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Fertility preservation in female patients

Murat Sonmezer[1,2] and Kutluk Oktay[2,3]

[1]Ankara University School of Medicine, Department of Obstetrics and Gynecology, Ankara, Turkey and [2]The Center for Reproductive Medicine and Infertility, Department of Obstetrics and Gynecology, Joan and Sanford I. Weill Medical College of Cornell University, 505 East 70th Street, HT-340, New York, NY 10021, USA

[3]To whom correspondence should be addressed. E-mail: kuo9001@med.cornell.edu

In the USA alone, >650 000 women will be afflicted by cancer in 2003, and 8% of these cases will be aged <40 years. Due to improvements in cancer therapy, cure rates of both adult and childhood cancers increased significantly over the past three decades. However, long-term consequences of cancer therapy and impact on quality of life are now being recognized. One of the major sequelae of cytotoxic chemotherapy is gonadal failure. Cytotoxic chemotherapy and/or radiotherapy are not only used to treat malignant diseases, but also non-malignant systemic conditions. Upon reviewing the extent and mechanism of gonadal damage due to chemo-/radiotherapy, this article discusses indications and the wide range of methods of fertility preservation in a comprehensive manner. All current, emerging, experimental as well as controversial approaches are reviewed. A comprehensive algorithm to manage fertility preservation through an individualized approach is presented.

Key words: chemotherapy/fertility preservation/ovarian failure/radiotherapy

Introduction

Over the past three decades, there has been a remarkable improvement in the survival rates due to progress in cancer treatment (Jemal *et al*., 2003). However, multi-agent chemotherapy regimens and/or radiotherapy are associated with significant long-term sequelae such as: growth disorders, cardiovascular problems, neurocognitive abnormalities, second malignant tumours and reproductive failure (Leung *et al*., 2000; Shusterman and Meadows, 2000; Bhatia *et al*., 2002; Tauchmanova *et al*., 2002). As a consequence of the increase in the number of patients surviving cancer, greater attention has been focused on the delayed effects of cancer treatments on the quality of future life of the survivor.

More than 650 000 new female cancer cases are estimated to be diagnosed in 2003 in the USA (Jemal *et al*., 2003). Approximately 8% of female cancer cases occur under the age of 40 years (Oktay and Yih, 2002). In women, the cancer death rates for all cancers combined decreased by 0.6% per year from 1992 to 1999, despite an increase in the incidence by 0.3% per year from 1987 to 1999 (Jemal *et al*., 2003). In 1997, the National Cancer Institute estimated the number of survivors of all childhood cancers to be 270 000; ~1 per 1000 population (Simone, 2003). By the year 2010, as many as 1 in 250 patients will have survived malignancies (Bleyer, 1990). Early loss of ovarian function, which is one of the long-term devastating consequences of combined chemotherapy and/or radiotherapy, not only puts the patients at risk for menopause-related complications at a very young age, but is also associated with loss of fertility. Even in those patients who were not sterilized by high dose chemo-/radiotherapy, there may be an increased risk for complications during pregnancy such as early pregnancy loss, premature labour and low birth weight (Sanders *et al*., 1996; Chiarelli *et al*., 2000; Green *et al*., 2003).

The picture is similar with paediatric cases. As the number of patients surviving childhood cancers increases, and as an increasingly higher number of these reach reproductive ages, paediatric patients and parents have begun to face some critical long-term cancer treatment-related issues. Among the concerns are: whether chemo-/radiotherapy will cause any growth problems, or whether future reproductive function and childbearing will be affected? If in fact this child grows to have children, will the offspring be healthy? All these are challenging clinical and psychosocial considerations for both parents and physicians in choosing the course of treatment.

Increased awareness of the effects of various cancer treatments on fertility resulted in a surge in the number of patients seeking help to preserve their fertility (Oktay *et al*., 2003a). This surge in demand is now mirrored by a proliferation of techniques to preserve fertility via assisted reproduction and cryopreservation. The available options range from clinically well-established techniques such as embryo cryopreservation to highly experimental ones such as ovarian tissue cryopreservation and xenografting. In this article we will review the current techniques of fertility preservation. Possible future options will also be discussed. An algorithmic approach will be presented.

Chemotherapy- and radiotherapy-associated gonadal damage

Chemotherapy

In many cancers, multi-agent chemotherapy constitutes the basis of the modern cancer treatment. Ovaries, which are endowed with an irreplaceable number of follicles, are extremely sensitive to cytotoxic drugs that induce an irreversible gonadal damage (Oktay *et al*., 2001a). The end result of the chemotherapy-induced damage to steroid-producing cells of the ovary (granulosa and theca cells), as well as to the oocyte, is premature ovarian failure leading to premature menopause and permanent infertility. Ultrastructurally it is associated with marked follicle loss (Familiari *et al*., 1993). Some chemotherapeutic agents are more commonly associated with gonadal damage; such as cyclophosphamide, chlorambucil, melphalan, busulfan, nitrogen mustard and procarbazine (Warne *et al*., 1973; Koyama *et al*., 1977; Fisher *et al*., 1979; Viviani *et al*., 1985; Mackie *et al*., 1996; Teinturier *et al*., 1998; Legault and Bonny, 1999; Meirow *et al*., 1999; Blumenfeld *et al*., 2000; Kenney *et al*., 2001; Tauchmanova *et al*., 2002). Among the moderately gonadotoxic agents are cisplatin and adriamycin (Hortobagyi *et al*., 1986; Wallace *et al*., 1989a; Maneschi *et al*., 1994; Tangir *et al*., 2003), while bleomycin, actinomycin D, vincristine, methotrexate and 5 fluorouracil are associated with mild or no gonadotoxicity (Van Thiel *et al*., 1970; Shamberger *et al*., 1981; Stillman *et al*., 1981; Sudman *et al*., 1992; Bines *et al*., 1996; Bower *et al*., 1998) (Table I).

Table I. Cytotoxic agents according to degree of gonadotoxicity

High risk
Cyclophosphamide
Cholarambucil
Melphalan
Busulfan
Nitrogen mustard
Procarbazine
Intermediate risk
Cisplatin
Adriamycin
Low/no risk
Methotrexate
5-Fluorouracil
Vincristine
Bleomycin
Actinomycin D

Cyclophosphamide is the most commonly implicated agent in causing damage to oocytes and granulosa cells in a dose-dependent manner (Warne *et al*., 1973; Sanders *et al*., 1988; Montz *et al*., 1991; Meirow *et al*., 1999; Kenney *et al*., 2001). In a recent mouse study, cyclophosphamide-induced follicular damage occurred in a dose-dependent manner, but destruction of primordial follicles was observed at all levels of the cyclophosphamide exposure, even at low cyclophosphamide doses of 20 mg/kg (Meirow *et al*., 1999). Relative risk of premature ovarian failure was reported between 4 and 9.3 in patients receiving cyclophosphamide (Byrne *et al*., 1992; Meirow and Nugent, 2001). Sanders *et al*. (1988) reported that the probability of having ovarian failure in patients receiving high dose cyclophosphamide prior to haematopoietic stem cell transplantation (HSCT) was 0.35 by 7 years.

Older women, with low primordial follicle pool, have higher risk to develop complete ovarian failure compared with young women with high primordial follicle number (Schilsky *et al*, 1981; Richardson *et al*., 1987; Rivkees and Crawford, 1988; Sanders *et al*., 1988; Brice *et al*., 2002; Tauchmanova *et al*., 2002). With advancing age, permanent gonadal damage can be induced with smaller doses of the implicated chemotherapeutic agent. Even though irregular menstrual pattern or amenorrhoea is highly likely to occur in a significant number of patients during the chemotherapy, sometimes lasting for a considerable period after completion of the chemotherapy, many patients return to a pre-chemotherapy menstrual pattern (Wallace *et al*., 1989a; Brewer *et al*., 1999; Low *et al*., 2000; Tangir *et al*., 2003). Hormonal reversal of a hypergonadotrohpic hypogonadal state that commonly occurs during the courses of chemotherapy to a normagonadotrophic state may also be expected, especially in young patients (Blumenfeld, 2001; Zanetta *et al*., 2001; Tauchmanova *et al*., 2002; Wikstrom *et al*., 2003). However, these women will always have a high risk of developing premature menopause later time during their reproductive life (Byrne *et al*., 1992; Larsen *et al*., 2003). The fact that ovulation may occur despite loss of half of the follicular pool in rodents (Meirow *et al*., 1999) indicates that indirect assessment of ovarian reserve is an unreliable tool. In the light of these data, patients should be advised to not to delay childbearing once cure is achieved.

Radiotherapy

Ionizing radiation is a well-recognized cause of ovarian damage and permanent infertility. Gonadal damage occurs not only by direct exposure to radiation such as in the case of pelvic or low abdominal irradiation, but also scatter radiation may cause considerable damage even if gonads are outside of the radiation field.

Radiation causes a dose-related reduction in the primordial follicle pool (Gosden *et al*., 1997). The human oocyte is extremely sensitive to radiation, and irradiation at ovarian dose >6 Gy usually causes irreversible ovarian failure (Howell and Shalet, 1998). Wallace *et al*. (1989b, 2003) demonstrated that <4 Gy is enough to destroy half of the oocyte population (LDL_{50} <4 Gy); however, very recently, using a revised mathematical model the same authors suggested that the LDL_{50} of the oocytes was <2 Gy. Age at the time of exposure to radiotherapy, extent and type of radiation therapy (e.g. abdominal, pelvic external beam irradiation, intracavitary brachytherapy) and fractionation schedule are important prognostic indicators for development of ovarian failure (Fisher and Cheung, 1984; Lushbaugh and Casarett, 1976; Tease and Fisher, 1991; Morice *et al*., 2000; Meirow and Nugent, 2001). In mice, radiation-induced chromosome damage in the oocytes was more evident in older animals compared with younger ones (Tease and Fisher, 1991). In general, irradiation is more toxic when given in single dose compared to fractionated doses.

Stillman *et al*. (1981) investigated the risk of ovarian failure among 182 long-term survivors of childhood cancers receiving abdominal radiotherapy. The mean follow-up was 16.4 years. When both ovaries were in the irradiation field, ovarian failure

occurred in 68% of the patients, and in 14% of the patients when both ovaries were at the edge of the treatment field. None of the 122 children developed ovarian failure when one or both ovaries were outside of the abdominal treatment field. In another study, failure in pubertal development or premature menopause was observed in 37 of 38 patients who received external abdominal irradiation in doses ranging from 20 to 30 Gy during childhood for intra-abdominal tumours (Wallace *et al*., 1989c). Sanders *et al*. (1988) reported the probability of ovarian failure in patients receiving cyclophosphamide and total body irradiation for HSCT as 1.00 at 1 year. Failure in pubertal development may be the first sign of ovarian failure in these patients who received radiotherapy during their childhood.

Total body irradiation used in conditioning regimens prior to HSCT to eradicate the host's pre-existing bone marrow (e.g. leukaemias) is commonly associated with ovarian failure (Sanders *et al*., 1996; Thibaud *et al*., 1998; Couto-Silva *et al*., 2001; Tauchmanova *et al*., 2002). Radiation therapy had also been used for the treatment of some metastatic germ cell tumours of the ovary, but it was replaced by multi-agent platinum-based chemotherapy in order to preserve fertility (Brewer *et al*., 1999).

Although successful full term pregnancies were reported (Giri *et al*., 1992; Maruta *et al*., 1995), the risk of ovarian failure is always high in women who received high dose abdominal or pelvic irradiation (Wallace *et al*., 1989c; Bath *et al*., 1999). In addition, if pregnancy is achieved, these patients may have increased risk for pregnancy complications including early pregnancy loss, premature labour, and low birthweight due to impaired uterine growth and blood flow (Critchley *et al*., 2002; Green *et al*., 2002a,b).

Who are the candidates for fertility preservation?

Cytotoxic chemotherapy and/or radiation therapy have been used to treat not only patients with malignant conditions, but also those with various nonmalignant systemic diseases. Patients who are under the risk of developing future ovarian failure may all benefit from fertility preservation technologies. Those suffering from benign ovarian diseases and undergoing radical surgery may also be added to this list. There is a growing list of diseases in which the treatment, or the disease itself, is associated with gonadal damage. Because each cancer patient's clinical situation is unique, not one technique alone would be suitable. In our centre we developed a comprehensive approach to fertility preservation, depending on the patient's age, presence or absence of ovarian involvement, available time, and the indication for fertility preservation.

Childhood cancers

Cancer is the second leading cause of death in children between the ages of 1 and 14 years (Jemal *et al*., 2003). The cure rates from childhood cancers have improved markedly over the last three decades, thanks to cancer treatment that includes combined chemotherapy and/or radiotherapy, and HSCT. Five-year survival is now ~80% for all cancers combined, which is between 80 and 86% for childhood acute lymphoblastic leukaemia (ALL), and >90% for Hodgkin's disease (Brenner *et al*., 2001; Jemal *et al*., 2003; Pui *et al*., 2003; Robison and Bhatia, 2003). Around 2000 patients are estimated to become long-term survivors of ALL each year, the most common childhood malignancy (Gurney *et al*., 1995). In addition to leukaemias, patients who face the risk of ovarian failure due to cytotoxic treatment are those with Hodgkin's lymphoma, neuroblastoma, non-Hodgkin's lymphoma, Wilm's tumour, Ewing's sarcoma and osteosarcoma of the pelvis and genital rhabdomyosarcoma (Nussbaum *et al*., 1999; Arndt *et al*., 2001; Franchi-Rezgui *et al*., 2003; Ozaki *et al*., 2003; Rodl *et al*., 2003).

Poirot *et al*. (2002) evaluated the feasibility of long-term ovarian tissue cryopreservation in 31 women with malignant and non-malignant disease who were at risk for ovarian failure due to treatment. In their study, the age of the patients ranged between 2.7 and 34 years, and 16 of them were <18 years old. The majority had the diagnosis of leukaemia, lymphoma, Ewing's sarcoma and neuroblastoma. The average number of follicles was higher in younger patients, and they proposed ovarian tissue cryopreservation in young patients at risk of ovarian failure as a result of gonadotoxic treatment. Fertility preservation is especially challenging in children, as embryo or oocyte cryopreservation is neither ethical nor practical. Currently the only option that could be offered to child patients is ovarian tissue cryopreservation, provided that the families are thoroughly informed about the experimental status of the technique.

Breast cancer

Breast cancer is the most common malignant disease in reproductive age women (Weir *et al*., 2003). In the USA, it is estimated that ~211 000 new breast cancer cases will have been diagnosed in 2003. The incidence of female breast cancer has increased since 1986, but the death rates decreased in the early 1990s; 2.5% per year in white women, and 1.0% per year in black women (Weir *et al*., 2003). One out of every 228 women will develop breast cancer before age 40 years, and ~15% of all breast cancer cases are estimated to occur at <40 years (Oktay and Yih, 2002; Jemal *et al*., 2003).

Many of these patients will be subjected to multi-agent, mainly cyclophosphamide-based, cytotoxic chemotherapy (Kaufmann *et al*., 2003). In breast cancer, because chemotherapy is usually initiated 6 weeks after the surgery, there is adequate time for controlled ovarian stimulation to preserve fertility by oocyte or embryo cryopreservation. Because conventional ovulation induction regimens are deemed risky for breast cancer patients due to resultant surge in estradiol levels, potentially safer regimens including tamoxifen or aromatase inhibitors have been introduced (Oktay *et al*., 2003b). These patients may also be candidates for ovarian tissue cryopreservation, as occult ovarian metastasis is extremely rare in non-metastatic breast cancer (Gagnon and Tetu, 1989; Hann *et al*., 2000).

Cancer of the cervix

Cancer of the cervix is a serious health problem affecting 500 000 women each year worldwide. In the year 2002, 13 000 new cervical cancer cases were diagnosed in North America and roughly half of them occurred before the age of 35 years (Waggoner, 2003). Over the past three decades, while the incidence of squamous cell carcinoma of the cervix decreased by 42%, the incidence of adenocarcinoma of the cervix increased by 29% (Smith *et al*., 2000). Ovarian involvement is extremely rare in squamous cell cervical carcinoma, but it is encountered in

up to 12% of the cases with adenocarcinoma and adenosquamous carcinoma of the cervix (Nakanishi *et al*., 2001; Yamamoto *et al*., 2001).

Ovarian transposition is commonly considered for patients with cervical cancer who will receive pelvic radiotherapy, but the success rates tend to vary with this procedure. If adjuvant radiosensitizing chemotherapy is scheduled, ovarian cryopreservation for future autotransplantation is another option. Alternatively, one ovary can be transposed, usually the one on the opposite site of the main tumour, and the other one could be cryopreserved. Even if there is enough time for a controlled ovarian stimulation, it is not considered safe to perform transvaginal oocyte aspiration in these patients, as there is risk of profuse bleeding from the friable, cancerous cervix.

Patients receiving pelvic radiation

Radiotherapy is utilized to improve prognosis or to achieve local tumour control in some solid tumours presenting in the pelvis such as Ewing sarcoma, osteosarcoma, retroperitoneal sarcomas, and in some benign bone tumours (Feigenberg *et al*., 2001; Ferguson and Goorin, 2001; Bacci *et al*., 2003; Ozaki *et al*., 2003; Pisters *et al*., 2003; Rodl *et al*., 2003). Radiation therapy also plays an important part in the management of rectal cancer (Kapiteijn *et al*., 2001). These patients can resort to ovarian, oocyte or embryo cryopreservation, or alternatively oophoropexy may be considered, especially if an abdominal surgery is already necessary for the treatment of the primary disease.

Benign ovarian diseases

Some benign ovarian diseases, either due to their extensive or progressive nature, or because of bilateral occurrence and repeated surgeries, may significantly compromise ovarian reserve (Oktay *et al*., 2001b). Healthy pieces of the ovarian tissue can be cryopreserved in women undergoing oophorectomy for benign conditions. If disease recurrence is likely, subcutaneous transplantation of the ovarian pieces is the preferred procedure, due to ease in monitoring and the presumed simplicity of removal in the case of recurrence.

Prophylactic oophorectomy

Inherited mutations, mainly BRCA-1 and BRCA-2, account for almost 10% of all epithelial ovarian carcinomas (Claus *et al*., 1996; Newman *et al*., 1998). In the USA, the carrier frequency of the germ-line BRCA mutations is 0.1%, although it is as high as 1% for each mutation in the Ashkenazi Jewish population (Struewing *et al*., 1995). The cumulative lifetime risk of developing ovarian cancer is ~60% in the presence of BRCA-1 mutation, and 10–20% in women with BRCA-2 mutation (Struewing *et al*., 1995, 1997; Liede *et al*., 2002). In addition, lifetime risk of breast cancer in female carriers of BRCA1 mutation is 80–90%.

While peritoneal cancer cannot be prevented in BRCA-positive patients, prophylactic oophorectomy is suggested as soon as childbearing is completed, or by the age 35–40 years, to decrease the risk of ovarian and breast cancer (Kauff *et al*., 2002; Rebbeck *et al*., 2002). Ovarian tissue cryopreservation might be offered in patients who wish to delay childbearing beyond the age of 35 years. When patients desire to conceive, ovarian tissue can be transplanted, preferably subcutaneously, so that it could be easily monitored, and removed once the pregnancy occurs in order to avoid further cancer risk. These patients may also be candidates for future *in vitro* maturation using oocytes obtained from cryopreserved ovarian tissue.

Systemic lupus ertythematosus and other autoimmune diseases

Systemic lupus ertythematosus (SLE) typically affects reproductive age women, with an overall incidence between 40 and 250 per 100 000 people (Michet *et al*., 1985). High dose cyclophosphamide, with or without HSCT, is sometimes used in the treatment of SLE, and can result in premature ovarian failure in these patients (Gladstone *et al*., 2002).

Other autoimmune diseases reported to benefit from cytotoxic therapy with alkylating agents are Behcet's disease, steroid-resistant glomerulonephritis, inflammatory bowel diseases, and pemphigus vulgaris (Russell *et al*., 2001; Langford *et al*., 2003; Nousari *et al*., 2003; Stallmach *et al*., 2003). These patients may also become candidates for ovarian, oocyte or embryo cryopreservation.

Haematopoietic stem cell transplantation

Autologous or allogeneic HSCT has been an important therapeutic tool in the management of some malignant and non-malignant systemic diseases. Among the non-malignant conditions reported to benefit from HSCT are some autoimmune diseases previously unresponsive to immunosuppressive therapy, diseases associated with genetically abnormal stem cells, and those associated with the deficiency of bone marrow stem cell products (Slavin *et al*., 2001; Burt *et al*., 2003).

Prior to HSCT, preconditioning regimens are used to ablate the pre-existing bone marrow. The most commonly used conditioning regimens for allogeneic and autologous HSCT in acute myelogenous leukaemia (AML) include cyclophosphamide/total body irradiation or busulfan/cyclophosphamide (Litzow *et al*., 2002). Both regimens are highly gonadotoxic; the risk of developing complete or partial ovarian failure may be >80% in children receiving a conditioning regimen for HSCT (Thibaud *et al*., 1998; Couto-Silva *et al*., 2001).

HSCT has also been used in patients with breast cancer, multiple myeloma and lymphoma (Einsele *et al*., 2003; Mazza *et al*., 2003). Among the non-malignant conditions suggested to benefit from HSCT are systemic lupus erythematosus, aplastic anaemia, sickle cell anaemia, autoimmune thrombocytopenia, progressive systemic sclerosis, rheumatoid arthritis, juvenile idiopathic arthritis and vasculitis (Olalla *et al*., 1999; Tyndall and Millikan 1999; Walters, 1999; Burt *et al*., 2003; Cohen *et al*., 2003). Adult patients, if there is sufficient time, can resort to conventional IVF for oocyte or embryo cryopreservation. Ovarian cryopreservation is the only choice to preserve fertility in paediatric patients, and in patients who cannot postpone their treatment.

Available options for fertility preservation

Currently embryo cryopreservation is the only well-established procedure that is commonly used in many infertility clinics worldwide. However, each cancer patient presents with a unique situation, and embryo cryopreservation may not always be applicable to every individual. We will outline the currently

available fertility preservation options and their applicability in different clinical situations.

Embryo cryopreservation

In cancer patients, IVF can be performed to store embryos for future use, if the patient has a partner and enough time prior to treatment. Survival rates per thawed embryo range between 35 and 90%, implantation rates between 8 and 30%, and cumulative pregnancy rates can be >60% (Al-Shawaf *et al*., 1993; Frederick *et al*., 1995; Selick *et al*., 1995; Senn *et al*., 2000; Wang *et al*., 2001; Son *et al*., 2003).

In breast cancer, there is typically a 6 week hiatus between surgery and chemotherapy, which would be adequate to perform ovarian stimulation and IVF. Nevertheless, since conventional ovulation hyperstimulation regimens in IVF cycles typically result in estradiol levels that may be 10-fold higher than peak estradiol levels seen in a natural cycle, they are not recommended in breast cancer patients (Pittaway and Wentz 1983; Adashi, 1996; Pena *et al*., 2002; Chen *et al*., 2003).

After its discovery in 1963, tamoxifen became an important part in the treatment of breast cancer, and has been tested for the chemoprevention of this disease (Harper and Walpole, 1966, 1967; Veronesi *et al*., 2003). While it was originally used as a contraceptive agent in the UK, it was later found to be a useful ovulation induction agent (Klopper and Hall, 1971). Exploiting its dual effect, we recently demonstrated that tamoxifen can be safely used to perform ovarian stimulation and IVF in breast cancer patients (Oktay *et al*., 2003a). In that study, 12 women with breast cancer received 40–60 mg tamoxifen for a mean duration of 6.9 days beginning on days 2–3 of their menstrual cycle. Patients underwent IVF and embryo transfer with either fresh or cryopreserved embryos, and were compared with a retrospective control group of breast cancer patients who had natural cycle IVF (NCIVF). Cycle cancellation was significantly less frequent in patients receiving tamoxifen, compared to those who underwent NCIVF (1/15 versus 4/9, $P < 0.05$). The mean numbers of mature oocytes (1.6 versus 0.7, $P = 0.03$) and embryos (1.6 versus 0.6, $P < 0.05$) per initiated cycle were higher in the tamoxifen group compared with NCIVF. In addition, tamoxifen stimulation resulted in the generation of an embryo in every patient (12/12) whereas only three of five patients had embryos following natural cycle IVF. The mean peak estradiol level in the tamoxifen group was significantly higher than in natural cycle IVF patients (442.4 versus 278 pg/ml).

Even though tamoxifen results in an increase in peak estradiol levels, it is well known that tamoxifen can block the effects of supraphysiological levels of estrogen on breast tissue, and inhibits the growth of breast tumours by competitive antagonism of estrogen at its receptor site. In fact, mean estradiol levels are chronically elevated in breast cancer patients who are on long-term tamoxifen treatment, and can be higher than the levels seen in patients undergoing ovarian stimulation with tamoxifen (Shushan *et al*., 1996; Klijn *et al*., 2000).

Endometrial cancer is another estrogen-sensitive malignancy, which can be seen in reproductive age women. Because tamoxifen is stimulatory on endometrium, it cannot be used in endometrial cancer for ovarian stimulation. For these patients, aromatase inhibitors can be used for ovarian stimulation, IVF and embryo cryopreservation (Oktay *et al*., 2003b). Aromatase P450 catalyses the reaction converting androgenic substances to estrogens. Letrozole is a potent and highly selective third generation aromatase inhibitor that was developed in the early 1990s. It competitively inhibits the activity of aromatase enzyme, and has a half-life of ~48 h (Pfister *et al*., 2001). Letrozole significantly suppresses plasma estradiol, estrone and estrone sulphate levels at doses ranging from 0.1 to 5 mg/day, and it was recently shown to be superior to tamoxifen in the treatment of advanced stage post-menopausal breast cancer (Dowsett *et al*., 1995; Buzdar *et al*., 2001; Mouridsen *et al*., 2003).

Aromatase inhibitors have recently been considered and tested as ovulation induction agents. In cycling bonnet monkeys, letrozole resulted in the formation of multiple follicles (Shetty *et al*., 1997). Clinical studies have also shown its benefit in ovulation induction alone or in combination with FSH, and letrozole has been suggested in the treatment of poor responders (Mitwally and Casper, 2002). Currently we are testing the safety of ovarian stimulation with aromatase inhibitors in breast and endometrial cancer patients.

Mature and immature oocyte cryopreservation

Embryo cryopreservation may not be an option for single women unless they choose to use sperm donation. In these patients, if they have time to complete ovarian stimulation prior to cancer therapy, freezing mature or immature oocytes can be considered instead.

The first human live birth from cryopreserved oocytes was reported by Chen (1986). Porcu *et al*. (1997) reported the first human live birth after transferring embryos generated by ICSI of cryopreserved oocytes. Later, additional successful human pregnancies were declared by several investigators using the same technique (Young *et al*., 1998; Quintans *et al*., 2002; Katayama *et al*, 2003; Yoon *et al*., 2003).

Unlike the cryopreservation of embryo and sperm, early results have been disappointing with low survival, fertilization, and pregnancy rates after IVF of thawed oocytes (Mandelbaum *et al*., 1988; Imoedemhe and Sigue, 1992; Oktay *et al*., 2001a). However, with recent studies suggesting increased success rates, the interest in cryopreservation of oocytes has been rekindled (Fabbri *et al*., 2001; Porcu, 2001; Katayama *et al*., 2003; Liebermann *et al*., 2003; Yoon *et al*., 2003).

In earlier reports, survival and fertilization rates of frozen–thawed mature oocytes varied between 25 and 95% (Al-Hasani *et al*., 1987; Gook *et al*., 1995; Kuleshova *et al*., 1999; Yoon *et al*., 2000; Porcu, 2001; Katayama *et al*., 2003) and between 13.5 and 71% (Imoedemhe and Sigue, 1992; Kazem *et al*., 1995; Porcu *et al*., 1997; Fabbri *et al*., 2001; Chen *et al*., 2002) respectively. When data from 21 studies in peer-reviewed journals were reviewed, we found a mean survival rate of 47%, mean fertilization rate of 52.5% and a mean pregnancy rate per thawed oocytes of 1.52%.

One of the factors cited in improvement of fertilization rates of frozen–thawed oocytes is the use of ICSI to overcome the zona hardening, which is believed to have been caused by the freezing process (Kazem *et al*., 1995; Tucker *et al*., 1996; Porcu, 2001; Katayama *et al*., 2003). It has also been proposed that ICSI results in embryos with greater cleavage rates (Gook *et al*, 1995). The safety record for oocyte cryopreservation is not extensive. The rate of abnormal fertilization ranges from 5 to 15.3% (Porcu, 2001). Of the 32 pregnancies where the perinatal outcome was reported

(Oktay *et al*., 2001a), there has been one ventricular septal defect (VSD) and one triploid pregnancy. The latter, however, resulted after ICSI of frozen–thawed testicular sperm into cryopreserved oocytes (Chia *et al*., 2000).

Recently, improved post-thaw survival and fertilization rates and live births were obtained with vitrification using ethylene glycol and dimethylsulphoxide (DMSO) as cryprotectants (Katayama *et al*., 2003; Yoon *et al*., 2003). Review of the existing data indicates a mean survival rate of 68.4%, fertilization rate of 48.5%, and pregnancy rate of 1.7% per vitrified–thawed oocytes.

It has been suggested that immature oocytes may be more resistant to cryodamage due to lower cell volume, and lack of metaphase spindle. Even though high rates of nuclear maturation have been reported with cryopreserved immature oocytes, the developmental capacity has been generally low (Toth *et al*., 1994; Van Blerkom and Davis, 1994; Wu *et al*., 2001). Spindle abnormalities, premature and partial condensation of chromosomes have also been observed after *in vitro* maturation of cryopreserved germinal vesicle GV stage oocytes (Park *et al*., 1997). Only a few pregnancies have been reported using frozen–thawed human immature oocytes thus far (Tucker *et al*., 1996; Wu *et al*., 2001).

Ovarian tissue cryopreservation

Ovarian cortex contains primordial follicles with oocytes arrested in the diplotene of prophase of first meiotic division. It has been suggested that relatively high surface/volume ratio, low metabolic rate and the absence of zona pellucida make primordial follicles less susceptible to cryodamage.

Ovarian tissue cryopreservation and transplantation studies date back to the 1950s. Initial studies were disappointing until the discovery of effective modern cryoprotectants and the availability of automated cryopreservation machines. Glycerol was the only available cryoprotectant in 1960s, but was found ineffective for cryopreservation of human oocytes and ovarian tissue (reviewed in Oktay, 2001). With the advent of more effective cryoprotectants such as ethylene glycol, DMSO and propanediol, animal studies were repeated and successful deliveries were reported in a number of species (Gosden *et al*., 1994a; Sztein *et al*., 1998; Liu *et al*., 2001). In humans, resumption of ovarian endocrine function could be demonstrated (Oktay and Karlikaya, 2000; Radford *et al*., 2001), and very recently, the first embryo following subcutaneous transplantation of cryopreserved ovarian tissue was reported (Oktay *et al*., 2004). There has not yet been a report of pregnancy after ovarian transplantation.

Xenografting human ovarian tissue to immunodeficient mice

Severe combined immunodeficiency disease (SCID) mice can harbour tissues from foreign species without graft-versus-host response, because of the T- and B-cell deficiency due to a gene mutation (Bosma *et al*., 1983; Gosden *et al*., 1994b). Ovarian tissue pieces can be grafted intramuscularly, subcutaneously, or placed under the kidney capsule to improve vacularization.

The SCID mouse model was first utilized to study follicle development in xenografted sheep and cat ovarian tissue (Gosden *et al*., 1994b). Thereafter we adapted this model to study human ovarian tissue xenografting (Oktay *et al*., 1998a). Approximately 1 mm^3 of ovarian tissue pieces were xenografted under the kidney capsule, animals were stimulated with FSH during the last 6 weeks of the 17 week grafting period. We demonstrated estradiol production, estrogenization of the uteri and antral follicle development in FSH-stimulated mice. In another study, we aimed to determine the long-term survival of frozen–thawed human ovarian tissue as xenografts in ovariectomized SCID mice (Oktay *et al*., 2000). We did not give exogenous gonadotrophins since these animals had anatomically intact hypothalamic-pituitary axis; however, follicles did not grow beyond the 2-layer stage in these xenografts. The latter study indicated that endogenous gonadotrophins in ovariectomized SCID mice were not sufficient to support human follicular growth. Similarly, several groups showed follicle development, ovulation and corpus luteum formation after stimulation of xenografted human ovarian tissue using gonadotrophins in immunodeficient mice (Weissman *et al*., 1999; Gook *et al*., 2001; Kim *et al*., 2002; Van den Broecke *et al*., 2002; Abir *et al*., 2003).

In summary, even though human ovarian xenografts provided a model to study human ovarian tissue autotransplantation, their use as a means to utilize banked ovarian tissue is in question. Concerns regarding cross-species retroviral infections should be addressed. Moreover, this technique will require large numbers of animals to be killed since only very small fragments of ovarian tissue can be xenografted. This may not only make the technique impractical, but may also raise further ethical concerns.

Development of human ovarian transplantation techniques

There have been two main approaches in autotransplanting ovarian cortical pieces in humans. Orthotopic transplants involve grafting these strips near the infundibulopelvic ligaments or possibly on a post-menopausal ovary. In the heterotopic transplant, tissues can be grafted subcutaneously at various locations including forearm and abdominal wall.

Orthotopic ovarian transplantation. While transplantation may allow a natural pregnancy to occur (Gosden *et al*., 1994a), it requires abdominal surgery and general anaesthesia. A laparoscopic approach makes this surgery less invasive but technically more challenging.

In the first case of laparoscopic orthotopic transplantation procedure with frozen ovarian tissue in a 27 year old woman, ovarian cortical pieces had been cryopreserved in 1.5 mol/l propanediol using a slow-freeze protocol. After tissues had been thawed, they were sutured to two triangular frames made from an absorbable cellulose membrane. Then we laparoscopically transplanted them beneath the left pelvic peritoneum of the ovarian fossa (Figure 1). With the expectation of improving vascularization, aspirin 80 mg/day p.o. and FSH 150 IU/day i.m. were given for a week after the operation. Fifteen weeks after grafting, the patient was stimulated with daily menopausal gonadotrophins, which was gradually increased from 150 to 675 IU/day, and ovulation was confirmed by elevated progesterone levels, ultrasonographic demonstration of a corpus luteum, free fluid in the cul-de-sac, and change in endometrial pattern on ultrasound. Ovarian function could not be demonstrated beyond 9 months of follow-up.

Radford *et al*. (2001) reported a 36 year old woman with stage IIIB nodular sclerosing Hodgkin's lymphoma who had had her right ovary cryopreserved before implementation of high dose chemotherapy for third recurrence of the disease. This patient had

Figure 1. Laparoscopic ovarian transplantation to the pelvic sidewall. Reprinted by permission from the American Society of Reproductive Medicine (Oktay et al., 2001, *Fertil. Steril.* 75, 1212–1216).

already been exposed to chemotherapy prior to ovarian cryopreservation. Nineteen months later, after she had experienced premature menopause, two ovarian cortical strips were thawed and transplanted onto the left ovary and another to the site of the removed ovary. Seven months after transplantation, the patient reported resolution of the menopausal symptoms. Five weeks later, serum estradiol rose up to 352 pmol/l and pelvic ultrasonograhy showed a 10 mm thick endometrium and a 20 mm follicle on the right side. However, progesterone levels were never >2 nmol/l, and no ovulation was detected. Gonadotrophin levels were in the post-menopausal range 9 months after the transplantation. Cortex of the frozen–thawed ovary, as well as biopsy samples of the retained left ovary, did not show evidence of neoplasia.

However, none of these cases can be considered as 'ideal' to judge the performance of this procedure, because the cryopreserved tissue was previously compromised due to ovarian surgery or chemo- and radiotherapy.

Heterotopic ovarian transplantation. Autotransplanting tissue to a heterotopic site is a well-known concept, and it has long been utilized for implanting fresh or frozen–thawed parathyroid tissue following total parathyroidectomy (Wells *et al.*, 1975, 1978). Heterotopic transplantation has significant advantages; this technique does not require general anaesthesia or abdominal surgery. In addition, it is easy to monitor follicle development, and remove the transplanted tissue from a subcutaneous site when necessary. In our initial reports, we utilized the subcutaneous space above the brachioradialis facia of the forearm, and recently, the lower abdomen to transplant ovarian cortical pieces (Oktay *et al*., 2001b,c, 2004).

We performed the first subcutaneous ovarian transplantation in a 35 year old woman with stage III squamous cervical carcinoma prior to pelvic radiation (Oktay *et al*., 2001b). Immediately after

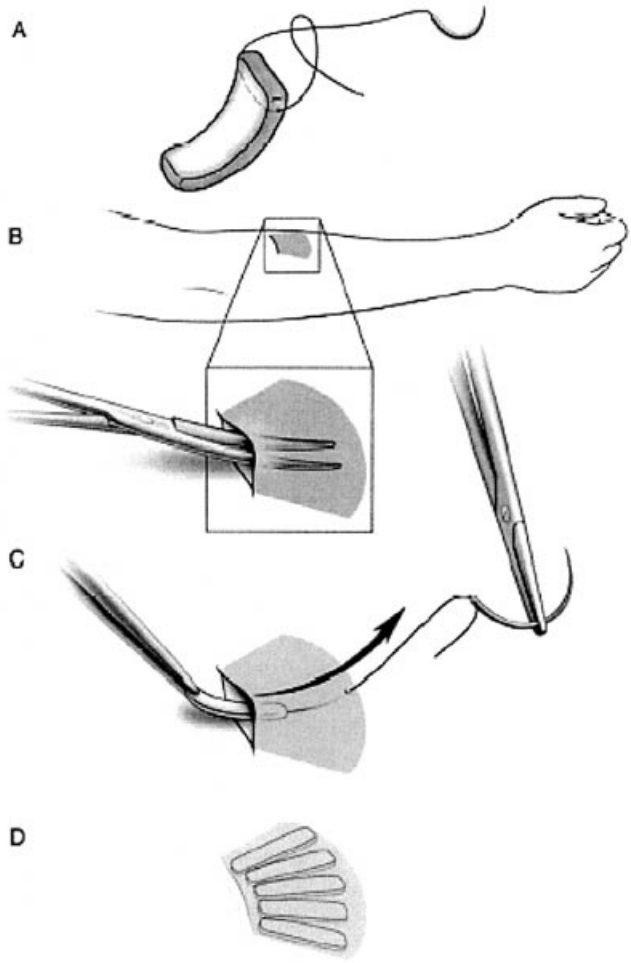

Figure 2. A surgical technique for ovarian transplantation to the forearm. (**A**) Preparation of the strips. (**B**) Creating a subcutaneous poket. (**C**) Wedging grafts underneath the forearm skin. (**D**) Final position of the grafts after transplantation. †Patients receiving only radiotherapy. ‡Ovarian tissue cryopreservation is currently the only option for pre-pubertal patients. Reprinted by permission from the American Society of Reproductive Medicine (Oktay et al., 2003, *Fertil. Steril*. 80, 193–198).

laparoscopic oophorectomy her FSH and LH levels indicated that she was in menopause. Six weeks after the transplantation, the patient reported a painless swelling at the site of the ovarian transplantation. Estradiol levels were elevated, and ultrasound revealed one dominant follicle measuring 15 mm and four other antral follicles. Restoration of normal testosterone levels also indicated normal stromal function. After controlled ovarian stimulation with hMG, three oocytes were retrieved, two of which were immature and one was in metaphase I. The metaphase I oocyte underwent *in vitro* maturation but ICSI of this oocyte did not result in fertilization. This patient had nearly 3 years of ovarian function but never ovulated spontaneously. In another 37 year old woman, we transplanted fresh ovarian tissue subcutaneously to the forearm after oophorectomy due to recurrent benign ovarian cysts (Oktay *et al*., 2001b). Resumption of menstrual periods and spontaneous ovulation occurred as early as 3 months after the transplant. However, the cycle length varied from 14 to 45 days, and the graft ceased function after 3 years (Figure 2).

Very recently, after transplantation of frozen-banked ovarian tissue underneath lower abdominal skin in a 36 year old breast cancer survivor, we were able to reverse menopause. Percutaneous oocyte aspiration resulted in the generation of a 4-cell embryo, the first after an ovarian transplantation procedure (Oktay *et al*., 2004).

One of the potential limitations of ovarian tissue cryopreservation and transplantation is loss of a large fraction of follicles during the initial ischaemia after transplantation. Previous work indicated that whereas the loss due to freezing is relatively small (Oktay *et al*., 1997a; Aubard *et al*., 1999; Baird *et al*., 1999), up to two-thirds of follicles are lost after transplantation. Because of this fact, we do not recommend ovarian tissue freezing in patients aged >40 years (Oktay, 2000), and we prefer this procedure in patients <35 years of age.

Risk of metastatic disease

Ovarian tissue can be grafted once the patient survives malignancy or is considered cured. It is naturally of concern that the frozen–thawed tissue might harbour malignant cells, and the cancer could be reseeded by ovarian transplantation. Fortunately, most of the malignant tumours of reproductive age women do not metastasize to ovaries, with the exception of some haematological malignancies such as leukaemias, Burkitt's lymphoma, and some advanced stage solid tumours such as breast and colon cancers (Chu *et al*., 1981; Wyld *et al*., 1983; Yada-Hashimoto *et al*., 2003). In children, ovarian metastasis was demonstrated in 25–50% of neuroblastoma cases in *post mortem* examinations (McCarville *et al*., 2001; Oktay *et al*., 2001a).

Breast cancer has a low-to-intermediate risk of ovarian involvement in early stages (Oktay and Sonmezer, 2004). In the absence of clinical and radiological evidence of distant metastasis, ovarian involvement is extremely rare, and most cases could be detected by a thorough clinical and radiological evaluation (Curtin *et al*., 1994). Most of the occult metastases belong to the less common histological type, the infiltrating lobular (15% of all breast cancer) as opposed to the invasive ductal cancer which constitutes >70% of all breast cancers (Young and Scully, 1987; Morrow, 2001; Perrotin *et al*, 2001; Li *et al*, 2003). Moreover, lobular cancer typically occurs in post-reproductive age women (Sastre-Garau *et al*., 1996; Li *et al*., 2003), and ovarian metastasis more commonly occurs in advanced stage cancer (Gagnon and Tetu, 1989; Hann *et al*., 2000). Incidence of ovarian involvement is exceptionally low in Wilm's tumour, Ewing's sarcoma, lymphomas, osteosarcomas, and extragenital rhabdomyosarcomas. In squamous cell cervical cancer, ovarian involvement is < 1.0%, whereas it is reported to be between 1.7 and 12.5% in adenocarcinoma of the cervix, (Woodruff *et al*., 1970; Sutton *et al*., 1992; Nakanishi *et al*., 2001). The risk of ovarian involvement according to tumour type is summarized in Table II.

Table II. The risk of ovarian metastasis according to cancer types

Cancers with low risk of ovarian involvement
Wilm's tumour
Ewing's sarcoma
Breast cancer
Stage I–III
Infiltrative ductal histological subtype
Non-Hodgkin's lymphoma
Hodgkin's lymphoma
Non-genital rhabdomyosarcoma
Osteogenic sarcoma
Squamous cell carcinoma of the cervix
Cancers with moderate risk of ovarian involvement
Adenocarcinoma/adenosquamous carcinoma of the cervix
Colon cancer
Breast cancer
Stage IV
Infiltrative lobular histological subtype
Cancers with high risk of ovarian involvement
Leukaemia
Neuroblastoma
Burkitt lymphoma

The risk of reimplanting cancer cells via transplanted ovarian tissue was investigated in some animal and xenograft studies. In a rodent study, most animals died of the disease after a small piece of fresh or frozen–thawed ovarian tissue had been transplanted from mice with a very aggressive form of lymphoma (Shaw *et al*., 1996). However, this type of aggressive lymphoma is extremely rare in humans, during reproductive age. In another study, human frozen–thawed ovarian tissue from patients with Hodgkin's disease (follicular B cell lymphoma, $n = 13$) and non-Hodgkin's lymphoma (NHL) ($n = 5$) were xenografted subcutaneously to NOD/LtSz_SCID mice (Kim *et al*., 2001). None of the animals grafted with ovarian tissue from lymphoma patients developed lymphoma, whereas in the positive control group, all three SCID mice grafted with lymph node tissue from NHL patients developed human B-cell lymphoma, as demonstrated by microsatellite DNA analysis. The latter indicated that ovarian transplantation is safe in NHL patients. The results were not completely reassuring for Hodgkin lymphoma patients, since, in this model, positive controls did not also transmit the disease. However, based on clinical experience, ovarian involvement is extremely rare in Hodgkin's patients.

Regardless of the magnitude of risk of ovarian involvement, a thorough histological evaluation should be performed on multiple samples taken from the ovarian tissue before and after cryopreservation. Additionally, molecular biology techniques and immunohistochemistry can be used to screen for the presence of cancer cells in the ovary (Oktay and Yih, 2001).

Ovarian transposition (oophoropexy)

Ovaries can be moved out of the radiation field so that direct effects of ionizing radiation may be avoided. It is more than three decades since this procedure was first put into practice to preserve ovarian function in patients with Hodgkin's disease receiving pelvic or para-aortic lymph node irradiation at staging laparotomy (Ray *et al*., 1970; Nahhas *et al*., 1971; Le Floch *et al*., 1976; Thomas *et al*., 1976). If the patient is to undergo an abdominal surgery, ovaries can be transposed simultaneously, or if she is to be treated non-surgically, laparoscopic transposition can be performed before the scheduled radiotherapy (Tulandi and Al-Took, 1998; Morice *et al*., 2000).

The success with fertility preservation by ovarian transposition prior to radiotherapy varies between 16 and 90% (Hunter *et al*., 1980; Anderson *et al*., 1993; Clough *et al*., 1996; Morice *et al*., 2000; Meirow and Nugent, 2001; Bisharah and Tulandi, 2003).

This variation in success rates is due to variations in the degree of scatter radiation, vascular compromise, the age of the patient, dose of radiation, whether the ovaries were shielded, whether concomitant chemotherapy is used, and whether vaginal brachytherapy or pelvic external beam irradiation plus brachytherapy was used (Thomas *et al*., 1976; Hunter *et al*., 1980; Anderson *et al*., 1993; Feeney *et al*., 1995; Williams *et al*., 1999; Morice *et al*., 2000; Meirow and Nugent, 2001). In a recent study, laparoscopic oophoropexy was performed to preserve ovarian function prior to pelvic irradiation in 10 patients with Hodgkin's disease (Williams *et al*., 1999). Pelvic radiation dose ranged from 1500 to 3500 cGy. All five patients who received minimal or no chemotherapy had evidence of ovarian function, four of whom achieved pregnancy. In contrast, four patients who received multiple courses of chemotherapy and one patient who received 3500 cGy to the femoral lymph nodes and pelvis with little central shielding had ovarian failure at follow-up. The length of follow-up was not clearly stated.

Even though ovarian transposition may decrease the risk of ovarian failure, ovaries are still subjected to a significant amount of radiation despite proper shielding. This is mainly due to scatter radiation and transmission through the shield, which may amount to as much as 8–15% of the total pelvic radiation dose (Le Floch *et al*., 1976). In addition, this surgical procedure is not without complications; Fallopian tube infarction, chronic ovarian pain, ovarian cyst formation, and migration of ovaries back to their original position before radiotherapy have been reported, some of which may require additional gynaecological surgeries (Gabriel at al., 1986; Williams *et al*., 1999; Meirow and Nugent, 2001). Anderson *et al*. (1993) reported subsequent oophorectomy in nine of 51 patients (17.5%) for the management of painful ovarian cysts after a mean duration of 46.8 months from the procedure.

When ovaries are transposed to an abdominal position, spontaneous pregnancy may not be possible, unless a second procedure is performed to relocate ovaries back to pelvis (Morice *et al*., 1998). In addition, should these patients need IVF in the future, oocyte retrieval may become technically more challenging. Therefore candidates for ovarian transposition should be selected carefully, accounting for all the variables that may affect its success rates. It should also be borne in mind that, when gonadotoxic chemotherapy is used along with radiation, there is no strong rationale to perform this procedure.

GnRH analogue co-treatment

It has been hypothesized, largely based on the debated role of gonadal suppression in men in preserving testicular function against chemotherapy, and partially the misbelief that pre-pubertal girls are not affected by gonadotoxic cancer treatment, that ovarian suppression can be protective.

Some animal studies demonstrated a protective role of GnRH analogue treatment against chemotherapy-induced gonadal damage (Glode *et al*., 1981; Ataya *et al*., 1989; Bokser *et al*., 1990). Ataya *et al*. (1995a) demonstrated that primordial follicle loss associated with cyclophosphamide treatment was significantly lower in Rhesus monkeys receiving GnRH analogue treatment, compared with GnRH analogue untreated animals (65 versus 29%). In another study (Ataya *et al*., 1995b) they failed to demonstrate any protective effect of GnRH analogue treatment against radiation-induced ovarian injury in rhesus monkeys. Germinal epithelium is extremely sensitive to irradiation, and despite some reports showing a protective effect, it seems unlikely that radiotherapy-induced gonadal damage can be prevented by gonadal suppression (Ortin *et al*., 1990; Ataya *et al*., 1995b; Gosden *et al*., 1997; Viviani *et al*., 1999).

A few non-randomized studies with short-term follow-up suggested a protective role for GnRH analogue treatment (Blumenfeld *et al*., 1996; 1999, 2000, 2002; Recchia *et al*., 2002) Blumenfeld *et al*. (1996) investigated the protective role of GnRH analogue co-treatment in 18 women treated with chemotherapy for Hodgkin's or Non-Hodgkin's lymphoma, compared with a historical control group of 18 women treated with similar regimens. Ten patients in the study and 11 in the control group also received mantle field irradiation after chemotherapy. Patients received GnRH analogue for a maximum of 6 months starting prior to chemotherapy. In the study group, 93.7% of the patients resumed spontaneous ovulation and menses within 3–8 months of termination of the combined chemotherapy/GnRH analogue co-treatment. In contrast, only 39% in the control group resumed ovarian cyclic activity, and 61% experienced premature ovarian failure. However, mean follow-up was 1.7 $\pm$ 1.0 years (range, 0.5–4) in the study, and 7.0 $\pm$ 4.9 years (range, 1.5–8.0) in the control group. In another study, the same authors studied the protective effect of GnRH analogue in 54 cancer patients and eight women with non-malignant diseases who received chemotherapy (Blumenfeld, 2001), and retrospectively compared these patients with 55 women who had been treated with similar chemotherapy. In almost all of the surviving patients in the GnRH analogue/chemotherapy group, spontaneous ovulation and menses occurred within 6 months. Less than 50% of the patients in the control group resumed ovarian function and regular cyclic activity. However, the methodology was not clear; whereas the control group was retrospective, the criteria for choosing the control patients were not mentioned. For the treatment group, the length of follow-up was not mentioned. From these studies, it cannot be determined whether the lower incidence of ovarian failure is due to GnRH analogue treatment or to the shorter follow-up.

In a rodent study, Meirow *et al*. (1999) demonstrated that, albeit the residual follicle count was related to the dose of chemotherapy, primordial follicle destruction occurred in all mice receiving different doses of cyclophosphamide irrespective of age. In that study, the dose of cyclophosphamide ranged from 20 to 100 mg/kg. It was notable that, despite significant follicle loss, short-term reproductive performance of cyclophosphamide-treated mice was not affected compared with controls. The authors concluded that immediate reproductive performance was not an accurate marker for assessment of chemotherapy-induced ovarian damage. In a small prospective randomized study, Waxman *et al*. (1987) demonstrated that GnRH analogue treatment was ineffective in preserving fertility in patients receiving chemotherapy for Hodgkin's disease. In that study, 30 men and 18 women were randomly allocated to receive GnRH analogue prior to, and for the duration of, cytotoxic chemotherapy. Twenty men and eight women received buserelin. After 3 years of follow-up, all men in both study and control groups became oligo/azoospermic. Among the women, four of eight in the treatment and six of nine female controls became amenorrhoeic.

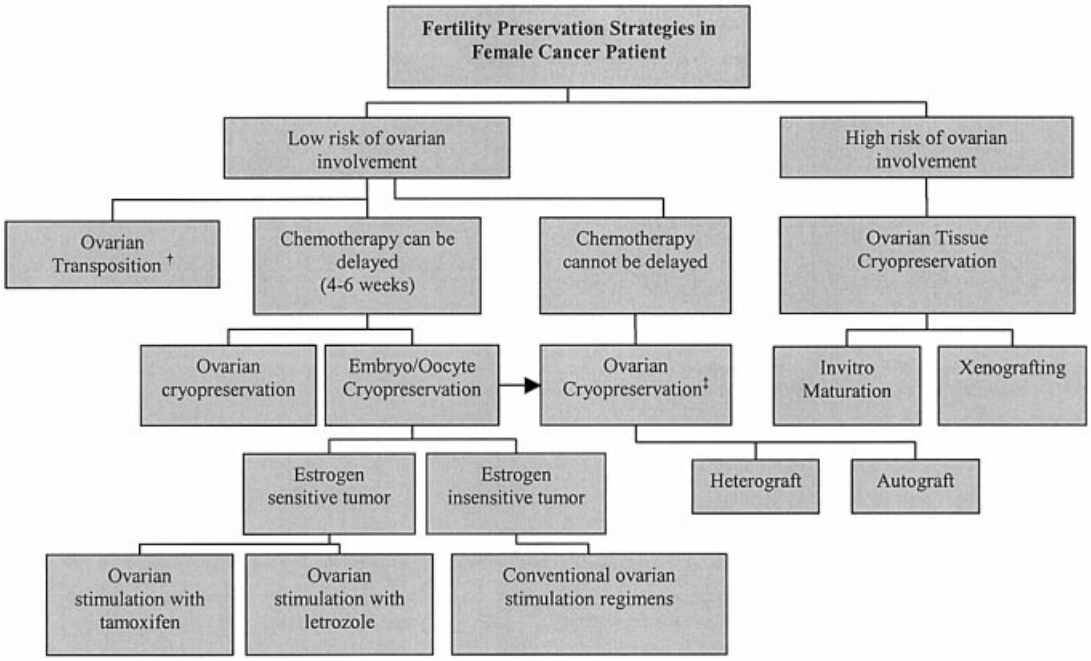

Figure 3. Fertility preservation algorithm. The patient's options vary depending on ovarian involvement, type of cancer treatment, time available, age, and estrogen sensitivity of the tumour.

In an adult ovary, ovarian reserve is made up of primordial follicles that constitute ~90% of the total follicle pool (Lass *et al*., 1997; Oktay *et al*., 1997a). These follicles are at resting stage with an oocyte arrested at the prophase of first meiotic division. Primordial follicles initiate follicle growth through an unknown mechanism which is FSH independent (Gougeon, 1996; Oktay *et al*., 1997b, 1998a; McNatty *et al*., 1999; Meduri *et al*., 2003). FSH receptors are not expressed in primordial and primary follicles, but the expression is uniformly present in as early as 3–4 granulosa layer preantral follicles. It is possible that ovarian suppression with GnRH analogues preserves these follicles that have initiated growth. However, growing follicles not only constitute <10% of all follicles at any given time in the ovary, but once growth has been initiated, they are destined either to become atretic or to ovulate. It is quite possible that GnRH analogue co-treatment delays the fate of these follicles, hence giving the impression that ovarian function is protected in the short run. It has also been suggested that the effects of GnRH analogues may be explained through direct actions of GnRH in the ovary (Blumenfeld, 2003). It was shown that luteinized immortalized granulosa cell lines express GnRH receptors (Cheng *et al*., 2002); however, to our knowledge, presence of these receptors on human primordial follicles or oocytes has never been demonstrated. A clinical example for why gonadal suppression may not protect ovaries is the fact that pre-pubertal children receiving heavy chemotherapy still suffer from ovarian failure (Teinturier *et al*., 1998). However, since younger patients have a larger ovarian reserve, absence of immediate ovarian failure does not mean that gonads are unaffected by the chemotherapy (Meirow *et al*., 1999). All of the patients who receive heavy gonadotoxic chemotherapy will eventually suffer from premature ovarian failure (Viviani *et al*., 1999; Grigg *et al*., 2000). In the absence of a prospective randomized study with sufficient power, we do not rely on ovarian suppression as an effective means of fertility preservation.

Other strategies for fertility preservation and future possibilities

When the risk of ovarian involvement with cancer cells is high, some other experimental options may be considered in the future. It has been possible to xenograft human ovarian tissue to immunodeficent mice and grow mature follicles in these xenografts (Oktay *et al*., 1998a,b; Gook *et al*., 2001). It has also been possible to retrieve oocytes from xenografted human ovarian cortical pieces (Revel *et al*., 2000). However, the possibility of trans-species viral infections has to be addressed. Primordial follicles can also be isolated from cryopreserved ovarian tissue, and it is theoretically possible to use these follicles for the purpose of *in vitro* maturation (Oktay *et al*., 1997a). Even though this has been partially successful in mice, the prospect for success in humans is not clear at the present time. A combination of oocyte and ovarian tissue cryopreservation has also been suggested as a new strategy (Revel *et al*., 2003).

Another possibility is *in vitro* growth of primordial follicles isolated from cryopreserved ovarian tissue. It has been possible to isolate primordial follicles from human ovarian tissue (Oktay *et al*., 1997a), but there has been no success in growing them *in vitro*. Progress has been made in rodents with this technique including production of oocytes competent of meiotic maturation, fertilization and preimplantation *in vitro* from primordial follicle (Cortvrindt and Smitz, 2001; O'Brien *et al*., 2003) but whether this will translate to human studies is currently unclear.

It has recently been postulated that the mechanism of age-related physiological as well as chemo- or radiotherapy induced

loss in the ovarian germ cell population is mediated by programmed cell death (Perez *et al*., 1997; Morita and Tilly, 1999). Sphingosine-1-phosphate (S1P), a bioactive sphingolipid metabolite formed by sphingosine kinase, is an important lipid mediator and has many actions both inside and outside the cell. Morita *et al*. (2000) showed that wild-type mice treated with S1P resisted both developmental and cancer therapy-induced apoptosis. Radiation-induced oocyte loss could be completely prevented by S1P therapy in wild-type mice. The same group investigated transgenerational genomic instability, and failed to demonstrate discernable propagation of genomic damage in mice pretreated with S1P before receiving ionizing radiation (Paris *et al*., 2002).

One of the limiting factors in ovarian transplantation is the loss of a large fraction of ovarian reserve due to initial ischaemia. In theory, cryopreservation of an intact ovary with its vascular pedicle would lend itself to whole organ transplantation with vascular anastomosis, thus enabling us to avoid the ischaemia. Even though preservation of ovarian architecture and restoration of the reproductive function after transplantation of fresh or frozen–thawed intact ovary to an orthotopic or heterotopic site using microvascular anastomosis was recently demonstrated in mice and in sheep (Jeremias *et al*, 2002; Wang *et al*., 2002; Bedaiwy *et al*., 2003; Chiu and Hu, 2003), it has not been technically possible to cryopreserve an entire human ovary with its vascular pedicle: first, human ovary is larger than the ovaries of the animals used in the aforementioned studies; and second, it may be challenging to devise a cryopreservation protocol that will optimally preserve both the follicles and vasculature. Nevertheless the research on cryopreserving whole human ovary is continuing, and this technique may one day become clinically possible.

Donor oocytes and surrogacy

IVF with donor oocytes is another alternative in patients who suffer from premature menopause or low ovarian reserve due to cancer treatment (Polak de Fried *et al*., 1998). The success rates with appropriate oocyte donors are now >60% per embryo transfer. Gestational surrogacy can also be employed in patients who had undergone hysterectomy, or received pelvic radiation for cervical cancer. Patients with breast cancer who are considered high risk for recurrence, or who have to be on lifelong therapy with aromatase inhibitors, may also resort to gestational surrogacy. However, laws and regulations regarding this procedure vary significantly between countries, and between each state in the USA.

Conclusions

The number of options to preserve fertility is growing. These options vary depending on the patient's age, the time available, type of cancer and whether the likelihood of ovarian involvement is high. Physicians should take a comprehensive approach in counselling their patients regarding fertility preservation procedures. The experimental nature of these procedures should be carefully discussed. With the exception of embryo cryopreservation, all of the fertility preservation options are considered experimental. In many cases, rapid referral to appropriate centres with adequate experience in fertility preservation will ensure that the patient has enough time for adequate counselling, and can resort to one or more of the procedures outlined in Figure 3.

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