Article

Preliminary experience of ovarian tissue cryopreservation procedure: alternatives, perspectives and feasibility

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Abstract

Chemotherapy and radiotherapy induce premature ovarian failure in many patients treated for oncological or benign diseases. The present paper reviews the risk of developing premature ovarian failure according to the type of treatment and the different options to preserve fertility, focusing on the cryopreservation of ovarian tissue. This technique constitutes a promising approach to preserve the fertility of young patients and offers the advantage of storing a large number of follicles that could be subsequently transplanted or cultured *in vitro* to obtain mature oocytes. Based on 34 requests, from which 19 were performed, the feasibility of the ovarian cryopreservation procedure is evaluated. The medical and ethical approaches of this protocol are also discussed. Cryopreservation of ovarian tissue constitutes new hope for many patients, but must still be kept for selected cases, with a significant risk of premature ovarian failure after treatments such as bone marrow transplantation.

Keywords: *chemotherapy, fertility, gonadotoxicity, premature ovarian failure*

Introduction

Chemotherapy and radiotherapy have enhanced the life expectancy of patients with cancer in many conditions. Survival rates have been particularly improved in patients with haematological malignancies, currently surpassing 80% at 5 years (Horwitz and Horning, 2000). This increase in disease-free life expectancy raises the question of the consequences of treatment on the long-term quality of life of these young patients, one of the most common late effects of the treatment being damage to the gonads.

The chemotherapy regimen and radiotherapy can induce massive destruction of the follicular reserve, resulting in premature ovarian failure (POF). The recovery of ovarian function varies according to the type of chemotherapy, the dose administered and the age of the patient. Intensive chemotherapy and/or total body irradiation (TBI) requested before bone marrow transplantation (BMT) are the combination of regimens presenting the highest risk of POF. **Table 1** summarizes the results of different studies published during the last 10 years regarding the effect of various conditioning regimens for BMT on the ovarian function. TBI and high-dose busulfan are both a major cause of POF even when given in the prepubertal period (Teinturier *et al*., 1998; Thibaud *et al*., 1998; Couto-Silva *et al*., 2001).

Young women who experience POF have to consider years of hormonal replacement therapy, which is effective in preventing menopausal symptoms. However, this substitutive treatment cannot fully replace the reproductive function of the ovaries and alternative approaches to parenting, e.g. oocyte donation or adoption, have to be considered as a last resort. Moreover, the prospect of definite infertility can increase the psychological distress related to the disease.

Table 1. Frequency of ovarian recovery after different conditioning regimens for bone marrow transplantation.

Authors	*Diseases*	*Treatment*	n	*Age in years (range)*	*Ovarian recovery (%)*	*Follow-up in months (median)*
Liesner *et al.* (1994)	AL-MDS	CT	16	Prepubertal	15[a] (93)	18–108
		CT + TBI	4		1[b] (25)	
Spinelli *et al.* (1994)	AL-CGL-IT-MM-MDS-LNH	CT + TBI	5	Premenarchal	4 (80)	4–108 (48.8)
				Postmenarchal		
			14	<18	6 (43)	
			60	>18	4 (6)	
Sanders *et al.* (1996)		Cy	103	28 (13–58)	56 (54.3)	12–204 (36)
		Bu/Cy	73	38 (14–57)	1 (1.3)	
		Cy +TBI	532	28 (11–58)	53 (10)	
Keilhotz *et al.* (1997)	AL	Cy + TBI	9	37 (21–52)	0 (0)	42–91 (69)
	HL	CT	1	20	1 (100)	
Sarafoglou *et al.* (1997)	AL	Cy + TBI	16	Prepubertal	9 (56)	
Teinturier *et al.* (1998)	NB, ES, HL, NHL, RBM, OD	CT	11	5.8 (2–14.8)	7 (73)	14–156 (84)
		CT (Bu)	10	12.7 (4.7–17.3)	0 (0)	
Thibaud *et al.* (1998)	HL, RB, OD, NE, ES, HL, NHL, AL, CL, SAA, FD	CT	8	10.3 (3.2–17.5)	3 (37.5)	14–138 (72)
		CT + TBI	23		3 (13)	
Schimmer *et al.* (1998)	AL-HL-NHL	CT	11	27 (19–48)	4 (36)	19–105 (52)
		CT + TBI	6		1 (16)	
Bath *et al.* (1999)	AL	CT + TBI	8	11.5 (5.9–15)	2 (25	
		CT + cranial I	12	6.7 (3.8–13.5)	12 (100)	
Couto-Silva *et al.* (2001)	AL, HL, LNH, NB, FD, SAA, CID, NPB	CT+ TBI	22	7.3 (1.5–13)	3 (13.6)	
		TLI	7	8 (5.2–13.1)	2 (28.5)	
		CT	5	5.3 (0.6–12.9)	2 (40)	
Tauchmanova *et al.* (2002)	AL, CL, SAA	Bu/Cy	21	13–45	2 (5)	12–62 (38)

[a]Most of the patients were still prepubertal at the time of the study.
[b]Prepubertal patient; AL: acute leukaemia; LNH: non-Hodgkin's lymphoma; HL: Hodgkin's lymphoma; CGL: chronic granulocytic leukaemia; IT: idiopathic thrombosis; MM: multiple myeloma; MDS: myelodysplasic syndrome; RB: retinoblastoma, OD: ovarian dysgerminoma; NE: neuroepithelioma; NB: neuroblastoma; ES: Ewing sarcoma; CL: chronic leukaemia; SAA: severe aplastic anaemia; FD: Fanconi disease; RBM: rhabdomyosarcoma; CID: congenital immunodeficiency; NPB: nephroblastoma; CT: chemotherapy regimens; TBI: total body irradiation; TLI: total lymphoid irradiation; Bu/Cy: busulfan/cyclophosphamide.

Clinical care of patients at risk of developing POF

Different options can be proposed to the patients at risk of developing POF. These are shown in **Figure 1**, and the various options will be considered briefly.

Reducing gonadotoxicity

The surgical option is ovarian transposition, which minimizes ovarian exposure to radiation by moving the ovaries within the peritoneum to a position behind the uterus or away from the field of radiation. It is a safe and effective procedure, allowing preservation of ovarian function in more than 80% of cases (Bisharah and Tulandi, 2003), but the indications are limited to patients treated by pelvic irradiation.

The pharmacological option is a recent promising approach to prevent ovarian failure during chemotherapy, by concomitant treatment with LHRH analogues (LHRHa). Studies on rats and monkeys treated by cyclophosphamide combined with LHRHa have proved their efficacy in inhibiting chemotherapy-induced ovarian depletion (Ataya and Moghissi, 1989; Ataya *et al.*, 1995). Limited data regarding the potential protective effect of LHRHa on human ovarian function are available (Blumenfeld, 2002). Spontaneous ovulation was observed in 94% of patients after combined treatment with chemotherapy/LHRHa for lymphoma (Blumenfeld, 2002). In the control group without LHRHa treatment, 56% of patients presented POF.

This promising study was, however, neither randomized nor prospective and the efficiency of this treatment is still controversial: other larger prospective randomized studies are necessary before clinical application of this treatment during chemotherapy.

Future perspectives regarding the treatment of POF rely on a deeper understanding of apoptosis, recently identified as one of the mechanisms responsible for oocyte loss induced by antineoplastic drugs (Morita *et al.*, 1999). The capacity to control early intracellular events that trigger the activation of

the different steps in the programme of apoptosis, as the sphingomyelin pathway, may constitute a new approach to prevent POF. Studies in mice showed that oocytes lacking the gene for acid sphingomyelinase or wild-type oocytes treated with sphingosine-1 phosphate resisted apoptosis induced by anti-cancer therapy (Morita *et al*., 2000). Sphingosine 1-phosphate also preserved the fertility of irradiated female mice without propagating genomic damage to the offspring (Paris *et al*., 2002).

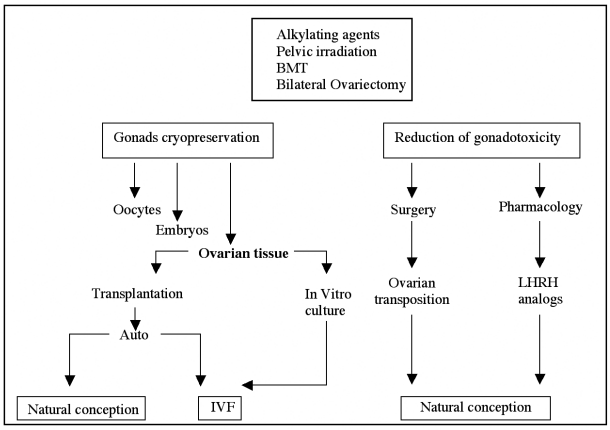
Gamete cryopreservation

The second option is to cryopreserve gametes or embryos before gonadotoxic treatment. This can be achieved by the cryopreservation of semen, prior to the initiation of therapy that is expected to compromise testicular function (Ragni *et al*., 2003). For women, cryopreservation of oocytes before gonadotoxic treatment is more difficult to perform, for multiple reasons. A recent study confirmed that despite a high survival rate after thawing, cryopreserved oocytes at both immature and mature stages presented a high rate of abnormal spindle and chromosome configuration (Boiso *et al*., 2002). The limited number of collected oocytes restricts the achievement of pregnancies.

Embryo cryopreservation is currently performed after IVF treatment. However, it is unsuitable for patients without a male partner, or before puberty. Moreover, the required ovarian stimulation is contraindicated for patients with oestrogen-sensitive cancers. Alternatives to ovarian stimulation, such as IVF during natural cycle or after tamoxifen treatment, have been proposed in those cases (Oktay *et al*., 2003). Finally, this technique is also difficult to perform due to the timing of the procedure: practically, in case of malignant disease, chemotherapy usually starts within 1 or 2 weeks of diagnosis, which is insufficient to perform an IVF cycle.

Despite their limitations regarding the results, or their feasibility, oocyte or embryo cryopreservation procedures have occasionally proven helpful in preserving fertility before gonadotoxic treatment (Lockwood, 2002).

The cryopreservation of ovarian tissue constitutes a new promising technique to preserve fertility before oncological treatment. The large number of primordial oocytes cryopreserved when ovarian tissue is frozen constitutes a significant advantage compared with the cryopreservation of full sized oocytes or embryos. In addition, it can be performed before puberty and at any time of the cycle without delay. Advances in cryotechnology now allow 70% primordial follicles in human ovarian tissue to survive cryopreservation (Newton *et al*., 1996). Oocytes from these primordial follicles are immature (prophase I) and cannot be fertilized. A period of growth and maturation of about 3 months in human is essential to obtain an antral follicle with a fully grown oocyte (Gougeon, 1996). After thawing the cryopreserved tissue, this maturation step can be performed *in vivo* by transplantation or, if techniques improve, by in-vitro culture. Both procedures are still experimental. The present paper aims at evaluating the feasibility and the perspectives of ovarian tissue cryopreservation based on clinical experience.



BMT : Bone Marrow Transplantation, IVF : In Vitro Fertilization

Figure 1. Different options to preserve fertility before oncological treatment.

Clinical experience on ovarian cryopreservation

Patients and methods

Cryopreservation of ovarian cortex is proposed for selected patients after multidisciplinary evaluation of the risk of iatrogenic POF. Approval was obtained from the Ethics Committees of the different hospitals concerned in this programme. Inclusion and exclusion criteria are detailed in **Table 2**. Options to preserve fertility must be considered for all patients who are likely to lose ovarian function prematurely for various medical or surgical reasons. The ovarian cryopreservation procedure was proposed only when the risk of premature ovarian failure was very high, for example after treatment with high doses of alkylating agents, BMT, pelvic irradiation or surgical castration. Indications for ovarian tissue cryopreservation are multiple and concern mostly patients with haematological diseases such as lymphoma, leukaemia, drepanocytosis or thalassaemia. Other cancers occurring commonly during childhood or adolescence are also often treated with sterilizing chemotherapy or radiotherapy, for example sarcoma, Wilm's tumour and medulloblastoma. Finally, patients affected by gynaecological diseases as breast cancer and borderline ovarian tumour could also benefit from this procedure when neither conservative treatment nor poor gonadotoxic chemotherapy can be proposed. All patients are informed about the experimental status of the procedure as well as its uncertain issue, and are required to sign an informed consent form.

Unilateral oophorectomy or ovarian biopsies were performed by laparotomy or laparoscopy under general anaesthesia. The ovarian specimens were rapidly transported to the laboratory in Leibovitz-L15 medium (Life Technologies, Merelbeke, Belgium), and carefully dissected in the same medium in order to obtain small slices of ovarian cortex (0.5–1 cm diameter, 1–2 mm thickness). The slices of ovarian cortex were transferred in vials (Simport, Merck Eurolab, Leuven, Belgium) containing 1.8 ml of the cryoprotectant solution composed of Leibovitz medium supplemented with 1.5 mol/l DMSO, 0.1 mol/l sucrose (both provided by Sigma-Aldrich, Bornem, Belgium) and 10% patient's serum at 4°C. The vials were gently agitated for 30 min at 4C°. The slices of ovarian

cortex were then cryopreserved using a slow freezing protocol: start at 0°C, 2°C/min to –7°C, at which temperature seeding was induced manually, followed by 0.3°C/min to –40°C, with continued cooling at –10°C/min to –140°C. The slices were then plunged into liquid nitrogen as described previously (Newton *et al*. 1996).

Results

During the period from 1999 to January 2003, 34 requests for ovarian tissue cryopreservation were evaluated and 19 were actually performed (**Table 3**). Sixty-four percent of requests concerned patients with haematological diseases, 20% of requests were related to patients with breast cancer and 14% of requests were related to patients with less common diseases (ovarian teratoma, sarcoma, medulloblastoma, melanoma).

Table 2. Inclusion and exclusion criteria for ovarian tissue cryopreservation.

Inclusion criteria	*Exclusion criteria*
<35 years old	Previous highly gonadotoxic treatment
Highly gonadotoxic treatment	Premature ovarian failure
Intact uterine cavity	Positive serology (hepatitis B and C, HIV, syphilis)
Presence of both ovaries	Operative contra-indication
Ability to give written informed consent (patient or responsible representative)	

The procedure was not performed for 15 patients for various reasons (**Table 4**). These patients sustained the inclusion criteria but presented one of the exclusion criteria. Last minute refusal also occurred commonly. This usually involved patients or parents who were so affected by the diagnosis of the primary disease, in all cases haematological diseases, that they could not accept the risk of the surgical act implied by the procedure. For two patients affected by acute leukaemia, the procedure was cancelled because of a rapid deterioration of health status, contraindicating surgery.

Nineteen patients from 4 to 32 years old (mean 21.5) underwent the ovarian cryopreservation procedure (**Table 3**). Most patients (73%) had never been pregnant before the oncological treatment. Three of them were pregnant at the time of diagnosis. The main indications for ovarian tissue cryopreservation were the need for BMT (42%) or of high doses of alkylating agents (31.5%). Thirty-one per cent of patients (six) underwent various chemotherapy regimens before ovarian tissue cryopreservation.

Laparotomy was performed in 47% of cases because of the young age of the patients (four), concomitant Caesarean

Table 3. Description of the 19 patients undergoing ovarian cryopreservation.

Disease	*Age (years)*	*Gravidity (G) and parity (P)*	*Indication*	*Ovariectomy*	*Associated surgery*	*Evolution disease*	*Ovarian function*
Breast cancer	30	G0P0	Bilateral ovariectomy	Bilateral	None	CR	POF
Ovarian teratoma	17	G0PO	Bilateral ovariectomy	Unilateral	None	CR	POF
AML	26	G0P0	BMT	Unilateral	PC	CR	POF
Sarcoma	18	G0P0	Alkylant	Unilateral	PC	Death	-
Breast cancer	32	G1P1	Alkylant	Unilateral	PC	CR	Normal
Medulloblastoma	23	G0P0	PI	Unilateral	OT	CR	Pregnant
HD	24	G0P0	BMT	Unilateral	PC	CR	POF
NHL	27	G0P0	BMT	Unilateral	None	CR	POF
Drepanocytosis	11	G0P0	BMT	Unilateral	PC	CR	POF
AML	25	G1P0	BMT	Unilateral	Caesarean section	CR	POF
ALL	4	G0P0	BMT	Unilateral	PC	Death	–
Breast cancer	29	G1P1	Alkylant	Unilateral	None	CR	Normal
NHL	28	G1P0	BMT	Unilateral	Caesarean section	CR	POF
NHL	21	G1P0	Alkylant	Unilateral	Caesarean section	CR	?
HD	28	G0P0	Alkylant	Biopsy	OT	CR	Normal
Drepanocytosis	10	G0P0	BMT	Unilateral	PC	CR	?
Drepanocytosis	14	G0P0	BMT	Unilateral	PC	CR	POF
HD	18	G0P0	Alkylant	Unilateral	PC	CR	Normal
Breast cancer	25	G0P0	Alkylant	Biopsy	Mastectomy	?	?

NHL: non-Hodgkin's lymphoma; BMT: bone marrow transplantation; CR: complete remission; POF: premature ovarian failure; AML: acute myelogenous leukaemia; HD: Hodgkin's disease; ALL: acute lymphoblastic leukaemia; PI: pelvic irradiation; OT: ovarian transposition; PC: port catheter; ? = unknown.

Table 4. Patients requesting cryopreservation of ovarian tissue for whom the procedure was not performed.

Disease	*Age (years)*	*Indication*	*Exclusion criteria*
NHL	25	BMT	POF
ALL	6	BMT	Last minute refusal
HD	29	BMT	Pelvic tuberculosis
AML	27	BMT	POF
HD	20	BMT	Operative contraindication (BMI >40)
AML	27	BMT	Last minute refusal
Cerebral neoplasia	29	Alkylant	HIV seropositivity
Melanoma	22	Alkylant	Operative contraindication (poor state of health)
Breast cancer	35	Alkylant	Pregnancy (refusal of abortion)
Breast cancer	35	Alkylant	Last minute refusal
Breast cancer	26	Alkylant	Low risk of POF
ALL	27	BMT	Last minute refusal
ALL	26	BMT	Operative contraindication (relapse)
ALL	32	BMT	Operative contraindication (relapse)
ALL	3	BMT	Last minute refusal

NHL: non-Hodgkin's lymphoma; BMT: bone marrow transplantation; POF: premature ovarian failure; AML: acute myelogenous leukaemia; HD: Hodgkin's disease; ALL: acute lymphoblastic leukaemia.

section (three) or difficulties in performing laparoscopy (two). No surgical complication was observed. For 15 patients (79%), the procedure was associated with other surgical acts such as Caesarean section (three), port catheter positioning (nine), mastectomy (one) and ovarian transposition (two).

An average of 31 cryotubes per patient (range 7–45) were stored following the procedure, each containing one or two pieces of ovarian cortex.

Sixteen patients were in complete remission after a minimum follow-up period of 6 months. POF occurred in more than half. Two patients died within 1 year after the diagnosis. One patient who had undergone ovarian transposition concomitantly to the ovarian cryopreservation procedure became pregnant spontaneously 6 months after the end of the radiotherapy.

Discussion

Chemotherapy drugs such as alkylating agents are frequently used in various combined regimens to treat neoplasic and benign diseases. These drugs are definitely associated with gonadal toxicity. A recent study showed that the patients treated with alkylating agents had a 3.98-fold higher risk of losing their ovarian function compared with those who were treated with other agents (Meirow and Nugent, 2001). Forty per cent or more of patients treated with alkylating agents required hormonal replacement therapy for chemotherapy-induced POF. Over the past 15 years, clinicians have aimed to reduce acute and chronic toxicity associated with chemotherapy. In the treatment of Hodgkin's disease (HD), the replacement of the MOPP regimen (mechlorethamine, vincristine, procarbazine, prednisone) with the ABVD regimen (adriamycin, bleomycin, vinblastine and dacarbazine) has considerably reduced gonadal toxicity: no case of permanent amenorrhoea has been observed after ABVD treatment among women younger than 25 years (Canellos *et al*., 1992; Brusamolino *et al*., 2000). On the other hand, treatment including high-dose chemotherapy with autologous stem cell transplantation is now an accepted curative therapy for young patients with aggressive lymphoma. Few pregnancies have been reported after this treatment (Brice *et al*., 2002). However, no pregnancy was obtained in patients older than 29 years following treatment with this regimen

Clinical information such as normal reproductive outcome and hormonal concentrations after treatment are not always reassuring and are not sufficient to conclude that the ovaries have not been damaged. Indeed, a reduced follicular reserve can predispose to the development of premature menopause. For survivors of childhood and adolescent cancer who still menstruated at the age of 21 years, the relative risk of menopause during their 20s was 9.2 after alkylating agent alone, 3.7 after radiotherapy alone and 27 after combined subdiaphragmatic irradiation and alkylating agent (Byrne *et al*., 1992).

All these patients and others affected by less common diseases such as sarcoma, thyroid cancer, kidney cancer, or autoimmune diseases such as rheumatoid arthritis and systemic lupus, requiring treatment by alkylating agents or pelvic/total irradiation, also must be informed of the risk of developing POF and the options to try to preserve fertility.

Cryopreservation of ovarian tissue is presented as the most promising technique to preserve fertility of many patients. Two different approaches, both experimental, are considered in the future to restore the fertility of the patient using frozen–thawed ovarian tissue: ovarian tissue transplantation or follicular in-vitro culture.

Restoration of normal reproductive lifespan (1 year) has been obtained following autologous ovarian tissue grafting in the mouse (Candy *et al*., 2000; Shaw *et al*., 2000). In sheep, cyclicity and fertility have been restored in the short term (<1 year), but the grafts have generally failed within 2–3 years after grafting (Gosden *et al*., 1994; Salle *et al*., 2002) probably because 65% of the follicles were lost during the revascularization period of the grafts. In monkeys, 50% of the animals had ovarian cyclicity up to 9 months after

subcutaneous transplantation of cryopreserved ovarian tissue (Schnorr *et al*., 2002). Transplantation, contrary to the cryopreservation procedure, induces a major primordial follicular loss, limiting the lifespan of the graft (Nisolle *et al*., 2000; Liu *et al*., 2002).

Encouraged by the results of animal autograft and human xenograft studies, the first ovarian tissue transplantations were initiated in humans. Two different approaches were investigated: heterotopic, where the ovarian tissue was grafted at a distance from its original place, and pelvic or orthotopic, where the ovarian tissue was grafted at the original place.

Several studies on the feasibility of heterotopic sites for ovarian transplantation have been reported (Leporrier *et al*., 1987; Nugent *et al*., 1998; Callejo *et al*., 2001). Recently, two cases of successfully heterotopic transplantation of frozen–thawed ovarian cortex in the forearm were reported (Oktay *et al*., 2001). Ovarian stimulation allowed retrieval of three oocytes by percutaneous puncture. Intracytoplasmic injection of spermatozoid into one of them failed to result in fertilization. The ovarian graft was still functional 10 months after transplantation.

Orthotopic reimplantation of cryopreserved ovarian cortical strips into a previously oophorectomized woman has recently been reported (Oktay and Karlikaya, 2000). Follicular development was observed in response to gonadotrophin stimulation 4 months after the procedure, confirming the long-term survival of the graft.

Autologous ovarian transplantation of frozen ovarian tissue was reported in a woman treated for HD (Radford *et al*., 2001). Resumption of ovarian function was observed after 7 months and persisted for 2 months. However, the ovary had already been exposed to gonadotoxic treatment at the time of cryopreservation, and thus the follicular pool had already been severely depleted. In this particular case, the benefits/risks ratio of the procedure caused controversy (Baird *et al*., 2001).

Gonadotrophin stimulation of human ovarian tissue grafted to severe combined immunodeficient mice allowed follicular development to the antral stage, with the most significant follicular growth when gonadotrophic stimulation started 14 weeks after grafting (Van den Broecke *et al*., 2002).

These results show that human frozen primordial follicles are able to grow and mature after transplantation. Their ability to be fertilized and to produce healthy embryos has still to be demonstrated. Moreover, a major concern of grafting cryopreserved ovarian tissue is the possibility to reintroduce malignant cells during the procedure. Shaw first reported transmission of lymphoma from a donor to a graft recipient with fresh and cryopreserved mouse ovarian tissue samples (Shaw *et al*., 1996). Recently, severely immunodeficient mice were xenografted with frozen–thawed ovarian tissue from a patient with lymphoma (Kim *et al*., 2001). None of the animals grafted with ovarian tissue developed the human disease. Control mice were grafted either with lymph node cells or with a cell line L1236 previously reported to transmit lymphoma in around 40% of cases. None of the animals grafted with the L1236 cell line developed the human disease. This may be due to the limited observation period (16 weeks) or to the low number of injected cells. These results were reassuring, but should not be interpreted as an absolute indication of safety. The transmission risk could be even higher for cancers such as leukaemia, which constitutes one of the main indications of ovarian tissue cryopreservation. These patients could always turn to in-vitro ovarian tissue culture in the future to conceive their own child.

The second option for clinical use of cryopreserved ovarian tissue is the in-vitro growth and maturation of primordial follicles in order to obtain fertilizable oocytes. The mechanism that regulates the exit of ovarian follicles from the primordial pool and their needs until the secondary stage is still poorly understood. Different growth factors have been already identified as responsible for the regulation of follicular growth initiation, for example growth differentiation factor-9 (GDF9), bone morphogenetic proteins (BMP), transforming growth factors (TGF) and kit ligand (Matzuk *et al*., 2002)

Up to recently, only one live offspring had been obtained after fertilization of in-vitro grown oocytes from primordial mouse follicles (Eppig and O'Brien 1996). Fifty-nine other offspring have been recently obtained by the same team after transfer of 1160 embryos derived from in-vitro grown, matured and fertilized oocytes (O'Brien *et al*., 2003).

The long period of growth necessary to obtain a preovulatory follicle from a primordial follicle in the human constitutes an additional limiting step in the in-vitro culture techniques. The other difficulty encountered during ovarian tissue culture is related to the structure of the cortex: the ovarian cortex of large mammals and humans is dense, and isolation of primordial follicles is difficult to perform. So far, secondary follicles have been obtained after in-vitro culture of human ovarian cortex, but further research is necessary in order to improve the in-vitro follicular techniques and to obtain fertilizable oocytes.

Promising results concerning ovarian transplantation, advances in in-vitro follicular culture and the capacity of the primordial follicles to survive the cryopreservation procedure have encouraged various teams to suggest storage of ovarian tissue for patients at high risk of POF (Donnez and Bassil, 1998; Gosden, 2001; Poirot *et al*., 2002). However, success is still hypothetical and must be proposed with caution (Bahadur, 2000; Van den Broecke *et al*., 2001). Moreover, the procedure implies surgery, which is not inherently dangerous for patients, but which is nevertheless not devoid of any complication. It has been found that the procedure was associated with other surgical acts in 79% of patients.

Other issues have been raised, such as the risk of genetic damage induced by the procedure. Live offspring obtained after grafting frozen–thawed ovarian tissue in large animals seem to be normal (Salle *et al*., 2002). However, definitive conclusions cannot be drawn based on this experiment because of the low number of pregnancies described.

In the present series, 31% of patients undergoing ovarian tissue cryopreservation had already received chemotherapy. The effect of chemotherapy prior to the freezing–thawing procedure is poorly known. Experiments on mouse showed that early fertilization post-chemotherapy could result in a high pregnancy failure and malformation rates (Meirow *et al*.

2001). Congenital or chromosomal malformation rate has been shown to be normal in the offspring of women exposed to oncological treatments (Sanders *et al*. 1996). However, most of these women conceived many years after treatment. This large period of time between drug exposure and conception could allow the DNA repair mechanisms to correct damage or to eliminate defective primordial follicles.

In conclusion, cryopreservation of ovarian tissue is an alternative to preserve the fertility of selected patients, but must be proposed after multidisciplinary evaluation of the risk of developing POF. The patients must be well informed about the experimental aspect of the procedure and that whatever the evolution of progress in research, safe clinical application is probably restricted to a couple of years. Alternative options to prevent POF must be always considered, offering additional possibilities to restore fertility after oncological treatment.

Even if no pregnancy has been reported to date in humans using these techniques, ovarian function has been restored by ovarian transplantation and encouraging results in animal studies suggest that this is a valid prospect for humans. Future trials must determine the safety and the efficiency of the procedure. Further experiments are also necessary to improve the in-vitro follicular culture system, offering an alternative to ovarian tissue autologous transplantation in order to restore fertility.

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