cryopreservation procedure and that especially the antral follicles, with a fluid-filled centre, are susceptible to cryoinjury by ice crystal formation. In one study, cortex samples from porcine ovaries were cryopreserved by programmed slow-freezing in either of four different CPAs (Borges et al. 2009) at set concentrations (GLY-10%; DMSO-1.5M, EG-1.5M, PROH-1.5M). The ovarian cortex pieces were subjected to short term incubation after thawing, and histological analysis including LM and TEM showed that the follicular viability was decreased after freezing, but that DMSO and EG were superior to PROH and GLY. This finding is in line with clinical observations of pregnancies in the human demonstrated with only DMSO and EG as CPAs (Donnez et al. 2004; Andersen et al. 2008). However, it should be mentioned that species differences exist in this regard, as shown in a comparative study between ovarian cortex of human, bovine and porcine ovaries (Gandolfi et al. 2006). In these experiments DMSO and PROH were equally effective to preserve primordial and primary follicles of the pig ovary which was also more resistant to cryoinjury than the bovine and the human ovary.

There are also reports on ovarian cortex cryopreservation by vitrification in the pig (Gandolfi et al. 2006; Moniruzzaman et al. 2009). Ovarian strips, obtained from ovaries of 15-day old pigs, were vitrified using 15% EG, 15% DMSO and 20% FCS supplemented with either 0M, 0.25M or 0.5M sucrose (Moniruzzaman et al. 2009). Histology after warming demonstrated about 50% of normal primordial follicles when CPA solution with 0.25M sucrose was used as compared to 20% and 35% of primordial follicle density at 0M and 0.5M, respectively. In addition, a higher rate of oocyte shrinkage was seen when the high sucrose supplementation (0.5M) was used. These findings can be attributed to insufficient cell dehydration obtained without sucrose as well as to excessive dehydration at 0.5M sucrose. To test the function in vivo vitrified ovarian tissues were xenografted under the kidney capsule in nude mice (Moniruzzaman et al. 2009)**.** Two months after grafting, the primordial follicle density was decreased by around 20% in the fresh grafts but several antral follicles were seen. In the vitrified grafts, the relative decrease of primordial follicle density was similar to that of fresh controls but follicles did not developed beyond the secondary stage. Thus, it may be that

follicular development is retarded after vitrification or that there is a follicular stage dependant blockage in the developmental capacity, due to cryoinjury. In the other study, vitrification of pig ovarian cortex showed low survival of primordial follicles with either EG or a combination of EG and DMSO as CPA (Gandolfi et al. 2006)

There is only one study that has evaluated the pig ovary for studies of whole ovary cryopreservation. Porcine ovaries, obtained from slaughterhouse, were perfused with 1.5M DMSO through the ovarian artery and cryopreserved with a slow freezing protocol (Imhof et al. 2004). After storage and subsequently thawing in 25ºC, the percentage of viable primordial follicles, assessed by LM, was slightly depleted in the frozen-thawed ovaries (84%) as compared to fresh controls (98%). In addition, only 20% of healthy follicles were seen when the ovaries were placed directly into LN without perfusion or immersion in DMSO. Examination by TEM did not demonstrate any major cellular difference between the fresh and slow-freeze groups. The results of that study clearly shows the importance of CPA perfusion before freezing and also indicates the possible benefits of slow freezing over vitrification, when a comparably large tissue mass is cryopreserved.

2.4.7 Sheep

Various in vivo and in vitro sheep models have been used extensively in the research on ovarian cortex cryopreservation with avascular transplantation as well as on whole ovary cryopreservation. This is most likely due to that the sheep is a traditional experimental animal in reproductive physiology research and that it is of comparably low cost in countries with a large wool-producing industry. There exist a large knowledge base concerning physiological changes of the ovine ovary and the similarities between the human and ovine ovary in regards to tissue architecture make it suitable as a research tool towards optimization of procedures for human use. The sheep ovary has, like the human ovary, a collagen-dense outer stroma layer containing the pool of primordial follicles (Arav et al. 2005). However, the size of the sheep ovary is only around 20% of the human premenopausal ovary (Munn et al. 1986; Gerritse et al. 2008) and this fact has to be taken into account, especially in comparative studies relating to whole ovary cryopreservation.

2.4.7.1 Avascular fresh ovary transplantation

Systematic studies on freshly transplanted sheep ovarian cortex with comparison to cryopreserved tissue appeared in the literature some years after demonstration that sheep

ovarian cortex that had been cryopreserved successfully could be transplanted back, with further demonstration of fertility (Gosden et al. 1994).

The first detailed study on fresh ovine ovarian cortex transplantation came in 1998 (Salle et al. 1998) The study utilized sheep ovaries that at backtable preparation were cleared from the central medulla and the cortex further divided into three pieces. The pieces were either cryopreserved followed by grafting orthotopically to the hilum of the previously dissected and removed contralateral ovary or used as a fresh non-transplanted cortex control. Evaluation of the fresh graft after six months revealed some ovarian fibrosis and also decreased number of primordial follicles as compared to fresh controls.

A heterotopic site (subcutaneously adjacent to carotid artery and jugular vein) with evaluation of endocrinological changes was also studied in the sheep (Campbell et al. 2000). After grafting, around 4-fold higher FSH levels than in the control group was seen and ovulation failure was seen in those with the highest FSH. Naturally, these animals had lower levels of E2 and inhibin and this was likely due to transplantation-related depletion of small antral follicles. In the same study, orthotopic autografting of ovarian strips was used as a model system to demonstrate hormonal influence on follicular development. Some of the results indicated that high levels of FSH and LH may accelerate follicle growth.

2.4.7.2 Vascular fresh ovary transplantation

Succerssful vascular transplantation of a whole sheep ovary was reported more than 40 years ago (Goding et al. 1967), with the ovary transplanted to a preformed subcutaneous pocket of the neck. The entire ovarian artery, including an aortic patch at its origin, and the middle uterine vein were anastomosed end-to-side and end-to-end to the carotid artery and jugular vein, respectively. The success rate, in terms of reestablished progesterone secretion was four out of six. Only two ovaries were assessed by histology and both ovaries showed normal morphology and one had corpus luteum. The aim of development of the technique was to perform studies on ovarian physiology (Harrison and Heap 1978; de Souza et al. 1998) but it has also recently been used in the research field of fertility preservation (Onions et al. 2009).

Orthotopic or near-orthotopic transplantation of a whole ovary may be more attractive from a clinical standpoint since this would make a natural conception possible. Microsurgical anastomosis was accomplished between ovarian vessels of the right ovary and the inferior epigastric vessels by the use of 9-0 and 10-0 sutures (Jeremias et al. 2002). To be able to anastomose the vessels of different sizes, “fish-mouth incisions” of the smaller vessels were

created in some instances. Even if immediate vascular patency was demonstrated in all grafts only 3/6 grafts showed vascular patency one week after grafting which was the endpoint of the experiment. Thrombosis was seen in all cases with fish-mouth technique and high FSH levels were also seen in this group. It is important to point out that these report shows that microvascular anastomosis on the ovarian vessel level is possible but the success rate of this procedure seems to be considerably lower than the patch technique described above. The latter method is a more risky surgery since it involves extensive retroperitoneal dissection with the retrieval surgery on the major vessels such as the aorta and vena cava. Another comment is that in the study by Jeremias and coworkers (Jeremias et al. 2002) the warm ischemic time was extremely long (more than 3 h) and it is well known from clinical transplantation surgery that these long warm ischemic times induces major damage to the organs and to the blood vessels.

2.4.7.3 Avascular frozen ovary transplantation

The pioneering work in ovarian cryopreservation is the study from 1994 from the Edinburgh group (Gosden et al. 1994), where live births, after ovarian cortex cryopreservation and transplantation in a large experimental animal, were demonstrated for the first time. This study really opened up the field for future clinical fertility preservation and also states in its conclusion "that frozen storage and replacement of a patient´s own ovarian tissue might be practicable when fertility potential is threatened by chemotherapy/radiotherapy". The cryoprotection protocol used in that study has thereafter been extensively used in research and clinical practice, although with small modifications in many instances. It should be mentioned that this slow freezing-rapid thawing protocol was adopted from a study on cryopreservation of mouse primordial follicles (Carroll and Gosden 1993) and it is also stated in the sheep paper that the authors "have no evidence whether it is optimal". In the Gosden study ovarian cortex pieces were placed in cryovials with Leibowitz L-15 solution containing donor serum and 1.5M DMSO and the cryovials were kept on ice for 15 min. The ovarian tissue pieces were then considered equilibrated in the CPA and placed in a programmable freezer and cooled at a rate of 2ºC/min to -7ºC and maintained at -7ºC for 10 min before seeding. Freezing was continued by reducing the temperature at a rate by 0.3ºC/min to -40ºC following by 10ºC/min until -140ºC before plunging in LN (-196ºC) and stored for 3 months. For thawing, frozen tissue was hold in air temperature for 2 min before placement in water bath at room temperature for an unspecified time. A second laparotomy was performed and the frozen-thawed slices were attached adjacent to the left ovarian pedicle. High progesterone, indicating reestablished cyclicity was first observed four months later. Eight months after

grafting, one animal delivered a lamb which was successfully bottle-reared. One more lamb gave birth by Cesarean section at gestational age of about 144 days.

In a follow up study it was concluded that the longevity of the transplants was at least 22 months, but at that time very few primordial follicles remained in the graft (Baird et al. 1999). The basal values of FSH and LH were increased as compared to controls, although all grafted animals showed normal cyclical pattern. This is in concordance with that levels of inhibin were lower and thus indicating reduced number of inhibin-producing small antral follicles. The authors in addition grafted both fresh and frozen-thawed ovarian slices under the kidney capsule of nude mice to evaluate whether the cryopreservation procedure per se or if the warm ischemic period post transplantation was the major cause of that the follicle pool was depleted already within 2 years. The follicular loss after grafting of fresh tissue was about 65% as compared to fresh non-grafted ovarian tissue. Additionally 7% was lost after grafting of frozen-thawed slices. Thus, this finding pointed out that ischemia rather than cryopreservation is the major cause of follicle loss.

The fact that there is a great loss of follicles during the ischemic post transplantation period was also confirmed in a recent study in the sheep, where fresh and frozen ovarian cortical strips were grafted onto either the uterine horn or subcutaneously in the anterior abdominal wall (Aubard et al. 1999). Cryopreservation was performed using a slightly modified method as described before (Gosden et al. 1994). After 7 months, a significant follicular loss was demonstrated in both fresh and frozen-thawed tissue and it was estimated that only 5% of primordial follicles survived grafting. None of the animals became pregnant after mating but mature oocytes were obtained after gonadotropin stimulation. A low fertilization rate and cleavage arrest at 4 cells was seen in oocytes from both fresh and frozen ovarian tissue. The later results open up the issue about cytoplasmatic maturation as well as quality of the oocytes obtained from grafted and frozen-thawed tissue.

Moreover larger parts of sheep ovaries, containing parts of the medulla, have been tested concerning viability after cryopreservation and avascular transplantation (Salle et al. 1998). Cortex was in that study frozen with the original slow freezing-rapid thawing technique utilizing 1.5M DMSO (Gosden et al. 1994) and at evaluation 6 month after transplantation to the ovarian hilus, well preserved morphology with follicles of all stages was seen (Salle et al. 1998) as well as restoration of progesterone secretion (Salle et al. 1999). In the latter study, cryopreservation of hemi-ovarian cortex and avascular transplantation resulted in live births (Salle et al. 2002). However, at this stage the cryoprotocol had been slightly modified with a

higher concentration (2M) of DMSO and with a temperature decreased by a rate of 2ºC/min to -35ºC followed by 25ºC/min to -140ºC. Semiautomatic seeding was initiated at -11ºC. Six to nine months after orthotopic transplantation and natural mating, four out of six ewes achieved pregnancy of which two delivered twins. The remaining four ewes were monitored further for two years (Salle et al. 2003). All ewes became pregnant and delivered offspring of which two gave birth for a second time. All recovered grafts displayed significant depletion of follicles. This finding also emphasizes that the avascular transplantation procedure per se induces major damage to the tissue.

Vitrification is today widely used for embryo cryopreservation in human IVF (Capalbo et al. 2011) and for human oocyte cryopreservation (Cobo et al. 2008). The method may present certain advantages and there is no need for expensive programmable freezers to obtain conditions for long-term cryostorage of the tissue/cells. Vitrification was applied to sheep hemiovaries with the VS1 solution as CPA consisting of DMSO (2.62M), acetamide (2.60M), PROH (1.31M) and polyethylene glycol (PEG) (0.0075M) in BM1 medium (Bordes et al. 2005). The ovarian tissue was equilibrated in concentration of 12.5%, followed by 25%, 50% and 100% of VS1 and then placed in cryovials containing 100% of VS1 to be plunged directly into LN at -196ºC. Warming was performed by placing cryovials in water at 37ºC for 10 min. Importantly, restored cyclicity was demonstrated 4 months to 1 year after grafting and 3/6 sheep became pregnant and delivered offspring. As expected, histological examination of the graft after delivery demonstrated very few follicles (6-58 follicles per graft) in the grafted tissue.

2.4.7.4 Vascular frozen ovary transplantation

A group from the Cleveland Clinic reported microvascular fresh ovary transplantation in the sheep (Jeremias et al. 2002) and later reported their results after autotransplantation of whole cryopreserved ovary using the identical method for anastomosis surgery as before (Bedaiwy et al. 2003) with comparison of results to avascular grafting of frozen-thawed cortical strips (frozen with Gosdens traditional protocol). These avascular grafts were placed in a similar anatomical location as the whole ovaries, close to the rectus abdominis The whole ovaries were perfused by a solution containing Leibowitz L-15 medium, 10% FCS and 1.5M DMSO followed by placement of the ovaries in cryovials which were further allocated to controlled-rate freezing. The temperature was reduced at 2ºC/min until -7ºC, when seeding was performed. Further reduction of temperature continued at 2ºC /min until -35ºC followed at 25ºC /min until -140ºC when cryovials were placed in LN. The thawing was done by placing

the cryovials in a water bath at 37ºC. Then, the ovaries were perfused with Leibovitz L15 medium containing 10% fetal calf serum for 20 min and end-to-end anastomosis on the deep inferior epigastric vessels was performed in all transplants. All vascular grafts showed immediate patency but only 27% of the transplants were patent at the end of the experiment 8 to 10 days after transplantation. Histology revealed large necrotic areas in the grafts of the non-patent group and the numbers of primordial follicles were severely reduced in the non-patent anastomosis group as compared to the patent group. An important finding was that the vasculature of the transplant showed signs of severe injuries with focal transmural necrosis of intraovarian vessels which may be due to cryoinjury of the vessels. The follicular viabilities, assessed by Trypan blue test, were around 80%, in the cortical transplantation and the patent whole ovary transplantation group, with a slightly higher density of apoptotic cells in the whole ovary group. It should be pointed out that this study only evaluated short-term (8-10 days) outcome.

The long-term results with the same microsurgical method were assessed in a study of ovarian viability approximately 5 months after transplantation of cryopreserved ovaries (Grazul-Bilska et al. 2008). A similar cryoprotocol was applied as in the previous study (Bedaiwy et al. 2003), apart from that the temperature from the seeding point was reduced at a rate 0.3 ºC/min until -40ºC in contrast to 2ºC/min until -35ºC, as in the previous study. Normal follicular development (assessed by histology) was demonstrated in only 25% of the transplanted ovaries and oocytes (n=3) from the larger follicles could be matured but fertilization in vitro did not take place The vasculature of the patent grafts appeared normal, with expression of marker proteins such as factor VIII, VEGF and smooth muscle cell actin (SMCA). Since the efficiency of the procedure was only 25%, and possibly even lower taking into account that mature oocytes did not fertilize, the study points to that major advances in this area are needed.

Key advances in this field may be both to the optimize the freezing procedure and also to modify the anastomosis technique as evaluated by an Israeli research group (Revel et al. 2004)**.** After perfusion through the ovarian artery with 1.4M DMSO in University of Wisconsin (UW) solution for 3 min, the ovary was slowly frozen by the directional freezing technology. This method secures identical cooling rate through the whole organ and allows constant cooling rate. The temperature was reduced by the rate of 0.6ºC/min until seeding and 0.3ºC/min to the temperature of -30ºC before plunging in LN. After storage rapid thawing was performed by placement of cryovials into a water bath at 68ºC for 20 s and 37ºC for 2 min. The anastomosis procedure was modified so that the ovary was transplanted to an orthotopic

site by end-to-end anastomosis to the remaining parts of the ovarian vessels. Three out of eight animals showed cyclicity at around 6 months after the procedure. At laparotomy 8 weeks after transplantation, adhesions were seen in only one sheep. In a follow up study presenting long-term results of this methodology (Arav et al. 2005), cyclicity in the three animals was demonstrated 2-3 years after transplantation. Oocytes collected from these animals could be parthenogenic activated with divisions until the 8-cell stage. In an extended long-term follow up study (6 years) of the three whole ovary transplanted sheep (Arav et al. 2010) 2/3 of ovaries responded to superovulation (FSH) and these ovaries were fully normal upon post mortem histological examination. The third animal that did not respond to gonadotropin stimulation had a fibrotic ovary with no follicles. The importance of the results of this study is that it shows that a cryopreserved whole ovary can survive for a long time and that it also indicates that this directional slow-freezing method can be beneficial. However, it should be pointed out that the ultimate end point of healthy offspring after an uneventful pregnancy has not been demonstrated with the use of this cryopreservation method.

There is only one reported live-birth after whole ovary cryopreservation and vascular transplantation in sheep (Imhof et al. 2006)**.** The freezing protocol of the ovaries used was essentially that of Gosden and coworkers (Gosden et al. 1994) but naturally the ovaries were cannulated and perfused with CPA prior to controlled-rate slow-freezing. Thawing was performed by exposure of the frozen ovaries in air for 2 min following by plunging in water bath at 25ºC for 7 min and perfusion by Roswell Park Memorial Institute (RPMI) medium to remove CPA. At the point of transplantation, the contralateral ovary was removed and the frozen-thawed ovary was orthotopicaly transplanted with microvascular anastomosis to the ovarian vessels using 9-0 sutures, which is a similar technique to that described above by the Israeli group (Arav et al. 2005). Four out of nine transplanted ewes demonstrated cyclicity and one pregnancy occurred with a healthy lamb delivered around 1.5 years after grafting. At examination of a number of ovaries 18-19 moths post transplantation, the primordial follicle pool was only 2-8% of that in non transplanted control ovaries. The authors suggested that the major follicle depletion occurred during freeze-thaw. However, this suggestion was based on results of histological evaluation, and as pointed out before this is probably an unreliable method to assess viability and should be combined with other methods. Nevertheless, this single large animal species live-birth after whole ovary cryopreservation is an important proof of concept, which should stimulate to further research in this area.

It is of importance to understand the mechanisms behind the low success rate of whole ovary cryopreservation and also what cell compartments are affected by the cryopreservation and

thawing procedures. It seems that vascular patency is directly correlated to follicular survival and ovarian function, as elegantly demonstrated in a study of heterotopic autotransplantation of the frozen-thawed whole ovary (Onions et al. 2009). The CPA and freezing method were essential to the traditional Gosden protocol (Gosden et al. 1994) and the anastomosis was by aortic patch and utero-ovarian vein to carotid artery and jugular vein as reported more than 40 years ago (Goding et al. 1967). Ewes that received fresh heterotopic transplants were used as controls. Seven out of eight cryopreserved transplants and three out of four fresh ovarian transplants showed patency at post mortem evaluation eight months after transplantation. However, despite vascular patency five out of these seven frozen-thawed ovaries with vascular patency did not demonstrate resumed cyclicity and 90% of follicles were lost in both fresh and frozen group 8 months after transplantation. The authors discussed the damage of endothelial cells by cannulation. The results further signify the need to improve both cryopreservation and surgical procedures.

After in vitro research on whole ovary cryopreservation of the sheep ovary by vitrification (Courbiere et al. 2005; Courbiere et al. 2006), the effectiveness of this method in vivo was evaluated. In the study only 1 out of 5 vitrified and thawed ovaries regained endocrine function (Courbiere et al. 2009) after orthotopic transplantation. Vitrification was obtained using VS4 solution (2.75M DMSO, 2.76M formamide and 1.97M PROH) in RPS-1 medium and the ovaries were perfused by stepwise increasing concentrations of CPA. At warming, the ovaries were held in LN vapor and then placed in water bath at 45ºC. The rationale behind this two step warming procedure was to avoid fractures during the glassy state (Pegg et al. 1997). A total follicular loss in the vitrified group was demonstrated one year after transplantation, with vascular thrombosis in 3/4 vitrified ovaries as compared to no thrombosis in the fresh group. The longer warm ischemic time in the vitrified group (median 287 min) as compared to the control group (median 129 min) may be one contributing factor but it is more likely that major injuries occurred during both freezing and thawing.

2.4.7.5 Cryopreservation research on ovine ovary

It is evident from the results presented above that there is a need for more systematic studies on the various stages of cryopreservation/transplantation procedures to improve the results. It will then also be required to evaluate the viability and function of the entire ovarian organ after each stage of the procedure.

The effect of different cryoprotocols with emphasis of CPA toxicity was studied utilizing the

sheep hemiovary model (Demirci et al. 2001). The hemiovaries were incubated for 10 min in different concentrations (2-10M) of DMSO and PROH and tested before and after slow-freezing-thawing (1, 1.5 and 2M CPA) concerning primordial follicle survival (Trypan blue test and histology). The follicular survival after incubation was higher as concentration of CPA decreased regardless of what kind CPA that was present. Thus, the results of this study support that relatively high CPA concentrations should be used but that there has to be an awareness concerning the intrinsic toxicity of the CPAs.

In vitro ovarian perfusion methodology, which was developed for assessment of intra-ovarian events of ovulation (Brannstrom et al. 1987) may be a method that can be used in these studies of frozen-thawed ovaries (Figure 8). This in vitro perfusion situation highly imitates the physiological in vivo situation, as shown by that such complex processes such as ovulation takes place during in vitro perfusion (Lofman et al. 1989). Recently, in vitro ovarian perfusion was applied in combination with live-dead assay, histology and cell culture to evaluate the viability of frozen-thawed whole sheep ovaries (Wallin et al. 2009). The ovaries had been cryopreserved in PROH by slow uncontrolled-rate freezing (Martinez-Madrid et al. 2004) and after that subjected to thawing in water bath at 37ºC. The in vitro perfusion results indicated that the function of the ovary was also compromised after cryopreservation in PROH, when comparison was done to fresh controls. It should be underlined that PROH was used as CPA and not DMSO, which is the most extensively used CPA in ovarian cryobiological research.

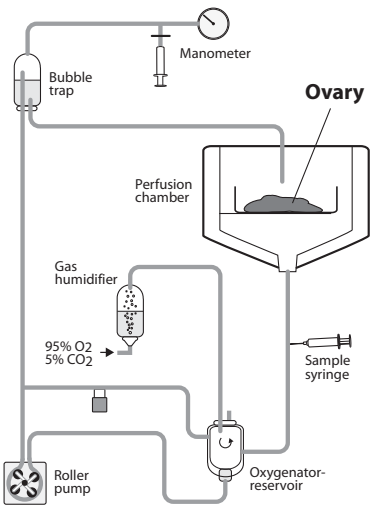

Figure 8 Schematic drawing of the in vitro perfusion system

As pointed out above, damage during cryopreservation may not only take place within the follicles but also in the vascular bed of the ovary or in the large vessels. It was speculated that perfusion with the anti-apoptopic agent sphingosine-1-phosphate (S-1-P) before cryopreservation-perfusion by 1.5M DMSO (Onions et al. 2008) may diminish this injury. Post thawing histology of the vascular pedicle tissue revealed arterial endothelial disruption in the cryopreserved ovaries, with most extensive damage in the area where the cannula had been placed, followed by the hilus region and with less extensive damage on the venous side. Addition of S-1-P did not demonstrate any protective effect. In conclusion, the study emphasizes the vulnerable state of the vasculature in whole ovary cryopreservation and that damage is mostly seen on the arterial side. By a battery of proliferation and apoptosis markers it was also shown that granulosa cells of antral follicles remain viable after cryopreservation.
In another study in the sheep, fresh ovarian cortical strips, previously incubated in S-1-P, were autografted in anterior abdominal wall (Hancke et al. 2009). The grafts were evaluated after 2 weeks by TUNEL test and histology. No protective effect of S-1-P was shown as compared to non treated grafts with anti-apoptotic agent.

As mentioned above, in vitro studies of vitrification of the whole sheep ovary preceded the trial in vivo. In an initial study it was demonstrated that the sheep ovary could be cryopreserved by vitrification and that the VS4 CPA solution (mixture of 2.75M DMSO, 2.76 M formamide and 1.97M PROH) was superior to the VS1 solution (mixture of 2.62M DMSO, 2.60M acetamide, 1.31M PROH and 0.0075M PEG) (Courbiere et al. 2005). Higher post thaw primordial follicular density (50% vs 23%) and percentage of histologically normal primordial follicles (53% vs 25%) were seen in VS4 group. However, it should be noted that endothelial damage of the vascular pedicle was more pronounced in VS4 group.

In a subsequent study, the thermodynamic properties of VS4 in RPS-1 medium was studied (Courbiere et al. 2006). The cooling rate was studied by differential scanning calorimeter when the thermocouples were connected to the ovarian cortex, the medulla and in the CPA solution. The estimated rate of cooling was above 300ºC/min, with the measured cooling rate of the cortex being slightly higher than that of the medulla. It was also shown that the mean cooling rate of CPA solution was higher as compared to the ovarian medulla and cortex. That can be explained by the differences in tissue architecture and vascularity of different compartments of the ovary that leads to uneven perfusion of the CPA. This finding also pointed out that it is unlikely that the ovarian tissue can be completely vitrified at the end of

the procedure. Ice crystal formation during warming was also detected, which indicated that the warming rate did not exceed the critical warming rate. However, contrary to the former study (Courbiere et al. 2005), damage of the endothelial layer of the ovarian vessels was not seen which possibly may be a result of the two steps thawing procedure to avoid ice crystallization (Pegg et al. 1997) as used in this specific study.

In a follow up study from the same group using VS4, it was reported that the cortex warms at the slightly higher rate than the medulla (Baudot et al. 2007). This again points towards irregular distribution of CPA and complexity of ovarian tissue. As in the previous, report, the primordial follicle survival rate of about 50% was demonstrated after warming. The authors detected in addition ice crystals during the cooling and discussed that maybe, a small proportion of ice can be acceptable for successful clinical application.

2.4.8 Non-human primates

Several non-human primate species have been used in research involving reproductive physiology and development of techniques later used clinically in reproductive medicine. The advantage of these experimental animals is that the physiology and anatomy are very close to the human. Even though many procedures are introduced in the human without appropriate tests in non-human primate species, it is advisable to include these animal models in preclinical research.

2.4.8.1 Avascular fresh ovary transplantation

The first life offspring after avascular transplantation of ovarian tissue in any primate species, including the human, was reported in the rhesus macaque, (Lee et al. 2004). After a laparoscopic bilateral oophorectomy small (1 x 3 x 4 mm) ovarian pieces were grafted into subcutaneous pockets or flaps next to muscle (arm, abdomen) or kidney. Follicle development occurred at several sites and mature oocytes were obtained after ovulation induction with human chorion gonadotropin (hCG). Oocytes were fertilized by ICSI and two of these were transferred laparoscopically to the oviducts of a surrogate mother with the result of pregnancy and live birth of healthy infant monkey. The arm and the abdomen were suggested by the authors as preferable sites for grafting due to easier procedure for oocyte pick-up.

Recently, a study on two cynomolgus monkeys evaluated the retroperitoneal iliac fossa, the omentum and subcutaneous tissue of the back as sites for autotransplantation of ovarian cortex (Igarashi et al. 2010). Both monkeys resumed menstrual cycle approximately 70 days after transplantation. After FSH stimulation for around 10 days, metaphase II oocytes could

be obtained both from the retroperitoneal and omental grafts in initial trials. These oocytes were fertilized by ICSI and resulted in five embryos of which four developed to the morula stage. Interestingly collection of mature oocytes could be performed from the omentum about 2.5 years after transplantation. Thus, the omentum as an alternative transplantation should be explored further.

2.4.8.2 Vascular fresh ovary transplantation

A technique to transplant an intact ovary by microsurgical vascular anastomosis in the rhesus macaque was reported 30 years ago (Scott et al. 1981). The ovarian vessels were dissected from their origin on the aorta/vena cava followed by creation of an aortic/caval patch and removal of the ovary. Four orthotopic autotransplantations were performed by anastomosis of the patches to either aorta/vena cava or to the common iliac vessels. During the post-operative transplantation period, menstruation was resumed 4-8 weeks after transplantation but no pregnancy was reported. Timed mating was performed during 6 month but pregnancy did not occur. One monkey received an allograft and was treated by azathioprine, but did not show resumed menstruation. The importance of this paper lies in that it demonstrates a surgical method that can be used in studies of whole ovary cryopreservation and transplantation.

2.4.8.3 Avascular frozen ovary transplantation

Surprisingly, there is only one non-human primate study that has evaluated function of cryopreserved ovarian cortex tissue in vivo. The tissue was cryopreserved by programmable freezing with 1.5M DMSO and both fresh and frozen-thawed strips were transplanted subcutaneously to the upper arm of cynomolgus monkeys (Schnorr et al. 2002)**.** Re-established menstrual cycle and ovarian steroidogenesis were found in 80% of the animals after fresh ovarian cortex transplants and in 50% of the animals after cryopreserved transplants, indicating that the cryopreservation technique may have detrimental effect on the tissue. In addition, the cryopreserved ovarian tissue responded to gonadotropin stimulation, but only one mature oocyte was harvested from subcutaneous ovarian tissue after trials of oocyte aspiration in 4 animals. In an attempt to provoke angiogenesis, one group of fresh grafted animals was treated locally at the transplantation site by VEFG for 2 weeks after grafting, but no beneficial effect could be demonstrated.

2.4.8.4 Cryopreservation research on non-human primate ovary

In a study on ovarian cortex from macaques (rhesus and cynomolgus) vitrification (3.4M GLY, 4.5M EG) and slow freezing (1.5M EG) were compared and tested whether post-

thawing coculture on feeder cells (mouse fetal fibroblast monolayer) and supplementation with FSH, insulin, transferrin and selenium would increase viability (Yeoman et al. 2005). The post-thaw viability (live-dead fluorescent staining) was comparable (around 70%) after the either of the two freezing methods, which was in addition only marginally lower than the follicular viability in fresh tissue (76%). Interestingly, post-thawing co-culture increased follicular viability, indicating rescue of partly damaged follicles. Taking into account the promising results of co-culture post-thawing, it is surprising that this approach has not been further explored in other studies of the field.

It may well be that conventional viability stains such as fluorescent stain (Wallin et al. 2009), Trypan blue (Fauque et al. 2007) and nuclear red (Kristensen et al. 2011) may not correctly determine whether frozen-thawed ovarian tissue is viable and that small follicles have the capacity to grow after thawing. In studies on ovarian cortex tissue of rhesus macaques, expressions of activin subunits and phosphorylated form of the signalling protein (Smad2) were investigated after controlled-rate slow-freezing with 1.5M EG (Jin et al. 2010). Activin subunits and the phosphorylated form of Smad2 are markers for early follicular development and these proteins were equally distributed in primordial and primary follicles of cryopreserved and fresh cortex. The evaluation was only based on immunohistochemistry (IHC) and in future studies in this field it would be beneficial to use quantitative methods such as immunoblotting. One interesting observation was that there was a higher rate of apoptosis in stromal cells as compared to oocytes and granulosa cells after cryopreservation. This finding supports the need for studies not only focusing on follicular survival but also on survival of other ovarian cell compartments that are necessary to support follicular development and ovarian function.

2.4.9 Human

Research activities on ovarian cryopreservation and transplantation are mainly aiming at optimization of the procedure for human use, even though the techniques have been suggested to be of importance for as well as preservation of endangered species (Santos et al. 2010) and for preservation of special strains of laboratory animals of rodents (Dorsch et al. 2004). Up until today, there have been no publications regarding transplantation of whole cryopreserved human ovaries, although a cryobank of whole human ovaries is established (Donnez et al. 2010).

2.4.9.1 Avascular fresh ovary transplantation

The first reports of human ovarian transplantation are dated more than century ago. Ovarian tissue from a donor was grafted on the uterine fundus of a patient with primary amenorrhea and in a second case, in a patient with severe pelvic inflammatory disease autografting on the stump of oviduct was performed (Morris, 1895). The first patient achieved pregnancy 4 months after grafting, but unfortunately this pregnancy resulted in miscarriage. In 1906, allografting into a peritoneal slit of the broad ligament was performed in a patient with polycystic ovaries that have been removed before grafting (Morris, 1906). This patient restored menstrual period after four months and subsequently delivered a healthy baby. To be noticed, no immunosuppression was administrated at that time. After this pioneering work and long time of quiescence, the research on human ovarian transplantation restarted in the 1990s.

The most extensive experience of this procedure is that of Sherman Silbers group, which conducted fresh ovary syngenic cortex transplantation in eight monozygotic twins (Silber et al. 2008). The donor and the recipient of each monozygotic twin pair were discordant for spontaneous premature ovarian failure. The cortical strips were around 1 mm thick and these strips were sutured to the remnant recipient ovary by 9-0 sutures. Ovulatory cycles resumed within 2.5 to 5 months in all cases and seven out of the eight patients have conceived naturally, with seven healthy babies from ten pregnancies. One report on autotransplantation of fresh tissue, is a case when both ovaries were inadvertently removed during caesarean hysterectomy due to placenta accreta in a 34-year old woman (Kodama et al. 2010). The surgeons grafted both ovaries by 3-0 sutures retroperitoneally on the psoas muscle at a position lateral to the external iliac artery. Initially E2 was undetectable but started to rise around four months after surgery. The patient was followed for up to 4.5 years and there were no further signs of hypogonadism during this period. Unfortunately, imaging diagnostics with evaluation of vascularisation were not performed. For research purposes the ovarian cortex of the left ovary was grafted on the contralateral ovarian medulla after the ovarian cortex of that ovary had been removed (Sanchez et al. 2007). Ultrasound and hormone evaluation demonstrated ovarian function in 9 out of 12 patients after 2 years. Although, the mean age of the patients was 41 years and the follicular pool was most probably depleted, this study points towards medulla as a possible orthotopic site for transplantation.

One case of allogenic avascular and orthotopic ovarian transplantation has been published (Mhatre et al. 2005). This case included transplantation of the ovarian tissue, which had been

divided into four equal parts, from a healthy sister to one woman with Turner´s syndrome and the initial idea was to perform whole ovary transplantation. However, the surgeons were not able to identify large enough veins for vascular anastomosis. The patient received standard immunosuppression with calcineurin inhibitor and corticosteroids. E2 levels showed a 2.5 fold increase after transplantation, as a sign of activity of the allogenic graft, but unfortunately no long-term follow-up data was reported.

2.4.9.2 Vascular fresh ovary transplantation

The group of around professor Silber has also been pioneers in transplantation of the ovary by vascular anastomosis (Silber, 1985). Transplantation was performed between monozygotic twins, where severe pelvic inflammatory disease of the recipient had destroyed the ovaries and Fallopian tubes. Since the recipient had scarred ovarian vessels, the tubal and ovarian artery was anastomosed to a distal ilical artery and the veins were connected to adjacent pelvic veins. No more information was published neither about the surgery nor postoperative period.

Vascular ovarian autotransplantation has also been used to move the ovary out of a position that will be covered by a radiation field in cancer treatment. In a patient with Hodgkin's lymphoma, who was planned to undergo pelvic irradiation, the left ovary with its vascular pedicle was removed and transplanted to the inner aspect of the patient's left arm (Leporrier et al. 1987) in order to remove it from the radiation field. The graft site was prepared by a subcutaneous implant to create a cavity. At surgery, the ovarian artery and veins (diameter of 0.8 mm and 1.5 mm, respectively) were isolated on a pedicle of about 5 cm. These vessels were anastomosed end-to-end on veins and artery of the left humoral vascular bundle. Ultrasound examination of the transplanted ovary demonstrated normal follicular growth, and menstrual cycles remained regular despite radiotherapy. Further long-term analysis showed that cyclicity has been normal for 16 years (Leporrier et al. 2002). However, the right ovary that was not evaluated and it is quite possible that this ovary was well protected by surgical transposition and maintained its functionality.

A similar approach for heterotopic ovarian autotransplantation was recently reported in a case of cervical carcinoma, where the ovarian artery and vein were anastomosed end-to-side to the brachial artery and basilic vein (Hilders et al. 2004). The diameter of the ovarian vessels was slightly higher than in the previous report (Leporrier et al. 1987) indicating individual anatomical variations. As in the previous study, the contralateral ovary was transposed from the pelvis. Doppler ultrasound scanning one year after surgery showed adequate blood flow in

both ovaries and cyclicity of the transplanted ovary was evident by monthly swelling of the upper arm.

Recently, microvascular transplantation to an orthotopic site was reported between monozygotic twins (Silber et al. 2008), discordant for premature menopause. End-to-end anastomosis to the ovarian artery and vein using 9-0 nylon sutures and 10-0 nylon sutures were used (Silber et al. 2008). The recipient became pregnant approximately fourteen months after surgery and delivered a healthy baby.

One report of allogenic vascular transplantation of a fresh whole ovary has been published and it involved transplantation between two sisters with the recipient having Turner's syndrome (Mhatre et al. 2005). The ovary, from the blood group-compatible and HLA-matched sister, was transplanted at the orthotopic site with arterial end-to-end anastomosis to the inferior epigastric artery and venous anastomosis end-to-side to the external iliac vein with concomitant treatment by cyclosporine and prednisolone. Follow-up for 2.5 years displayed regular ovarian cyclicity and normal development of secondary sexual characteristics.

2.4.9.3 Avascular frozen ovary transplantation and research on human ovarian cortex cryopreservation

Cryopreservation of ovarian cortex strips, followed by thawing and avascular autotransplantation, has emerged as a method of clinical use in fertility preservation in females. To date, at least 13 children have been born after ovarian cortex cryopreservation and avascular transplantation (Donnez et al. 2011). In addition, live births have been reported after frozen ovarian cortex transplantation between monozygotic twins (Silber et al. 2008). Furthermore, allografting of ovarian cortex between genetically non-identical sisters was performed with resumed ovarian function. Three patients, who were treated by chemotherapy and bone marrow transplantation from HLA-compatible sisters, received ovarian cortex from the same donors, with no need for immunosuppression due to chimerism (Donnez et al. 2010). Some of those 13 women with reported live birth received both heterotopic and orthotopic transplant (Demeestere et al. 2007; Andersen et al. 2008), and embryo development has in addition been demonstrated after oocyte retrieval from subcutaneous frozen-thawed graft (Oktay et al. 2004).

It seems as some degree of regained ovarian function is seen in most attempts of human ovarian cortex transplantation (Bedaiwy et al. 2008), with the function of the grafts persisting occasionally up to 4-5 years (Kim et al. 2009; Oktay and Oktem 2010). The limited survival

time of the graft may be attributed to the restricted amount of tissue that is transplanted, uneven follicular distribution (Schmidt et al. 2003) as well as suboptimal cryopreservation/thawing protocols. Also, the chemotherapy before ovarian tissue harvesting compromises the longevity of the graft (Donnez et al. 2011).

The major factor behind the short life-span of the graft seems to be injuries during the extended time (several days) of warm ischemia after autotransplantation. There exist several experimental studies that have evaluated the detrimental effects of ischemia on the ovarian cortex. In comparative study of human fresh and frozen cortical pieces that were grafted into nude mice, there was a similar decline in follicular density, a fact that demonstrates that ischemia is the major factor behind follicular depletion (Nisolle et al. 2000). Thus the period of warm ischemia before neovascularisation is essential for follicular survival and the major follicular loss takes place after grafting rather than after freezing, which is in line with results of several studies using animal models (Candy et al. 1997; Gunasena et al. 1997; Baird et al. 1999; Gosden 2000; Liu et al. 2002; Liu et al. 2008).

Neovascularisation of a transplanted ovary is a process which takes at least 2-3 days, as indicated by comprehensive studies of human ovarian tissue transplanted onto the chick chorioallantoic membrane (Martinez-Madrid et al. 2009) and reports of avascular transplantation of rat ovaries (Dissen et al. 1994; Israely et al. 2006). However, although vascularization seems to be completed within less than five days, the local oxygen pressure in the tissue continues to increase for a period of 10 days after xenotransplantation of human ovarian tissue (Van Eyck et al. 2009).

In general, human cortical grafts that have undergone cryopreservation with the use of modern protocols demonstrate survival rates of primordial follicles of > 50% (Newton et al. 1996) as well as normal morphology (Hovatta et al. 1996) based on evaluation by light microscopy. Follicular development has been demonstrated after grafting of human frozen-thawed ovarian tissue into immunodeficient mice. In these studies, folliculogenesis up to the antral follicle stage and with normal morphologic follicular appearance (Gook et al. 2001; Maltaris et al. 2007), development of corpora lutea to indicate completed ovulation (Kim et al. 2002) and presence of metaphase II oocytes (Gook et al. 2005) were demonstrated. However, the ultramorphology of the tissue may be influenced, since TEM revealed asynchrony between oocyte and granulosa cells of secondary human ovarian follicles developing in immunodeficient mice (Nottola et al. 2008). Nevertheless, the same research group demonstrated ultrastructurally normal primordial follicles, vascular and stromal tissue

after autotransplantation of human cortical strips (Camboni et al. 2008) and it is possible that the asynchrony was a consequence of the xenotransplantation. This finding is in addition corroborated by abnormal nuclear and cytoplasmic oocyte maturation in frozen-thawed human ovarian tissue transplanted into immunodeficient mice (Kim et al. 2005).

Several methods to decrease the damage of the ovarian tissue to ischemia-reperfusion have been suggested (Nugent et al. 1998; Sagsoz et al. 2002; Schnorr et al. 2002; Sapmaz et al. 2003; Kim et al. 2004) and it was discussed under sections where relevant research of each animal is discussed.

It should be noted that in the cases of live births after avascular human ovarian transplantation, the ovarian pieces were grafted orthotopically either into a previously created peritoneal window near to the ovarian hilus (Donnez et al. 2004; Demeestere et al. 2007; Roux et al. 2010) or onto the ovarian medulla (Silber 2009; Donnez et al. 2011). Since the majority of children (8/13) were born after natural conception, the orthotopic site should be regarded as a first choice for ovarian transplantation since assisted reproduction techniques are not needed then (Donnez et al. 2011). The rationale behind creation of a peritoneal window before grafting is that induction of the granulation tissue would increase the levels of locally produced angiogenic factors. There is indications from results after experiments in a rat model that this physical manipulation of the transplantation site shortens the ischemic period of transplanted ovaries (Israely et al. 2006).

Another issue that has been debated regarding human ovarian cortex cryopreservation is whether slow-freezing or vitrification is the optimal method to be used. Up until today, all children born after avascular ovarian cortex transplantation are derived from ovarian cortex that have been cryopreserved by the traditional slow-freezing protocol as used in the sheep (Gosden et al. 1994) or slight modifications of this method. Vitrification has been suggested to be a superior option but few comparative studies on human tissue between the methods concerning graft viability and function have been performed. In a comparative study using human ovarian tissue the outcome after slow freezing and vitrification protocols (PROH, EG, DMSO, PVP) were evaluated by LM and TEM (Keros et al. 2009). The results indicated that vitrification is superior regarding stroma protection, although similar follicular survival was demonstrated in both groups. However, another group presented better follicular survival and higher in vitro production of AMH and E2 in the slow freezing group as compared to vitrification (PROH, EG; sucrose) group (Oktem et al. 2011).

Even if 5 out of 13 live births were demonstrated after IVF (Donnez et al. 2011), it should be pointed out that IVF with oocytes from orthotopically grafted frozen-thawed ovarian cortical grafts in the human show low fertilization rates and higher rate of empty follicles (Dolmans et al. 2009).

2.4.9.4 Research on whole human ovary cryopreservation

There is a paucity of published reports regarding whole human ovary cryopreservation. In the initial study in this field three premenopausal human ovaries were subjected to uncontrolled-rate slow freezing with 1.5M DMSO as CPA (Martinez-Madrid et al. 2004). Follicular viability in frozen-thawed ovaries, as judged by vital staining, was decreased by about 25% as compared to fresh ovaries. However, as commented previously, this sole evaluation of viability is not fully appropriate to evaluate any tissue after cryopreservation. Thus, in a subsequent study TEM was used to evaluate the tissue after thawing, but surprisingly no major ultrastructural changes were demonstrated in any follicular cell type (Martinez-Madrid et al. 2007) as well as in cells of the vasculature. In addition, apoptosis, as assessed by TUNEL test and active caspase-3 IHC, could not be seen.

The outcome of slow freezing has been compared between cryopreserved-thawed whole human ovaries and ovarian cortical strips of two perimenopausal women (Bedaiwy et al. 2006). Standard freezing protocol utilizing DMSO for whole ovary (Bedaiwy et al. 2003) as well as that for cortical strips (Gosden et al. 1994) were used with viability assessed by Trypan blue exclusion test. Similar primordial follicle viability of about 80% were seen in both groups and this survival rate corresponds to that of the initial study on whole human ovary cryopreservation (Martinez-Madrid et al. 2004). IHC markers of apoptosis (Bcl-2, p53) as well as CD34 (marker of vascular endothelium) demonstrated similar expression of those proteins in frozen-thawed and fresh control tissue. However, it should be underlined that even if the ovaries in this study were perfused with CPA through the ovarian artery, they were subsequently bisected for cryopreservation as hemi-ovaries. This fact has also been commented by other researchers in the field (Martinez-Madrid and Donnez 2007).

A problem with uncontrolled-rate slow-freezing as used in the these studies mentioned above may be that the cooling rate is not controlled and in attempt to optimize cryopreservation of a whole human ovary the directional multi-gradient freezing device was used to create a set cooling rate of 0.3°C/min (Bromer and Patrizio 2009). The authors used a slightly modified sheep ovary cryoprotocol including 10% EG as a CPA as described before (Revel et al. 2004). Histological evaluation of thawed tissue revealed similar morphology and follicle numbers as

compared to fresh control. Subsequently, the same group cryopreserved three human ovaries together with their Fallopian tubes (Bromer and Patrizio 2009) by the same technique and post thawing histology showed unaltered tissue structure as compared to fresh controls. The findings of this study open up the possibility of cryopreservation of the entire adnexa, which could be transplanted by microsurgical techniques, as previously demonstrated for freshly transplanted adnexae. IHC analysis (caspase-3, p53) of eleven premenopausal human ovaries cryopreserved with the technique as in the previous experiments indicated slightly increased rate of apoptosis as compared to fresh control (Bromer and Patrizio 2009).

There are several possibilities regarding suitable blood vessels to perform whole ovary transplantation in the human. In a pursuit to identify adequate recipient vessels for this, the deep circumflex iliac and deep inferior epigastric pedicle were examined in fresh female cadavers (Ploteau et al. 2011). It was found that mean diameters of ovarian, deep circumflex iliac and deep inferior epigastric artery are 1mm, 1.25 mm and 1.51 mm, respectively. The mean diameters of ovarian and deep circumflex iliac vein were almost the same, 1.20 mm and 1.18 mm, respectively. Based on previous data best results after end-to-end anastomosis are obtained when the ratio between suturing vessels is not higher then 1:1.5 (Lopez-Monjardin and de la Pena-Salcedo 2000) and the authors proposed the deep circumflex iliac pedicle as a first choice for recipient vessels if the ovarian artery is not available. Importantly, the ovarian pedicle at ovarian harvesting should be at least 5 cm in length since at this point the vessels converge into a wider artery and one major vein.

Criteria for a first-class transplantation site should be based on easy surgical approach for microvascular surgery, adequate protection of the graft, similar calibre between recipient and gonadic vessels as well as possibility for ultrasound monitoring and oocyte pick-up (Bedaiwy and Falcone 2007). Furthermore, a laparoscopic technique for harvesting of the human ovary with its vascular pedicle for whole ovary cryopreservation has been reported (Jadoul et al. 2007). The conclusion that a great length of the infundibulopelvic ligament should be retrieved is based on results obtained from a study on female cadavers (Ploteau et al. 2011).

2.5 RISK FOR CANCER CELL REIMPLEMENTATION

An important issue that should be considered in both whole ovary cryopreservation and ovarian cortex cryopreservation is the risk of reimplantation of malignant cells which may be present in the frozen-thawed tissue. The risk of ovarian metastasis is high in leukemia, neuroblastoma and Burkitt´s lymphoma and moderate in adenocarcinoma/adenosquamous carcinoma of the cervix, colon cancer and breast cancer of infiltrative lobular histological

subtype (Sonmezer and Oktay 2004). One retrospective study analyzed 5571 autopsy findings of females younger than 40 years of age and found ovarian metastasis of various rates in patients dying of gastric carcinoma (56.6%), colon cancer (27%), lung cancer (23.4%), lymphoma (13.3%), endometrial cancer (13.1%) and in 8.4% of leukemia patients (Kyono et al. 2010). Regarding lymphoma, metastatic cells were demonstrated in 4.3% (5 out of 115) of patients with Hodgkin's disease and in 9.8% (5 out of 51) of those with non-Hodgkin's lymphoma. It should be mentioned that the stage/spread of the disease in these patients most certainly was higher than in patients requesting fertility preservation procedure. On the other hand, it is known from work on sentinel-node procedure in vulvar cancer (Oonk et al. 2010) that multiple sectioning and IHC against specific markers of tumor cells may be needed to detect micrometastasis, and the study mentioned above did only use conventional pathology techniques.

There are some specific studies on risk and detection of ovarian metastasis in patients with Hodgkin's lymphoma. In one study, IHC staining against Reed-Sternberg cells was not able to identify any metastatic cells (Seshadri et al. 2006). Similar findings of lack of metastasis in human ovarian tissue were obtained in a study when frozen-thawed tissue was grafted into nude mice (Kim et al. 2001). Recommended techniques of reasonable sensitivity to identify metastatic cells are polymerase-chain reaction (PCR) towards any gene that are specifically expressed in cancer cells (Meirow et al. 2008) and western blot analysis to detect the protein (Silasi et al. 2008). These investigations can be focused on special areas of the ovarian tissue by combined application of laser-capture micro-dissection of the tissue.

In a recent study, using disease specific PCR technique, leukemic cells were found in the ovarian tissue in 33% and 70% of chronic myeloid leukemia and acute lymphoblastic leukemia patients, respectively (Dolmans et al. 2010). In the same study, xenografting of frozen/thawed ovarian tissue of the patients suffering from acute lymphoblastic leukemia into nude mice demonstrated malignant cells in 5 out of 12 grafted animals. Also, PCR technique used in another study demonstrated cancer cells in thawed ovarian tissue in 2 out of 6 patients with acute lymphoblastic leukemia and in 7 out of 10 patients that suffered from chronic myeloid leukemia (Rosendahl et al. 2010). According to these findings, autotransplantation of ovarian tissue in leukemia patients cannot be recommended. It should be also underlined that the risk of reintroduction of malignant cells will be impossible to completely exclude and transplantation of the ovarian tissue will always be associated with a minimal risk of cancer cell transmission in all cases (Kim et al. 2001; Meirow et al. 2008). New emerging in vitro

maturation techniques, as mentioned in the Discussion section, may minimize the risk of retransmission.

3 AIMS OF THE STUDY

The general aim of this thesis was to develop and to use different model systems, with the developed methodology and the acquired results forming the base for further research in ovarian cryopreservation and transplantation.

The specific aims were:

- to evaluate the DMSO slow-freezing protocol for whole ovary cryopreservation in different models
- to compare different viability tests after whole ovary cryopreservation;
- to evaluate different intra-abdominal sites for ovarian cortex transplantation.

4 METHODS

4.1 PATIENTS

Human material was only used in Paper II. Five postmenopausal women subjected to hysterectomy for benign uterine disease were included in the study after written informed consent had been obtained. The inclusion criteria were to be at a clear postmenopausal state (> 2 years after last menstrual period), no previous ovarian surgery and macroscopically normal ovaries at surgery.

4.2 ANIMALS

Three different animal models were utilized. In Paper I, female adult (2-4 years old) sheep weighing 45-65 kg were used. In Paper III, female immature Sprague-Dawley rats (Harlan Laboratories, B.V.,Venray, The Netherlands) aged 26-28 days with a weight of 50-60 g were used. Paper IV utilized female adult olive baboons (*Papio Anubis*) weighing 11-14.3 kg at initial surgery.

4.3 HORMONES, CHEMICALS, REAGENTS AND MEDIA

Equine chorion gonadotropin (eCG; Intervet, International, Boxmer, Netherlands), human chorionic gonadotropin (hCG; Schering-Plough, Stockholm, Sweden), forskolin (7ß-acetoxy-8, 13-epoxy-1α, 6ß, 9α-trihydroxylabd-14en-11one; Sigma-Aldrich, St. Louis, MI, USA), medroxyprogesterone (Sigma-Aldrich) and RPMI cell-culture medium (Gibco, Invitrogen, Paisley, UK) were only used in Paper I. HEPES (Invitrogen-Gibco, Carlsbad, CA, USA), glutamate (Invitrogen-Gibco, Carlsbad, CA, USA), neutral red (2-amino-3 methyl-7-dimethyl-aminophenazoniumchloride; Sigma-Aldrich, Schnelldorf, Germany), fetal calf serum (FCS; Invitrogen-Gibco, Paisley, UK) and follicle stimulating hormone (FSH; Schering-Plough, Stockholm, Sweden) were used only in Paper III.

Human serum albumin (HSA; Sigma-Aldrich St. Louis, MI, USA) and M199 with Earl's salts (Invitrogen, Carlsbad, CA, USA) were used in Papers I and II. Collagenase (Sigma-Aldrich, Stenheim, Germany) and liberase (Liberase Blendzyme 3; Roche Diagnostics, Inidianapolis, IN, USA) were utilized in Papers I and III. Ringer-Acetate (RA; Braun Melsungen AG, Melsungen, Germany) and Leibovitz L-15 medium (Gibco, Invitrogen, Paisley, UK and Gibco, Invitrogen, Oslo, Norway) were used in Papers I, II and III. Dimethylsulphoxide (DMSO; Merck AG, Darmstadt, Germany), heparin (Leo Pharma AB, Malmö, Sweden and Astra Zeneca AS, Oslo, Norway) and xylocaine (Astra Zeneca, Mölndal, Sweden and Astra Zeneca AS, Oslo, Norway) were used in Papers I, II and IV. Bovine serum albumin (BSA;

Roche Diagnostic GmbH, Penzberg, Germany) was used in Papers I, II, III and IV and. Antibody against caspase-3 (AB4051; Abcam, Cambridge, UK), Tween 20 (Scharlau, Barcelona, Spain), MACH 3 rabbit alkaline phosphatase-polymer detection kit (Biocare Medical Inc. LLC, Concord, CA, USA) and chromogen Vulcan Fast Red (Biocare Medical Inc. LLC, Concord, CA, USA) were reagents in Papers III and IV.

4.4 ANESTHESIA, OVARIAN RETRIEVAL, OVARIAN VESSEL CANNULATION AND CORTEX PREPARATION

The ovaries were obtained by laparotomy (Papers I, II, III and IV), laparoscopy (Paper II) and also through the vaginal route (Paper II). In Paper I, the sheep was given diazepam (0.2 mg/kg) and pentothal (12 mg/kg) iv for anesthesia induction and 2-4% isoflurane was used for maintenance. In Paper IV, the baboons were induced into anesthesia by ketamine (100 mg for body weight <10 kg and 200 mg for body weight > 10 kg, Agraket, Agrar Holland BV; Soest, Holland) in combination with 10 mg xylazine (Ilium Xylazil, Troy lab Pty Limited, Smithfield, Australia) with maintenance of anesthesia by halothane. Isoflurane (4% for induction and 1% for maintenance; Ohmed Isotech, Stockholm, Sweden) in a mixture of air (600 mL/min) and oxygen (600 mL/min) was used for anesthesia in Paper III.

After ovarian retrieval, ovaries of Papers I and II were placed into RA at 4ºC and a Teflon cannula (22/24G) (Becton Dickinson Therapy AB, Helsingborg, Sweden) was inserted into the open end of the ovarian artery and tied in place with two sutures. The ovary was then gently flushed by a hand-held syringe with RA (4ºC) supplemented with 50 IU/ml heparin (Astra Zeneca AS, Oslo, Norway) and xylocaine 0.4 mg/ml (Astra Zeneca AS, Oslo, Norway). Cannulation in Paper III was obtained in situ through the aorta by 26G Teflon cannula (Terumo Sweden AB, Västra Frölunda, Sweden) followed by flushing, by a hand-held 1ml syringe, with RA supplemented with 50 IU/ml heparin (Leo Pharma AB, Malmö, Sweden) and 0.2 mg/ml xylocaine (Astra Zeneca, Mölndal, Sweden). In paper IV, the ovaries were placed in a glass-containing chilled (4ºC) Hank's Buffered Salt Solution (HBSS, Invitrogen AB, Lidingö, Sweden) and then divided into two hemi-ovaries with the division along the long axis of the ovary. The main part of the medulla was then removed using fine forceps and microsurgical scissors (S&T Microsurgery, Neuhausen, Switzerland). Each hemi-ovary was cut again twice and the remaining medulla was removed from the cortex using a surgical blade tip and fine forceps. Eight cortex fragments measuring approximately 4 mm length x 2 mm width x 0.8 mm depth were obtained from each ovary. Surgical loupes with magnification x3.5 and magnification x4 were used in Papers II and IV and operating

microscope (x6 to x40 magnification; Leica Microsystems, Wetzlar, Germany) was used in Papers I and III during the ovarian cannulation/preparation procedures.

4.5 CRYOPRESERVATION AND THAWING

After cannulations in Papers I and II, the ovary was flushed with either a solution containing 1.5M of DMSO (Merck AG, Darmstadt, Germany) with supplementation of 0.1M sucrose and 2% human serum albumin (HSA) (Sigma-Aldrich St. Louis, MI, USA) in Leibovitz L-15 medium (Gibco, Invitrogen, Paisley, UK) or an identical solution, but with the same volume of RA instead of DMSO. The two different solutions were in infusion bags at 4˚C and a pressure-infusion-device (Rudolf Riester GmbH & Co. KG, Jungingen, Germany) was used at 80 mmHg to perfuse the with a resulting flow rate between 1-2 ml/min.

The ovary was then placed in a 60 ml autoclavable straight-side, wide-mouth polypropylene jar (Nalgene Nunc, Rochester, NY, USA) with 5 ml of the same respective solution. This cryovial was placed inside a pre-cooled (4ºC) container (Cryo Freezing Container®, Nalgene Nunc International, Rochester, NY, USA) containing isopropanolol, and then placed inside a -80ºC freezer for 24 h, as described for slow freezing of the human ovary (Martinez-Madrid et al. 2004). The container was at that time placed in LN and stored (> 2 weeks) until thawing. The same protocol with minor modifications was applied in Papers II and III. The only differences in Paper II from Paper I was that DMSO solution was not supplemented with sucrose and HSA was purchased from Gibco, Invitrogen, Oslo, Norway. The differences in Paper III from Papers I and II were that the ovaries were frozen in either 1.5M or 7M DMSO in Leibovitz L-15 medium (Gibco, Invitrogen, Paisley, UK) without HSA and CPA was administered to the ovaries at increasing concentrations by 0.325M, 0.75M and 1.5M for 5 min each for those with final concentrations of 1.5M DMSO. Concerning ovaries with final concentration of 7M DMSO, the steps of DMSO concentrations (5 min each) consisted of 0.875M, 1.75M, 3.5M and 7M. After administration of CPA in the increasing manner as for slow freezing, the ovaries of the vitrification groups of Paper III were placed individually inside 60 ml autoclavable straight-side, wide-mouth polypropylene jars (Nalgene Nunc, Rochester, NY, USA) with 2 ml of either 1.5M DMSO or 7M DMSO. The container, with the cannulated ovary, was then plunged into LN and positioned there for 2 min before transfer to the cryotank.

In Papers I and II the ovary was thawed for 10 min by positioning the frozen vial in a 37ºC water bath. Each ovary was flushed for 10 min with flow rate of 1-2 ml/min at room temperature with three different solutions of 2% HSA in Leibovitz L-15 (Gibco, Invitrogen,

Paisley, UK or Gibco, Invitrogen, Oslo, Norway) containing 0.1M, 0.05M sucrose and no sucrose before the ovary was subjected to further experiments. In Paper III, in both the slow freezing and vitrification groups, the cryovials were placed into a water bath at 37 °C for 2-3 min to allow complete thawing. The ovary was then dissected out from the bursa and placed in Leibovitz L-15 medium at room temperature followed by rinsing in Leibovitz L-15 (Gibco, Scotland, UK) medium supplemented with sucrose at decreasing concentrations (1.0, 0.5, 0.25, 0.1M) with around 5 min at each washing step.

4.6 METHODS FOR EVALUATION OF OVARIAN TISSUE

4.6.1 In vitro perfusion

In vitro ovarian perfusion, as a method to test post-thaw viability of the ovary, was applied in experiments of Papers I and II. This method (Bjersing et al. 1981), is a closed circuit system where perfusion medium is pumped through the ovarian vasculature at a determined pressure. The perfusion medium consists of M199 with Earl's salts (Invitrogen, Carlsbad, CA, USA) plus 2% BSA (Roche Diagnostic GmbH, Penzberg, Germany) and is continuously equilibrated with 5% CO_2 in 95% O_2. The perfusion pressure was maintained at 80 mm Hg (Paper I) and 40-90 mm Hg (Paper II) which resulted in a flow rate through the ovary of around 2-2.5 ml/min. The ovaries were perfused for 2 h in Paper I and for 4 h in Paper II. In Paper I, the ovaries were stimulated after 1 h of perfusion by addition of the adenylate cyclase stimulator forskolin. The addition of forskolin, is a way to bypass effects through membrane receptors, and leads to a marked increase in cyclic adenosine monophosphate (cAMP) levels in the perfusion medium during in vitro perfusion of rabbit ovaries with this increase of the second messenger of luteinizing hormone (LH) also leading to induction of ovulations (Holmes et al. 1986).

4.6.2 Steroid assay

In Paper II, androstenedione was analyzed by an ELISA method (DRG International Inc., Mountainside, NJ USA) and testosterone was assayed by an immunofluorometric method (DELFIA, Wallac OY, Turku, Finland). The androstendione-assay had a detection limit of 0.019ng/mL. The intraassay-CVs were 9% at 0.3 ng/mL, 5.6% at 2.6 ng/mL and 4.7% at 4.7 ng/mL. The interassay-CVs were 9.6% at 0.2 ng/mL, 12.1% at 2.3 ng/mL and 8.8% at 4.4 ng/mL. The testosterone-assay had a detection limit of 0.3 nmol/L with intraassay CVs of <6% at 1.21, 10.2 and 27.6 nmol/L. Interassay-CVs were 14.2% at 1.21 nmol/L, 8.7% at 10.2 nmol/L and 5.6% at 27.6 nmol/L. E2 concentration was analyzed in Papers I, III and IV and kits were purchased from DELFIA; Wallac OY, Turku, Finland and from Abbot Architect,

Abbott Scandinavia AB, Solna, Sweden. The mean inter-/intra-assay coefficients of variations for E2 are 6.6 % /4.1 % for kit in Papers I and III and 1.4 /6.4% in Paper IV. Progesterone levels were assessed in Papers I and IV, with commercially available kits (DELFIA; Wallac, Turku, Finland and Abbot Architect and Abbott Scandinavia AB, Solna, Sweden). The mean inter-/intra-assay coefficients of variations for progesterone were 6.9% /13.0% in Paper I and 1.8% /5.5% in Paper IV

4.6.3 cAMP assay

In Paper I, the cAMP content of the perfusion medium was assessed using a modified DELFIA method together with a high sensitivity (detection limit 46 pmol) acetylation protocol (Wallac OY, Turku, Finland). All standard dilutions were performed in perfusion medium. The standard curve was diluted to give 2.8-180 fmol of cAMP in 50 µl of the final volume. Acetylation was accomplished by addition of 5 µl acetylation reagent to 180 µl of perfusion medium sample/standard. The samples were left for 10 min at room temperature and then 27 µl of a mixture of 1ml H_2O and 3.5 ml of 10x concentrated buffer for standards were added to all samples. The inter/intra-assay variations were 8.5/3.8% for 87.8 fmol (n=10), 9.6/8.6% for 9.4 fmol (n=9) and 12.2/14.4%, % for 3.3 fmol (n=10) cAMP.

4.6.4 Light microscopy

Light microscopy (LM) was used in Papers I, II, III and IV. The ovarian tissue was fixed in 4% buffered formaldehyde and stained with hematoxylin-eosin and then examined independently by two (Papers I, II and III) and three investigators (Paper IV) blinded to the experimental data.

The density of small follicles (primordial and primary) in Paper I was estimated by counting the number of these follicles within 8 square grids (0.16 mm^2) occupying a total area on the section of 1.28 mm^2. The grids were placed with one edge on the ovarian surface epithelium and the opposite side inside the cortex, with the area containing primordial/primary follicles within the grid. A total of 4-6 sections per ovary were counted and the mean of this was used as a data point.

4.6.5 Transmission electron microscopy

The small biopsies to be used for transmission electron microscopy (TEM) in Paper II were fixed in 3% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer. They were then postfixed in 1% osmium tetroxide, dehydrated and embedded in epoxy resin. Three non-consecutive

ultrathin sections contrasted with uranyl acetate and lead citrate were studied in an electron microscope (Philips 400).

4.6.6 Immunohistochemistry

Immunohistochemistry (IHC) to detect active caspase-3 was performed in Paper III and IV Active caspase-3 is a marker of early events in cells undergoing apoptosis (Porter and Janicke 1999). Biopsies were taken from the ovaries and fixed in 4% buffered formaldehyde, dehydrated and embedded in paraffin. Sections were made every 4 µm. The staining of the samples were done using a rabbit anti-human polyclonal antibody against caspase-3 (AB4051; Abcam, Cambridge, UK) according to the methodology previously described by Martinez-Madrid (Martinez-Madrid et al. 2007) with slight modifications. Sections were deparaffinized in xylene, rinsed in ethanol and brought to water through a series of decreasing concentrations of ethanol. Antigen retrieval with 10 mM citrate buffer (pH 6.0) was performed using a pressure cooker. The slides were incubated using a 1:200 dilution of the primary antibody in 0.02M Tris buffered saline containing 0.05% Tween 20 (Scharlau, Barcelona, Spain). For detection of the antibody, a MACH 3 rabbit alkaline phosphatase-polymer detection kit (Biocare Medical Inc. LLC, Concord, CA, USA) with the chromogen Vulcan Fast Red (Biocare Medical Inc. LLC, Concord, CA, USA) was used. The slides were co-stained with hematoxylin. Human tonsil tissue was used as a positive control. Ovarian biopsies incubated without the primary antibody were used as negative controls. Sections were assessed at 50 µm by two or three blinded observers using a Nikon EFD-3 microscope (Nikon, Tokyo, Japan) and a calibrated eye-grid at x100 magnification. The area of the grid was 2 mm^2.

4.6.7 Viability assays and follicle staining

In Paper I, a live/dead assay (Live/Dead® viability/cytotoxicity kit; Molecular Probes, Eugene, OR, USA) was performed to evaluate the proportion of cells surviving the freeze-thaw procedure. The ovarian sample was cut into small pieces and washed free from DMSO/RA. Enzymatic digestion was performed for 90 min at 37ºC with 5 ml of 0.04 mg/ml Liberase Blendzyme 3 (Roche Diagnostics, Indianapolis, USA) according to a protocol previously used for human ovarian tissue (Dolmans et al. 2006). The cell suspension was strained through a 100µm mesh cell-strainer (Falcon, BD Bioscience, MA, USA) and centrifuged (200xg). The pellet was then suspended in 100µl phosphate buffered saline (PBS) and incubated for 30 min at 37ºC with 100 µl of live/dead reagent at a concentration of 4 µmol/l calcein acetomethylester and 12.5 µmol/l ethidium homodimer-I in PBS. A small

volume of the incubated cell suspension was transferred on to a glass slide and covered with a cover glass. A minimum of 200 stained cells were counted under ultraviolet (UV) light in a fluorescence microscope and the proportion of live (green) and dead (red) cells was calculated independently by two observers and the mean of these was taken as a data point. The inter-observer variance was < 10 %.

The density of immature follicles (primordial and primary) in paper III was estimated in the areas of the cortex/cortico-medullary border in fresh and cryopreserved ovaries. Hemiovaries were divided by scissors into small pieces (around 0.5 x 0.5 mm) and these pieces were rinsed in PBS at room temperature. The pieces were transferred into a 35 mm culture dish (Nunclon; Nunc AS, Roskilde, Denmark) with 3 ml of McCoy´s 5a culture medium containing sodium bicarbonate and supplemented with 25 mM HEPES (Invitrogen-Gibco, Carlsbad, CA, USA), 0.1% BSA (Roche Diagnostic, Penzberg, Germany) and 2 mM glutamate (Invitrogen-Gibco, Carlsbad, CA, USA). This culture medium was then supplemented with 50 ug/ml of neutral red (2-amino-3 methyl-7-dimethyl-aminophenazoniumchloride; Sigma-Aldrich, Schnelldorf, Germany) solution and the ovarian tissue pieces were incubated at 37°C for 2-3 h in a humidified incubator. Neutral red is a cationic supravital dye, which at slightly acid pH gives a deep red colour. It has been demonstrated that neutral red readily passes the cell membrane and that it concentrates in lysosomes of viable cells (Allison and Young 1964), so that viable and non-viable cells can be distinguished (Borenfreund and Puerner 1985). After the incubation period, the medium was supplemented with a 60 µl of a mixture of liberase (Liberase Blendzyme 3; Roche Diagnostics, Indianapolis, IN, USA)) and collagenase IV (Sigma-Aldrich, Stenheim, Germany) to final concentrations of 0.2 mg/ml of liberase and 0.04 mg/ml of collagenase IV, in order to dissolve the tissue further. The pieces were incubated in this mixture for 30 min and the digestion was arrested by addition of 0.5 ml FCS (Invitrogen-Gibco, Paisley, UK). The ovarian tissues were then transferred by a pipette with a small volume of medium onto a glass slide. Several areas with small follicles of each ovary were counted within a squared grid area of 0.06 mm^2.

4.6.8 Cell culture

Cell culture was performed in Papers I and III, with slightly differences. In Paper I, dispersed ovarian cells were obtained by a method previously described for cultures of human ovarian cells, with collagenase and DNAse to digest the ovarian tissue after initial mechanical digestion (Runesson et al. 2000). The suspension of cells was passed through a 100 µm mesh cell-strainer (Falcon, BD Biosciences, Franklin Lakes, NJ, USA) and centrifuged (200xg) for

5 min. The pellet was washed two times in RPMI cell-culture medium (Gibco, Invitrogen, Paisley, UK) supplemented with 10% FCS. Cell viability was assessed by Trypan blue exclusion test and 5-10·10^5 live cells per well were placed in a 24-well plate. The cells were left for 24 h to attach to the plastic surface and after washing hCG (Schering-Plough, Stockholm, Sweden) was added. The cells were left for another 24 h after which the cell culture supernatant was collected and assayed.

In Paper III, the ovarian tissue was cut by scissors into small (approximately 1x1mm) pieces and transferred into separate wells of a 24-well plate (Nunc AS, Roskilde, Denmark) with 500 ul McCoy´s 5a culture medium containing sodium bicarbonate and supplemented with 25mM HEPES (Invitrogen-Gibco, Carlsbad, CA, USA), 10 % FCS (Invitrogen-Gibco, Carlsbad, CA, USA) and 2 mM glutamate (Invitrogen-Gibco, Carlsbad, CA, USA) in each well. The ovarian tissues were subjected to a preincubation period of 24 h and after change of medium, with addition of recombinant FSH (Schering-Plough, Stockholm, Sweden) at a concentration of 5 ng/ml, to another 24 h period.

4.7 STATISTICS

In Paper I, statistical analysis was made by Student´s t-test where a p-value of < 0.05 was considered significant. The data was also later revaluated with a non-parametric test as described in the Results and Comments section (see below). In Paper III, the data is expressed as medians, 25%/75% quartiles and ranges. Statistical comparisons were performed with the non-parametric Kruskal-Wallis test followed by multiple comparisons. A p-value of < 0.05 was considered significant. In Paper IV, hormonal levels and follicular densities are expressed as median and ranges. The follicular densities for each type of follicle were compared using the Friedman test. Multiple paired comparisons were done using the PAIRSetc module (I1) of WINPEPI (Abramson 2011). Categorical variables were compared using the McNemar test. Intra and inter-observer reliability analysis were performed on the follicular count using the intra-class correlation coefficient for absolute agreement and consistency using PASW 18.0 (IBM, Somers, NY, USA).

5 RESULTS AND COMMENTS

5.1 PAPER I

5.1.1 Background

Research and clinical development of ovarian cryopreservation as a fertility preservation method has been ongoing for many years, with also live-births demonstrated (Donnez et al. 2011). The method that has been used clinically is preservation of small slices or pieces of ovarian cortex, which have been frozen by a slow freezing protocol and then after cancer cure been transplanted back to the woman. Even though live births have been demonstrated with this method certain problems exist. The major difficulty is that there is a massive loss of primordial follicles during the warm ischemic period after vascular retransplantation of the cortex tissue (Baird et al. 1999)**.**

Another principle of ovarian cryopreservation that has been suggested is whole ovary cryopreservation (Bromer and Patrizio 2009) which would enable the ovary be connected to the vascular tree be immediately after thawing and then perfused by blood. Warm ischemic time could then be reduced from days to less than 1 h. Several animal models could be used for research towards an optimization of the whole ovary cryopreservation procedure that can be used clinically in the human.

In the present study we evaluated the sheep model for this research, since this species has been used for a long time in ovarian cryopreservation research and given that the ovarian vessels have reasonable sizes. Moreover, the studies have demonstrated live-birth after cryopreservation of a whole sheep ovary (Imhof et al. 2006) although with a low yield.

A critical aspect on experimental cryopreservation research is to really ascertain that the cryoprotected tissue is in fact viable after thawing. Several methods have been used. Concerning whole ovarian cryopreservation we proposed that it was important to demonstrate function of the whole ovary, since ovarian function obviously is dependent on cooperation between all ovarian cell compartments. We used an in vitro perfusion system, but for the restricted time of 2 h. However, the final and critical end point in whole ovary cryopreservation research is to evaluate the function and viability after retransplantation.

In cryopreservation there are several different CPAs that have been used, with PROH, EG and DMSO being the most widely used in research. In the present study we used DMSO to test and compare against a solution without CPA. Both of the solutions would be able to equilibrate with the tissue. In one previous study by the same group, PROH had been

evaluated utilizing the same protocol and it was found that PROH had some advantages (Wallin et al. 2009).

5.1.2 Results

In this study of sheep ovaries, with 4-5 ovaries in each group, we first evaluated the function during in vitro perfusion. Statistical analysis was made by Student's t-test. However, since a normal distribution could not be ascertained in this small material statistical evaluation has since then also been performed with the non-parametric Mann-Whitney's U test. The same statistical differences were found when this test was used. The ovaries were perfused for a pre-perfusion period of 60 min and then an exogenous adenylate cyclase stimulator was added to induce high intracellular levels of cAMP. The stimulation of this second messenger system of the gonadotropins would bypass any temporary defects occurring on the gonadotropin cell membrane receptor. After 60 min of perfusion we found higher mean levels of both cyclic AMP and progesterone than in the group that had been cryopreserved without DMSO. However, the differences were not statistical significant. A cell dispersate of the ovary was cultured for 48 h in the presence of different concentration of hCG. Both E2 and progesterone levels were higher although only progesterone levels were significantly higher than in the group cryopreserved in RA. Live-dead analysis also showed higher portion of live ovarian cells in the DMSO group (around 80%) than in the group without CPA (60%). LM was used to evaluate general morphology and small follicles in ovaries that had been perfused for 2 h. It was shown that the density of primordial/primary follicles was higher in those preserved with DMSO than in RA. It could also be seen that the RA ovaries were oedematous after perfusion, but the DMSO cryopreserved ovaries showed a fairly normal histology

5.1.3 Comments

The paper points towards the practicability to use the sheep ovary for further cryopreservation research. Although the sheep ovary is smaller in size than the human ovary it may be useful in initial studies. We were surprised that the viability and steroid production from those ovaries cryopreserved only in RA was reasonable. This shows that the equilibration process had worked but the results also demonstrate the additional benefit of addition of DMSO. Concerning the in vitro perfusion results, both the cAMP and progesterone levels were higher after 60 min in the presence of forskolin. It is likely that longer perfusion times would lead to that the difference would be larger and this is something which should be explored in the future. In previous studies we have used a similar perfusion method for perfusions up to 20 h concerning rat (Brannstrom et al. 1987) and rabbit (Holmes et al. 1985) ovaries and this

should of course also be possible in the sheep. The cell culture studies of ovarian cells showed that progesterone production was fairly high in the DMSO preserved ovaries. The production of progesterone was as high in the presence of hCG as under basal conditions. Since progesterone is produced mostly in luteal cells this implies that also the corpus luteum is partly protected by DMSO at cryopreservation. In the present study we used a well-characterized and widely used method to determine viability of cells. The live-dead assay (Martinez-Madrid et al. 2004) is an alternative to Trypan blue test (Fauque et al. 2007), which has also been used for studies of viability after cryopreservation. The reason to evaluate viability in a cell dispersate was to test not only the follicles but also the viability of other ovarian cells since the study concerns whole ovary cryopreservation with the importance of cryopreservation of all ovarian cells. Obviously further studies should be designed to study all the different cell compartments, including different follicular stages, also with diverse viability test such as used in Papers I and IV. Histology was evaluated by LM. Although we saw some differences it may well be that further major difference would be seen after longer perfusion times. It would also be interesting to evaluate the cellular morphology with TEM as used in Paper II. It should be mentioned that we did not specifically assay the vascularity in this paper, but since the in vitro perfusion method makes use of the vascular tree we assume that this was not damaged in a major way.

In conclusion, it was demonstrated that this standard DMSO slow-freezing cryopreservation protocol gives adequate equilibration of a whole ovine ovary. Furthermore, the results of this study could demonstrate that certain viability tests can be applied on the sheep ovary. This study and our previous study (Wallin et al. 2009) could be a starting point for further studies in whole ovary cryopreservation using the sheep with systematic evaluations with different in vitro techniques, including whole ovary in vitro perfusion during extended times. The aim would be to find optimal protocols for a high viability in vitro and then to test the optimal protocols, based on the in vitro findings, in vivo by vascular autotransplantation and long-term follow up.

5.2 PAPER II

5.2.1 Background

Whole ovary cryopreservation and auto-transplantation, as a method in fertility preservation, is still at a research stage. The research mostly involves animal studies in vitro or in vivo, even though a restricted number of studies in the human exist (Martinez-Madrid et al. 2004; Bedaiwy et al. 2006; Martinez-Madrid et al. 2007; Bromer and Patrizio 2009). The proposed

advantage of the whole ovary cryopreservation and transplantation procedure in comparison to avascular transplantation of cryopreseved ovarian cortex is that you would lose a minimum of the ovarian reserve up on retransplantation. However it poses special problems since the technique to effectively cryopreserve such large tissue mass as the entire human ovary is not yet fully developed and also that vascular anastomosis of the small ovarian arteries is a procedure which requires great microsurgical skills. Microsurgical skills are today uncommon among gynaecologist, but when tubal reanastomosis and other fertility surgery were more frequent, these skills were more common. Another obstacle with whole ovary cryopreservation is the increased risk of reintroducing malignant cells after the patient has been cured from her disease. The entire ovary is in this sense a fairly large tissue with possibilities for small metastatic foci.

Concerning whole ovary cryopreservation the most widely used animal models are the rat (Wang et al. 2002), rabbit (Chen et al. 2006), sheep (Revel et al. 2004) and bovine (Zhang et al. 2011). In some of these species successful retransplantation of cryopreserved whole ovaries have been accomplished (Imhof et al. 2006). When evaluating the suitability of these species for whole ovary crypopreservation research it should be stated that the only animal research model ovary that has a larger or comparable size to the human ovary is the bovine. However, folliculogenesis differs from the human, since there are several waves of follicles growing in the bovine cycle (Adams et al. 2008) in comparison to the human with one wave. For natural reasons premenopausal ovaries would be a very limited source for ovarian cryopreservation research in the human, since these ovaries are and should be very infrequently removed at gynaecological surgery for non-ovarian disease. One possible source of premenopausal ovaries would be those that are removed as prophylactic oophorectomy because the patient is a carrier of a BRCA1 or 2 mutations. In those cases it is of course very important to quickly evaluate the ovary histologically with multiple sections which would make whole ovary cryopreservation research unethical to perform on these ovaries. A perimenopausal ovary is a type of ovary which is more frequently removed at elective surgery. Patients undergoing hysterectomy may choose to perform a prophylactic oophorectomy due to future risk of ovarian cancer or because they want to avoid unnecessary benign ovarian surgery later in life because of ovarian cyst formation. However, the primordial follicle pool of these perimenopausal ovaries would still be very restricted. In this study we tested whether human postmenopausal ovary may be useful in cryopreservation research. The advantage with a postmenopausal ovary is of course that it is human tissue, but since the aim of the ovarian cryopreservation is preservation of the follicle pool the model is

not useful to evaluate all aspects of the cryopreservation protocol. Nevertheless much research could be done with the human postmenopausal ovary and several aspects of the ovary will still remain the same as a premenopausal ovary. This includes the stroma of the ovary and the vasculature.

5.2.2 Results

In this initial study of the postmenopausal ovary we used in vitro perfusion, LM and TEM to assess the viability of the tissue. Patients were between 57 and 79 years and were all operated for benign uterine disease. An initial observation was that it was more difficult to cannulate the ovary than expected. The cannulation time of the first patient (60 and 80 min) was long and it was also impossible to cannulate 2 of the ovaries of the following patient. In this study in vitro perfusion was used to assess ovarian stromal secretion androgens, which is the main steroid types that are produced in the stroma cells of a postmenopausal ovary (Fogle et al. 2007). The ovaries were not stimulated but secretions of steroid were assayed. No statistical comparisons were made due to the low number of ovaries included. However, the results of the study indicated that especially androstenedione secretion was higher from those ovaries adequately cryopreserved. The LM on tissue taken immediately after thawing confirmed that these were postmenopausal ovaries but there were no major difference in the tissue architecture after cryopreservation when the two groups were compared. One reassuring fact was that the TEM evaluation showed that there was no obvious ultrastructural damage after DMSO-preservation and it was also a general image that this ovary was better preserved.

5.2.3 Comments

This is the initial study to evaluate the use of the human postmenopausal ovary as a tool for cryopreservation research. Research in whole ovary cryopreservation has come to include many species, but the general aim is of course to cryopreserve human ovaries for fertility preservation. Thus research in the human has to be included during the process of development. The in vitro perfusion test showed that the ovary secreted steroids and it was indicated that the secretion was higher from those ovaries preserved with DMSO. This would indicate that the ovarian stroma and vasculature of the ovary was not extensively damaged during the cryopreservation process. Moreover, LM showed normal blood vessels and TEM showed that normal morphology was observed within in the endothelial cells. This initial study on human post menopausal ovary could be the starting point for further research. We suggest that larger groups should be included with longer perfusion times and that also the cells of the main ovarian artery as well as those of the intraovarian vascular network should

be examined closely to evaluate whether cryopreservation injuries may occur. It would also be feasible to include hormonal stimulation with hCG during ovarian perfusion and to compare different cryopreservation regiments.

5.3 PAPER III

5.3.1 Background

The rat and the mouse are the animal species which are most extensively used in biomedical research. This is due to that they are fairly inexpensive experimental animals and that they are not space-demanding for laboratory housing and caging. Since rat and mouse have been used extensively in research there is ample knowledge about the physiology and the biochemistry of all organs of these species. Concerning, the ovary knowledge about ovarian function and especially follicular development is probably larger in the mouse and in the rat than in any species. Due to the reasons described above, we thought it would be important to test whether a rat ovary would be practicable in research on whole ovarian cryopreservation. The drawback of using a rat ovary, when aiming to optimize procedures for the human, is of course that the size of the rat ovary does not correspond to the human ovary. Nevertheless, the rat ovary may be useful in this research because you can conduct experiments with large number of observations and also since inbred rats are used and the intra-individual variation should be fairly small. Previously we had experiments of in vitro perfusion of rat ovaries (Brannstrom et al. 1987) and the method to harvest the ovary with its connecting vasculature was based on what previously have been used in the laboratory. Since it has been shown in several studies that it is mostly the smaller follicles that survive the cryopreservation protocols we utilized immature rats that had not been stimulated by gonadotropins to initiate cyclic follicular growth. Preliminary studies had shown that these ovaries contained occasional small antral follicles but mostly smaller non-antral follicles. In this initial study we wanted to compare both vitrification and slow freezing with a low and high concentration of CPA. We are aware of that these may be far from optimal concentrations, especially concerning vitrification, but the reason we used these concentrations was to acquire useful background data of the extreme concentrations, for further use.

5.3.2 Results

In initial experiments we evaluated different methods to possibly obtain both of the bilateral ovaries from one rat. This included trials of cannulation from a caudal and from a cranial direction and different ligation techniques (Figure 9). However, due to anatomic reasons we

were not able to achieve an acceptable success rate concerning this and the experiments were designed to assess the right ovary in one specimen from each rat. The viability was determined by staining with nuclear red, which is taken up by viable follicles. The technique has been used in studies of follicular viability in the human (Kristensen et al. 2011) and in other species (Chambers et al. 2010). The viability was very low in the vitrified ovaries and that highest density of viable follicles was seen in the slow freezing group with low concentration of DSMO. However the density of these viable primary/primordial follicles was remarkably lower than in fresh ovaries. The E2 production detected was most likely from the secondary and tertiary follicles, since the smaller follicles would not have this capacity. Thus, the results indicate that some of these follicles survived the slow freezing procedure. Histological analysis showed that ovarian tissue was well preserved after incubation for 48 h, and importantly no blood was seen in the blood vessels. Apoptopic cells were however seen, but it was not major differences between the groups.

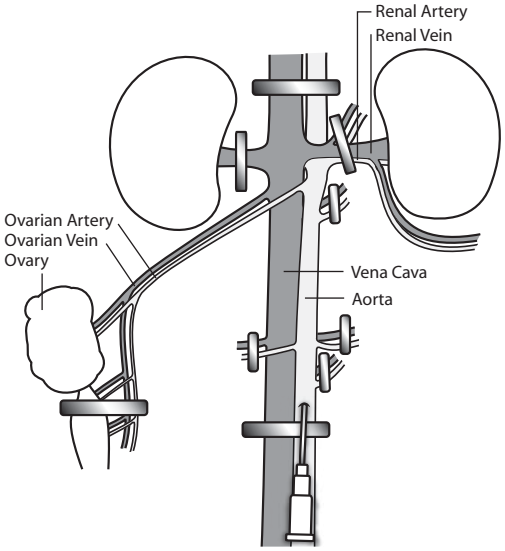

Figure 9 Schematic picture of the vascular anatomy of the rat. Ligations are indicated by schematic clips

5.3.3 Comments

Previously whole ovary cryopreservation has been accomplished in rats since cyclicity has been demonstrated after a vascular transplantation of whole ovaries (Qi et al. 2008) and also of avasculcar transplantation of whole ovaries (Dorsch et al. 2007). These studies have been

done with standard techniques and have all been done with slow freezing protocols. The CPAs that have been used are DMSO (Yin et al. 2003), PROH (Maltaris et al. 2006) and EG (Maltaris et al. 2006) for these studies in the rat. The rationale behind the present study was to evaluate whether the rat ovary could contribute in ovarian cryopreservation research to be a model system for tests of different freezing protocols. In this initial study we could demonstrate that ovaries cannulated and flushed well and were also preserved at least after slow freezing in a low/standard concentration of DMSO. In this study we used a novel viability test which proved to show very distinctly the density of viable follicles. However it remains to clarify by ultramorphological analysis or follicular culture with assessment of follicular protein/steroid secretion that these follicles truly represent viable follicles. The result indicated that vitrification used in the present study was not efficient. This will of course be evaluated more closely in future studies, where the more commonly used vitrification CPAs will be used.

5.4 PAPER IV

5.4.1 Background

Live-births in the human have been reported after avascular ovarian cortex transplantation of fresh ovarian grafts and of cryopreserved grafts (Donnez et al. 2011). These have been orthotopic transplantations to an existing but follicle-depleted ovary. In a non-human primate species one pregnancy with live offspring is reported after heterotopic ovarian cortex transplantation to a subcutaneous site of the abdomen (Lee et al. 2004).

Even though the clinical procedure of ovarian cortex cryopreservation and transplantation has been established there are several matters which may be studied more in detail and optimized until the procedure has reached a satisfactory level of effectiveness. This includes ways of minimizing warm ischemic injury after transplantation (Abir et al. 2011), but also to determine what the best site for avascular transplantation is. In some cases in the human an orthotopic transplantation site may not be available. These may be when there do not exist any remnant of an ovary to transplant the cortex on or there may be cases with extensive pelvic diseases or reconstructed pelvic organs, such as a continent ileostomy. Thus, in these cases an orthotopic transplantation site may be impossible to achieve or be unlikely to be successful. The present study represents the first systematic study in a primate species that has evaluated different intra-abdominal transplantation sites for ovarian cortex. We chose to start with non-cryopreserved fresh cortex, to avoid the possible extra damage by the cryopreservation and thawing procedures. The baboon was chosen as a suitable species

because of the relatively large size of the ovaries and also that tissue was available after a uterus retrieval procedure in ongoing uterus transplantation experiments. Another advantage with the baboon is that it is possible to monitor cyclicity by simply day-to-day observations of the perineal skin changes (Stevens 1997).

5.4.2 Results

The ovaries were subjected to around 10 minutes of warm ischemia and around 3 h of cold ischemia. The study that evaluated whether the cortex graft had any follicles after 3-6 months post transplantation and the follicle density in this graft showed that preservation after transplantation to the omental sites were better than the pelvic wall and especially the pouch of Douglas. The density of primary and primordial follicles was decreased in comparison to the fresh tissue. After transplantation to omental sites cyclicity was resumed and development of large antral follicles was seen, showing that the grafts had regained functionality.

5.4.3 Comments

This represents the first study in a primate species that has systematically looked at the suitability of different intra-abdominal transplantation sites for ovarian avascular cortex transplantation. The results of the study indicate that the omental sites were excellent transplantation sites. This result was not surprising since the greater omentum is a well vascularised location. An appropriate question is of course whether the greater omentum could be a suitable intra-abdominal transplantation site also in the clinical setting. We speculate that the transplantation procedure would be fairly easy when an omental location is used. A minimal invasive procedure for transplantation and oocyte pick up through the umbilicus would be possible. It would also be quite feasible to perform transabdominal ultrasound monitoring to detect follicular growth, which would be advantageous to the patient in comparison to the transvaginal route at orthotopic ovarian transplantation. Further studies should be conducted to compare different sizes of the ovarian transplants and also to evaluate the pelvic wall and the omental site specifically in other non-human primate species. An important future step should be to assess the potential for fertilisation of oocytes retrieved from an omental transplantation site in the baboon.

6 GENERAL DISCUSSION

This research project was initiated at Department of Obstetrics and Gynecology, Sahlgrenska Academy after discussions among senior scientists/clinicians, which identified the lack of a research program in the novel clinical area of fertility preservation. Although ovarian biopsies had been obtained locally from patients newly diagnosed with cancer during a period of at least 15 years (personal communication PO Janson), there had been no patient's request to retransplant any ovarian tissue and the cryopreservation protocols may not have been optimal since they were largely adopted from those used in embryo cryopreservation. Of importance was also that there had been no internal quality control studies to test the viability of the cryopreserved human ovarian pieces by the technique of retransplantation into immunodeficient mice (Aubard 2003) or by other means, in order to assess whether the tissue obtained at each time was still viable after freezing, storage and thawing. As pointed out in the introduction, the efficiency of the currently used ovarian cortex cryopreservation procedure with avascular transplantation and the associated extended warm ischemia is most likely low. Thus, there is a need for research models on other techniques and systematic research efforts in this area.

Due to the novelty of this basic research in fertility preservation, within the department, the thesis was largely designed to explore different models and viability tests, that later can be used in subsequent research within the research group. Whole ovary cryopreservation was chosen as suitable model system, since this was an identified future option in fertility preservation (Bedaiwy et al. 2006; Courbiere et al. 2006) and since it had not yet reached the stage of clinical introduction.

The animal models of the sheep and the baboon were used since tissue was naturally available as part of an ongoing uterus transplantation project within the department. Sheep ovaries of Paper I were removed as an element of the surgical retrieval procedure, after which the uterus was retransplanted with only one uterine horn together with the ipsilateral adnexae as an ovine utero-oviductal-ovarian autotransplantation procedure (Dahm-Kahler et al. 2008). The surgery in this sheep model was designed in this way so that vascular connections only had to be established on one side, thereby making the transplantation procedure easier. The functionality of the remaining graft, composed of a one-sided uterine horn and the common uterine cavity with its adnexae, has recently been demonstrated. Thus, in experiments involving natural mating after transplantation, pregnancies and offspring from the utero-tubal-ovarian autotransplanted ewes were demonstrated (Wranning et al. 2010). Ovarian tissue was

also available from baboons that were used in allogenic uterus transplantation research, as a continuation of the initial autotransplantation studies (Enskog et al. 2010). In the allogenic uterus transplantation procedure, the ovarian veins are used for vascular anastomosis together with the internal iliac arteries (L. Johannesson, personal communication). Since the ovarian veins are used jointly with the retrieved uterus, the ovaries and oviduct were excised early on during the retrieval procedure. Nevertheless, it was important for this non-human primate allogenic uterus transplantation research to ascertain that the uterine donors survived the surgery and that the postoperative health was fine, despite an extended surgical time of around 5-7h. Thus, the ovarian transplantation procedure was suitable in this setting of oophorectomized uterus donors, which were subjected to long-term follow up.

6.1 WHOLE OVARY VERSUS OVARIAN CORTEX CRYOPRESERVATION AND TRANSPLANTATION

In Papers I, II and III, whole ovary experiments on cryopreservation was performed in an attempt to find appropriate models that can be used in further research in this field. The long-term aim of the research is obviously to find a method that would enable efficient and safe use of whole ovary cryopreservation as a clinical human fertility preservation method. In Paper IV, ovarian cortex transplantation, which is today a clinically used method, was evaluated regarding different transplantation sites.

A major challenge regarding whole organ cryopreservation is to effectively cryopreserve such a large organ (Fahy et al. 2006) as the ovary. As a general rule, the success of cryopreservation is inversely related to the complexity of the biological system being frozen (Woods et al. 2004). It is of great value to minimize toxic injuries caused by CPAs and at the same time to protect the tissue from freeze-induced injury, as further discussed in the Introduction. In addition, cells inside the large tissue being frozen dehydrate in a slower rate as compared to superficial cells (Levin et al. 1977), which can have implications on survival of the ovary after thawing. Regarding cryopreservation of a whole human ovary, a major technical problem is to obtain satisfactory distribution of CPA in the entire ovary, which contains germ cells, granulosa cells, theca cells, stroma and blood vessels (Baudot et al. 2007). Furthermore, the oocyte and the granulosa cells are dependent on diffusion through the lamina propria because they are not directly surrounded by capillaries. Thus, distribution of CPA in and around these cells will be comparably slow. In contrast, ovarian cortex cryopreservation involves diffusion of the CPA from the external surface of the graft.

One comparative study using the sheep model has looked at outcome after cryopreservation by a similar cryopreservation method of cortical strips and whole ovary (Bedaiwy et al. 2003). The cryopreservation method that was used was controlled-rate slow freezing with 1.5M DMSO. It was found that the follicular viability was around 80% in both groups, with evaluation performed by Trypan blue test. In another study, with identical design using human ovarian tissue, primordial follicle count were comparable between the study groups as well as apoptosis that was also similar with fresh ovarian control tissue (Bedaiwy et al. 2006). Evaluation after thawing of the whole human ovary showed adequate follicular survival and absence of apoptosis after cryopreservation (Martinez-Madrid et al. 2004; Bedaiwy et al. 2006; Martinez-Madrid et al. 2007).

In contrast to the some results (Hreinsson et al. 2003; Kim et al. 2004) showing that the ovarian stroma is more sensitive than the follicles to cryoinjury during ovarian cortex cryopreservation, histological evaluation of stroma after whole human ovary cryopreservation did not reveal any abnormalities (Martinez-Madrid et al. 2004; Bromer and Patrizio 2009). This finding is in line with results obtained in Papers I and II, showing normal morphology of the stroma as evaluated by LM (Paper I) and LM and TEM (Paper II). Furthermore, androgen production after thawing of human postmenopausal whole ovary during subsequent in vitro perfusion was demonstrated in Paper II, where the stromal compartment is the only steroid producing site within the ovary.

It has been proposed that the major benefit of whole ovary cryopreservation followed by vascular transplantation, in comparison to avascular transplantation of ovarian cortical tissue, would be that the long period of warm ischemia at ovarian cortex transplantation (Arav et al. 2005) may be avoided and as a consequence there may be a higher proportion, than the calculated around 30% (Baird et al. 1999) of the primordial follicle pool in ovarian cortex transplantation that would survive the procedure. In whole ovary transplantation, the entire follicle pool of one ovary would be transplanted, as compared to the limited number of follicles at ovarian cortex transplantation (Bedaiwy et al. 2006). Recent data utilizing the whole ovary sheep model, demonstrated fairly poor follicular survival (0-8%) after vascular transplantation of frozen-thawed ovaries (Imhof et al. 2006; Courbiere et al. 2009). In those studies follicular survival was controlled 12-18 months after retransplantation and it may well be that the follicular pool survived the first weeks after transplantation but that other mechanisms led to a later follicular loss, regardless of that the vascular anastomosis may have worked. It is known from other studies of whole sheep ovary autotransplantation that common ischemic times of around 2-4.5 h (Courbiere et al. 2009) exist at vascular anastomosis of an

ovary, and it may well be that a large extent of the follicular loss occurs during these first hours of ischemia. Although not mentioned in the papers (Imhof et al. 2006; Courbiere et al. 2009) it is likely that the ovary was not kept chilled during the anastomosis procedure. In the experimental situations of Papers I and II, the ischemic times before connection to the perfusion apparatus were kept short. In Paper I, where the sheep ovary was used, follicular viability was assessed after perfusion for 2 hours. Follicular viability was higher after cryopreservation in DMSO than in RA. This experimental situation represents a short term reperfusion period after a very short warm ischemic time. Partly reassuring was the finding of increasing secretion of E2 and progesterone during the perfusion time.

Ischemia-reperfusion injury is the common name for these injuries, which can occur during cold ischemia, warm ischemia or during reperfusion of the organ with oxygenated blood. The mechanisms behind ischemic injury involve energy depletion (Wedenberg et al. 1995; Stoica et al. 2003) and at reperfusion oxidative stress (Kayyali et al. 2001; Zhang et al. 2007) which may cause cell death by creating alterations of lipids, DNA, enzymes and structural proteins (Jaeschke and Lemasters 2003; Kim et al. 2003). Expression of several inflammatory factors are initiated by hypoxia-sensitive response elements as well as NF-kappa beta, resulting in invasion of inflammatory cells (Vollmar et al. 1995) that may cause further tissue destruction and fibrosis. An extended time of cold ischemia over several hours is considered fairly undamaging but the warm ischemia is known to be the injurious time period. In solid organ transplantation surgery, the warm ischemic time is kept very short by flushing the organ with cold preservation solutions through the blood vessels to decrease the metabolic demands of the tissue. Moreover, care is taken to keep the ovary chilled when vascular anastomosis is established in situ in the recipient.

In the present study in vitro viability of a whole ovary was tested after fairly short ischemic times of 30-60 min in Papers I and II. Upon start of in vitro perfusion, the whole ovary of Papers I and II was flushed with oxygenated medium, thus ending the ischemic period but inducing possible injuries at reperfusion. The cold ischemic time at ovarian cortex transplantation of Paper IV was around 3 h. During this time ovarian tissue was hold at 4°C. It is of course not known how long the warm ischemic time after transplantation in Paper IV was but it can be assumed to be around at least 2-3 days, based on findings utilizing vascular corrosion cast methodology in a rat model (Dissen et al. 1994) and human ovarian cortex grafting onto avian chorioallantoic membrane (Martinez-Madrid et al. 2009). It may also be somewhat longer with ischemia, since ischemic times of around one week, was demonstrated in a study detecting appearance of new vessels by histology and magnetic resonance imaging

on rat ovarian tissue grafts into nude mice (Israely et al. 2004). However, a recently published study performing xenotransplantation into nude mice of human ovarian cortex tissue, with separate immunostaining of mice and human vessels, showed progressive formation of new vessels from day 5 followed by total vascularisation of central parts of the grafts by day 10 (Van Eyck et al. 2010). Angiogenesis that was visualized on day 5 originated from the host (nude mice) and was followed by appearance of human vessels indicating that both host and graft vessels are responsible for revascularisation. In the previous study of the same group, using an identical xenotransplantation model, a stepwise increase of local tissue oxygen pressure was demonstrated from day 5 (Van Eyck et al. 2009), pointing towards partial hypoxia during the first 5 days after grafting. In the experiments of Paper IV, the cortex tissue that was transplanted was thin which would be beneficial for early neovascularisation of the stroma around the follicle pool. It was also noted in the results of Paper IV that the follicular survival was different, according to the transplantation site. The results could imply that local factors in the omentum may be beneficial for ovarian neovascularisation, in comparison to the other transplantation sites. It may also be that the high density of large vessels that are clearly visible in the omentum is an advantage since the distance to arteries and veins of the omentum will always be fairly short.

A major difference between whole ovary cryopreservation and ovarian cortex cryopreservation is that two vascular anastomosis sites have to be established at vascular transplantation. Concerning the vascular surgery, common use of free vascular flaps in plastic surgery with the use of modern operating microscopes and the concomitant advancements in microsurgical skills have advanced the field of microvascular surgery. Older studies in humans have demonstrated that microsurgical digital replantation, using vessels with a caliber < 1 mm, may yield > 90% graft viability (Cheng et al. 1991). Typically, the vascular anastomosis surgery of ovarian transplantation would mean anastomosis of the fine caliber ovarian artery and one of the 2 to 3 ovarian veins. The diameter of the human ovarian vessels has been discussed in the Introduction**.** In Paper II, difficulties with cannulation of the ovarian arteries were encountered. However, it should be mentioned that the study was performed on postmenopausal ovaries with smaller ovarian vessels. The veins may however pose a significant problem. Even if the veins have much larger caliber than the ovarian arteries, they may be more difficult to manage, due to thin vessels walls and the poorly defined lumen, an experience encountered in the research on uterus transplantation in the mouse (Racho El-Akouri et al. 2002)**,** rat (Wranning et al. 2008) and pig (Wranning et al. 2006). In the studies of Papers I and II, the ovarian veins were simply opened and any problems related to the thin

walls and difficulties to define vein lumen were not encountered. The only human live-birth demonstrated after orthotopic microsurgical fresh whole ovary transplantation between identical twins utilized end-to-end anastomosis (Silber et al. 2008) of ovarian artery and vein. It should be mentioned that several reports of vascular sheep ovary transplantation, have shown that the rate of patent ovarian vessel anastomosis is fairly low (Bedaiwy et al. 2003). According to experience with experimental and clinical transplantation of the oviduct, end-to-side anastomosis of the ovarian vessels to larger vessels may be a better technique for long-term patency than end-to-end anastomosis (Cohen 2009). The vessel size discrepancy can be managed also by oblique cut, sleeve anastomosis and grafts (Lopez-Monjardin and de la Pena-Salcedo 2000). It is also important to point out that a huge quantity of training is necessary to obtain good skills in microsurgery for vascular anastomosis, including back table preparation of the specimen.

In relation to preservation of the ovarian vessel compartments at whole ovary cryopreservation, adequate protection of these was demonstrated after directional freezing (Arav et al. 2005), although arterial endothelial and smooth muscle damage were reported when slow freezing with manual seeding was performed (Onions et al. 2008).

Another problem in the research setting and later clinical introduction of whole ovary transplantation may be thromboembolism of the delicate vasculature and especially at the site of vascular anastomosis (Bedaiwy et al. 2003; Yin et al. 2003; Courbiere et al. 2009). It is not known for what duration treatment with low-molecular heparin or similar anticoagulants should be used after whole ovary cryopreservation. Moreover, addition of aspirin or another antiplatelet drugs may be beneficial in clinical vascular surgery (Vivarelli et al. 2007; Gao et al. 2010) .In the studies of whole ovary cryopreservation in the sheep treatment with anticoagulation drugs in form of low-molecular weight heparin were applied for either 3 (Grazul-Bilska et al. 2008) or 10 days (Courbiere et al. 2009).

As compared to vascular transplantation, surgery of cortex transplantation is technically much easier and may be carried out by laparoscopy (Donnez et al. 2008) or robotic-assisted laparoscopy (Akar et al. 2011). However, due to the restricted life span of the graft after avascular cortex transplantation, the transplantation procedure may have to be repeated several times. However, new data suggest that regardless of follicular loss due to ischemia and the cryoprocedure, the functionality of the graft remains up to 4-5 year (Kim et al. 2009; Oktay and Oktem 2010; Donnez et al. 2011). This absolute time is in the same range (6 years) as maximum follow up after whole sheep frozen-thawed ovarian transplantation (Arav et al.

2010). That study on the sheep ovary most likely represents the only attempt of long term follow up after whole ovary transplantation. The longevity of this transplant is reassuring, in the light of that the age of the sheep at ovary retrieval and transplantation was around 9 months and that the predicted reproductive life span of the sheep is about 6 years.

It is estimated that live birth can be obtained in about 20% of patients receiving frozen-thawed grafts (Smitz et al. 2010). Since 2004, live birth of 13 babies were reported after vascular grafting of frozen-thawed ovarian tissue (Donnez et al. 2004; Meirow et al. 2005; Demeestere et al. 2007; Andersen et al. 2008; Silber et al. 2008; Roux et al. 2010; Sanchez-Serrano et al. 2010) as a proof of concept of ovarian cortex freezing and grafting. This proof of concept in the human setting does not exist for whole ovary cryopreservation.

It should be underlined that one complete ovary is today usually removed for the purpose of ovarian cortex cryopreservation (Rosendahl et al. 2008; Jadoul et al. 2010). Thus, the follicular loss of the female patient would be similar when cortex cryopreservation or whole ovary preservation is performed. However, when low risk treatment is planned, the cortical biopsies should be considered (Jadoul et al. 2010). It is important to note that the gonadotoxicity of the treatment cannot be accurately determined and when one ovary is left undisturbed in situ, there is still the chance for remained function in the future. Concerning functionality after animal whole ovary transplantation, only a total of two pregnancies have been reported after transplantation of cryopreserved animal ovaries. These were accomplished in the rat (Yin et al. 2003) and the sheep (Imhof et al. 2006).

There also exist a risk of reimplantation of cancer cells in both ovarian cortex and whole ovary transplantation procedure as discussed in the Introduction section. The risk may be higher in whole ovary cryopreservation since more tissue, including the well perfused ovarian medulla, is included.

Taken together, the theoretical advantages of cryopreservation and transplantation of whole ovary have to be confirmed before clinical application. If (when) whole ovary cryopreservation and transplantation will be implemented in clinical praxis, it will be reasonable to cryopreserve some pieces of cortex in case of unsuccessful transplantation.

6.2 RESEARCH MODELS FOR OVARIAN CRYOPRESERVATION AND TRANSPLANTATION – ADVANTAGES AND DISADVANTAGES

A great deal of research on whole ovary cryopreservation, ovarian cortex transplantation and transplantation has been performed in animal models as presented in the introduction section

in detail. The majority of these research studies aimed at clarify questions of relevance for fertility preservation in human. However, some research had the purpose to demonstrate principals important for strategies to rescue endangered species (Santos et al. 2010) and rescue of certain specific strains of animals (Dorsch et al. 2004). Due to the scarcity of human premenopausal ovarian tissue for research purposes as well as ethical barriers in such research there is a need to find model that are reasonably comparable to the human. In general, animal models have to be similar in biochemical, physiological and anatomical characteristics to the human so that the results can be applicable to human conditions (VandeBerg 2004). Criteria for high suitability as a research tool for the human would be similar tissue architecture and size as of the ovary as well as being a monoovulatory species with the primordial follicles distributed superficially in cortex (Gerritse et al. 2008).

The rodents, the mouse and the rat, are utilized extensively in research on ovarian cryopreservation and transplantation as exemplified by early (Gunasena et al. 1997) and recent (Qi et al. 2008) studies. It should be pointed out that these are the species, where research on folliculogenesis has been most extensively carried out. The rat was also explored as a possible research model for whole ovary cryopreservation in Paper III. The rodents are of course small animals with proportionally tiny ovaries, as compared to the large experimental animals and the human. The ovarian size will thus be a limiting factor, when results concerning ovarian cryopreservation are extrapolated to the human. The volume of mouse a ovary is about 2 mm^3 (Candy et al. 2000) and the weight of the immature rat ovary is about 20 mg (Paper III). However, the ease of avascular transplantation of a whole ovary to several heterotopic sites as well as into the bursae, as extensively described in the Introduction section, is advantageous. An apparent advantage concerning the rodents is the low cost and the fact that several strains with certain characteristics exist. All the inbred strains would have a low inter-individual variation, which of course is important when comparative analyses are performed. In addition, transgenic and gene-deletion techniques are readily available in the mouse and these techniques are slowly getting available in the rat model. Specifically, conditional transgenic or gene-deleted animals will be useful in this research, since that will create a possibility to overexpress or turn off different genes in the ovary or at the site of transplantation. The ovarian tissue of both the mouse rat is softer than that of larger animals, including the human, which is not only depending on the size difference (Smitz et al. 2010). The reason for this dissimilarity in tissue texture is most likely that the collagen content of the rodent ovary is fairly low. In the events of that animals are used when they are young (prepubertal) the ovaries will not contain any large antral follicles, a fact that was utilized in

Paper III. Albeit the rats in that study was small the size of the animal allowed isolation of the vascular tree of the ovary, with subsequent cannulation for flushing and perfusion (Figure 9). It is of importance to emphasize that the rodents would be suitable experimental animals at a first step in ovarian cryopreservation research and that results obtained in rodents then should be tested in large animal models (Paper I), including a primate species (Paper IV) as the last step before clinical introduction of a procedure.

The rabbit ovary has been used extensively in research on ovarian physiology (Wallach et al. 1984; Hesla et al. 1997) and the amount of knowledge concerning follicular development and oocyte maturation is great in regards to the rabbit species. The rabbit has also been applied in the research field of ovarian cryopreservation (Neto et al. 2008; Gosden et al. 2010) and transplantation (Denjean et al. 1982) but it is less commonly used than the rodents in this research. The rabbit ovary is elongated in shape and measures about 15 x 10 x 5 mm (Dahm-Kähler, personal communication). Rabbits are considerably more expensive than rodents but they are regarded as easy to handle. Due to relative short interval between generations (5-6 months), it would be possible to study potentially negative impact of cryopreservation on the second generation (Neto et al. 2008). Another advantage with the rabbit is that the vasculature is larger than that of the rodents with possibilities to cannulate the ovarian artery directly (Holmes et al. 1986), so that two ovaries from each animal can be obtained for research on whole ovary cryopreservation. In that way the contralateral ovary could be used as a control ovary and this would be an advantage to minimize natural inter-ovarian variations.

The bovine ovary may be an alternative as a research tool in ovarian cryopreservation. One paper compared the volume of the bovine ovary (Gerritse et al. 2008) to the volume of human ovaries (6.5+/-2.9 cm^3) (Munn et al. 1986). Regardless, the larger size of the bovine ovary (14.3+/- 5.7 cm^3) as compared to the human ovary, the authors recommended this model due to monthly and mono/diovulatory cycle as well as similar tissue architecture to the human ovary. In that study distribution of Indian ink in the small vessels was used as an index of extent of perfusion of the ovary. It was demonstrated that the bovine ovary exhibited superior perfusion rate during follicular phase as compared to the luteal phase (Gerritse et al. 2008). Another advantage of bovine ovaries for this type of research is that slaughterhouse material can easily be obtained, although you have to be aware of that data on cycle stage is not available.

The sheep ovary has been extensively used in previous research on ovarian cortex cryopreservation and whole ovary cryopreservation (Bromer and Patrizio 2009). This experimental model was also used in Paper I. Although the sheep ovary is considerable smaller (1.0+/-0.4 cm^3) (Gerritse et al. 2008) than the human ovary (Munn et al. 1986), it has been stated that they have a comparable tissue composition with a thin fibrous stromal cortex rich in primordial follicles (Revel et al. 2004). However, the sheep is a seasonal breeder (Salle et al. 2002) showing cyclicity only parts of the year and this has to be taken into account when performing experiments that assess cyclicity after transplantation.
The pig is used extensively in biomedical research and this model has also been evaluated for ovarian cryopreservation research. The porcine ovary has volume (7.3+/- 2.2 cm3) (Gerritse et al. 2008) which is fairly similar to the human but the ovary seems to be less fibrous than the human ovary. It should also be mentioned that the pig ovary is multi-ovulatory. Pig ovaries are easily available from local abattoirs and should be useful tools at optimization of cryoprocedures as well as microsurgical training.

There only exist few reports utilizing non-human primate ovarian tissue for research on ovarian cryopreservation. Thus there is certainly a place for further non-human primate research in this area, as also carried out in Paper IV. The rhesus macaque was used for fresh whole ovary transplantation (Scott et al. 1981), and the cynomolgus macaque was used for experiments involving cortex transplantation (Igarashi et al. 2010). The size of the females of these subspecies is from around 4 to 8 kg, with a corresponding size of the ovary. The larger (12-15 kg) baboon female was used in experiments involving in vitro follicle maturation (Xu et al. 2011), but also ovarian tissue transplantation (Paper IV). There exist similarities of the baboon to the human regarding internal genital anatomy and ovarian steroid secretion (Stevens 1997) and baboons have been used extensively in research about reproductive physiology (Pepe et al. 2006; Nyachieo et al. 2007; Bocca et al. 2008), endometriosis (D'Hooghe et al. 2009) as well as uterine transplantation (Enskog et al. 2010). Baboons are readily available for research in certain areas of Africa (D'Hooghe et al. 2009) and they should be regarded as good modes in research towards whole ovary cryopreservation and transplantation as well to improve results after ovarian strips cryopreservation and transplantation. In general, non-human primate ovaries are the most suitable testing systems before application of any new treatment in the human because of the great similarities to the human.

Paper II introduces the human postmenopausal ovary as an alternative experimental model. Naturally, one is aware of the main drawback that there is a lack of follicles in this ovary. However, this model can be applied to evaluate stroma and the vascular network within the ovary and of the ovarian vascular pedicle. The ovarian stroma is essential for ovarian function as a source of theca cells during the premenopausal stage as well as to provide 3D physical environment around the follicles (Woodruff and Shea 2011). Moreover, any cryoprotocol has to be optimized to protect the ovarian vessels (Kim 2010). Human ovarian tissue of women in reproductive age was utilized in previous research on ovarian cryopreservation (Gook et al. 1999; Martinez-Madrid et al. 2004; Bedaiwy et al. 2006; Sanchez et al. 2007; Bromer and Patrizio 2009; Keros et al. 2009) but it is only at special circumstances that human premenopausal ovaries can be used in research. Examples of these are ovaries remained due to that the patient is carrying BRCA I or II mutations or ovaries removed from male-to-female transsexuals as part of the sex-change surgery (Van Den Broecke et al. 2001). Ovarian tissue surrounding cysts (Schubert et al. 2005) has also been proposed as suitable for research.

6.3 ALTERNATIVE METHODS FOR FREEZING OF WHOLE OVARY

Several methods have been used in attempts to successfully cryopreserve a whole ovary and in this research; ovaries of different animal species were used.

Thus, the whole ovary have been subjected to either controled-rate slow freezing (Candy et al. 2000; Wang et al. 2002; Bedaiwy et al. 2003; Bedaiwy et al. 2006; Chen et al. 2006; Imhof et al. 2006; Onions et al. 2008), uncontrolled-rate slow freezing (Martinez-Madrid et al. 2004; Wallin et al. 2009; Zhang et al. 2011), directional slow freezing (Revel et al. 2004; Bromer and Patrizio 2009) or vitrification (Courbiere et al. 2005; Deng et al. 2009; Zhang et al. 2011). Papers I, II and III utilized uncontrolled-rate slow freezing with the ovaries placed in -80ºC freezer for 24 h, with expected cooling rate of 1ºC/min.

Vitrification is an ultrarapid freezing method using a highly concentrated CPA solution to create a glass like state without formation ice crystals. It has been proposed as a better alternative for large tissue cryopreservation (Fahy et al. 2006) than slow freezing. However, the results after vascular transplantation of whole vitrified ovary were poor (Courbiere et al. 2009). In that study a total follicular loss was demonstrated 1 year after transplantation of vitrified sheep ovaries. However, in a recently published study on bovine ovaries comparing vitrification and uncontrolled-rate slow freezing showed, the results indicated superior results of vitrification (Zhang et al. 2011). This was demonstrated by higher follicular viability,

higher number of morphologically normal follicle and larger steroid secretion from cultured ovarian tissue. In the present study, vitrification was used in the experiments of Paper III. It was a pilot study towards optimization of protocols and evaluation of DMSO toxicity. One is aware that high concentration of CPA and rather combination of permeable and non permeable CPAs is preferable (Fahy et al. 2004). The plan in the future is to use the rat whole ovary system and to add permeable and non -permeable CPAs in an attempt to neutralize toxicity of the whole solution (Fahy 2010).

The species that have been used in experiments involving controlled-rate slow freezing are the mouse (Candy et al. 2000), rat (Wang et al. 2002), rabbit (Deng et al. 2007), sheep (Grazul-Bilska et al. 2008), pig (Imhof et al. 2004), cow (Zhang et al. 2011) and human (Bromer and Patrizio 2009). To my knowledge there do not exist any comparative studies between the different slow freezing procedures for whole ovaries.

The most widely used slow freezing method is based on a study on mouse oocyte (Carroll and Gosden 1993) and one on sheep cortical tissue (Gosden et al. 1994). The animal tissue characteristics differ from the humans, as stated by Gosden in a recent paper (Gosden et al. 2010). This method of controlled-rate freezing includes formation of extracellular ice, induced by seeding, as an integral part of slow freezing procedures for small samples, that may be detrimental when freezing multi-cellular systems (Kim 2010). It is known that it is much easier to achieve appropriate freezing and thawing rates in cell suspensions than in larger tissue mass (Karlsson and Toner 1996). However, this protocol, with minor modifications, was extensively used for whole organ cryopreservation with reported pregnancy after transplantation (Yin et al. 2003; Imhof et al. 2006). The pregnancy rates in these studies were low, with a pregnancy rate of 1/9 in the sheep (Imhof et al. 2006) and 1/7 in the rat (Yin et al. 2003).

Uncontrolled-rate freezing was used in Papers I, II and III. This slow freezing protocol was applied for bone marrow cell cryopreservation (Stiff et al. 1987; Almici et al. 2003). The comparative study between control-rate and uncontrolled-rate freezing on peripheral blood progenitor cells demonstrated similar results regarding engraftment and clinical outcome (Montanari et al. 2003). When mouse ovarian tissue were cryopreserved with 1.5M DMSO with controlled and uncontrolled freezing rate techniques followed by grafting under the kidney capsule, histological evaluation demonstrated similar follicular survival in both groups (Cleary et al. 2001). This method was later utilized for whole human ovary freezing (Martinez-Madrid et al. 2004) and resulted in high follicular survival (75%) as evaluated by

viability test and normal ultrastructure as well absence of apoptosis (Martinez-Madrid et al. 2007). The estimated cooling rate of the human ovary would be around 1°C/min (Martinez-Madrid et al. 2004) and it is likely that the cooling rate of the experimental ovaries of Papers I, II and III differ and that they are higher, since these ovaries are smaller than the human premenopausal ovary.

A high proportion of live cells after thawing were demonstrated in Papers I and III. Steroid production during in vitro perfusion as well as after cell culture pointed toward that uncontrolled freezing can be applicable in clinical work (Papers I, II and III). However, the main theoretical disadvantages of uncontrolled-rate freezing are ice formation at unpredictable temperature and that of a supercooling state, which may be detrimental for living cells (Arav and Natan 2009). The latter can disturb cell dehydration and at same time extracellular ice crystals can provoke mechanical damage.

Based on basic knowledge in cryobiology directional freezing may be the most appropriate alternative for slow freezing of whole organs such as the ovary (Arav and Natan 2009; Arav et al. 2010). Ovarian function after transplantation in sheep lasted for 6 years when directional freezing method had been applied (Arav et al. 2010). This method allows equal heat transfer and propagation which is not possible to obtain during vitrification (Baudot et al. 2007). Importantly, the ice formation occurs in correspondence to the freezing point of the CPA t so that supercooling is prevented (Arav and Natan 2009). It would be interesting to perform a study which compared outcome after directional and uncontrolled-rate freezing of a whole ovary. It should be stated that both vitrification and uncontrolled-rate freezing are in fact a much simpler and much less expensive methods than directional freezing.

Others parameters such as CPA concentration and administration, duration of perfusion with CPA, cannulation, design of cryovials and thawing should also optimized for each specific freezing technique. Paper III presents a pilot study toward optimization of each step in cryopreservation protocol.

6.4 THAWING

The basic principles of thawing are presented in the Introduction section and there is no established golden standard protocol for thawing. Understandably, different freezing techniques require specific thawing techniques. The exact mechanisms behind cell damage during the thawing procedure is not known (Karlsson and Toner 1996), especially for a larger tissue mass. However, rapid thawing rate may be helpful for rapidly cryopreserved specimens

and in opposite, slow thawing is more appropriate for slow frozen samples (Mazur 1984; Karlsson and Toner 1996). Thawing/warming rate must exceed critical thawing/warming rate for a cryoprocedure to be successful (Baudot et al. 2007) and research should be focused to determine this for each specific tissue.

In the present thesis, thawing was carried out in a water bath at 37ºC for 10 min (Papers I, II) and 2-3 min (Paper III) following by flushing with Leibovitz L-15 and sucrose at decreasing concentrations. Positioning of frozen ovaries in water bath at 37ºC was accepted from previously reports (Bedaiwy et al. 2003). Decreasing concentration of solution was used to reduce osmotic stress during removal of CPA as has previously been described (Courbiere et al. 2005; Grazul-Bilska et al. 2008; Wallin et al. 2009).

To my knowledge, there are no studies comparing different thawing protocols after whole ovary freezing. The rat model (Paper III) may be appropriate for initial studies to test different principles of thawing. However, there exist several examples of different thawing protocols that have been used in whole ovary cryopreservation research. Concerning whole sheep ovaries that had been cryopreserved with slow freezing protocols, thawing was performed in a water bath at temperature of 25°C (Imhof et al. 2006), 37°C (Bedaiwy et al. 2006; Onions et al. 2008) or 60°C (Martinez-Madrid et al. 2004) and with different duration. Further, another type of rapid thawing after controlled-rate freezing was tested on mouse ovary by holding the frozen specimen in -120ºC in the vapour phase of LN for 2 min, followed by placing them in 37ºC water bath for 5-10 s (Cox et al. 1996).

When directional freezing was applied, the sheep ovary was placed in water bath at 68°C for 20 s and subsequently at 37°C for 10 min (Arav et al. 2005). The authors motivate the use of this rapid thawing technique to avoid recrystallisation (Arav et al. 2010). Also concerning the sheep ovary that had been cryopreserved with other procedures, a two step thawing procedure was used to avoid recrystallization and it was shown that this procedure resulted in a better protection of the vasculature (Courbiere et al. 2006) when compared to one step rapidly warming in water bath at 37°C (Courbiere et al. 2005). The two step technique was based on a previous report (Pegg et al. 1997). The principle of the method is that the frozen ovary is exposed to hot air convection followed by conduction and natural convection at 45°C (Courbiere et al. 2006).

7 CONCLUSION AND FUTURE DIRECTIONS

The research and clinical activities within the field of female fertility preservation are high and the whole field is advancing at great pace. Several major improvements and new techniques may be introduced into the clinic within the next few years.

In the future we will be better to predict the individual risks of premature ovarian failure for women after various types of gonadotoxic treatments, also taking into account the individual ovarian reserve. The basis for this will be prospective long-term follow up studies with detailed information about exact doses of treatment and various measurements of ovarian reserve before, during and after treatment.

Today ovarian cortex cryopreservation with avascular transplantation (Paper IV) is the only accepted method for fertility preservation in females with childhood cancer and in many cases of women with gonadotoxic treatment during the reproductive period. There are great research efforts put into several alternative methods of fertility preservation to improve the efficiency and safety as compared to ovarian cortex cryopreservation.

Whole ovary cryopreservation (Papers I, II and III) is developed as a technique that may increase the longevity of an ovarian transplant since a larger pool of follicles would be transplanted and particularly in view of the fact that ischemic damage to the transplant would be avoided.

A strong research line is that of full in vitro maturation of cryopreserved ovarian tissue. The advantage of this type of procedure is that retransplantation of ovarian tissue can be avoided. Thereby the surgery and the associated surgical risks will be avoided and more importantly, the risk of retransmission of malignant cells that may be present in the ovarian tissue is circumvented. The general principle of this method is to culture the pieces of ovarian cortex under conditions that will allow follicle growth from the primordial stage until the antral stage. This in vitro folliculogenesis does not occur spontaneously but various factors have to be added to promote it. It has recently been shown that phosphatase and tensin homolog **(**PTEN**),** a negative regulator of phosphoinositide 3 kinase (PI3K) signaling, suppresses primordial follicles from growing (Reddy et al. 2008) and it may well be that human primordial follicles can be activated to growth in vitro by the use of PTEN inhibitors. In one study it was shown that exogenous activin was able to promote the growth in vitro of human follicles up to the preantral and antral stage (Telfer et al. 2008). Another principle is that of

promotion of in vitro follicle maturation by placement of isolated secondary follicles within tissue engineered three-dimensional alginate matrix (Xu et al. 2006; Xu et al. 2009). The above mentioned methodology or combinations (Figure 10) of these could lead to that an almost unlimited source of mature follicles could be made available after in vitro growth and risk of reintroduction of metastatic cells would be avoided (Brannstrom and Milenkovic 2008).

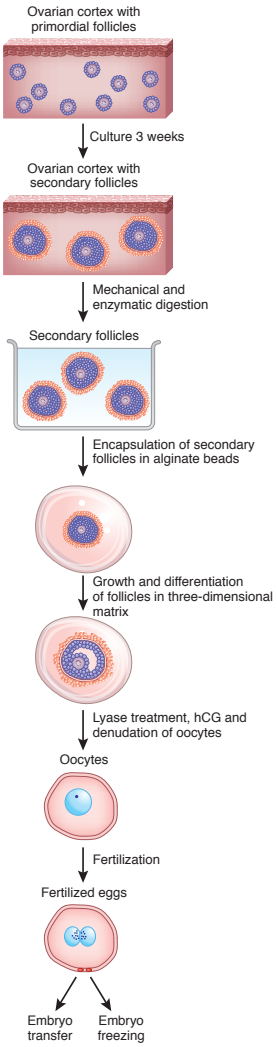

Figure 10 Frozen-thawed ovarian tissue strips would be cultured in vitro for 3 weeks to allow growing of follicles from the primordial to the secondary stage. These follicles could then be isolated and further cultured individually in vitro in three–dimensional alginate gel until antral stage following by retrieval from alginate beads and exposure to hCG to induce oocyte maturation. IVF of mature oocyte would give a large number of embryos (Brännström and Milenkovic, Nat Med 2008, published with permission from Nat Med).

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