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Ovarian tissue cryopreservation and transplantation: a review

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The review covers current options for ovarian tissue cryopreservation and transplantation and provides a systematic review of the existing literature from the last 10 years, taking into account all previously published reviews on the subject. The different cryopreservation options available for fertility preservation in cancer patients are embryo cryopreservation, oocyte cryopreservation and ovarian tissue cryopreservation. The choice depends on various parameters: the type and timing of chemotherapy, the type of cancer, the patient's age and the partner status. The different options and their results are discussed, as well as their putative indications and efficacy. The review concludes that advances in reproductive technology have made fertility preservation techniques a real possibility for patients whose gonadal function is threatened by premature menopause, or by treatments such as radiotherapy, chemotherapy or surgical castration.

Key words: fertility preservation/ovarian tissue cryopreservation/transplantation

Introduction

In 2006, 1 399 790 new cancer cases are expected in the United States, of which 679 540 will be women (Jemal *et al*., 2006). According to previous reports, 8% of these women will be under the age of 40. By 2010, it is estimated that one in every 250 people in the adult population will be childhood cancer survivors (Blatt, 1999). Advances in the diagnosis and treatment of childhood, adolescent and adult cancer have greatly increased the life expectancy of premenopausal women with cancer but have resulted in a growing population of adolescent and adult long-term survivors of childhood malignancies (Blatt, 1999), who may experience infertility problems due to induced premature ovarian failure (POF).

Aggressive chemotherapy and radiotherapy, and bone marrow transplantation (BMT), can cure >90% of girls and young women affected by such disorders (Ries *et al*., 1999). However, the ovaries are very sensitive to cytotoxic treatment, especially to alkylating agents, which are classified as high risk for gonadal dysfunction (e.g. cyclophosphamide, busulfan, melphalan, chlorambucil, dacarbazine, procarbazine, ifosfamide, thiotepa and nitrogen mustard) (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). Doxorubicin and the alkylating-like agents cisplatin and carboplatin fall into the medium-risk category (Hortobagyi *et al*., 1986; Wallace *et al*., 1989; Maneschi *et al*., 1994; Tangir *et al*., 2003), whereas methotrexate, bleomycin, 5-fluorouracil, actinomycin-D, mercaptopurine and vincristine are considered as low risk for gonadal dysfunction (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; Sonmezer and Oktay, 2004; Wallace *et al*., 2005a) (Table I). The type and dose of chemotherapeutic agent influence the progression to ovarian failure, with alkylating agents increasing the risk of POF by a factor of 9 (Byrne *et al*., 1992).

Cyclophosphamide is the agent most commonly implicated in causing damage to oocytes and granulosa cells in a dose-dependent manner (Warne *et al*., 1973; Sanders *et al*., 1996; Meirow *et al*., 1999; Kenney *et al*., 2001). Since Meirow *et al*. (1999) demonstrated primordial follicular depletion in mice treated with cyclophosphamide, tremendous progress has been made towards elucidating the cellular and molecular mechanisms responsible for mediating oocyte death and follicular depletion under normal and pathological conditions. Perez *et al*. (2000) demonstrated that doxorubicin induces apoptosis in pregranulosa cells, and Takai *et al*. (2003) showed that bax and caspase-2 and caspase-3 are functionally important mediators of vinylcyclohexene diepoxide-induced ootoxicity in mice.

This follicular destruction generally results in the loss of both endocrine and reproductive functions, depending on the dose and the age of the patient. Indeed, Larsen *et al*. (2003) reported a fourfold increased risk of POF in teenagers treated for cancer, and a risk increased by a factor of 27 in women between 21 and 25 years of age. Complete amenorrhoea was reported after a dose of 5 g of

Table I. Cytotoxic agents according to the degree of gonadotoxicity

High risk	Intermediate risk	Low/no risk
Cyclophosphamide	Doxorubicin	Methotrexate
Busulfan	Cisplatin	Bleomycin
Melphalan	Carboplatin	5-Fluorouracil
Chlorambucil		Actinomycin-D
Dacarbazine		Mercaptopurine
Procarbazine		Vincristine
Ifosfamide		
Thiotepa		
Nitrogen mustard		

cyclophosphamide in women over 40 years of age, and after doses of 9 and 20 g in women of 30–40 and 20–30 years of age, respectively (Shalet, 1980). A combination of various chemotherapeutic agents further increases gonadal toxicity. After chemotherapy with MOPP/ABV (chlorethamine, vincristine, procarbazine, prednisone, doxorubicin, bleomycin, vinblastine) hybrid chemotherapy, Schilsky *et al*. (1981) found that amenorrhoea developed in 89 and 20% of patients >25 and <25 years of age at the time of treatment, respectively. The median age of patients who became amenorrhoeic after therapy was significantly higher than that of patients who maintained normal menses (26 versus 20 years; $P = 0.008$).

Abdominal ionizing radiation associated with alkylating agents often induces POF, rendering patients infertile in almost 100% of cases. Indeed, for radiotherapy, it has been stated that a dose of 5–20 Gy administered to the ovary is sufficient to completely impair gonadal function (Wallace *et al*., 2005b), whatever the age of the patient. The dose of radiation required to destroy 50% of the oocyte reserve has been found to be <2 Gy (Wallace *et al*., 2003). Moreover, uterine irradiation at a young age reduces adult uterine volume (Larsen *et al*., 2004). Radiation doses between 14 and 30 Gy have been reported to result in uterine dysfunction (Critchley *et al*., 1992; Bath *et al*., 1999; Critchley and Wallace, 2005). The practitioner should be aware of this effect of radiotherapy on the uterus, which could interfere with the implantation capacity of embryos.

In the field of oncological indications for ovarian tissue cryopreservation, there have been no major modifications since our review published in 1998 (Donnez and Bassil, 1998) (Table II). In countries where surrogacy is not legal, a conservative fertility approach in case of gynaecological malignancy is only valuable if the uterus can be spared during surgery. This includes cases of early cervical carcinoma (Dargent *et al*., 2000; Burnett *et al*., 2003; Tanguay *et al*., 2004), early vaginal carcinoma (Hicks and Piver, 1992), early endometrial adenocarcinoma (Jadoul and Donnez, 2003), ovarian tumours of low malignancy (Donnez *et al*., 2003; Boran *et al*., 2005; Fauvet *et al*., 2005; Tinelli *et al*., 2005) and some selected cases of unilateral ovarian carcinoma (stage IA) (Donnez and Bassil, 1998; Kleine, 1996). The choice of a possible conservative surgical approach in these patients and the question of implementing such treatment alone remain controversial, as all the published results were obtained on the basis of retrospective studies and/or case reports. The fertility outcome is conditioned by the adjuvant therapy, i.e. local radiotherapy and/or chemotherapy.

Intensive chemotherapy and/or total body irradiation required before BMT constitute the treatment combination presenting the greatest risk of POF. Indeed, such high doses of chemotherapy (commonly using the highly cytotoxic cyclophosphamide/busulfan regimen) and/or radiotherapy lead to subsequent ovarian failure in almost all cases, children and adults alike (Sanders *et al*., 1996; Meirow and Nugent, 2001; Lobo, 2005). The risk of POF was estimated to be 92% in the study by Meirow and Nugent (2001), while

Table II. Indications for ovarian tissue cryopreservation in case of malignant and non-malignant diseases

Malignant	Non-malignant
Extrapelvic diseases	Uni/bilateral oophorectomy
Bone cancer (osteosarcoma—Ewing's sarcoma)	Benign ovarian tumours
Breast cancer	Severe and recurrent endometriosis
Melanoma	BRCA-1 or BRCA-2 mutation carriers
Neuroblastoma	
Bowel malignancy	
Pelvic diseases	Risk of premature menopause
Non-gynaecological malignancy	Turner's syndrome
Pelvic sarcoma	Family history
Rhabdomyosarcoma	Benign diseases requiring chemotherapy: autoimmune diseases
Sacral tumours	(systemic lupus erythematosus, rheumatoid arthritis, Behçet's disease and Wegener's disease)
Rectosigmoid tumours	
Gynaecological malignancy	
Early cervical carcinoma	
Early vaginal carcinoma	
Early vulvar carcinoma	
Selected cases of ovarian carcinoma (stage IA)	
Ovarian borderline tumours	
Systemic diseases	Bone marrow transplantation
Hodgkin's disease	Benign haematological diseases: sickle cell anaemia, thalassaemia major and aplastic anaemia
Non-Hodgkin's lymphoma	Autoimmune diseases unresponsive to immunosuppressive therapy
Leukaemia	
Medulloblastoma	

Teinturier *et al*. (1998) actually reported 0% of ovarian recovery after busulfan treatment before BMT. A large retrospective survey of pregnancy outcomes after haematopoietic stem cell transplantation (HSCT) (peripheral blood or BMT) involving 37 362 patients revealed that only 0.6% of patients conceived after autologous or allogenic SCT (Salooja *et al*., 2001; Lutchman Singh *et al*., 2005). It is thus obvious that high doses of alkylating agents, irradiation and advancing age increase the risk of gonadal damage.

Cryopreservation should not be reserved solely for women with malignant disease (Donnez *et al*., 2006). Indeed, HSCT has been increasingly used in recent decades for non-cancerous diseases, such as benign haematological disease (sickle cell anaemia, thalassaemia major and aplastic anaemia) and autoimmune diseases previously unresponsive to immunosuppressive therapy (systemic lupus erythematosus and autoimmune thrombocytopenia) (Slavin *et al*., 2001; Mattle *et al*., 2005; Wolner-Hanssen *et al*., 2005; Donnez *et al*., 2006). Other benign diseases, such as recurrent ovarian endometriosis or recurrent ovarian mucinous cysts, are also indications for ovarian cryopreservation. Patients undergoing oophorectomy for prophylaxis may potentially benefit from ovarian cryopreservation too. The indications for cryopreservation of ovarian tissue in case of non-malignant disease are summarized in Table II.

Fertility preservation in cancer patients: different cryopreservation options

Several options are currently available to preserve fertility in cancer patients and give them the opportunity to become mothers when they have overcome their disease: embryo cryopreservation, oocyte cryopreservation or ovarian tissue cryopreservation (Gosden *et al*., 1997; Donnez and Bassil, 1998; Oktay *et al*., 1998a, 2004; Donnez *et al*., 2000, 2005; Oktay and Karlikaya, 2000; Meirow and Nugent, 2001; Torrents *et al*., 2003; Sonmezer and Oktay, 2004; Gosden, 2005; Lobo, 2005; Meirow *et al*., 2005; Kim, 2006). The choice of the most suitable strategy for preserving fertility depends on different parameters: the type and timing of chemotherapy, the type of cancer, the patient's age and the partner status.

The only established method of fertility preservation is embryo cryopreservation, according to the Ethics Committee of the American Society for Reproductive Medicine (2005), but this option requires the patient to be of pubertal age, have a partner or use donor sperm, and be able to undergo a cycle of ovarian stimulation, which is not possible when the chemotherapy has to be initiated immediately or when stimulation is contraindicated according to the type of cancer.

Cryopreservation of oocytes can be performed in single women who can undergo a stimulation cycle, although the effectiveness of this technique is very low, with pregnancy and delivery rates ranging from 1 to 5% per frozen oocyte (Stachecki and Cohen, 2004; Borini *et al*., 2006; Levi Setti *et al*., 2006).

Cryopreservation of ovarian tissue is the only option available for prepubertal girls, and for woman who cannot delay the start of chemotherapy. Ovarian tissue can theoretically be frozen using three different approaches: as fragments of ovarian cortex, as entire ovary with its vascular pedicle or as isolated follicles. Human ovarian cryopreservation and transplantation procedures have so far been almost exclusively limited to avascular cortical fragments, both in experimental and in clinical studies, and, for now, this is the only procedure that has yielded live births in humans after autologous transplantation (Donnez *et al*., 2004; Meirow *et al*., 2005).

Embryo cryopreservation

Embryo cryopreservation has become a routine technique in all IVF centres and has proven its efficacy in terms of pregnancy and 'take-home-baby' rates. Although this method has already been used for young cancer patients (Winkel and Fossum, 1993), there are significant drawbacks to its use.

First, medical reasons might impede its application (a) if the beginning of cancer treatment cannot be delayed and there is no time to complete ovarian stimulation or (b) when the stimulation procedure may be theoretically harmful to patients with hormone-sensitive tumours, such as breast cancer. Even if IVF can theoretically be undertaken on the basis of a spontaneous ovarian cycle (Brown *et al*., 1996), the small number of obtainable oocytes (and subsequently viable embryos for transfer) makes it extremely unlikely that any live births will be achieved in these conditions.

Ginsburg *et al*. (2001) showed that women undergoing IVF after chemotherapy had a poor response to gonadotrophins and, more recently, Dolmans *et al*. (2005) clearly demonstrated the poor results of IVF and embryo cryopreservation, even if the attempt is carried out after only one or two regimens of chemotherapy. Recently, tamoxifen and letrozole were employed to stimulate the ovaries for IVF and embryo cryopreservation with some success, whilst possibly providing a safe alternative to traditional ovarian stimulation methods in these patients (Oktay *et al*., 2005).

The partner status of the patient may also impede embryo cryopreservation. If the patient has no partner or is an adolescent, the only available solution is using donor sperm to ensure fertilization of her oocytes. Finally, this technique is inappropriate for children, who have not reached puberty.

In conclusion, embryo cryopreservation is an efficient technique, but only an option for patients from whom mature oocytes can be collected and who have a partner (or are willing to use donor sperm).

Oocyte cryopreservation

Oocyte cryopreservation is an alternative option for patients with the same characteristics as those described above for embryo cryopreservation but who are not with a partner and do not wish to use donated sperm. In this case, IVF of their oocytes to produce embryos to be frozen for future implantation is not possible. Thus, the oocytes themselves must be cryopreserved either as mature or as immature oocytes. Human oocyte cryopreservation has been rapidly incorporated into clinical practice in several centres (Porcu *et al*., 1997, 2004; Fabbri *et al*., 2001; Porcu, 2005).

Mature oocyte cryopreservation

Mature oocyte freezing appears, at least in theory, to be the most logical way of storing female germ cells, comparable to the routinely performed sperm banking. It is an attractive option for women without a partner, if they have time to complete ovarian stimulation before cancer therapy. However, ovarian stimulation and oocyte collection are not applicable for children (Oktay *et al*., 1998a; Torrents *et al*., 2003; Akar and Oktay, 2005).

Still, this procedure has proved largely disappointing. Since the first report of a live birth from a frozen oocyte (Chen, 1986), the results of this procedure worldwide have been variable, with a reported success rate of <2% per thawed oocyte, despite an improved success rate when combined with ICSI (Porcu *et al*., 1997, 2004; Fabbri *et al*., 2001; Lutchman Singh *et al*., 2005; Porcu, 2005). Techniques developed in recent years to improve survival rates of mature oocytes after slow-freezing protocols include increased sucrose concentrations (Fabbri *et al*., 2001), longer pre-equilibration and thawing times (Yang *et al*., 2002) and the use of sodium-depleted media (Boldt *et al*., 2003; Stachecki and Cohen, 2004). Data on frozen-thawed mature oocytes from 21 studies in peer-reviewed journals were examined by Sonmezer and Oktay (2004), who reported a mean survival rate of 47%, a mean fertilization rate of 52.5% and a mean pregnancy rate per thawed oocyte of 1.52%. Very recent data indicate that, although the combination of slow cooling and high sucrose concentrations ensures high rates of oocyte survival, it is not sufficient to guarantee a high standard of clinical efficiency (Borini *et al*., 2006). Indeed, 18 clinical pregnancies were obtained from 927 oocytes in Borini's study. In another very recent study, 2900 oocytes were cryopreserved, of which 1087 were subsequently thawed. In this series, 18 pregnancies were also obtained (Levi Setti *et al*., 2006). Even in the very latest studies, the mean pregnancy rate per thawed oocyte does not exceed 1.8% (Borini *et al*., 2006; Levi Setti *et al*., 2006).

The metaphase II (MII) oocyte is a large and highly specialized cell that is extremely fragile. Oocyte freezing is accompanied by various types of cell injury, which may explain the low survival rate (Van der Elst, 2003). There are two main reasons for these poor results. First, the zona pellucida hardens during the freezing process, probably as a consequence of premature exocytosis of the cortical granules. It could then act as a fence, impairing sperm penetration and normal fertilization, although micromanipulation techniques (ICSI) can, to a certain extent, bypass this problem (Porcu *et al*., 1997; Fabbri *et al*., 2001; Gook *et al*., 1993, 1995). Second, in the mature oocyte, the metaphase chromosomes are lined up by the meiotic spindle along the equatorial plate, but the spindle apparatus is easily damaged by intracellular ice formation during the freezing or thawing process (Pickering *et al*., 1990) (for review, see Mandelbaum *et al*., 2004). The cellular cooling process induces depolymerization of the meiotic spindle, which is a dynamic structure (microtubules being continually assembled at one of its ends and separated at the other). The cell is thus at risk of losing chromosomes and suffering aneuploidy.

There are two main steps in the process of cryopreservation: (i) chilling, i.e. lowering the temperature from the physiological temperature to the point of freezing and (ii) freezing, i.e. further reducing the temperature to the storage temperature (liquid nitrogen at –196°C). Chilling injury can modify the structure of membranes and therefore their integrity (Ghetler *et al*., 2005). It also affects oocyte microtubules (Albertini and Eppig, 1995), cytoskeletal organization (Overstrom *et al*., 1990) and the zona pellucida (Vajta *et al*., 1998).

Chilling injury is temperature dependent; it is caused by changes in membrane properties and integrity and is responsible for the extensive cell damage that occurs during the process of cryopreservation. Inappropriate handling of oocytes at room temperature appears to pose the greatest threat (Mandelbaum *et al*., 2004).

In conclusion, mature oocyte banking is still limited by its low success rate; oocytes are sensitive to chilling, often fail to survive freeze-thawing processes, and are susceptible to cytoskeletal damage and aneuploidy. Currently, even the most optimistic success rates offer patients only a slim chance of pregnancy, if few oocytes are available (Gosden, 2005). Ultrarapid freezing with vitrification may offer advantages over conventional slow cooling protocols by improving post-thawing survival rates but needs to be investigated further. Despite the few promising studies on vitrification (Yoon *et al*., 2000; Katayama *et al*., 2003), even less is known about the potentially detrimental effects of this process compared with conventional cryopreservation techniques; to date, slow freezing and rapid thawing is the protocol of choice for freezing human oocytes (Falcone *et al*., 2004). Better evaluation of sucrose concentrations in the freezing medium could lead to improved results in the future (Porcu, 2005; Borini *et al*., 2006).

Immature oocyte cryopreservation and in vitro *maturation*

Oocytes at the diplotene stage of prophase I, or germinal vesicle (GV) stage, survive the cryopreservation procedure better than those frozen at the MII stage (Boiso *et al*., 2002). These cells have reached full size and complete meiotic competence but have not yet resumed their maturation process and initiated their second metaphase. Although the risk of hardening of the zona pellucida or damage to the cytoskeleton cannot be avoided, it is probable that the absence of a meiotic spindle and the presence of a nuclear membrane protecting the chromatin guarantees the absence of cytogenetic anomalies during further cellular divisions.

Freezing immature oocytes followed by *in vitro* maturation thus offers practical and theoretical advantages (Gosden, 2005), but this method is still suboptimal. Frozen–thawed immature oocytes have to follow a process of *in vitro* maturation before they are ready to be fertilized. Oocyte maturation is considered as the reinitiation and completion of the first meiotic division from the GV stage to the MII stage, and the accompanying cytoplasmic maturation phase for fertilization and early embryonic development (Cha and Chian, 1998). The co-ordination of nuclear and cytoplasmic maturation *in vitro* has proved very difficult to achieve.

Although there are several reports of pregnancies achieved after *in vitro* maturation of fresh GV-stage oocytes (Cha *et al*., 1991; Trounson *et al*., 1994), only one live birth has resulted from an immature oocyte cryopreserved at the GV stage, with subsequent *in vitro* maturation (Tucker *et al*., 1998). Two other teams have described non-ongoing pregnancies. Kan *et al*. (2004) reported one pregnancy from an immature frozen oocyte which ended in a blighted ovum at 12 weeks, and Wu *et al*. (2001) reported one biochemical pregnancy. According to a recent review by Kim, the cryopreservation of GV-stage oocytes will not be a practicable strategy until *in vitro* maturation of these oocytes becomes more reliable (Kim, 2006).

Ovarian tissue cryopreservation

For patients who need immediate chemotherapy, ovarian tissue cryopreservation is the only possible alternative (Gosden *et al*., 1994; Donnez and Bassil, 1998; Meirow *et al*., 1998; Oktay *et al*., 1998a; Donnez *et al*., 2000, 2005). The main aim of this strategy is to reimplant cortical ovarian tissue into the pelvic cavity (orthotopic site) or a heterotopic site like the forearm or the abdominal

wall once treatment is completed and the patient is disease-free (Donnez and Bassil, 1998; Oktay *et al*., 1998a, 2004; Oktay and Karlikaya, 2000; Radford *et al*., 2001; Donnez *et al*., 2004, 2005, 2006; Kim *et al*., 2004a; Meirow *et al*., 2005; Schmidt *et al*., 2005; Demeestere *et al*., 2006) (Table III).

Other strategies, such as the transplantation of isolated cryopreserved primordial follicles and the transplantation of cryopreserved whole ovary, will also be discussed here.

Fragments of cortical ovarian tissue

Lessons learned from auto- and xenografting of cryopreserved animal ovarian tissue

To date, ovarian tissue has been successfully cryopreserved and transplanted into rodents, rabbits, sheep, and marmoset monkeys (Candy *et al*., 1995, 2000; Salle *et al*., 2002; Almodin *et al*., 2004). Successful fertilization and pregnancy after oocyte collection from fresh transplanted ovarian tissue have been described in a primate (Lee *et al*., 2004); the grafted tissue functioned without any surgical connection to major blood vessels.

The generation of live young from xenografted mouse ovarian tissue into a rat recipient was described by Snow *et al*. (2002). Fresh mouse ovarian tissue was xenografted under the kidney capsule of rat recipients. Mature oocytes were produced from the xenografted tissue and subsequently fertilized; they developed into fertile adult mice. The authors state that xenotransplantation may thus prove to be a potentially promising method for the conservation of rare and endangered species of animals. The use of this strategy in human assisted reproduction should be considered with caution, however (Snow *et al*., 2002).

In a recent review of their experimental studies, Baird *et al*. (2004) observed a significant increase in the proportion of growing follicles from <20% in ovaries before grafting to >70% at 7, 30 and 60 days after grafting. According to the authors, the massive recruitment of primordial follicles, which also occurs in cultured fragments, suggests the removal of some inhibitory mechanisms regulating FSH. Following autotransplantation, the number of antral follicles and the secretion of inhibin A are reduced, resulting in raised basal levels of FSH, which could account for the massive recruitment, although the early stage of folliculogenesis can occur in the absence of FSH and LH.

Experimental studies have indicated that the fall in the number of primordial follicles in grafted tissue is due to hypoxia and the delay that occurs before reimplanted cortical tissue becomes revascularized. The loss of primordial follicles in cryopreserved ovarian tissue after transplantation is estimated to be 50–65% in some studies (Baird *et al*., 1999; Nisolle *et al*., 2000) and >90% in one study (Aubard *et al*., 1999).

A very recent study in mice concluded that the graft site affects the number and quality of oocytes produced from ovarian grafts (Yang *et al*., 2006). The study used a mouse ovarian grafting model to investigate whether the graft site (bursal cavity, kidney capsule or site) influences the number, fertilization rate and developmental potential of oocytes recovered from grafts. Graft retrieval and the number of oocytes found in each graft were lowest from the s.c. graft site. The number of 2-cell embryos produced was significantly higher with oocytes from grafts to the bursa, compared with the other sites.

In a recent article, Israely *et al*. (2004) analyzed angiogenic events following ovary xenotransplantation. Rat ovaries were transplanted into the muscle of castrated nude mice. The characterization of the neovasculature by dynamic contrast-enhanced magnetic resonance imaging (MRI) confirmed that the graft was devoid of any blood supply. Functional vessels within the graft were detected by MRI and histology from day 7 onwards. By 2–3 weeks, both the blood volume fraction and the permeability were measured using albumin-based MR contrast material and were found to be higher in the graft than in adjacent muscle. The same team recently demonstrated that transplantation into angiogenic granulation tissue created during wound healing shortened the ischaemic interval (Israely *et al*., 2006). This confirms the benefits of the technique of induced angiogenesis and neovascularization that was used in the first case of successful orthotopic transplantation of cryopreserved ovarian tissue (Donnez *et al*., 2004).

The assessment of tumour oxygenation by electro-paramagnetic resonance (EPR) was recently reviewed by Gallez *et al*. (2004). In this review, different methods were used to estimate the oxygenation of tumours and compare EPR oximetry with nuclear magnetic resonance and MRI. The evaluation of oximetry in human ovarian xenografts was initiated to improve the revascularization process, which remains one of the main limitations of ovarian cortical strip use (Donnez *et al*., 2005).

Lessons learned from xenografting cryopreserved human ovarian tissue

Human ovarian tissue can be successfully cryopreserved, showing good survival and function after thawing. Hovatta (2005) arrived at this conclusion after reviewing all relevant studies since 1996, when the first case of cryopreservation of human ovarian tissue was described. In these studies, survival and morphological and functional recovery of ovarian tissue after thawing were proved by live/dead assays, organ culture, xenotransplantation to severe combined immunodeficient (SCID) mice and normal light and transmission electron microscopy (TEM) analysis.

Adequate penetration of cryoprotectant through the stroma and granulosa cells to the oocytes is necessary, however, while at the same time avoiding possible cryoprotectant toxicity. Indeed, Newton *et al*. (1996, 1998) demonstrated the importance of the diffusion rate and the diffusion temperature. Ice crystal formation must also be minimized by choosing optimal freezing and thawing rates. The choice of cryoprotectant with maximum permeation capacity but minimum toxicity and ice crystal formation potential is specific to each cell and tissue type (Fuller and Paynter, 2004). Thus, in the ovary, it is a compromise between the stroma, the follicular cells and the oocytes (Hovatta, 2005). On the basis of current knowledge, the standard method for human ovarian cryopreservation is slow-programmed freezing, using human serum albumin-containing medium, and propanediol, dimethylsulphoxide or ethylene glycol as a cryoprotectant, combined or not with sucrose (Hovatta, 2005).

Kim *et al*. (2004b) showed that a correlation exists between ischaemic tissue damage and the duration of ischaemia. It has been demonstrated that vascular endothelial growth factor does not accelerate revascularization, either after s.c. (Schnorr *et al*., 2002) or peritoneal (Donnez *et al*., personal data) implantation of ovarian tissue. According to Kim *et al*. (2004b), the ovarian cortex can tolerate ischaemia for at least 3 h at 4°C. They also demonstrated

Table III. Autotransplantation of cryopreserved human ovarian tissue

Reference	Age before freezing	Chemo before freezing	Indication	Graft site	Graft size	Recovery of ovarian function	Outcome
Oktay and Karlikaya (2000)	29	No	Intractable menometrorrhagia	Orthotopic: pelvic peritoneum	8 pc (5–10 × 2 mm)	16 weeks	↑ E_2, FD after stimulation, ovulation, menses
Radford *et al*. (2001)	36	Yes	Hodgkin's lymphoma	Orthotopic: ovarian	2 pc (10 × 5 × 1 mm)	8 months	↓ FSH and LH, ↑ E_2, FD, ovulation, menses (1 cycle)
Callejo *et al*. (2001)	47	No	Uterine leiomyoma	Heterotopic: rectus abdominis muscle	40–45 pc (2–3 mm^3)	3–4 months	↑ E_2, FD after stimulation (follicle of 20 mm)
Kim *et al*. (2004a)	37	No	Squamous cell cervical carcinoma (Ib)	Heterotopic: breast [between breast tissue and pectoralis muscle (a)] + abdominal [rectus muscle (b)]	40 pc (5 × 5 × 1–2 mm): 20 (a) + 20 (b)	14 weeks	↑ E_2, ↓ FSH, FD (follicle of 11–16 mm) only in (b), ovulation
Oktay *et al*. (2004)	30	No	Breast cancer	Heterotopic: s.c. abdominal wall	15 pc (from 5 × 5 × 1 to 15 × 5 × 2 mm)	3 months	FD after gonadotrophin stimulation, oocyte retrieval, 20 oocytes, 8 IVF–ICSI, 4-cell embryo transfer + 1 aneuploidic embryo
Donnez *et al*. (2004)	25	No	Hodgkin's lymphoma	Orthotopic: ovarian fossa peritoneum	1 pc (12 × 4 × 1 mm) + 67 (35 + 32) pc (2 × 2 × 1 mm)	$4^1/_2$ months	↑ E_2, ↓ FSH and LH, FD (follicle of 22 mm), regular cycles (± 5 weeks), pregnancy, live birth
Meirow *et al*. (2005)	28	Yes	Non-Hodgkin's lymphoma	Orthotopic: ovarian	3 pc (15 × 5 × 1–2 mm) + tiny fragments injected	8 months	↓ FSH, ↑AMH and inhibin B, FD, ovulation, menses, modified natural cycle, oocyte retrieval, 1 metaphase II oocyte IVF–ICSI, 4-cell embryo transfer, pregnancy and live birth
Schmidt *et al*. (2005)	28	No	Hodgkin's lymphoma	Orthotopic: ovarian (a) + peritoneum (b) and heterotopic: abdominal wall (c)	12 pc: 4 (a) + 4 (b) + 4 (c)	19/22 weeks	↑ E_2, ↓ FSH, FD (19 weeks), menses (22 weeks), no stimulation, empty follicle (of 20 mm)
	25	No	Hodgkin's lymphoma	Orthotopic: ovarian (a) + peritoneum (b) and heterotopic: abdominal wall (c)	12 pc: 4 (a) + 4 (b) + 4 (c)	18/25 weeks	↑ E_2 ↓ FSH, FD (18 weeks), menses (25 weeks), 2 IVF cycles, 2 metaphase II oocytes, 2-cell embryo
	32	No	Non-Hodgkin's lymphoma	Orthotopic: ovarian	6 pc	8/14 weeks	↑ E_2, ↓ FSH, FD (8 weeks), menses (14 weeks), 1 IVF cycle, 1 metaphase II + 1 GV oocyte, 4-cell embryo
Wolner-Hanssen *et al*. (2005)	30	No	Pure red cell aplasia + BMT	Heterotopic: s.c. forearm	10 pc (1–2 × 1–2 × 0.5–1 mm)	18 weeks	FD (12.6 mm follicle and 6.7 mm follicle) after local gonadotrophin stimulation
Donnez *et al*. (2006)	21	No	Sickle cell anaemia + BMT	Orthotopic: ovarian (a) + ovarian fossa peritoneum (b)	69 pc (2 × 2 × 1 mm): 45 (a) + 24 (b)	$4^1/_2$ months	↑ E_2, ↓ FSH and LH, FD (follicle of 20 mm), menses
Oktay (2006)	29	Yes	Hodgkin's lymphoma	Heterotopic: s.c. abdominal wall	Left ovary in pieces	2 months	↑ E_2, FD, ovulation, 6-week pregnancy and miscarriage, and pregnancy with live birth from native ovary
Demeestere *et al*. (2006)	24	Yes	Hodgkin's lymphoma	Orthotopic: ovarian (a) + ovarian fossa peritoneum (b) and heterotopic: s.c. abdominal wall (c)	18 pc (5 × 5 × 2 mm): 3 (a) + 9 (b) + 6 (c)	4 months	↑ inhibin B and E_2, ↓ FSH, FD in all sites: (a) large follicles in ovarian site, (b) only one dominant follicle and (c) follicles <13 mm, 6 ovulations, natural conception, pregnancy, miscarriage at 7 weeks (aneuploidy)
Donnez *et al*. (unpublished data)	24	Yes	Non-Hodgkin's lymphoma	Orthotopic: ovarian	5 pc (10 × 4–5 × 1 mm)	5 months	↑ E_2, ↓ FSH and LH, FD (follicle of 21 mm), menses

AMH, anti-Müllerian hormones; BMT, bone marrow transplantation; E_2, estradiol; FD, follicular development; GV, germinal vesicle; pc, pieces.

that an antioxidant (ascorbic acid) reduces apoptosis in ovarian cortex by up to 24 h in case of incubation *in vitro*. In their study, significant ischaemic damage was evidenced by decreased oxygen consumption and increased apoptosis. Moreover, it appeared that stromal cells were more vulnerable to ischaemia than primordial follicles. The apoptosis rate of stromal cells was higher in the frozen-thawed group than in the fresh group, regardless of the duration of incubation, which may reflect a degree of freeze-thawing injury to ovarian cortex.

Several immunodeficient animal strains can be used as xenograft recipients, with SCID and non-obese diabetic-SCID mice best suited to xenografting to obviate potential rejection problems, especially if the graft is to be borne for a long period (Weissman *et al*., 1999; Aubard, 2003). All reports on human ovarian tissue grafting to mice (for review, see Aubard, 2003) that have studied the implantation site have shown peritoneal transplantation, either under the peritoneum (Nisolle *et al*., 2000) or under the kidney capsule (Abir *et al*., 2003; Hernandez-Fonseca *et al*., 2004) to be better than s.c. transplantation in terms of follicular survival and development. After xenografting cryopreserved human ovarian tissue into the kidney capsule of SCID mice, Oktay *et al*. (1998b) achieved follicular development up to the antral stage, and (Gook *et al*. 2003, 2005) obtained MII-stage oocytes. The ovulatory capacity of frozen-thawed human follicles in xenografts was evidenced by the formation of morphologically normal corpora lutea and elevated progesterone levels in immunodeficient mice (Kim *et al*., 2002; Gook *et al*., 2003).

Very few data are available on the final maturation of follicles in xenografts and therefore the quality of oocytes obtained. In a recent study, Kim *et al*. (2005) assessed the integrity of human oocytes obtained after s.c. xenografting of cryopreserved ovarian tissue into SCID mice and *in vitro* maturation of retrieved cumulus–oocyte complexes. By immunocytochemical analysis of microtubules and DNA, some oocytes showed abnormal nuclear and cytoplasmic maturation. Nevertheless, the authors could not determine whether these alterations were due to freeze–thawing injury, the lack of optimal ovarian stimulation protocols, suboptimal conditions of animal hosts for the growth of human follicles, inadequate *in vitro* maturation techniques or the choice of a s.c. transplantation site. It has therefore not been established whether human oocytes matured in a xenograft are ultrastructurally normal and functionally competent.

Autotransplantation of cryopreserved human ovarian tissue

Reported cases of autotransplantation of cryopreserved ovarian tissue, either to an orthotopic or to a heterotopic site, are summarized in Table III, detailing in each case the age of the patient before freezing, whether the patient received chemotherapy before freezing, the indications for cryopreservation, the graft site and size, the interval before recovery of ovarian function after grafting and the outcome of transplantation (Oktay and Karlikaya, 2000; Callejo *et al*., 2001; Radford *et al*., 2001; Kim *et al*., 2004a; Oktay *et al*., 2004; Oktay, 2006; Donnez *et al*., 2004, 2006; *unpublished data;* Meirow *et al*., 2005; Schmidt *et al*., 2005; Wolner-Hanssen *et al*., 2005; Demeestere *et al*., 2006).

Orthotopic autotransplantation of cryopreserved human ovarian tissue. In theory, natural pregnancy may be achieved via orthotopic tissue transplantation if the fallopian tubes remain intact.

In 2000, Oktay and Karlikaya (2000) reported laparoscopic transplantation of frozen–thawed ovarian tissue to the pelvic side wall in a 29-year-old patient, who had undergone bilateral oophorectomy for a non-malignant disease. Pieces of cryopreserved ovarian tissue were thawed and transplanted. The patient was stimulated by gonadotrophins once after 15 weeks and then again after 10 months. Follicular development was demonstrated by ultrasonography and ovulation occurred in response to HCG administration.

Radford *et al*. (2001) reported a patient with a history of Hodgkin's disease treated by chemotherapy, whose ovarian tissue had been biopsied and cryopreserved 4 years after chemotherapy and later reimplanted. In this case, histological section of the ovarian cortical tissue revealed only a few primordial follicles because of the previous chemotherapy. Estradiol (E_2) was detected and the FSH and LH levels decreased 8 months after reimplantation. The patient had one menstrual period, but 9 months after reimplantation, her LH and FSH concentrations returned definitively to menopausal levels.

We reported the first successful transplantation of cryopreserved ovarian tissue (Donnez *et al*., 2004) resulting in a pregnancy and live birth. In 1997, a 25-year-old woman presented with clinical stage IV Hodgkin's lymphoma. Ovarian tissue cryopreservation was undertaken before chemotherapy. By laparoscopy, we took five cortical biopsies, about 12–15 mm long and 5 mm wide, from the left ovary. The removal of the whole ovary was not an option because one can never completely exclude recovery of ovarian function after chemotherapy.

After laparoscopy, the patient received hybrid chemotherapy from August 1997 to February 1998, followed by supradiaphragmatic radiotherapy (38 Gy).

According to Schilsky *et al*. (1981), the risk of POF after such a regimen in a woman of 26 years of age is >90%, whereas according to Wallace *et al*. (2005a) and Lobo (2005), the risk of subfertility after Hodgkin's treatment with alkylating agents is >80%. Indeed, not only the type of drug and dose but also the age are important factors when evaluating the risk of POF after chemotherapy.

In 2003, once the patient had been declared completely disease-free, transplantation went ahead. A large strip and 35 small cubes of frozen–thawed ovarian tissue were implanted into a furrow created by the peritoneal window very close to the ovarian vessels and fimbria on the right side. Four months after transplantation, a laparoscopy was carried out to check the viability of the orthotopic graft and to reimplant the remaining 32 ovarian cortical cubes. A follicular structure was visible in the area where the tissue had been reimplanted, clearly outside the native ovary. Biopsy and analysis by vital fluorescent probe staining and histology revealed the presence of viable primordial follicles and a follicular structure with inhibin A-marked cells (Donnez *et al*., 2004). From 5 to 9 months after reimplantation, concentrations of FSH, E_2 and progesterone showed the occurrence of ovulatory cycles. At 11 months, the patient became pregnant and subsequently delivered a healthy baby.

Several lines of evidence lend support to our assertion that the origin of the pregnancy was indeed the autotransplanted cryopreserved tissue. The possibility that the egg was derived from the native ovary is highly unlikely, because vaginal echography demonstrated the development of a follicle of 18×22 mm in size outside the native ovary during the cycle which led to the pregnancy. The same day, the E_2 level was 156 pg/ml and progesterone 0 ng/ml. This was extensively explained in the publication itself (Donnez *et al*., 2004), as well as in a letter later published by Donnez and

Dolmans (2004) in response to comments by Oktay and Tilly (2004). Another of our main arguments was that laparoscopy performed 4½ months after reimplantation proved, by direct visualization, the development of a follicle from the grafted tissue and, on histological examination, the biopsy samples indicated not only the survival of primordial follicles in the grafted tissue but also the maturation of a follicle (granulosa cells marked by inhibin A). It was the first histological proof of follicular maturation from reimplanted cryopreserved ovarian tissue. After delivery, the patient experienced ovulatory cycles every 5 to 6 weeks. Each time, an increase in FSH levels was observed (Figure 1). Figure 1 demonstrates that the ovarian graft is still functioning almost 3 years after transplantation.

In 2006, the restoration of ovarian function after orthotopic (intraovarian and paraovarian) transplantation of cryopreserved ovarian tissue was reported in a woman treated by BMT for a non-cancerous disease (sickle cell anaemia) (Donnez *et al*., 2006). Thirty-nine small cryopreserved cubes were thawed and grafted into the ovary itself (24 cubes) and a peritoneal window (15 cubes). Vaginal echography and sequential measurement of FSH, LH, E_2 and progesterone concentrations revealed the onset of an ovulatory cycle 4½ months after the reimplantation of ovarian tissue, demonstrating the efficacy of orthotopic transplantation and confirming, once again, the time interval between reimplantation and the onset of ovulation. The patient experienced 3 cycles in total, evidenced by the development of a follicle and raised E_2 levels. It should be noted that the E_2 peaks never exceeded 55 pg/ml and that FSH rose to 40 mIU/ml between the cycles. However, after these 3 cycles, LH and FSH concentrations returned to castrated levels. We then decided to reimplant the 30 remaining cubes into the ovary, the patient being under GnRH agonist to decrease LH and FSH levels. She experienced a first ovulatory cycle 4 months after reimplantation. The pre-ovulatory E_2 level was 120 pg/ml. The follicle measured 20 mm before the LH peak and progesterone was at 14.7 ng/ml in the mid-luteal phase (Figure 2).

Very recently, we applied a technique, similar to that used by Silber *et al*. (2005) for the transplantation of fresh ovarian cortex between monozygotic twins, in a woman who had also undergone BMT and two regimens of alkylating agents in 2000 for non-Hodgkin's lymphoma (Table III). Ovarian tissue cryopreservation was carried out 1 year after first-line chemotherapy. One ovary was removed and biopsies of cortical ovarian tissue revealed the presence of histologically normal primordial follicles. Six ovarian cortical pieces measuring 10 × 4–5 mm were then grafted onto the remaining ovary after the cortex of this ovary had been removed (Figure 3). It was 5 months before a mature follicle (21 mm) developed and an increase in E_2 levels (194 pg/ml) was noted. The patient experienced an ovulatory cycle every 5 weeks, the pre-ovulatory E_2 level reaching values between 210 and 356 pg/ml.

The analysis of these cases raises some important points for discussion. First, in all three cases, it took between $4^1/_2$ and 5 months after reimplantation before a follicle could be seen. The process of folliculogenesis takes ~4–6 months, during which time the oocyte and surrounding somatic cells undergo a series of changes that eventually result in the development of a large antral follicle, capable of producing a mature oocyte (Gougeon, 1996). Thus, the appearance of the first follicle originating from the grafted tissue 5 months after reimplantation, proved by laparoscopy in one case, is totally consistent with the expected time course. This time interval between implantation of cortical tissue and the first E_2 peak is also consistent with data obtained from sheep (Baird *et al*., 1999, 2004) and human beings (Table III), although some variations may be observed. Indeed, as summarized in Table III, the delay between transplantation and follicular development was found varying from 8 weeks to 8 months. Such a variation could be explained by a difference in follicular reserve at the time of cryopreservation.

Another very interesting finding is the persistence of relatively high FSH levels during the follicular phase. FSH levels remained

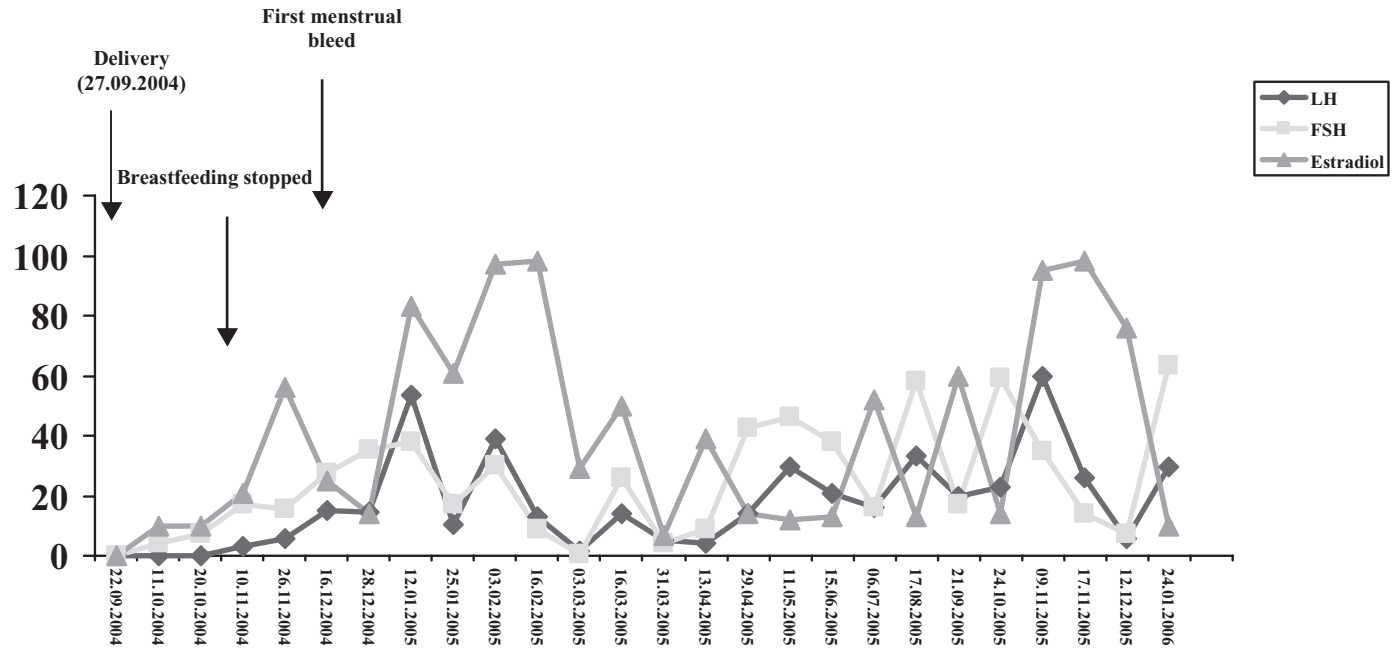

Figure 1. Post-partum LH (mIU/ml), FSH (mIU/ml) and estradiol (pg/ml) levels in the first case of successful cryopreserved ovarian tissue transplantation, demonstrating that the graft is still active (pre-pregnancy hormone levels were published in Donnez *et al*., 2004).

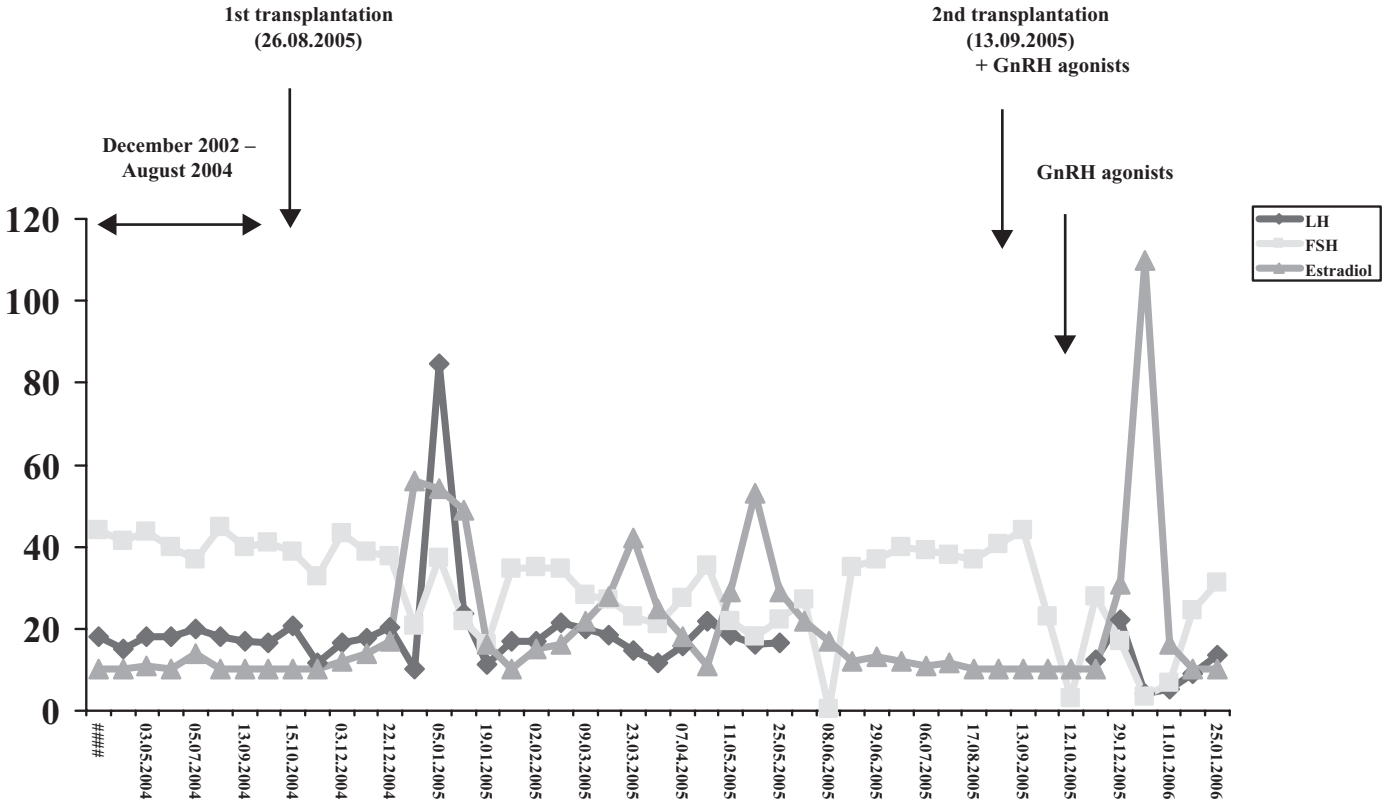

Figure 2. LH (mIU/ml), FSH (mIU/ml) and estradiol (pg/ml) levels in our second case of cryopreserved ovarian tissue transplantation. Four-and-a-half months after reimplantation, the patient experienced 3 consecutive ovulatory cycles, evidenced by the development of a follicle and raised estradiol levels (published by Donnez *et al*., 2006). Subsequently, FSH returned to castrated levels, and a second reimplantation was carried out. Four months later, the patient experienced a further ovulatory cycle (unpublished data).

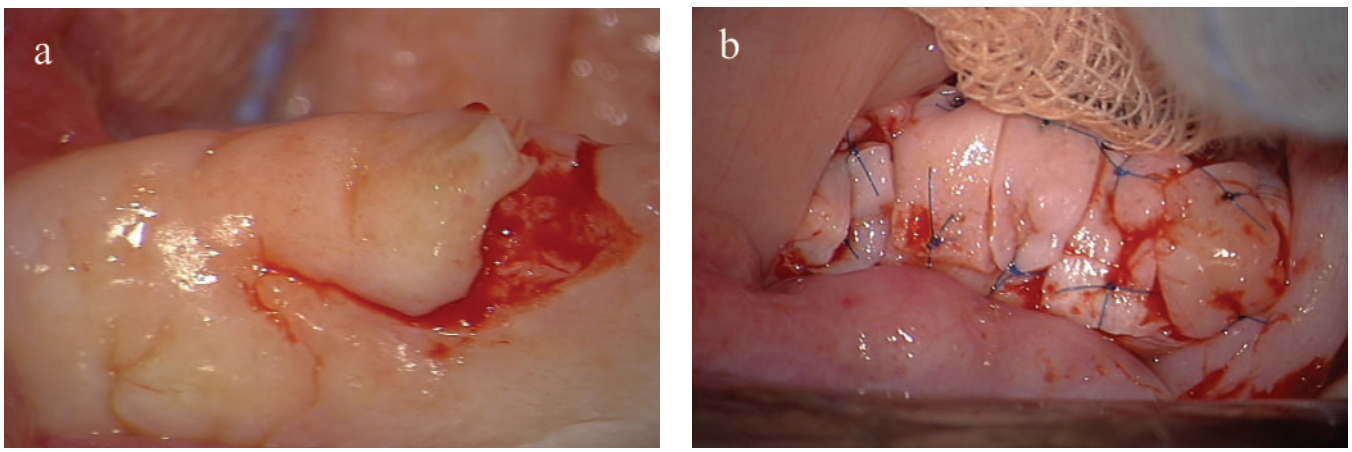

Figure 3. Ovarian cortical pieces measuring 4–5 mm to 1 cm in size were grafted onto the remaining ovary after the cortex of this ovary had been removed. (**a**) Cortex of the remaining ovary was removed. (**b**) Cortical pieces were sutured with 7-0 stitches.

as high as 25 mIU/ml during the follicular phase until ovulation and then decreased to <15 mIU/ml during the luteal phase. This may constitute an argument against the use of gonadotrophin injections. The relatively high FSH levels may be explained by the relatively low number of surviving primordial follicles in the graft. The patient should be considered a poor responder, with reduced inhibin B secretion. These results are in agreement with those obtained in sheep by Campbell *et al*. (2000).

A further significant observation is the return to an FSH level of >35 mIU/ml immediately after each menstrual bleed, which supports the theory suggested by Baird *et al*. (2004) that some inhibitory mechanisms, such as inhibin or anti-Müllerian hormones (AMH) normally produced by developing follicles in intact human ovaries, are probably almost non-existent in transplanted tissue. After transplantation, the patient would have been regarded a poor responder because, of the 500–1000 primordial follicles that

would have been transplanted, >50% would have been lost owing to hypoxia (Donnez *et al*., 2004). This raises the question of the evaluation of the ovarian reserve. There is a lack of data on the ovarian reserve in cancer. Qu *et al*. (2000), Gook *et al*. (2005) and Schmidt *et al*. (2003) have all demonstrated an unequal distribution of primordial follicles in ovarian cortex.

In 2005, Meirow *et al*. also published a live birth after orthotopic autotransplantation of cryopreserved ovarian tissue in a patient with POF after chemotherapy. Eight months after orthotopic transplantation, the patient spontaneously menstruated. The rise in AMH and increased inhibin B levels were consistent with the presence of early growing follicles and ovulation, respectively. Nine months after transplantation, the patient experienced a second spontaneous menstrual period. After a modified natural cycle, a single mature oocyte was retrieved and fertilized. Two days later, a 4-cell embryo was transferred. The patient became pregnant from this embryo transfer and delivered a healthy infant weighing 3000 g. The possibility that the oocyte was derived from the native ovary is highly unlikely given the consistent evidence of POF after high-dose chemotherapy in this patient, from whom ovarian tissue was harvested after administration of a first-line conventional chemotherapy regimen, before second-line high-dose chemotherapy.

Schmidt *et al*. (2005) recently reported the results of three cases of ovarian tissue transplantation. All three patients with autotransplanted ovarian tissue regained ovarian function, as confirmed by the recovery of menses, follicles visible on ultrasonography and normal hormone levels. Two embryos were obtained from three MII oocytes and one GV oocyte, but no pregnancy resulted from embryo transfer.

Demeestere *et al*. (2006) very recently reported a pregnancy after natural conception in a woman who had undergone orthotopic and heterotopic transplantation of cryopreserved ovarian tissue. They observed follicular development in all three transplantation sites: large follicles in the ovarian site, only one dominant follicle in the peritoneal site and follicles <13 mm in size in the heterotopic site. Detectable HCG levels and ultrasonography confirmed the presence of a viable intrauterine pregnancy. Unfortunately, this pregnancy, obtained by natural conception, ended in miscarriage at 7 weeks due to aneuploidy. Interestingly, Demeestere *et al*. (2006) observed normal FSH values after orthotopic and with heterotopic transplantation of cryopreserved ovarian tissue. As stressed by the authors, this may have been due to the young age of the patient and the large number of tissue fragments transplanted (Demeestere *et al*., 2006), which could have yielded a rich follicular reserve in the graft.

Heterotopic autotransplantation of cryopreserved human ovarian tissue: a better option than orthotopic transplantation?. There are only six existing reports on this subject (Table III). Callejo *et al*. (2001) evaluated the long-term function of cryopreserved heterotopic grafts, but no conclusions could be drawn because the patient was perimenopausal at the time of ovarian biopsy for cryopreservation.

In 2004, Kim *et al*. (2004a) reported a case of a 37-year-old woman who underwent heterotopic (rectus and pectoralis muscle) transplantation of cryopreserved ovarian tissue. By 14 weeks of transplantation, the restoration of endocrine function was demonstrated but, ~28 weeks after transplantation, the cessation of ovarian function was evidenced by very high FSH levels (62–99 IU/l) and very low E_2 levels.

The same year, Oktay *et al*. (2004) reported transplantation of frozen–thawed ovarian tissue beneath the skin of the abdomen. A 4-cell embryo was obtained from 20 oocytes retrieved from an ovarian graft, but no pregnancy occurred after transfer. Oocyte quality might have been compromised by transplantation to a heterotopic site.

In 2005, Schmidt *et al*. reported two cases of mixed (heterotopic and orthotopic) transplantation, as did Demeestere *et al*. in 2006. These cases are discussed in the section on orthotopic transplantation.

Wolner-Hanssen *et al*. (2005) reported s.c. transplantation of frozen–thawed tissue to the forearm. Two follicles developed, but only to a maximum diameter of 12.6 and 6.7 mm, respectively, and the tissue survived 7 months. The authors suggested that the risk of graft exposure to suboptimal temperatures or mechanical stress may depend on transplantation site, and thus tissue transplanted under the skin of the forearm will probably be exposed to both higher pressure and lower temperature than ovaries in their normal location.

Very recently, Oktay (2006) reported a pregnancy after heterotopic transplantation of cryopreserved ovarian tissue, but ovulation occurred from the native ovary.

Papers describing heterotopic transplantation have all reported follicular development, but with follicles always <15 mm in size. As stressed by Wolner-Hanssen *et al*. (2005) and Oktay *et al*. (2004), differences in temperature and pressure could interfere with follicular development in heterotopic sites.

Isolated primordial follicles

The primordial follicle is resistant to cryoinjury, because the oocyte it contains has a relatively inactive metabolism, as well as a lack of meiotic spindle, zona pellucida and cortical granules. The small size of primordial follicles also greatly facilitates penetration of cryoprotectant. Oktay *et al*. (1997) developed an isolation technique for human primordial follicles using enzymatic digestion and microdissection and obtained high follicular viability rates with both fresh and frozen ovarian tissues.

Nevertheless, the procedure to isolate primordial follicles remains difficult (Martinez-Madrid *et al*., 2004a); to date, it has not been possible to grow human isolated primordial follicles *in vitro* to the mature oocyte stage (Torrents *et al*., 2003).

Although safe xenotransplantation of ovarian tissue from lymphoma patients has been reported in SCID mice (Kim *et al*., 2001), the possibility of reintroducing tumour cells into cancer patients by autografting of ovarian tissue cannot be excluded. To avoid transferring malignant cells, ovarian tissue culture with *in vitro* follicle maturation could be performed. Culturing isolated follicles from the primordial stage is another particularly attractive proposition because they represent >90% of the total follicular reserve and show high cryotolerance (Smitz and Cortvrindt, 2002). However, isolated primordial follicles do not grow properly in culture (Hovatta *et al*., 1999; Abir *et al*., 2001), and further studies are clearly needed to identify factors sustaining follicular growth and maturation in humans (Smitz and Cortvrindt, 2002) and to assess the contribution of stromal cells to these processes. Encouraging results were achieved by Hovatta (2004) when human primordial follicles were grown in organ culture. Follicle isolation, or partial follicle isolation, severely impairs follicular viability in culture and, after isolation, primordial and primary follicles degenerate within the first 24 h of culture (Hovatta *et al*., 1999; Abir *et al*., 2001). Only more advanced, multilaminar pre-antral follicular

stages can survive in short-term culture, a few reaching the early antral stage (Roy and Treacy, 1993; Abir *et al*., 1997).

Another approach could be to transplant a suspension of isolated follicles. As the follicular basal lamina encapsulating the membrana granulosa excludes capillaries, white blood cells and nerve processes from the granulosa compartment (Motta *et al*., 2003; Rodgers *et al*., 2003), grafting fully isolated follicles could be considered safer. Moreover, this would allow the introduction of a high and known number of follicles into the host, obtaining faster angiogenesis and minimizing ischaemic and reperfusion damage (Laschke *et al*., 2002). The transplantation of frozen–thawed isolated primordial follicles has indeed been successfully achieved in mice (Carroll and Gosden, 1993), yielding normal offspring. For human primordial follicles, however, mechanical isolation is not possible due to their size (30–40 μm) and their fibrous and dense ovarian stroma, and therefore enzymatic digestion with collagenase or Liberase has to be used (Dolmans *et al*., 2006). To enhance the chances of follicular survival and reproductive function restoration, enzymatic digestion procedures for human ovarian tissue need to be optimized and standardized.

As we recently demonstrated (Dolmans *et al*., 2006), Liberase treatment allows the isolation of highly viable follicles with an unaltered morphology and ultrastructure (Figure 4). This purified endotoxin-free enzyme preparation is a promising alternative to collagenase preparations for the reproducible isolation of intact primordial follicles for culture and grafting purposes. Preliminary results after xenotransplantation of isolated human primordial and primary follicles are encouraging. Indeed, the survival of grafted isolated primordial follicles in nude mice was estimated to be >60%, leading us to consider the possibility of grafting isolated follicles in the future, if the risk of reintroducing malignant cells into cancer patients by autografting ovarian cortical fragments cannot be excluded.

Whole ovary

As previously discussed, the main drawback of ovarian tissue cryopreservation followed by avascular transplantation is that the graft is completely dependent on the establishment of neovascularization and, as a result, a large proportion of follicles are lost during the initial ischaemia occurring after transplantation (Newton et al., 1996; Candy et al., 1997; Gunasena et al., 1997; Aubard et al., 1999; Baird et al., 1999; Nisolle et al., 2000; Liu et al., 2002). Reducing the ischaemic interval between transplantation and revascularization is therefore essential to maintaining the follicular reserve and extending the life span and function of the graft. In theory, the best way to achieve this is by transplantation of intact ovary with vascular anastomosis, allowing immediate revascularization of the transplant.

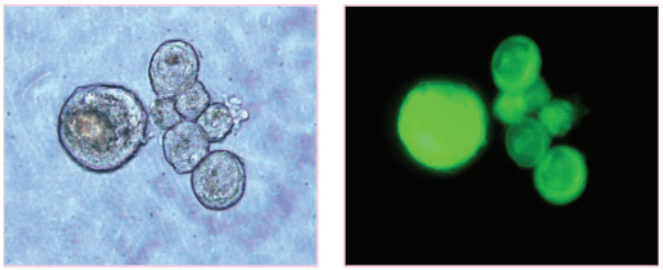

Figure 4. Enzymatically isolated follicles (between 30 and 110 μm) visible under an inverted fluorescence microscope after fluorescent viability staining (calcein-AM and ethidium homodimer-1). Follicles are visible on light microscopy (left) and fluorescence microscopy (right), which show all of them to be viable. (Original magnification ×400.)

Ovarian vascular transplantation has already been successfully performed using intact fresh ovaries in rats (Wang *et al*., 2002; Yin *et al*., 2003), rabbits (Winston and Browne, 1974), sheep (Goding *et al*., 1967; Jeremias *et al*., 2002), dogs (Paldi *et al*., 1975), monkeys (Scott *et al*., 1981) and humans (Leporrier *et al*., 1987; Hilders *et al*., 2004; Mhatre *et al*., 2005). In the last few years, attempts at freezing and grafting whole ovaries in rats (Wang *et al*., 2002; Yin *et al*., 2003), rabbits (Chen *et al*., 2005) and sheep (Bedaiwy *et al*., 2003; Arav *et al*., 2005; Imhof *et al*., 2006) have also yielded encouraging results.

The first case of restoration of fertility after whole frozen ovary transplantation was described by Wang *et al*. in 2002. They described successful vascular transplantation of frozen–thawed rat ovaries and reproductive tract in four of seven (57%) transplants, which survived for ≥60 days, were ovulatory and resulted in one pregnancy. Chen *et al*. (2005) showed that frozen–thawed rabbit ovaries remained functional for at least 7 months after microvascular transplantation in 13 of 15 (86.7%) animals.

It appears that, in large mammals and humans, cryopreserving such a large-sized intact ovary may prove more problematic than in small animals because of the difficulty of adequate diffusion of cryoprotective agents into large tissue masses and vascular injury caused by intravascular ice formation. Nevertheless, Arav *et al*. (2005) reported progesterone activity 36 months after vascular transplantation of frozen–thawed sheep ovaries in three of eight transplants, and retrieval of six oocytes, resulting in embryonic development up to the 8-cell stage after parthenogenic activation. Bedaiwy *et al*. (2003) reported the restoration of ovarian function after autotransplantation of intact frozen–thawed sheep ovaries with microvascular anastomosis, but it should be noted that 8 of 11 ovaries were lost due to thrombotic events in the reanastomosed vascular pedicle. Imhof *et al*. (2006) recently demonstrated that the autotransplantation of whole cryopreserved sheep ovaries with microanastomosis of the ovarian vascular pedicle could lead to pregnancy and delivery. Moreover, in this study, six of eight ovaries showed major ovarian vessels to be free of thrombosis, with the structural integrity of the ovarian stroma largely retained 18–19 months after transplantation.

Recently, Martinez-Madrid *et al*. described a cryopreservation protocol for intact human ovary with its vascular pedicle and proved high survival rates of follicles (75.1%) (Figure 5), small vessels and stroma, and a normal histological structure in all the ovarian components after thawing (Martinez-Madrid *et al*., 2004b).

After freeze–thawing whole human ovaries using this protocol, no induction of apoptosis was observed in any cell types, assessed by both the terminal deoxynucleotidyl transferase biotin-dUTP nick-end labelling (TUNEL) method and immunohistochemistry for active caspase-3 (Martinez-Madrid *et al*., 2005). TEM confirmed that the majority (96.7%) of primordial follicles were intact after cryopreservation (Camboni *et al*., 2005) (Figure 6). Particular attention was paid to the evaluation of the endothelial cells: TEM revealed that 96.3% of these cells had a completely normal ultrastructure, and the percentage of active caspase-3-positive endothelial cells was <1%.

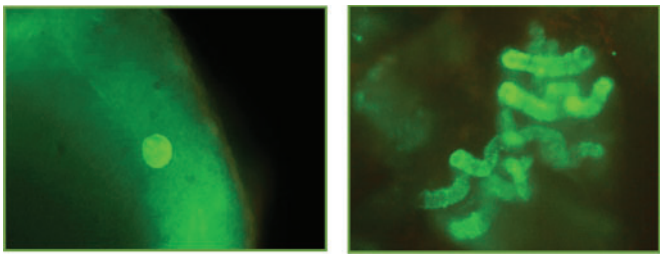

Figure 5. High survival rate of stromal cells, follicles and vessels in a cryopreserved whole ovary (viable cells stained green with calcein-AM and dead cells stained red with ethidium homodimer-1). (Original magnification ×200.)

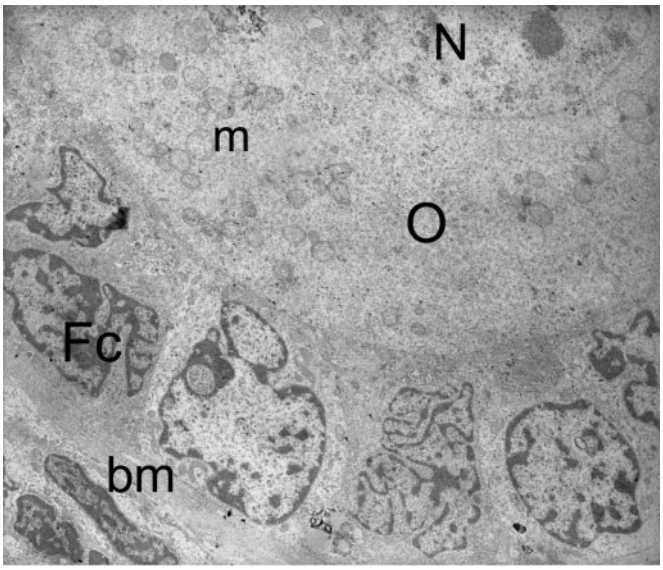

Figure 6. Primary follicle in a frozen–thawed whole ovary. The oocyte (O) is surrounded by a single layer of cuboid follicular cells (Fc) on a continuous basal membrane (bm). Note the presence of rounded mitochondria (m) with a pale matrix and peripheral cristae in the oocyte cytoplasm. Follicular cells show indented nuclei containing peripheral patches of heterochromatin and numerous rod-shaped mitochondria in the cytoplasm. N, oocyte nucleus. Original magnification: transmission electron microscopy (×4400).

Our results in humans have led us to seriously consider proposing this option to women in the future, when there is no risk of transmitting malignant cells via the graft after transplantation. So far, in our department, three whole ovaries have been cryopreserved with a view to future reimplantation (grafting) and vascular anastomosis.

Developing new cryochambers and improving protocols for whole ovary cryopreservation must therefore be considered as vital directions in ongoing research to make the transplantation of an entire ovary a feasible objective (Donnez *et al*., 2005; Martinez-Madrid and Donnez, 2005). Research and development of technology to cryopreserve whole organs, as well as surgical techniques for the autotransplantation of an entire ovary with its vascular pedicle, should be encouraged. This could lead to the transplantation of intact ovaries with microvascular anastomosis carried out to restore immediate vascularization and minimize post-transplantation ischaemia, responsible for the reduction in follicular density.

Safety and ethical issues

The transmission of lymphoma via grafts of ovarian tissue from diseased donor mice to healthy recipients was reported by Shaw *et al*. (1996). This study highlighted the risks of clinical transplantation of ovarian biopsy samples to women recovering from cancer, especially a blood-borne cancer (Shaw *et al*., 1996; Shaw and Trounson, 1997). However, there are certain circumstances where the risk of cancerous involvement of the ovary is absent or minimal (Meirow *et al*., 1998) and where autografting would present little or no danger (Gosden *et al*., 1997; Moomjy and Rosenwaks, 1998; Kim *et al*., 2001). Future experiments should help us address questions about the relevance of replacing residual malignant cells with grafted tissue in such cases. Screening methods must be developed to eliminate the risk of cancer cell transmission with reimplantation. In some diseases, other options must be considered, such as the transplantation of isolated follicles. Meanwhile, the debate rages on.

The Practice Committee of the American Society for Reproductive Medicine (2004) has summarized some important points to be taken into consideration, and Dudzinski (2004) recently underlined the need to develop policies to protect the patient's right to self-determination with respect to her gametes. She conducted a normative analysis of ethical issues in the context of oocyte and ovarian tissue cryopreservation for adolescent cancer survivors and concluded that more research is required before adolescents can ethically be enrolled in clinical trials.

We do not fully agree with this conclusion. Indeed, approximately one-third of young women exposed to chemotherapy develop ovarian failure. In 2006, we believe it is our ethical responsibility to propose ovarian tissue cryopreservation under Institutional Review Board protocols to all adolescents and young women under having to undergo chemotherapy with alkylating agents. Indeed, is it ethical to simply accept the existing discrepancy between males and females with regard to their chances of preserving their fertility following cancer treatments? What do we then say to young women facing POF following chemotherapy, knowing that ovarian cryopreservation has been an option for >10 years? It will be too late to say 'we should have done something—we should at least have tried'.

This is why, since 1996, we have systematically proposed cryopreservation to all women <35 years of age before chemotherapy, when there is a risk of POF. We accept that ovarian tissue cryopreservation is a more innovative and invasive procedure than sperm cryopreservation and that all possible applications in adolescents are ethically complex. But we wholeheartedly agree with Revel and Schenker (2004), who contributed to a debate published in *Human Reproduction*, arguing that ovarian cortex banking should be offered before chemotherapy in all cases where emergency IVF is not possible.

One of the most important ethical issues is to ensure that the intervention does not harm the patient by dangerously delaying cancer treatment and that no remnant cells are reintroduced by subsequent transplantation. Taking these points into account, we agree with Dudzinski (2004) that policies to protect the patient's future rights to her gametes should be developed, as well as policies addressing the disposition of the gametes if the patient dies.

Although an adolescent is more vulnerable when consent is sought in the rush to begin chemotherapy, she must be mature enough to understand the risks and benefits of the procedure. Consent must then be discussed extensively, the discussion including both the adolescent patient and her parents to minimize the risk of conflict of interest or inadvertent caution (Bukovsky, 2005). Respecting the code of good practice, all patients who may become infertile have the right to receive proper consideration of their interests for future possibilities in the field of ovarian function preservation. The selection of cases should be carried out on the basis of a multidisciplinary staff discussion including oncologists, gynaecologists, biologists, psychologists and paediatricians. Counselling should be given and informed consent obtained from the patient. Cancer treatment takes priority over potential restoration of fertility, but offering the chance to preserve fertility may greatly enhance the quality of life for cancer survivors.

Conclusion

Advances in reproductive technology have made fertility preservation techniques a real possibility for patients whose gonadal function is threatened by premature menopause, or by treatments such as radiotherapy, chemotherapy or surgical castration. Decision-making in this area is particularly difficult because of the experimental nature of some of these techniques. With their continued development and optimization, however, it may one day be possible to offer an individualized approach to management, be it through embryo cryopreservation, oocyte cryopreservation or cryopreservation of ovarian tissue (isolated follicles, cortical fragments or whole ovary).

Cryopreservation of ovarian tissue should be seriously considered for any patient undergoing treatment likely to impair future fertility, the indications being pelvic, extrapelvic and/or systemic malignant diseases, as well as non-malignant diseases. The age of the patient should be taken into consideration, because the follicular reserve of the ovary is age dependent. Because a decline in fertility is now well documented after the age of 38 years, the procedure should probably be restricted to patients below this limit. In any case, irradiation and chemotherapy appear to be less harmful to the gonads of prepubertal than postpubertal patients (Haie-Meder *et al*., 1993; Sanders *et al*., 1996; Meirow and Nugent, 2001).

It has been demonstrated that cryopreserved primordial follicles can survive the thawing process. Research must now focus on investigating current options and new alternatives in the field, to identify the best way of using tissue after thawing. It is probable that the answer lies in the use of culture environments adapted to each stage of follicular development. If autografting is the aim of cryopreservation of ovarian tissue, testing for malignant cells in the tissue must be carried out using adequate techniques, especially in case of haematological malignancies.

In conclusion, live births obtained after transplantation of frozen-thawed ovarian tissue in humans give hope to young cancer patients, but there is still much work to be done. Research programs need to determine whether active angiogenesis can be induced to accelerate the process of neovascularization in grafted tissue, if isolated human follicles can be grafted, or indeed if microvascular reanastomosis of an entire cryopreserved ovary is a valuable option.

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