

Transplantations of frozen-thawed ovarian tissue demonstrate high reproductive performance and the need to revise restrictive criteria

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Objective: To report the single-center results of orthotopic retransplantations of cryopreserved ovarian tissue in cancer survivors and evaluate the validity of commonly accepted procedure limitations.

Design: Prospective cohort study.

Setting: Tertiary university-affiliated assisted reproduction technology (ART) and oncology centers.

Patient(s): Twenty cancer survivors who underwent ovarian transplantation of frozen-thawed ovarian tissue with the aim to conceive.

Intervention(s): Ovarian tissue cryopreservation (OTCP) and transplantation, endocrine monitoring, in vitro fertilization (IVF).

Main Outcome Measure(s): Endocrine profile, IVF, pregnancies, live births.

Result(s): The patient ages at tissue harvesting ranged from 14 to 39 years. Fifteen women had hematologic malignancies, and two had leukemia (chronic myelogenous leukemia and acute myelogenous leukemia). Ten patients were exposed to nonsterilizing chemotherapy before OTCP. After transplantation, the endocrine recovery rate was 93%. Fourteen patients underwent IVF treatments with a fertilization rate of 58%. Sixteen pregnancies were achieved (10 after IVF, 6 spontaneous), resulting in 10 live births, two (twins) after harvesting from the mother at the age of 37. Two pregnancies are currently ongoing. After transplantation, 53% of patients conceived, and 32% delivered at least once. One patient conceived four times. Preharvesting chemotherapy exposure was not associated with inferior outcomes. All patients, including two leukemia survivors, remained cancer free.

Conclusion(s): Orthotopic transplantation of thawed ovarian tissue is a highly effective measure to restore fertility in sterilized cancer patients. Chemotherapy exposure before harvesting and age >35 is a realistic option in selected patients. Retransplantation in leukemic patients is possible after application of maximal safety measures. These results have led the national ethical and professional authorities to decide for the first time not to consider OTCP as an experimental modality for fertility preservation.

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Key Words: Fertility Preservation, Cryopreserved Ovarian Tissue, Transplantation, Pregnancy, Safety

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Ovarian tissue cryopreservation (OTCP) aims to provide a chance for future fertility for young women and prepubertal girls who are at major risk for significant ovarian injury and sterility, most

commonly as a result of radiation/chemotherapy induced loss of ovarian follicular reservoir (1). It has been more than a decade since the first reports of live births after OTCP and retransplantation (2, 3), and to date, more than 60 live births have been described by multicenter reports (4, 5), single-center studies (6, 7), and case reports (8–10) from dozens of centers worldwide. Nevertheless, significant heterogeneity exists among different groups regarding selection criteria for

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OTCP, such as age limits for tissue collection and ineligibility due to previous exposure to chemotherapy (11) or specific cancer diagnoses that might preclude retransplantation. Some of these exclusion criteria were adapted into practice long before data on true procedural limitations became available (12). Tissue preparation, freezing and transplantation techniques are also diverse, applied differently worldwide.

A major challenge is estimation of true transplantation success rates in cancer patients. Reports on pregnancies and deliveries are currently compiled from multiple centers, some of which do not conduct complete pretransplantation endocrine monitoring, therefore possibly including transplantations performed in patients who were not sterilized in the first place. To accurately assess fertility outcomes after transplantation of cryopreserved-thawed ovarian tissue we describe here the results of a single-center study of OTCP outcomes. Transplantations were performed using a uniform technique by one team, which consistently performed pre/posttransplantation endocrine monitoring, fertility treatments, and in vitro fertilization (IVF) procedures in all patients. This study provides an accurate evaluation of procedure success rates and allows for a careful clarification of procedure limits and risks.

MATERIALS AND METHODS

Patients

From January 2004 to March 2015, 20 cancer patients who had stored ovarian tissue before sterilizing chemotherapy underwent autotransplantation in our center to restore fertility and conceive. The patients' ages at the time of cryopreservation were between 14 and 39 years (mean 28.8 years). Fifteen patients had hematologic malignancies and an additional five from solid tumors (Table 1). Ten patients had been treated with nonsterilizing chemotherapy before tissue harvesting. In patient no. 8, ovarian tissue was harvested during a surgery of large abdominal tumor removal and after several chemotherapy cycles. In the remaining nine patients, OTCP was performed when switching to second-line protocols was required due to disease refractoriness (patients no. 2, 3, and 20) or a later relapse (patients no. 1, 4, 6, 14, 16, and 19). In seven of nine cases, collection of tissue was performed before second-line therapy had begun. Two of nine patients (patients no. 1 and 19) underwent chemotherapy cycles right before OTCP took place. Patient no. 1 required immediate salvage therapy on relapse due to poor medical condition incompatible with general anesthesia, and patient no. 19 underwent OTCP after completing second-line chemotherapy when the

TABLE 1

Patient characteristics and pre/posttransplantation endocrine profiles.

Patient	Diagnosis	Pre-OTCP chemotherapy ^a	No. of children	Age (y)		Ovarian function before Tx			Ovarian function after Tx		
				OTCP	Tx	Menses	FSH (mIU/mL)	E ² (pmol/L)	Menses ^b	FSH (mIU/mL)	E ² (pmol/L)
No previous chemotherapy											
5	CML	—	0	19	27	No	116.4	<37	Yes (6M)	7.2	147
5 ^c	CML	—	0	21	28	No	44	174	Yes (4M)	100	360
7	Hodgkin's	—	0	19	35	No	87	<100	Yes	12.4	107
9	Breast cancer	—	1	37	45	Yes	8.4	100	Yes	5.7	177
10	NHL	—	0	39	41	No	99	<100	Yes (1M)	96	<100
11	Hodgkin's	—	0	32	36	No	54.6	<73.4	Yes	27.6	126
12	Breast cancer	—	0	33	38	Yes	4.8	858	Yes	6.8	380
13	NHL	—	1	34	42	No	30	100	Yes (6M)	26.4	<100
15	Hodgkin's	—	0	37	40	No	41.8	137	Yes	7.8	645
17	Breast cancer	—	0	37	45	Yes	6.8	232	Yes	14	<185
18	Ewing's sarcoma	—	0	24	28	No	107	<70	Yes	24.3	<100
Previous chemotherapy											
1	NHL	VACOP_B, MINE/ESHAP	1	30	32	No	100	<70	Yes	7	342
2	NHL	VACOP_BX6	1	38	42	No	120	<100	Yes	11	220
3	Hodgkin's	ABVDX4	1	31	36	Yes	10.3	380	Yes	9.5	99
4	Hodgkin's	ABVDX6	1	24	27	No	81	<70	Yes	12.2	<73.4
6	Hodgkin's	ABVD	0	23	26	No	60.7	146	Yes	13.8	<73.4
8	Ewing's sarcoma	VCAIE	0	14	21	No	60	69	No	94	<100
14	Hodgkin's	ABVDX6	0	29	32	No	108	70	Yes	27	288
16	Hodgkin's	ABVDX6	0	32	36	No	33.1	99	Yes	16	95
19	Hodgkin's	ABVDX5, MOPPX1	0	23	28	No	68	70	Yes	44	134
20	AML	Doxorubicin + ARA-C	0	19	31	No	37	180	Yes	29	70

Note: AML = acute myeloid leukemia; CML = chronic myelogenous leukemia; E₂ = estradiol; FSH = follicle-stimulating hormone; NHL = non-Hodgkin's lymphoma; OTCP = ovarian tissue cryopreservation; Tx = transplantation.

^a VACOP_B = etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin; MINE/ESHAP = mesna, ifosfamide, mitoxantrone, etoposide/etoposide, methylprednisolone, high-dose cytarabine, cisplatin; ABVD = doxorubicin, bleomycin, vinblastine, dacarbazine; MOPP = mechlorethamine, vincristine, procarbazine, vincristine; VCAIE = vincristine, cyclophosphamide, arabinoside/decytosine, idarubicin.

^b Numbers in brackets represent duration of menses in case menses ceased during follow-up observation.

^c Results after the second transplantation.

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absence of leukemic cells in the blood was confirmed but before high-dose chemotherapy conditioning for heterologous stem cell transplantation.

At the time of transplantation, on average 5.6 years (range: 2–16 years) after tissue cryopreservation, the patients' mean age was 34 years (range: 21–45 years). Sixteen patients had no menses for a minimum of 1 year before transplantation and demonstrated a hormone profile consistent with a menopausal state (see Table 1); four women (patients no. 3, 9, 12, and 17) did show some evidence of ovarian activity but were infertile. Two of those (patients no. 3 and 12) clearly had ovarian insufficiency as indicated by low antimüllerian hormone levels ($<0.08\text{ng/mL}$, $<0.05\text{ng/mL}$) and IVF performance. Each underwent four IVF cycles, all of which resulted in only one embryo transfer, and no pregnancy was obtained. The two other women (patients no. 9 and 17) had normal hormone activity but were both aged 45. Patient no. 9 conceived naturally three times, with all pregnancies resulting in early abortions. Patient no. 17 went through several IVF cycles which resulted in dozens of embryos, but no pregnancies resulted. The poor reproductive outcomes could be explained by advanced age, so both patients requested transplantation of ovarian tissue to overcome age-related infertility. For all 20 patients, institutional review board and national ethics committee approval for ovarian tissue autotransplantation were obtained.

Ovarian Tissue Extraction and Cryopreservation

In 17 patients, ovarian tissue harvesting was performed by laparoscopic removal of one-half to two-thirds of one ovarian cortical cortex, as previously described elsewhere (3). Three additional patients had their tissue removed and stored in other medical centers. Patient no. 5 and patient no. 20 underwent unilateral laparoscopic oophorectomy, and patient no. 8 underwent bilateral oophorectomy during a surgery for abdominal sarcoma removal.

Tissue was prepared in pieces of $5 \times 10\text{ mm}^2$, and 1–2 mm in thickness for all 17 cryopreservations performed by our team. For the three patients who were initially treated elsewhere, the tissue was prepared and stored differently, either in smaller fragments of 1–2 mm^2 (patients no. 8 and 20), or in much larger pieces measuring $30 \times 20\text{ mm}^2$ (patient no. 5). In all cases the tissue was cryopreserved using a slow-freezing protocol (13). Two fresh ovarian tissue samples of both cortex and medulla were histologically evaluated to

rule out the presence of malignant cells and confirm the presence of primordial follicles (14). The latter was especially important in patients who had been treated with chemotherapy shortly before harvesting, for whom histology was the only option to prove that the ovary was potentially active. Additional small cortical strips were stored separately for future pretransplantation evaluation (14).

Thawing and Transplantation

When patients expressed their desire to restore fertility, approval of the treating oncologist/hematologist was obtained, and evaluation of a fragment of thawed tissue followed. The presence of primordial follicles with normal histologic features was confirmed within the sampled fragments. Various methods (multiple section histology, immunostaining, fluorescence in situ hybridization, polymerase chain reaction, and animal studies) were applied to rule out possible infiltration by cancer cells, guided by the patients' background malignancy (Table 2). A multidisciplinary team of a pathologist, cancer researchers, and fertility preservation team evaluated the cumulative results and approved transplantation.

Thawing was followed by a minilaparotomy for autotransplantation in all 20 patients (13) and took place in the laboratory adjacent to the operating theater. A single laboratory and operating team performed all procedures, and the operating time ranged between 60 and 120 minutes. In 18 patients, thawed tissue was inserted into subcortical tunnels (3). In brief, pairs of 5-mm parallel transverse incisions were made through the tunica albuginea bilaterally, and tunnels were created underneath the cortex stripe, as defined by the incisions. Thawed ovarian cortical stripes were gently placed in the cavities, and additional strips were inserted into blinded subcortical pockets (15), as dictated by ovarian gross architecture. All incisions were closed using 5/0 Prolene sutures. When there was insufficient space within the atrophic ovaries or in cases when both ovaries had been removed, the tissue was placed beneath the peritoneum in the broad or ovarian ligaments adjacent to the ovary.

In the two patients who had shown normal endocrine activity but had age-related infertility (patients no. 9 and 17), a different surgical technique was implemented: a total decortication of one ovary was performed, while the second ovary remained untouched. Thawed tissue fragments were placed onto the remaining medulla, using 5/0 Prolene sutures (16).

TABLE 2

Methods for detection of tissue involvement with cancer cells.

Cancer	Patients (n)	Histology	Immunohistochemistry	Molecular biology	SCID mice transplantation
Hodgkin's disease	9	9	9		
Non-Hodgkin's lymphoma	4	4	4		
Breast cancer	3	3	3		
Ewing's sarcoma	2	2	2	1	
Chronic myeloid leukemia	1	1		1	
Acute myeloid leukemia	1	1	1	1	1

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This technique was designed to ensure the preservation of patients' preexisting endocrine activity while also allowing for the possible collection of younger oocytes from the unilaterally transplanted tissue.

Follow up and Assisted Reproduction

Close monitoring of ovarian function was conducted after transplantation, including documentation of any menstrual bleeding, repeated measurements of follicle-stimulating hormone (FSH) and estradiol (E_2) hormone levels, and sonographic evaluations. Antimüllerian hormone levels are not good predictors of graft function, so they were not measured after transplantation (17). In patients 9 and 17 repeated ultrasound examinations were performed by a single physician to clearly monitor follicle development from each ovary separately. Once an increase in E_2 levels and follicular growth were sonographically demonstrated, proceeding to assisted reproductive technologies (ART) was considered. In 12 patients, IVF was performed using the modified natural cycle protocol (13); gonadotropin releasing-hormone antagonist and a daily gonadotropins support (225 IU/d of FSH + LH) was started once the leading follicle had reached a size of about 13–14 mm. When the follicle size had reached 18–20 mm, recombinant human chorionic gonadotropin was added, and follicle aspiration was performed. In two additional patients (patient no. 9 and 15), the antral follicular count and endocrine profile indicated the use of a standard controlled ovarian hyperstimulation gonadotropin releasing-hormone antagonist protocol.

RESULTS

Graft Function

Evidence of ovarian function was observed after transplantation in 19 of the 20 patients. Of the 16 patients who lacked any endocrine activity before transplantation, 15 regained their menses (93.7%), and 8 had FSH levels below 16mIU/mL (50%).

Among the 10 patients who had been treated with chemotherapy before tissue harvesting, 9 had amenorrhea and showed a menopausal endocrine profile before transplantation. After transplantation, eight of these patients regained menses (89%) and five showed FSH levels below 16mIU/mL. One patient (patient no. 8) who underwent bilateral salpingo-oophorectomy on diagnosis was lost to follow-up evaluation 4 months after transplantation after being diagnosed with a secondary malignancy of breast cancer at the age of 21 years.

Among the 10 patients who had not been treated with chemotherapy before tissue harvesting, seven had amenorrhea and showed a menopausal endocrine profile before transplantation. After transplantation, all seven patients regained their menses (100%), and three showed FSH levels <16mIU/mL. In one patient (patient no. 5) whose ovaries had been harvested abroad at the age of 19 and cryopreserved in very large pieces, the menses ceased after 6 months and a rise in FSH levels was observed. A second transplantation also resulted in limited ovarian activity for a period of 4 months.

ART Results: Pregnancies and Live Births

A total of 14 patients underwent ovarian stimulation and IVF cycles (Table 3). Among them were eight patients who had shown full recovery of hormone activity after transplantation and four patients who had undergone OTCP due to ovarian insufficiency or age-related infertility. Two additional patients did not demonstrate a normal hormone profile after transplantation but did display reasonable follicular growth on sonographic evaluation (patients no. 18 and 20). In 12 patients the natural modified cycle was used, as described.

Table 3 presents the IVF results of each of the 14 patients. A total of 56 cycles were performed, resulting in an average oocyte yield of 1.47 (0–3) per cycle, and in a cumulative total of 84 aspirated oocytes. In 23.2% of cycles, attempts to perform oocyte retrieval revealed empty follicles. The rate of empty follicles was similar in patients who had or had not been treated with chemotherapy before tissue collection (22.7% vs. 23.5%). Forty-nine of the 84 aspirated oocytes were fertilized (58% fertilization rate). The average oocyte yield per cycle (1.55 vs. 1.47) and fertilization rates (see Table 3) were comparable in patients who had been exposed to chemotherapy before harvesting and those who had not. A total of 38 embryos were transferred to 12 of the 14 patients, resulting in 10 pregnancies.

The pregnancy rate was 18% per cycle and 26% per transfer. The pregnancy rate per cycle was 20% in case of preharvesting chemotherapy versus 13.6% in case of no chemotherapy. Of the 10 IVF pregnancies, one was biochemical, one was ectopic, and two were terminated in early miscarriages. One IVF pregnancy (patient no. 20) is still ongoing (third trimester), and five pregnancies were carried to term (four singleton pregnancies, one twin pregnancy), resulting in six live births. The live-birth rate was 10.7% per cycle and 16% per transfer. The live-birth rate per cycle was 8.8% in cases of preharvesting chemotherapy versus 13.6% in cases of no chemotherapy.

In four patients who had been treated with chemotherapy before harvesting, an additional six pregnancies occurred spontaneously, resulting in four live births. Three of these patients (patients no. 1, 4, and 6) spontaneously conceived after already giving birth to healthy IVF babies. Patient no. 4 conceived four times after transplantation: two IVF pregnancies resulted in one live birth and one early abortion, and two spontaneous pregnancies both ended in live births. The unexpected fourth spontaneous pregnancy occurred in spite of visualization of atrophic ovaries during a cesarean delivery for the third pregnancy (Fig. 1). In patient no. 14, who had repeatedly demonstrated evidence of ovarian failure long before and immediately before transplantation (see Table 1), a spontaneous pregnancy was discovered 7 weeks after transplantation. The implantation date was estimated to be close to the transplantation date. Because this pregnancy and birth originated from the native, nontransplanted ovarian tissue, these data were not included in the presented results.

Safety

None of the patients have experienced cancer recurrence, including the two women who had leukemia at the time of

TABLE 3

Results of autotransplantation: IVF outcomes, pregnancies, and live births.

Patient	No. of cycles	Empty follicles (%)	Oocytes	2PN embryos	Fertilization rate (%)	Embryo transfer	Pregnancies		Live birth	
							IVF	Spontaneous	IVF	Spontaneous
No previous chemotherapy										
5+5'	4+1	0+1	7+0	2	29	2	0	0	0	0
7	2	0	4	1	25 ^a	1	0	0	0	0
9	5	2	8	4	50	4	0	0	0	0
10	—							0		0
11	—							0		0
12	6	2	7	6	86	5	1 (biochemical)	0	0	0
13	—							0		0
15	1	0	5	4	80	3	1 (twins)	0	2	0
17	1+ 1 (canceled)	0	1	0	0	0	0	0	0	0
18	1	0	2	2	100	2	1	0	1	0
Total	22	5 (22.7%)	34	19	55.8	17	3	0	3	0
Previous chemotherapy										
1	1	0	1	1	100	1	1	1 (miscarriage)	1	0
2	2	0	2	0	0	0	0	0	0	0
3 ^a	10	3	10	6	60	6	1 (miscarriage)	0	0	0
4	11	5	8	5	63	5	2(1 miscarriage)	2	1	2
6	3	0	5	3	60	3	1	2 (1 ongoing)	1	1
8	—							0		0
14	—							1 ^b		1 ^b
16	5	0	18	11	61	4	1 (ectopic)	0	0	0
19	—							1		1
20	2	0	6	4	66	2	1 (ongoing)	0	0	0
Total	34	8 (23.5%)	50	30	60	21	7	6	3	4
Total	56	13 (23.52%)	84	49	58	38	10	6	6	4

Note: 2PN = 2 pronuclei.

^a Male factor infertility.^b Pregnancy originating from native ovary not included (see article).Meirow. Ovarian transplantation in 20 cancer survivors. *Fertil Steril* 2016.

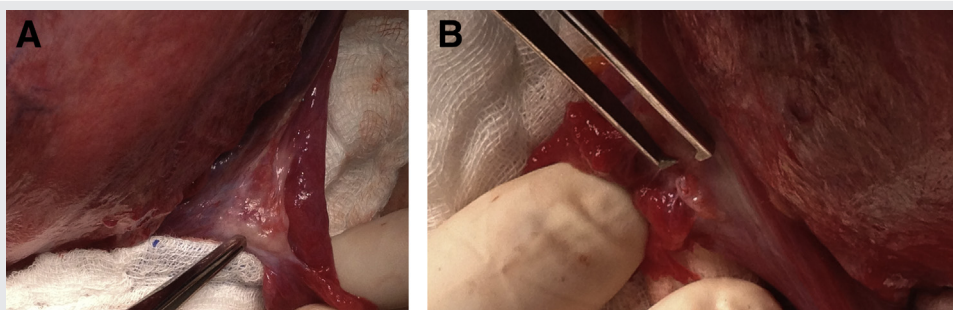
tissue harvesting (patients no. 5 and 20). The time of follow-up evaluation after transplantation ranged from 7 to 141 months (mean 3.18 years). For patient no. 5 who had chronic myelogenous leukemia, the time to follow-up evaluation was 5 years. Patient no. 20, who had acute myelogenous leukemia, has been observed for a period of 8 months thus far. Patient no. 8, who had Ewing sarcoma at tissue collection, received a diagnosis of breast cancer shortly after transplantation, which in this case cannot be regarded as consequence of transplantation. All patients delivered healthy babies, other

than one patient in whom fetal arthrogryposis was discovered during prenatal evaluations, which was confirmed after delivery.

DISCUSSION

This study reports the comprehensive results of 20 patients who underwent ovarian tissue transplantations and were continuously monitored over a period of 12 years by a single team, thereby allowing critical evaluation of procedure

FIGURE 1



(A) Right and (B) left residual ovarian tissue after transplantation visualized during cesarean delivery of the third pregnancy of patient no. 4, after which a spontaneous fourth pregnancy occurred.

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success rate and reassessment of previous recommendations and procedure limits. The results presented demonstrate that OTCP is a highly effective technique for fertility preservation, providing a realistic chance for future pregnancy even in patients who have been treated with chemotherapy before tissue harvesting. Ten (53%) of 19 patients conceived at least once (when excluding patient no. 8 who was diagnosed with a secondary malignancy). Six of these patients (32%) delivered, and one patient conceived four times and delivered three babies.

Out of the 10 live births, one newborn had a major malformation (arthrogryposis), but all other 9 newborns were healthy. A malformation rate of about 1% to 2% is commonly quoted in the literature. However, this case could be attributed to the occurrence of other limb malformations in the patient's family. With more than 60 live births obtained thus far worldwide (18), this has been the first malformation to be reported, which obviously highlights the importance of further data collection on offspring safety and health.

Eligibility and Indications for OTCP and Transplantation

Chemotherapy before tissue collection. Chemotherapy treatment before OTCP is deliberately performed when patient's medical status on admission carries notable surgical or anesthesiology risks associated with the OTCP procedure. In cases of hematologic malignancy, chemotherapy before OTCP also clears the peripheral blood and reduces the risk of possible cancer cells present in the graft. This is highly relevant for leukemic patients (19), who can be harvested before high-dose chemotherapy and bone marrow transplantation (13, 20).

The results of 10 patients (50%) whose tissue was collected after prior chemotherapy treatment or in between chemotherapeutic cycles underline the efficacy of OTCP after chemotherapy exposure. In all but one, menstruation and ovarian endocrine function were documented after transplantation. Seven patients conceived (13 pregnancies), and four gave birth (7 live births). Such outcomes are clearly not inferior to those obtained in chemotherapy-naïve patients, showing that previous recommendations (12) to limit the procedure for prechemotherapy patients should be abandoned.

Transplantation in leukemia survivors. This is the first report on transplantation of ovarian tissue in leukemia survivors. Patients with leukemia are at high risk for transfer of malignant cells with the transplanted tissue (21). Yet early age at diagnosis and high ovarian failure rates after treatment put them at a high need for OTCP (22, 23), especially as immediate chemotherapy is required on admission, precluding the use of other fertility preservation techniques (14). To provide maximal safety and avoid reintroduction of malignancy on transplantation, both postchemotherapy tissue harvesting and an intense search for leukemic cells were performed.

In one chronic myelogenous leukemia patient, the tissue was harvested after treatment with Imatinib and before bone marrow transplantation. The pretransplantation tissue evaluation, including a reverse-transcription polymerase

chain reaction bcr-abl marker search (24), was negative for tissue involvement with leukemic cells (see Table 2). The transplantation was performed more than 5 years ago, and continuous monitoring indicates that the patient is presently disease free.

In one patient with acute myelogenous leukemia, transplantation was performed almost a year ago, after all tests for cancer cell detection were negative (see Table 2). After 12 years of prior amenorrhea, the patient resumed menstruation, continued to IVF treatments, and recently, after her second IVF cycle, conceived; she is now in her third trimester. To the best of our knowledge, this ongoing pregnancy represents the first OTCP related pregnancy to be reported in leukemia survivor.

In additional two leukemia survivors treated by our team, molecular biology tests revealed cancer cells in the thawed tissue, precluding transplantation. Neither of them were treated with chemotherapy before tissue collection. The use of the described approach significantly increases transplantation safety, potentially overcoming previous restrictions for transplantation (22, 23).

Even the most advanced tests cannot entirely ensure the absence of cancer cells within the transplanted tissue. Patients should be aware that in case of relapse after transplantation there is no way to verify whether the relapse occurred due to disease natural biology or as a result of transplantation. This should be emphasized, especially when counseling leukemia survivors. Nevertheless, continuous efforts should be taken to improve methods for minimal residual disease detection and account for its clinical significance. Further long-term follow-up evaluation on transplanted patients, especially in case of past leukemia, is obligatory.

Upper age limit for transplantation. An upper age limit (<35 years) beyond which OTCP should not be performed was previously suggested (11, 25). In our fertility preservation program, the upper age limit was set at 40 years. All patients demonstrated clear presence of tissue premordial follicles on histologic evaluation during harvesting. Our experience with patient no. 15, whose tissue was harvested at the age of 37 years, advocates the redefinition of patients' eligibility for harvesting in terms of age. At the age of 40, she showed clear laboratory and clinical evidence of ovarian failure. After transplantation, her FSH levels dropped to normal (see Table 1), and conventional stimulation for IVF followed, resulting in birth of healthy twins (see Table 3). In patient no. 2, whose tissue was harvested at the age of 38 years, recovery of endocrine function was also observed (see Table 1). In keeping with the reports from other groups (26), it appears that OTCP should not be restricted to girls or young women only; in fact, it can be considered also in patients at their late 30s in selected cases.

Transplantation for overcoming age related infertility. In two breast cancer patients who demonstrated normal endocrine function, transplantation aimed to overcome age-related infertility alone. Such a concept was previously proposed but has never been tested (27). Ovarian tissue was transplanted to one ovary after full decortication (28), while

the other ovary was left intact for future hormone production. However, in both cases, controlled ovarian hyperstimulation mostly resulted in follicle development within the native ovary and not within the transplanted tissue that contained “young oocytes.”

It is plausible that the native nontransplanted ovary that was actively functioning prevented follicle growth and maturation at the transplanted site. If this observation is confirmed, the idea of using stored ovarian tissue to overcome age-related infertility without affecting preexisting endocrine activity (29) might prove to be unrealistic. Further research using animal transplantation studies should be conducted to explore the interactions between a normally functioning ovary and the transplanted one.

Surgical and Medical Aspects of OTCP and Transplantation

High success rates together with technical simplicity encourage using dimensions of $5 \times 10 \text{ mm}^2$ in area and 1–2 mm in thickness for tissue preparation (3, 30), as was used for most patients in this study (17 of 20). There still remain insufficient data with regard to the effectiveness of storing very small or large tissue fragments. Although ovarian tissue can be cryopreserved using either the slow-freezing or the vitrification method, the former enabled satisfactory outcomes in our experience as well as in most of the other worldwide reports (30). Various transplantation techniques have been described since OTCP was introduced into practice, and different transplantation sites have been under continuous evaluation (28, 31, 32). Heterotopic transplantation of the tissue outside the abdominal cavity has failed to restore fertility, but orthotopic transplantation to the ovary or its surroundings has resulted in recovery of endocrine activity and fertility.

When transplanting tissue into the ovarian site, two main techniques are employed. The first includes the removal of atrophic cortex and subsequent attachment of thawed strips onto the resultant bold surface (30). The second, initially described by us (3), involves the creation of subcortical tunnels into which tissue strips are inserted to enable revascularization from beneath and above. In 19 of the 21 transplantations described, the latter technique was used, which allowed for several tissue strips to be transplanted in a short operating time. Added to other reports on subcortical ovarian transplantations (9), this study demonstrates that subcortical grafts can yield a greater than 90% ovarian graft recovery rate, together with a substantial number of pregnancies, both spontaneous or after IVF. As such, surgical removal of atrophic cortex followed by meticulous suturing of the ovarian strips onto the ovarian surface with the cortical side above is not necessary.

After transplantation, an active approach was adopted, and once monitoring showed evidence for graft functioning, attempts to conceive via IVF were initiated. Such an approach required close endocrine and sonographic monitoring to detect developing follicles, allowing for early oocyte retrieval and IVF, which resulted in multiple pregnancies as described. Additionally, IVF enabled an early initiation of progesterone

support, which was shown to be imperative due to a relative corpus luteum insufficiency in ovarian grafts. Nevertheless, spontaneous pregnancies were achieved in this series before, during, and after IVF attempts, which obviously demonstrates the potential of ovarian grafts to resume fertility without additional interventions.

Recovery of natural menstruation emerged as the most important factor to predict the outcome of IVF treatments. Follicular growth and aspiration of mature oocytes was commonly achieved even when day 3 FSH levels were high (up to 27 mIU/mL), ultimately resulting in oocyte collection in 77% of follicle aspiration cycles performed. The setting of an imbalanced endocrine function, also reported by other investigators (9, 17), and a much delayed follicular growth, account for the ineffectiveness of conventional controlled ovarian hyperstimulation protocols. Although known to result in low yield of about one oocyte per cycle and a high rate of empty follicles (33, 34), the modified natural protocol (11, 35) was used in most cycles (49 of 55) in this study, allowing for an initial natural pituitary-graft balance while resulting in an average oocyte yield of 1.5 per cycle, as expected.

Over the years, criticism has been raised regarding the uncertain origin (native ovary vs. transplanted ovary) of the pregnancies obtained after transplantation. In the setting of close pre/posttransplantation follow-up observation, such uncertainties can be easily clarified, allowing for a more accurate portrayal of a graft's reproductive potential. Indeed, we showed that spontaneous pregnancy may in fact originate from the native ovary, even in the setting of prolonged amenorrhea and persistent high FSH levels long before transplantation. In this specific case (patient no. 14), pregnancy was not attributed to the transplantation procedure and accordingly was not included in the final results presented. Existing data clearly show that about 5% to 10% of young patients suffering from premature ovarian insufficiency can be expected to spontaneously conceive (36). Yet the high posttransplantation pregnancy rates in this group and in other groups as well (4), together with the proximity of conceptions to transplantation dates, indicate that the vast majority of pregnancies are attributable to the graft itself.

CONCLUSIONS

At present, OTCP and transplantation are gaining increasing recognition as a valid method for fertility preservation. In this study we used a uniform transplantation technique that was frequently followed by IVF treatments. One-third of graft recipients fulfilled their wish to have their own biological child. Some managed to conceive and deliver several times, demonstrating clear evidence of long-term graft survival. In contrast to previous recommendations, this study shows that patients who were previously exposed to chemotherapy are good candidates for OTCP and can expect good outcomes after transplantation. In addition, we recommend that OTCP be considered in patients at their late 30s.

We suggest that leukemia survivors may also benefit from OTCP provided that maximal safety measures (including harvesting after chemotherapy exposure and an intense search

for leukemia cells within the graft) are meticulously taken. These patients should be informed that no measures to date can entirely rule out tissue involvement with cancer cells.

This study confirms the effectiveness and safety of OTCP and transplantation, challenges previous restrictions made before real data existed, and extends the role of this effective method in current fertility preservation practice. Together with the accumulated worldwide success rates, our results have led the national ethics and professional authorities to decide for the first time not to consider OTCP procedures as an experimental modality for fertility preservation.

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