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Feasibility of Ovarian Tissue Cryopreservation for Prepubertal Females With Cancer

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Background. Loss of fertility is one of the long-term adverse effects of high-dose chemotherapy or total body irradiation for cancer, even in children. Ovarian tissue cryopreservation (OTC) may make it possible for survivors of childhood cancer to have children. We evaluated the feasibility of this technique for prepubertal girls. ***Methods.*** Between September 2000 and February 2005, 49 prepubertal girls were referred to the Reproductive Biology Unit for OTC before sterilizing treatment. ***Results.*** One ovary each was collected from 47 patients, by laparoscopy in 24 patients and laporotomy in the others. In 16 cases, the ovary was harvested during laparotomy to resect a residual abdominal tumor. No complications occurred after operations. Ovarian tissue was frozen by a slow-cooling protocol, using DMSO and sucrose as cryoprotectants. An mean of 17.6 ± 6.5 ovarian tissue fragments was cryopreserved per patient. Follicle concentration was evaluated histologically for 46 patients and a strong correlation was found between age and follicular density. None of the cases had visible ovarian tumor components. Ovarian cryopreservation was not carried out for two patients. ***Conclusion.*** The cryopreservation of ovarian tissue could be systematically offered even to prepubertal girls at risk of sterility due to gonadotoxic treatment. Pediatr Blood Cancer 2007;49:74–78.

Key words: fertility preservation; gonadotoxic treatment; ovarian cryopreservation; ovarian follicle; prepubertal girl

INTRODUCTION

In the 1970s, many children diagnosed with malignant cancers died from their disease. Nowadays, three quarters of children and adolescents diagnosed with cancer survive for at least 5 years [1]. This progress has been achieved largely by increasing the intensity of therapy. Consequently, many children treated for cancer suffer long-term adverse effects, such as the loss of fertility. Some treatments are known to be highly deleterious for ovarian function. These treatments include high dose alkylating agents, total body irradiation and abdominopelvic irradiation [2,3]. For prepubertal girls, ovarian tissue cryopreservation (OTC) is currently the only available means of potentially preserving gonad function and fertility [4].

This promising approach to preserving the fertility of young patients involves the storage of a large number of follicles, which could subsequently be transplanted or cultured to obtain mature oocytes. The method remains experimental, but there have been significant scientific advances in this field. Oktay reported the first demonstration of ovarian function restoration after grafting, in 2000 [5]. He obtained a single embryo after an ectopic ovarian graft in 2004 [6]. This embryo was transferred to the uterus of the patient but no pregnancy occurred. The first baby conceived following ovarian cortex freezing, thawing and orthotopic autograft was born in September 2004, [7]. A second such baby was born in July 2005 [8]. These advances have made it increasingly important to offer OTC to children undergoing sterilizing treatment. We therefore initiated an OTC program from prepubertal girls in 2000. This paper reports our experience of OTC for prepubertal females, focusing on the feasibility of the technique and specific features of this procedure for prepubertal children.

METHODS

Patients

From September 2000 to February 2005, 49 prepubertal girls were referred by oncologists to the Reproductive Biology Unit at Pitié-Salpêtrière Hospital for OTC. OTC has been offered to patients before cancer treatment at high risk of ovarian failure, such as high-dose busulfan or total body irradiation. The patient and/or at least one parent were informed of the risks of the planned treatment for subsequent fertility. Information about ovarian tissue preparation, freezing and cryopreservation was provided, and the experimental nature of OTC emphasized informed consent forms for OTC were signed by patients and/or their parents in cases of agreement to undergo the procedure patients were also seen by the surgeon and the anesthetist. The procedure was approved by the ethics committee of Limoges Hospital, France.

Collection of Ovarian Tissue

Ovarian tissue harvesting was programmed to take place immediately before the sterilizing treatment, after induction chemotherapy. Ovarian tissue was collected during another surgical procedure, such as the removal of a primary abdominal tumor, in

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some cases. In all cases, one entire ovary was collected. For other patients not undergoing another surgical abdominal procedure, ovarian tissue was collected by means of a short supra-pubic laparotomy or laparoscopy as described below [9].

Laparoscopy was carried out in day surgery whenever possible, using only two ports. A 10-mm port was inserted through an open laparoscopy in the inferior abdominal crease, for the introduction of a 10 mm 0° laparoscope. CO_2 pressure was maintained at 10–12 mmHg. A 5-mm port was inserted symmetrically into the same crease and an atraumatic grasper was used to grasp the ovary and to present it to the first port. The laparoscope was then removed, CO_2 was exsufflated and the ovary was gently pulled out of the abdominal cavity. Ovariectomy was performed, and the mesovarium was sutured with a single absorbable stitch (5/0 PDS, Ethicon, UK). The fallopian tube was returned to the abdomen and the 10 mm port site was carefully closed. The median duration of this technique was 30 min (range: 20–45 min).

Cryopreservation of Ovarian Tissue

The protocol used was as previously described [10]. Briefly, after isolation, fragmentation and transfer to a Nunc cryovial (Polylabo, Strasbourg, France), ovarian fragments were slowly frozen, in an automated freezer down to temperature of liquid nitrogen, in which, they were stored.

Histological Analysis

For each patient, one sample of ovarian cortex selected at random, before freezing, from the fragments prepared for freezing and the medulla were fixed in formaldehyde. Each specimen was measured in three dimensions and embedded individually in paraffin. Sections (5 μm) were cut perpendicularly to the surface of the ovary and stained with haematoxylin-eosin-saffron. The follicles were counted, with classification according to the modified Oktay system [11]. We checked for malignant tumor cells in both the cortex and the medulla. For each patient, we determined the number of primordial and primary follicles per mm^2 within the ovarian cortex.

Statistical Method

We investigated the relationship between age and follicular density using a linear regression model. We used the logarithm of density, rather than density itself, to normalize the residuals. The assumptions of the model were checked, by plotting the residuals.

RESULTS

Ovarian fragments were collected and cryopreserved for 47 prepubertal girls, with a median age of 5 years (range 10 months to 15 years) (Table I). All patients suffered from malignant cancer: metastatic neuroblastoma (n = 20), rhabdomyosarcoma (n = 6), medulloblastoma (n = 3), Ewing sarcoma (n = 4), osteosarcoma (n = 2), nephroblastoma (n = 2), leukaemia (n = 6), lymphoma (n=2), neuroectodermal tumor (n = 1) and Hodgkin's disease (n = 1). All patients underwent several courses of chemotherapy before OTC, the number of courses depending on the disease. This chemotherapy was not known to have a sterilizing effect.

OTC was carried out during first-line treatment or after relapse. The treatment sequence associated with a high risk of sterility was high-dose busulfan (33 patients) high-dose thiotepa (2 patients) total body irradiation (7 patients) or abdominopelvic irradiation including both ovaries (5 patients). OTC was not carried out for two patients. In one case, the patient herself refused and in the other, parents refused OTC for their daughter. OTC was refused in these two cases because it was too new and uncertain.

Ovarian Collection and Cryopreservation

Treatment by chemotherapy and/or radiotherapy was not delayed due to OTC. Surgical procedures were performed as soon as possible after informed consent was obtained. The entire ovary was collected by laparoscopy for 24 patients (51%) and by laparotomy for the remaining patients 23 (49%). Sixteen girls underwent a laparotomy for the resection of a residual abdominal tumor (15 neuroblastomas, 1 Ewing metastasis), with collection of the ovary during this procedure. For seven other girls with rhabdomyosarcoma (n = 2), medulloblastoma, Ewing sarcoma, osteosarcoma, nephroblastoma and neuroblastoma, the ovary was collected by short suprapubic laparotomy. No complications occurred after these operations. A mean of 17.6 ± 6.5 ovarian tissue fragments was cryopreserved per patient (range = 7–41).

Follow-Up

Median follow-up time was 30 months (range 10–60 months). Thirty-seven of the girls are still alive. Ten girls died of their disease. Five these patients suffered from metastatic neuroblastoma, one from medulloblastoma, two from acute leukaemia, one from osteosarcoma and one from Ewing sarcoma.

Histological Analysis of Ovarian Tissue

In one case, the ovary was too small and the entire cortex was cryopreserved. In 46 cases, the cortex and medulla were analyzed. None of the patients had visible ovarian tumor components. In three cases, only fibrinoid tissue was characterized, corresponding to a bias in sampling. In one of these three cases, a few follicles were identified in the medulla (Case 29). In the other cases, the total number of primordial and primary follicles per square millimeter showed a strong inverse correlation dependent on the age of the patient. The relationship between log-density and age was $\log(\text{density}) = 3.201 - 0.011 \times \text{age}$. The *P*-value for the age effect was 0.0011 (Fig. 1).

DISCUSSION

In the light of major improvements in the treatment of childhood cancer, and consequently in the survival rates, the long-term side effects of cytotoxic therapies, such fertility loss, must now be considered. Ovarian damage is drug- and dose-dependant and increases with age at treatment [12]. Nevertheless, some regimens are gonadotoxic even if administered to young children [2,3]. This is the case for high-dose chemotherapy including busulfan or thiotepa and radiotherapy including both ovaries.

For prepubertal girls, OTC is the only possible means of preserving fertility available [13].

This describes a large report concerning a large number of prepubertal girls undergoing OTC to preserve fertility. Given the young age of the patients concerned, ovarian cortex use is likely to

TABLE I. Characteristics of the 47 Prepubertal Females who had an Ovarian Tissue Cryopreservation

Case no.	Pathology	Age (years)	Treatment with high risk of sterility	Surgical procedure	No. of cryopreserved fragments	Primordial and primary follicles (no./mm^2)	Follow-up
1	Medulloblastoma	4	HD busulfan	Laparoscopy	24	1	Deceased
2	Neuroblastoma	2.5	HD busulfan	Laparotomy	21	10	Deceased
3	Leukaemia	5	HD busulfan	Laparoscopy	24	20	Healthy
4	Lymphoma	9	HD busulfan	Laparoscopy	20	3.9	Healthy
5	Neuroblastoma	2	HD busulfan	Minilaparotomy	17	12.9	Healthy
6	Ewing sarcoma	11	HD busulfan	Minilaparotomy	16	31	Healthy
7	Neuroblastoma	8	HD busulfan	Laparotomy	28	1.5	Healthy
8	Neuroblastoma	5	HD busulfan	Laparotomy	29	4.8	Healthy
9	Neuroblastoma	2	HD busulfan	Laparotomy	17	7.9	Deceased
10	Ewing sarcoma	9	HD busulfan	Laparotomy	7	ND	Healthy
11	Rhabdomyosarcoma	2	API	Laparoscopy	14	25.6	Healthy
12	Neuroblastoma	3	HD busulfan	Laparotomy	13	9	Healthy
13	Neuroblastoma	1.5	HD busulfan	Laparotomy	12	21	Deceased
14	Neuroblastoma	5	HD busulfan	Laparotomy	16	30.5	Healthy
15	Leukaemia	12	TBI	Laparoscopy	31	3.9	Healthy
16	Osteosarcoma	14	HD busulfan	Minilaparotomy	14	1.6	Deceased
17	Leukaemia	7	TBI	Laparoscopy	19	6.3	Deceased
18	Neuroblastoma	5	HD busulfan	Laparotomy	15	14	Deceased
19	Neuroblastoma	7	HD busulfan	Laparotomy	13	40.5	Healthy
20	Hodgkin disease	15	TBI	Laparoscopy	25	5.7	Healthy
21	Neuroblastoma	6	HD busulfan	Laparoscopy	20	24.3	Deceased
22	Neuroblastoma	2.5	HD busulfan	Laparotomy	17	86.7	Healthy
23	Neuroblastoma	3.5	HD busulfan	Laparotomy	17	19.3	Deceased
24	Neuroblastoma	1	HD busulfan	Laparoscopy	8	76.7	Healthy
25	Lymphoma	4.5	TBI	Laparoscopy	11	23.2	Healthy
26	Leukaemia	6	TBI	Laparoscopy	18	8.7	Healthy
27	Neuroblastoma	1.5	HD busulfan	Laparotomy	12	8.8	Healthy
28	Rhabdomyosarcoma	2	HD busulfan	Minilaparotomy	14	Fibrinoid	Healthy
29	Ewing sarcoma	8	HD busulfan	Laparoscopy	13	Fibrinoid	Deceased
30	Rhabdomyosarcoma	4	API	Laparoscopy	16	0	Healthy
31	Medulloblastoma	4.5	HD busulfan	Laparoscopy	13	34	Healthy
32	Leukaemia	13	TBI	Laparoscopy	41	3.3	Deceased
33	Neuroblastoma	0.10	HD busulfan	Laparotomy	11	8	Healthy
34	Nephroblastoma	9	API	Minilaparotomy	18	8	Healthy
35	Leukaemia	14	TBI	Laparoscopy	17	3.5	Healthy
36	Rhabdomyosarcoma	9	HD busulfan	Laparoscopy	15	3.8	Healthy
37	Neuroblastome	1.5	HD busulfan	Laparotomy	13	130	Healthy
38	Ewing Sarcoma	14	HD busulfan	Laparoscopy	20	2	Healthy
39	Neuroblastoma	1.5	HD busulfan	Laparoscopy	11	Fibrinoid	Healthy
40	Rhabdomyosarcoma	1.5	API	Laparoscopy	16	4	Healthy
41	Osteosarcoma	12	HD Thiotepa	Laparoscopy	27	2.7	Relapse
42	Neuroblastoma	6	HD busulfan	Laparotomy	18	3.4	Healthy
43	Medulloblastoma	9.5	HD busulfan	Minilaparotomy	23	0.1	Healthy
44	Neurectodermal tumor	12.5	HD thiotepa	Laparoscopy	13	6.7	Healthy
45	Neuroblastoma	2.5	HD busulfan	Laparoscopy	11	38.3	Healthy
46	Nephroblastoma	4.5	API	Laparoscopy	20	35	Healthy
47	Rhabdomyosarcoma	5	HD busulfan	Minilaparotomy	11	33.3	Healthy

TBI, total body irradiation; HD, high dose; API, abdominopelvic irradiation; ND, not done.

be more efficient by the time these patients decide to try to start a family.

Little is known about the number of follicles in the ovarian cortex required to restore fertility. However, the probability of restoring fertility should be higher for younger girls, as their ovarian cortex clearly contains a large number of follicles. As shown here, the number of follicles per mm^2 was inversely related to the age of the patient. OTC is particularly relevant in children, because young girls have a large number of primordial and primary follicles and the survival rates for such follicles after freezing and thawing are high [14].

None of the patients in our study had visible ovarian tumor components. Nevertheless, the possibility of ovarian metastatic tumors in patients with lymphoma, leukaemia, neuroblastoma or rhabdomyosarcoma should be borne in mind [15,16], because histological analysis was carried out on only three sections of a

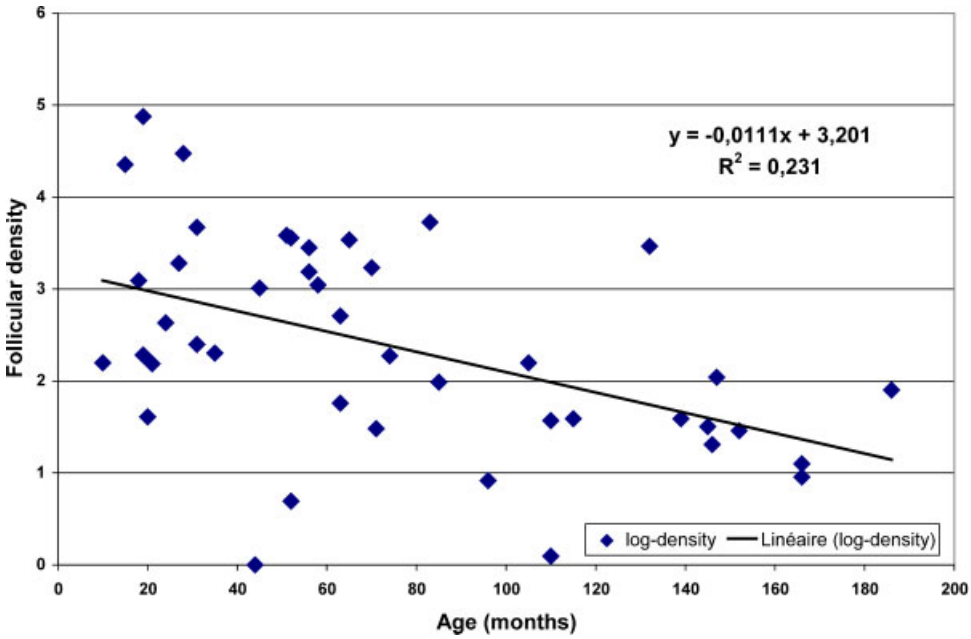

Fig. 1. Relationship between age and follicular density.

randomly selected ovarian fragment. Even in more extensive studies, it would not be possible to analyze the entire ovary. Another explanation for the lack of tumors in the ovaries was that our patients had received chemotherapy before the ovary collection. One way to reduce the risk of inducing a relapse of the initial disease by grafting [17], is to mature follicles from ovarian biopsy samples in vitro. This technology remains experimental. It involves culturing ovarian follicles from the primordial follicle stage to the preovulatory stage. This work is most advanced in mice [18,19]. In humans, this procedure is still experimental [20]. The young patients reported in this paper, with a median age of 5 years, are unlikely to want to use their ovarian cortex in the near future. However, the use of their cryopreserved ovarian tissue provides them with a possible means to restores their fertility when they reach reproductive age.

The timing of ovary collection is important. We chose to remove the ovary for cryopreservation just before sterilizing treatment. OTC after several chemotherapy regimens probably carries no risk of toxicity to future offspring. The major risk is premature ovarian failure rather than mutagenic effects [21]. Moreover, the second birth after OTC and grafting was obtained for a patient for who ovarian tissue was harvested and frozen after first- and second-line chemotherapy, before high-dose chemotherapy [8]. This progress provides very good reasons for trying to preserve fertility by OTC for prepubertal patients, even if chemotherapy is administered before OTC.

Another specific feature of this management is that decision to perform OTC was taken by the parents alone in most cases. Parents do not wish to have regrets and regard this technology as a source of hope. OTC forces them to think about the future fertility of their daughters, projecting them into the future. The refusal rate is lower (2.2%) than that reported in a previous study (12%) [22].

In conclusion, this study reports on ovarian cryopreservation in a large number of prepubertal females. There are many lines of evidence favoring OTC for prepubertal girls, including the large number of primordial follicles in ovarian tissue, and the young age of the patient with respect to the experimental nature of the procedure. OTC seems to be highly feasible and safe, does not delay cancer treatment and is acceptable to most patients and/or their families. This technology also seems to be very promising in terms of the probability of a child being born after autologous grafting. It is, therefore, important to offer OTC to prepubertal girls before sterilizing treatment.

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