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Fertility Preservation for Pre-Pubertal Girls and Young Female Cancer Patients

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1. Introduction

New protocols in the early diagnosis and treatment of cancer have led to major improvements in the long-term survival of patients. However, aggressive chemotherapy or radiotherapy of the pelvic region, often lead to infertility, due to the damage of the follicles and/or oocytes that are present in the ovaries. In women the probability of sterilization due to cancer therapy varies with age, the type of treatment, and the follicular reserve in the ovary. Safeguarding their reproductive potential is a very important issue for women that have not yet started or completed their family, and even more so in pre-pubertal girls. Several options, some of which are still in the experimental phase, can now be offered to these women to (partially) preserve their fertility.

In this review, we will, after briefly describing the anatomy and physiology of an ovary, discuss the detrimental effects of chemotherapy and radiation on ovarian function. Subsequently, the various options that are currently available or are still in an experimental phase, for preserving fertility in women and pre-pubertal girls, will be discussed. These options (with the exception of option (i)), deal with cryopreserving either oocytes, embryos or ovarian tissue until the patient has been cured.

i. Minimizing the effects of radiation of the inner pelvic region by transposing the ovaries from the radiation area.

ii. Standard IVF procedures can be offered to women who are awaiting chemotherapy and radiotherapy for neoplastic disease. This procedure results in the generation of embryos that can be transferred after recovery of the disease. This option has its limitations, since it not only requires the presence of a male partner, but also delays cancer treatment during ovarian stimulation. In addition, the number of embryos that can be produced is restricted, and the chance of achieving a pregnancy after transfer of a cryo-preserved embryo is only 8-30%. Furthermore, the presence of estrogen-sensitive tumors is a contra-indication for this type of treatment, as high estradiol levels are induced during a normal IVF procedure, although alternative stimulation protocols with aromatase-inhibitors are nowadays available for these specific patients. Most importantly, this

treatment is not an option for pre-pubertal girls, or for post-pubertal girls who are not yet involved in a stable relationship.

iii.   Aspiration of oocytes, followed by cryopreservation and IVF (if necessary preceded by *in vitro* maturation). This option has already been applied to a number of patients. Although the same drawbacks that apply to standard IVF are applicable, this procedure is mainly aimed at the treatment of post-pubertal girls/young women without a stable relationship. Only limited scientific data are currently available to substantiate its efficacy and long-term safety.

iv.   As an alternative, cryopreservation of small ovarian cortex strips containing primordial follicles can be offered. After the patient has been cured, these cortex strips can subsequently be retransplanted either heterotopically or orthotopically. This procedure has been successfully used to re-establish female fertility in humans in a limited number of cases. A major problem with these avascular implants however is their relative short life expectancy and follicular loss due to long term ischemic injury directly after reimplantation.

v.   Cryopreservation and subsequent reimplantation of intact ovaries may be a valuable addition to the existing array of options, especially for pre-pubertal girls and post-pubertal girls/women without a stable relationship. An important safety issue of this procedure is obviously the chance of reintroduction of malignant cells that may be present in the cryopreserved intact ovary. For this reason, patients with solid types of tumor and diffuse types of cancer such as leukemia that have a high chance of metastasizing to the ovaries, will have to be excluded from this kind of therapy. The cryobiological and surgical aspects of the preservation and retransplantation of an organ *in toto*, is technically clearly more challenging than the cryopreservation and transfer of isolated cells or tissue strips. The advantages of this approach are obvious; immediate revascularization of the transplanted ovary ensures that less ischemic damage is inflicted to the ovarian tissue post-thawing, and that more follicles will survive. In addition menses, normal long term reproductive functions, and normal hormonal status will be restored.

Finally, we will go into the safety of the procedure. Inevitably the autotransplantation of cortical strips or intact ovaries carries the risk of reintroducing malignant cells from the graft into the recipient.

The increase in knowledge of the biology and treatment of cancer has been accompanied by an increase in the efficacy of cancer therapies. Long term survival rates for many cancer types have therefore increased accordingly (Gatta et al., 2009). Consequently, the quality of life of cancer survivors is becoming an important issue.

The possibility to have genetically concordant progeny is for many people an event that is essential for an unrestricted quality of life as an adult (Schover, 2009). The loss of fertility that may result from cancer therapy, is therefore an additional complication on top of an already difficult period spent on conquering a devastating disease.

With this in mind, it is of the utmost importance to explore the possibilities for fertility preservation in patients that are to be treated with a gonado-toxic therapy. For post-pubertal boys and men, this can be achieved relatively easy via the cryopreservation of their semen prior to start of the therapy. For pre-pubertal boys this is not an option, as semen production is initiated during puberty. Also for this group of patients options for fertility preservation are being developed.

In this paper we confine ourselves to fertility preservation for female patients. We discuss the causes of anti-cancer therapy-related infertility, and review the current options for fertility preservation. We illustrate this matter with two case reports from our own clinical practice. In addition we discuss some as yet experimental procedures, that may in the future be offered to patients requiring fertility preservation.

2. Ovaries, oocytes and female reproduction

The human ovary is spherical structure with a mean volume of 7 cm^3 (range 2-15 cm^3; Munn et al., 1986). The inner ovarian mass, the medulla, consists mainly of stromal cells and contains the larger blood vessels. The outer layer of the ovary consists of the cortical tissue, spanning 2-3 mm. This tissue is rich in extra-cellular matrix proteins and poor in capillaries , and contains the vast majority of the follicles containing oocytes that comprise the ovarian reserve. The most important role of the follicle is to protect the oocyte, and support its development. Follicles are comprised of layer(s) of theca cells and granulosa cells. Different stages of follicles can be distinguished, ranging from primordial follicles to primary follicles, and via secondary finally to tertiary (antral) follicles.

In contrast to males in whom spermatogenesis is a continuous process resulting in the uninterrupted generation of fresh spermatozoa, in women a fixed number of oocytes is formed during embryogenesis from 1000-2000 germ cells. These germ cells are present in the human embryo at 30 days after conception. After 9-10 weeks, these cells transform to oogonia (Baker, 1972), that degenerate for the greater part between 10 and 20 weeks of gestation. After 5 months of gestation, the first meiotic division is initiated in the remaining oogonia, resulting in the differentiation to primary oocytes. At this stage the meiotic division process is arrested, and the oocytes enter a stage of dormancy (Wandji, 1996). At birth only 300.000 to 400.000 oocytes remain in the ovaries. From birth, the number of oocytes gradually decreases, and at the beginning of puberty around 200.000 oocytes remain. Under the influence of pituitary gonadotropic hormones (Gougeon, 1996; Oktay, 1997), each month a cohort of primary oocytes is recruited, and resumes development. Usually only one primary oocyt completes the first meiotic division. This secondary oocyt again enters a stage of dormancy, and is ovulated. The second dormant stage is only lifted after fertilization by a sperm cell. Around the age of 50 years, the total oocyte reserve is almost depleted and the woman enters menopause. In addition to age, several factors may affect the follicular reserve, leading to an early exhaustion and to premature ovarian insufficiency (POI). These factors include fertility-threatening therapies that are discussed in more detail in the next section.

3. Effects of radio- and chemotherapy on female fertility

3.1 Chemotherapy

Cytotoxic therapy may affect all components of the follicle, including granulosa cells, theca cells, and of course the oocyt itself (Sobrinho et al., 1971; Blumenfeld et al., 1999). In addition, interactions between these cell types that are required for oocyt development may be disturbed, resulting in the demise of the oocyt. Damage may become manifest by reduced ovarian weight, stromal fibrosis and in a reduction in the number of oocytes and ovarian follicles (Warne et al., 1973; Meirow et al., 1999; Oktem & Oktay, 2007).

The effect of chemotherapy on fertility is dependent on the type of the cytotoxic agent, the dose, and the duration of the therapy. Alkylating agents such as cyclophosphamide, L-

phenyalanine mustard, and chlorambucil permanently damage ovarian tissue by interacting with DNA (Meirow et al., 1999; Manger et al., 2006; Oktem & Oktay, 2007). Analysis of a group of 138 young females receiving the alkylating agent busulfan as a preparative regimen for indicated that 83% of these women showed signs of fertility impairment, demonstrating the potentially very severe effects of this type of compounds (Borgmann-Staudt et al., 2011). Mertens et al. (1998) showed an even higher percentage of 99% in gonadal dysfunction for women receiving allogeneic haematopoietic stem cell transplantation. The cumulative dose of the cytotoxic drug being administered is an important factor in determining the level of ovarian insufficiency (Goldhirsch et al., 1990). Permanent ovarian insufficiency was more often induced when high dosages of drugs were administered during a short period of time, compared to low doses given over a longer time (Koyama et al., 1977).

In addition the age of the patient is pivotal in determining the amount of damage that is inflicted to the ovary. Older women, with an already decreased number of primordial follicles, have a higher risk of developing acute complete POI, compared with young women who still possess numerous primordial follicles (Schilsky et al., 1981; Sanders et al., 1996; Tauchmanova et al., 2002). Prepubertal girls seem less vulnerable to cytotoxic drugs than adults (Chiarelli et al., 1990). This may be explained by the fact that several chemotherapeutical drugs affect DNA replication and/or RNA and protein synthesis, and are therefore targeted at metabolically active cells. In prepubertal ovaries all follicles are in a dormant, metabolically quiescent state, and therefore less prone to chemotherapy induced damage. In contrast, in adult ovaries a number of follicles will be in an active state, and therefore more prone to chemotherapy induced damage. Nicosia et al. (1985) actually showed in ovarian autopsy material derived from patients having received chemotherapy, that the number of growing follicles was reduced, whereas the number of primordial follicles remained the same.

3.2 Radiotherapy

Similar to the effects of chemotherapeutical agents on DNA integrity, ionizing radiation, amongst other effects, also interferes with DNA function. As a consequence, also radiotherapy may negatively affect the ovarian reserve. Analogous to chemotherapy, the (cumulative) dose and the fractionation schedule determine the degree of damage to the ovary (Gosden et al., 1997). The human oocyte is exceptionally sensitive to radiation (Howell & Shalet, 1998) and the estimate of the LD50 (the lethal dose need to kill half the total number of oocytes) seems to be less than 2 Gy (Wallace et al., 2003). Also for radiation therapy, the age of the patient is an important factor in determining the level of damage. A dose of 4 Gy leads to sterility in 30% of young women, and in 100% of women over 40.

Not surprisingly, the combination of radiotherapy with chemotherapy increases the risk of POI (Williams et al., 1999; Wallace et al., 2005; Chemaitilly et al., 2006). Abdominal radiotherapy in combination with alkylating agents increased the risk of POI 27-fold (Byrne et al., 1992). By the age of 31, 42% of patients treated with this combination therapy, was postmenopausal, compared with 5% of women in the normal population.

3.3 Effects on pregnancy and health of newborns

In addition to their effects on oocytes and follicles, chemotherapy and radiotherapy may also influence uterine function. Radiation may lead to impaired uterine growth in premenarchal girls and failure of uterine development during pregnancy, leading to

miscarriages, premature births and intrauterine growth retardation (Ogilvy-Stuart et al., 1997; Critchley, 1999; Critchley et al., 1992; 2002; Wallace et al., 2005). Comparable results were described by Salooja et al. (2001), who showed that in women that had received total body irradiation prior to autologous or allogeneic stem cell transplantation, are at high risk for maternal and fetal complications. These problems are probably a consequence of uterine vascular damage and reduced elasticity of the uterine musculature.

4. Current options for fertility preservation

4.1 Ovarian transposition

An way to prevent damage to the ovaries caused by ionizing radiation therapy applied to the pelvic region, is to surgically move the ovary temporarily to a location outside the field of radiation (Hadar et al., 1994; Howard, 1997). This procedure, referred to as oophoropexy, can be performed laparoscopically. Potential ovarian insufficiency following transposition may occur if the ovaries are not entirely moved outside the field of radiation, or when they spontaneously migrate back to their original position. Ovarian failure can also occur when the ovarian vascular pedicle has been compromised by the surgical procedure (Feeney et al., 1995). Oophoropexy is a safe and effective procedure, allowing preservation of ovarian function in 80% of cases (Bisharah & Tulandi, 2003).

4.2 Vitrification of oocytes

Cryopreservation of mature or immature oocytes is an obvious approach to preserve fertility. As no fertilization of the oocytes is yet required, this is option is especially suitable for women without a partner. The collection of mature oocytes requires stimulation with follicle stimulating hormone (FSH). This procedure, that may have to be repeated to obtain a sufficient number of oocytes, takes at least two weeks, and is therefore only suitable for women for whom it is safe to postpone their cancer treatment. The use of high doses of FSH makes this option unsuited for women with oestradiol-sensitive breast tumors, as high levels of oestradiol are induced by the FSH treatment (Sonmezer & Oktay, 2006) This caveat may be circumvented by the simultaneous use of aromatase inhibitors / anti oestrogens such as letrozole or tamoxifen (Oktay et al., 2005b; Sonmezer & Oktay, 2006). Alternatively, immature oocytes can be collected without prior stimulation. This procedure may also be used for young (prepubertal girls). Evidently, these immature oocytes must be matured in vitro (IVM) before they can be fertilised (Gosden, 2005).

After collection of the oocytes, they have to be cryopreserved in liquid nitrogen for long term storage. The formation of ice crystals during the freezing process may severely damage the oocyte, rendering it useless for further use. This is especially the case for mature oocytes, as they possess a fragile and sensitive meiotic spindle. Immature oocytes are in this respect less sensitive. Cryodamage can be prevented by freezing the oocytes in the presence of cryoprotective agents via specific protocols, either by slow freezing, or via vitrification (Cao et al., 2009; Chian et al., 2009; Kuwayama et al., 2005). During the latter procedure, that appears to result in more oocytes surviving the process undamaged, the oocytes are frozen extremely rapidly (> 12.000 °C/minute), in the presence of high concentrations of cryoprotectant, resulting in the prevention of ice crystal formation.

A consequence of the cryopreservation procedure (either slow freezing or vitrification) is hardening of the zona pellucida. Therefore, cryopreserved oocytes can only be fertilized via

intracytoplasmatic sperm injection (ICSI). As the pregnancy rate per cryopreserved oocyte is approximately 3% (Kuwayama et al., 2005; Cobo et al., 2007; Homburg et al., 2009), a large number of oocytes, equivalent to several stimulation cycles and/or oocyte retrieval procedures, are required to achieve a reasonable chance of progeny. The exact number of children conceived with cryopreserved oocytes is unknown, but it is estimated to be over 500 worldwide. Postnatal parameters such as birth weight and incidence of congenital anomalies, were comparable with the reference population, indicating the safety of this procedure (Borini et al., 2007; Chian et al., 2008).

4.3 Cryopreservation of embryos

For women with a partner, the generation and cryopreservation of embryos is a suitable option. Obviously, this option will generally require ovarian stimulation, and is therefore subject to the same limitations as mentioned previously for the collection and cryopreservation of mature oocytes – the cancer treatment has to be postponed to allow for one or more ovarian stimulation(s), and extreme caution has to be taken when stimulating women with hormone sensitive tumors. The factor time may be circumvented by skipping the stimulation with FSH and collect immature oocytes instead. Evidently, in that case IVM has to be performed prior to fertilisation of the oocytes. Employing tamoxifen or letrozole based stimulation regimes may be used in the case of hormone sensitive tumors (see previous paragraph) (Oktay et al., 2005a, 2005b; Sonmezer & Oktay, 2006). Oktay et al. (2005b) has shown that in cancer patients who had been stimulated with this compound, recurrence rates were not elevated compared to cancer patients who had not been receiving any ovarian stimulation. Although we should keep in time that the follow up period was confined to only a limited number of years.

Theoretically, in women with hormone sensitive tumors oocytes can also be collected in a spontaneous (non-stimulated) cycle. However, the very limited number of oocytes that can be collected this way (one or two per cycle) makes this a very inefficient option and is therefore not advisable (Brown et al., 1996).

Embryo cryopreservation is an established and efficient technique, with reported implantation rates per thawed embryo between 8 and 30% (Frederick et al., 1995; Selick et al., 1995; Wang et al., 2001; Son et al., 2002; Senn et al., 2006), that has resulted in the birth of tens of thousands of children worldwide. In the future, new cryopreservation techniques such as vitrification may further improve the efficiency of this technique (Kuwayame et al., 2005).

4.3.1 Case report A: Emergency IVF in a patient with breast cancer

Mrs. X was diagnosed 3 years ago with breast cancer. She then underwent a lumpectomy of the right breast, and received radiotherapy. Shortly thereafter a unilateral recurrence was found, and a mastectomy with lymph node dissection was performed. Pathologic examination revealed an invasive ductal carcinoma, positive for estrogen and progesterone receptors. No tumor cells were found in the lymph nodes, and no other indications for metastatic disease were found. Additional chemotherapy courses were planned.

At this stage the patient, now 35 years of age, and her partner visited the Centre for Reproductive Medicine of our hospital, and expressed their interest in fertility preservation. After establishing that the current reproductive status of herself as well as of her partner showed no abnormalities, the possibilities for fertility preservation were discussed. Although oocyte vitrification and ovarian tissue banking were in theory viable options, the couple was counseled to proceed with an emergency IVF-

ICSI attempt, followed by cryopreservation of the embryos, as this would probably give the highest chance of progeny within the time limit set by the oncologist.

The ovarian stimulation protocol was started one month after the mastectomy. Regarding the hormone receptor positive status of the tumor, a regimen combining FSH and letrozol was selected in order to avoid the high oestradiol levels associated with ovarian hyperstimulation. The treatment eventually resulted in the retrieval of 13 oocytes, 12 of which could be inseminated via ICSI. Of the 7 resulting embryos, 3 were eligible for cryopreservation. The efficacy of the letrozol treatment was demonstrated by the finding that during the stimulation with FSH, oestradiol levels did not rise beyond 1000 pmol/L.

The patient than completed 5 cycles of chemotherapy. In addition, she received adjuvant hormonal therapy. Menses had stopped and the patient suffered from hot flushes. Two years later at age 37, the patient wanted to achieve pregnancy. After discontinuation of medication, the hot flushes diminished and menses did resume. Five months later the patient conceived spontaneously, but unfortunately the pregnancy ended in an abortion. As further spontaneous conceptions did not occur, two cycles of fresh IVF were performed. Although the second cycle resulted in a pregnancy, this again ended in an abortion. The patient is now being prepared to receive the embryos that were cryopreserved prior to the start of her chemotherapy.

4.4 Cryopreservation of ovarian cortical tissue strips

As mentioned previously, each fertility preservation option is aimed at a specific group of patients. When the patient is prepubertal, when there is no partner is available for the generation of embryos, or when the cancer treatment cannot be postponed in order to perform ovarian stimulation, cryopreservation of ovarian tissue strips may be an alternative approach. Silber et al. (2005) showed previously that transplantation of fresh (non cryopreserved) ovarian cortex strips between identical twin sisters was actually feasible. The development of efficient freezing and thawing protocols for cortex strips has rendered this technique applicable for fertility preservation purposes and has recently led to the thirteenth live birth (Donnez et al., 2011).

Although still experimental, this option is nowadays being performed on an increasing scale. Cortical fragments can be obtained laparoscopically, and slow frozen using DMSO as a cryoprotectant. Care should be taken to minimize the thickness of the cortical strips to 1 mm, to facilitate diffusion of the cryoprotectant into the tissue. In addition, thin fragments will suffer less from ischemic damage, which is a serious problem after retransplantation. A significant proportion (60-95%) of (growing) follicles that survive the freezing and thawing process, is actually lost due to warm posttransplantation ischemia (Baird et al., 1999; Nisolle et al., 2000; Candy et al., 2000; Aubard et al., 1999, Aubard, 2003; Liu et al., 2002). Cortical strips can be autotransplanted heterotopically (for instance subcutaneous in the forearm), or orthotopically. Thus far, only orthotopic transplantation has led to the birth of a number of healthy offspring (Donnez et al.,2004; Meirow et al.,2005, 2007; Demeestere et al., 2006; 2007; 2010; Andersen et al.,2008; Schmidt et al., 2011; Ernst et al., 2010).

Cryopreservation of ovarian cortical strips is applicable for a wide range of different patients. Conception may require artificial reproductive techniques like IVF or ICSI, but may occur spontaneously as well. An additional advantage of this technique is resumption of the regular hormonal processes, leading to the reversal of the postmenopausal status that many patients experience after their cancer therapy. Follicular development and restoration of ovarian function usually occur 4-5 months after a transplantation procedure (Donnez et al., 2006a; 2008), as more than 120 days are required to initiate follicular growth and

approximately 85 days to the reach final maturation stage from a pre-antral follicle (Gougeon, 1985, Oktem & Oktay, 2008). Unfortunately, the survival time of a single autotransplanted number of strips is usually limited to a few months, with exceptions of survival up to approximately 3 years (Kim et al., 2009; Meirow et al., 2007; Silber et al., 2008a), requiring another surgical intervention to transplant a new set of cortex strips.

4.4.1 Case report B: Ovarian tissue cryopreservation in a patient with Hodgkin's lymphoma

Mrs. Y was diagnosed with Hodgkin's lymphoma at the age of twenty. As she was to start with six cycles of chemotherapy the next month, she visited our fertility Centre to discuss the options for fertility preservation.

Although the patient was at the time in a steady relationship, she regarded herself to young to start emergency IVF, as this would confront both herself and her partner with the definitive choice of having children together in the future. Ovarian hyper stimulation followed by cryopreservation of the retrieved oocytes was not considered an optimal option, as the time to the start of her chemotherapy was relatively short, allowing for only one cycle of hyperstimulation. As a consequence, only a limited number of oocytes would be obtained.

Eventually the choice was made for cryopreservation of ovarian cortical strips. At that time we could not offer her this procedure ourselves so we referred her to another centre. Biopsies of both ovaries were taken via a laparoscopic procedure, and 13 strips were cryopreserved. She then started with the chemotherapy. Since then, she has had two relapses, that were treated with chemotherapy, radiotherapy, and stem cell transplantation.

At the age of 27, the patient and her partner visited our Centre as she wished to conceive. She had now been in complete remission for 3 years. Hormonal examination showed that she was postmenopausal, indicating that both spontaneous conception as well as IVF treatments were no options to achieve pregnancy. The couple was referred back to the clinic where her ovarian tissue was cryopreserved, and is now considering autotransplantation of the ovarian cortical strips.

5. Future options for fertility preservation

Several alternative procedures are being evaluated to expand the current array of fertility preservation options. These include the isolation and cryopreservation of follicles from ovarian tissue that is harvested laparoscopically (Bedaiwy & Falcone, 2007; Feigin et al., 2007). However, isolation of follicles by either mechanical or enzymatic means is difficult, especially from human ovaries (Dolmans et al., 2006). In addition, this approach requires different cryopreservation techniques then for oocytes and embryos, and sophisticated *in vitro* maturation protocols to obtain oocytes that can be fertilized *in vitro* by IVF or ICSI.

A more promising future option may comprise the cryopreservation of an intact ovary, including its vascular pedicle. The vascular pedicle can be used to reconnect the thawed ovary to the circulation, thereby preventing the devastating effects of warm ischemia that is known to deplete the follicles in ovarian tissue transplanted without vascular anastomosis (Newton et al., 1996; Nisolle et al., 2000; Candy et al., 1997; Aubard et al., 1999; Baird et al., 1999; Aubard, 2003; Liu et al., 2008). However, the successful cryopreservation of an intact organ represents an immense technical challenge. Pioneering work by Parrot (1960) on murine ovaries provided proof of principle. Later reports showed that also in other mammalian species this proved to be a viable approach. Freezing and autologous grafting of whole ovaries has now been performed in rabbits (Chen et al., 2005), pigs (Imhof et al.,

2004), and sheep (Bedaiwy et al., 2003; Arav et al., 2005; Imhof et al., 2006), yielding promising results. In rats (Wang et al., 2002) and sheep (Imhof et al., 2006), this procedure has actually resulted in live offspring. In humans, transplantation of fresh (non-cryopreserved) intact ovaries has also been performed successfully. Ovarian autotransplantation in the upper arm was performed before pelvic irradiation (Leporrier 1987, Hilders et al., 2004). Over a period of 16 years, the ovary remained functional (Leporrier et al., 2002). A first full-term pregnancy was obtained using orthotopic fresh whole ovary transplantation between identical twin sisters (Silber et al., 2008b).

Cryopreservation of an intact human ovary with its vascular pedicle has been described previously (Martinez-Madrid et al., 2004, 2007; Bedaiwy et al., 2006). These authors showed that perfusion of the ovary with cryoprotectants led to a certain degree of protection from cryodamage. The subsequent autotransplantation of frozen and thawed human ovaries, however, has thus far not been performed. Major obstacle in this respect is the much larger volume of human ovaries compared to murine and ovine ovaries (Gerritse et al., 2008). This larger volumes hampers the sufficient diffusion of cryoprotectant into the tissue (Donnez et al., 2006b). In addition, the freezing kinetics in a bulky organ are bound to be completely different from those in a small volume organ (Pegg, 2005). Finally, all components of the organ, including the vascular pedicle, the inner vasculature, the stromal tissue and of course the follicles, should be verifiably protected before retransplantation to human subjects can be even considered. This requires the development of biologically relevant assays that are able to quantify cryodamage in a reliable fashion. Understandably, efforts have focused mainly on the survival of the follicles within the intact cryopreserved ovary. This has been done by conventional histology (Bedaiwy et al., 2003, Arav et al., 2005 Courbiere et al., 2005, 2006; Martinez-Madrid et al., 2004; Imhof et al., 2006; Baudot 2007), immunohistochemistry (Arav et al., 2005; Bedaiwy et al., 2006), determining the frequency of apoptosis (Bedaiwy et al., 2003, 2006; Martinez-Madrid et al., 2007), using survival/viability/proliferation assays (Bedaiwy et al., 2003, 2006; Martinez-Madrid et al., 2004, Arav et al., 2005, Courbiere et al., 2005, 2006; Imhof et al., 2004; Baudot et al., 2007, Onions et al., 2008), transmission electron microscopy (Martinez-Madrid et al., 2007) and estradiol assays (Huang et al., 2008; Isachenko et al., 2007; Gerritse et al., 2010). These studies have produced relevant information on the prevention of cryodamage in follicles, but have largely left out the main component of the ovary, namely the stromal cell compartment that constitutes over 95% of the ovarian mass. An additional reason to focus also on survival of stromal cells is the observation that these cells are vital for optimal follicular development (McLaughlin and McIver, 2009). Finally, the metabolically active stromal cells have been described to be more sensitive to cryodamage than the quiescent primordial oocytes (Kim *et al.*, 2004). These observations emphasize the need for a cryopreservation protocol that not only efficiently preserves the follicles/oocytes, but the stromal cell compartment as well.

We therefore decided to develop an assay that is capable of quantifying the basal metabolism of the bulk of the tissue as a measure of cryodamage. For this purpose we measured the uptake of glucose and the release of lacate by cultured ovarian tissue fragments. We used bovine ovaries as a model system, as they are comparable to human ovaries with respect to size, monthly cycle, and number of follicles that mature per cycle (Gerritse et al., 2008). In this model system we were able to test different cryopreservation protocols. Our results show that both immersion of the bovine ovary in cryoprotectant, combined with perfusing it for a prolonged period of time, resulted in a nearly complete

protection of the ovarian metabolism. This procedure did not affect the endothelium of the vascular pedicle and the inner vasculature (Gerritse et al., submitted). We plan to xenotransplant optimally cryopreserved bovine ovaries into immune deficient rats, in order to test the ability of the follicles to develop *in vivo* and produce mature oocytes.

6. Safety aspects of ovarian tissue autotransplantation

A major point of concern when autotransplanting ovarian tissue to cured cancer patients, is the possibility that (metastasized) tumor cells are present in the ovarian graft and are reintroduced to the patient (Shaw et al., 1996). Thus far a limited number of patients has received an autransplantation, and up to now no relapses have been reported. It should be noted, however, that most patients receiving an autotransplantation suffered from early stage cancer when their tissue was harvested. In addition, the follow up period after the transplantation has been relatively short. As a consequence, the experience with this matter is only limited, and retransplantation of the malignancy can never be ruled out completely. Shaw et al. (1996) actually showed that lymphoma could be transmitted via cryopreserved ovarian tissue in a mouse model. The physician therefore has the responsibility to counsel the patient comprehensively on the risk of malignant cells being present in the ovarian tissue, and the possible consequences after autotransplantation. Two different approaches can be used to draft an advice.First, one can extrapolate on statistical data describing the frequency with which a certain tumor in a certain stage will metastasize to the ovary. For a number of solid tumor types, ovarian metastases have been described for advanced stages but not for early stage tumors (Rosendahl et al., 2011). These include Hodgkin's disease (Khan et al., 1986), renal cell carcinoma (Insabato et al., 2003) and breast cancer (Horvath et al., 1977). It should be noted, however, that systematically collected data are missing for most tumor types, giving only a limited idea of the risk of tumor dissemination to the ovary. In contrast to solid tumors, diffuse malignancies such as leukemia are likely to be present in all blood-filled organs, including the ovary. Therefore, patients suffering from these kind of diseases should probably be excluded from using (cryopreserved) ovarian tissue as a means for fertility preservation. The second, and probably preferable, option, is to tailor a patient specific approach, i.e. analyzing (part of) the tissue that is to be autotransplanted in the future for the presence of (residual) disease. In an ideal situation, sensitive and specific tests would be available for the detection of each tumor type in a tissue. Techniques that have been used to asses the presence of malignant cells in ovarian tissue include conventional histology (Azem et al., 2010; Donnez et al., 2011), immunohistochemistry (Rosendahl et al., 2011), PCR amplification of tumor specific RNA/DNA (Rosendahl et al., 2010), and xenotransplantation of ovarian tissue fragments to immune deficient mice (Dolmans et al., 2010). In real life, however, these approaches encounter several obstacles. Histology is relatively non-sensitive as individual tumor cells can be missed, and usually only a limited number of sections is analyzed. Whereas immunohistochemistry is generally more sensitive than histology, it requires specific tumor cell markers that are not available for most type of cancers. While PCR in itself is a very sensitive technique, the ratio between the few malignant cells that are potentially present in the graft and the large number of normal ovarian cells impairs the reliability and sensitivity of this test. PCR results that indicate the presence of residual tumor cells are therefore mostly qualitative and not quantitative. Furthermore, PCR detects only the presence of relatively short stretches of specific RNA/DNA sequences and not viable cells. Xenotransplantation experiments may provide

biologically relevant information, but are expensive and cumbersome and probablyl not routinely applicable. Apart from these practical issues, positive tests results raise some more questions. First, we do not know when a positive signal becomes biologically significant,. i.e. predictive of relapse after transplantation. Examples of this notion are a positive PCR signal that may be derived from a number of deceased cells and may therefore not be of clinical relevance. We currently we do not know exactly how many malignant cells are required for reintroduction of the tumor. In animal models as few as 200 lymphoblasts were sufficient to introduce leukemia (Hou et al., 2007), but the same may not apply to the human situation. Next, the ovarian tissue fragment that is being analyzed for residual disease, is evidently no longer available for transplantation. The outcome of the analysis will therefore not necessarily be applicable to the cortical fragments that are actually transplanted. The importance of this notion was substantiated by the finding that malignant cell DNA was found in an ovarian cortex fragment by PCR analysis, whereas the adjacent cortex fragment from the same ovary was found to be PCR-negative (Rosendahl, 2010). Finally, the autotransplantation of small volume cortex fragments is much less likely to reintroduce the malignancy then the autotransplantation of an intact ovary.

7. Concluding remarks

The last decade has seen the development of a number of options for fertility preservation for cancer patients. All the options that are currently available have their own specific indications and contraindications. The choice for the appropriate option will be a shared decision of both the patient and her physician, requiring a careful evaluation and counseling. Increasing the awareness of physicians to address the issue of fertility preservation before starting gonadotoxic therapy should be an integral part of medical education.
Current research, including intact ovary cryopreservation, may lead to several exciting new options for fertility preservation. It should be noted that this option is not intended to replace the current possibilities, but will rather have its own specific patient population that may benefit most from this procedure. The obvious risk of intact ovary autotransplantation is reintroduction of the malignancy. Evidently, more research into the development of valid and biologically relevant tumor detection methods in ovarian tissue, as well as in the prevalence of ovarian metastases in cancer patients with different types of primary tumors, is urgently needed.

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9. References

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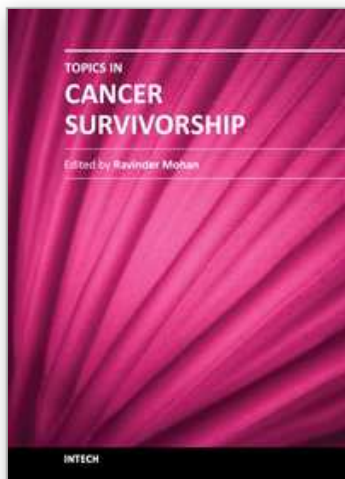
Topics in Cancer Survivorship

Edited by Prof. Ravinder Mohan

Cancer is now the leading cause of death in the world. In the U.S., one in two men and one in three women will be diagnosed with a non-skin cancer in their lifetime. Cancer patients are living longer than ever before. For instance, when detected early, the five-year survival for breast cancer is 98%, and it is about 84% in patients with regional disease. However, the diagnosis and treatment of cancer is very distressing. Cancer patients frequently suffer from pain, disfigurement, depression, fatigue, physical dysfunctions, frequent visits to doctors and hospitals, multiple tests and procedures with the possibility of treatment complications, and the financial impact of the diagnosis on their life. This book presents a number of ways that can help cancer patients to look, feel and become healthier, take care of specific symptoms such as hair loss, arm swelling, and shortness of breath, and improve their intimacy, sexuality, and fertility.

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