Ovarian tissue cryopreservation in girls undergoing haematopoietic stem cell transplant: Experience of a single centre

Title**: OVARIAN TISSUE CRYOPRESERVATION IN FEMALE CHILDREN UNDERGOING HAEMATOPOIETIC STEM CELL TRANSPLANT: EXPERIENCE OF A SINGLE CENTRE**

Running title: Ovarian tissue cryopreservation in children

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Abstract

Fertility after childhood cancer is a major concern. The risk of subfertility depends on the type of malignant disease and its treatment. Conditioning regimens before haematopoietic stem cell transplant (HSCT) present a high risk (>80%) of ovarian failure. Since 2000 we proposed to female patients undergoing HSCT at our Centre cryopreservation of ovarian tissue to preserve future fertility. After clinical and haematological evaluation the patients underwent ovarian tissue collection by laparoscopy. The tissue was analyzed by histologic examination to detect any tumor contamination and then frozen following the slow freezing procedure and cryopreserved in liquid nitrogen.

Since August 2000 to September 2013, 47 patients planned to receive HSCT, underwent ovarian tissue cryopreservation. The median age at diagnosis was 11.12 years and at the time of procedure was 13 years. Twenty-four patients were not pubertal at time of storage, while 23 patients had already experienced menarche. Median time between laparoscopy and HSCT was 25 days. Twenty-five out of 27 evaluable patients (93%) developed hypergonadotropic hypogonadism at a median of 23.30 months after HSCT. One patient requested autologous orthotopic transplantation that resulted in one live birth. Results show a very high rate of iatrogenic hypergonadotropic hypogonadism highlightening the need for fertility preservation in these patients.

Introduction

Survival after childhood cancer has substantially improved during the last decades and is now up to 80% considering all diseases, and nearly 75% of the patients will be living 10 years after diagnosis (1). Even more, long-term survival rate of children undergoing haematopoietic stem cell transplant (HSCT) is constantly increasing.

It is now well known that improving in survival presents, on the other side, an increase in mortality and morbidity in long term survivors (2-3-4).

Among all the late effects, infertility is reported as a major concern, especially in female cancer survivors (5).

Cancer treatment often involves aggressive radiotherapy or chemotherapy, which may permanently impair reproductive function. Ovarian damage after HSCT is determined by conditioning regimen that can include chemotherapy and/or radiotherapy. This effect could be increased by previous exposure to gonadotoxic treatment (6).

In particular, total body irradiation (TBI) and older age at time of HSCT can negatively affect the persistence of ovarian function and the onset of premature ovarian failure (POF) (7-9). When administered before puberty, TBI is less gonadotoxic, with 40-60% of patients experiencing spontaneous recovery versus 10-14% in post-pubertal girls (6). The protective effect of younger age might be related to the higher number of nongrowing follicles (10), to the higher resistance of primordial follicles to vascular phenomena and fibrosis or to paracrine factors (11).

Moreover, a model has been evaluated to predict the age of onset of menopause according to radiation dose and age at irradiation (12).

Loss of ovarian function after chemotherapy that includes an alkylating agent (cyclophosphamide, busulfan) could result in both sterilization and endocrine function deficiency as ovarian hormonal production is closely related to the presence of oocytes and maturation of the primary follicles (13-14).

Due to all these factors the risk of infertility in patients undergoing conditioning regimen for HSCT has been defined as >80% (10).

Fertility preservation is a key component of POF management in young people and should be considered for all young people undergoing potentially gonadotoxic cancer treatments or at high risk for ovarian failure.

Cryopreservation of ovarian tissue is the main option available to preserve fertility in women who require cancer treatment but cannot delay the chemotherapy and in prepubertal patients (15). The advantage is that it requires just few days to plan and perform the laparoscopic surgery and, as the retrieval of ovarian tissue is not dependent on the menstrual cycle, no delay in treatments is required. Moreover this technique allows the storage of a great number of primordial follicles that are relatively resistant to cryodamage (about 70%–80% survival) (16).

In our Centre, since 2000, we enrolled female patients at high risk for subsequent infertility in the “Fertisave” protocol, consisting of laparoscopic ovarian tissue cryopreservation.

Patients and methods

After obtaining the approval of the ethical committee we proposed to female patients candidate to HSCT who were at high risk of subsequent infertility, ovarian tissue cryostorage to preserve their fertility.

Informed consent was signed by patients or parents/legal guardians. Data on previous chemo/radiotherapy exposure, conditioning regimen, gonadal function have been collected. After clinical and hematological evaluation the patients were referred to surgery. Ovarian tissue retrieval was frequently scheduled at the same time of another procedure such as bone marrow harvest or placement of a central line for chemotherapy administration. Ovarian tissue collection was performed by laparoscopic surgery. We collected mono or bilateral ovarian cortex biopsies. The amount of cortex to cut is a compromise between the need to cryopreserve as much tissue as possible and the need

to maintain an ovarian volume permitting the future transplantation: usually about 50% of ovarian cortex was removed (17). Atraumatic scissors were used to perform the ovarian cortex explant, and electro-coagulation was avoided as much as possible in order to preserve the tissue to be cryopreserved as well as the remnant ovary. After retrieval the ovarian tissue was immediately rinsed in in vitro fertilization (IVF) buffered medium and transported in ice to the IVF laboratory, where the freezing procedure took place.

At the same time we performed histological examination before storage, to detect any tumor contamination in all patients affected by malignant disease.

In our Centre rapid transport of the removed tissue to the laboratory was performed, anyway it has been demonstrated that transport from the place of removal to the tissue bank is also possible over a longer period of time (18). Once the sample reached IVF laboratory ovarian cortex was enucleated from medullary compartment with sharp scalpel dissection, and then it was cut in small thin cubes and placed in Petri dishes containing slow freezing media (19). Then ovarian cortex was stored in liquid nitrogen following slow-freezing procedure (20). Cryoprotectants used for the freezing procedures were the same used for oocyte cryopreservation although time of exposure was significantly increased, as cryoprotectant penetration into ovarian strips requires several minutes. After completing partial dehydration of the fragments, they were placed in 1.5 ml cryovials (4/5 each), loaded in a programmable vertical freezer (Kryo Planer) and frozen at -196°C. While ovarian tissue was prepared for cryopreservation, a small piece of the ovarian cortex was sent to pathologist in order to evaluate the number and density of primordial follicles and the possible presence of tumoral cells (21-23).

Prior to perform reimplantation, a small piece of frozen-thawed tissue has been analyzed to check the presence and density of morphologically normal primordial follicles. At the time of transplantation a small fraction of the bilateral remnant atrophic ovaries were collected in order to detect possible presence of follicles. Thawing procedure required

removal of cryoprotectant from the ovarian strips. Cryovials were exposed to room temperature for few minutes, plunged in a 30°C water bath and exposed to decreasing stepwise solutions of cryoprotectants. The fragments were placed in a Petri dish filled with IVF buffered medium equilibrated at room temperature and carried to the operating room. Transplantation took place into the pelvic cavity (orthotopic transplant). The advantages of orthotopic transplantation include the possibility of natural conception, the favorable environment for follicular development and the proven efficacy in restoring fertility (24).

Results

Since August 2000 to September 2013, 228 female patients underwent HSCT in our Center. Forty-seven (20.61%) underwent ovarian tissue cryopreservation. The other patients were excluded due to parents' refusal, poor prognosis or lower risk of subsequent infertility.

Patients' characteristics are summarized in Table 1.

Patients were affected by: Blackfan Diamond Anemia (n=1), Ewing Sarcoma (n=3), immunodeficiency (n=2), Acute Myeloid Leukemia (n=11), Acute Lymphoblastic Leukemia (n=14), Chronic Myelogenous Leukemia (n=5), Non Hodgkin Lymphoma (n=2), Myelodisplastic Syndrome (n=2), Thalassemia (n=7).

The median age at diagnosis was 11.12 years (range: 0-17.49 years). The median age at the time of procedure was 13 years (range: 2.7-20.3 years).

Twenty-four patients (51%) were not pubertal at time of storage, while 23 patients (49%) had already experienced menarche.

Laparoscopic surgery resulted in no acute or chronic complications.

Histological examination revealed no tumor contamination in all patients affected by malignant disease (n=36).

Eleven patients (23.4%) had cryopreservation before undergoing any treatment except for iron chelation treatment in thalassemic patients (n=7) while 36 patients (76.6%) had already received chemotherapy.

Median time between laparoscopy and HSCT was 25 days (range: 14-595 days).

Conditioning regimens were TBI-based (12 Gy) in 23 patients (48.9%), Busulfan-based in 21 patients (44.7%) and without TBI or Busulfan in 3 patients (6.4%).

Five patients (10.6%) received additional treatment after HSCT: basin radiotherapy 54Gy (n=2), lower limb radiotherapy 54 Gy (n=1), imatinib (n=1), second HSCT (n=1).

Median follow up time was 6.54 years (range: 0.30-13.68 years), median age at last follow up was 18.6 years (range: 5.46-29.36 years).

Forty patients (85.1%) were alive at last follow up while 7 (14.9%) were dead due to progression of disease or transplant related mortality.

Hypogonadism

Thirteen patients were not evaluable for hypogonadism due to early age (10 patients), lost to follow up (2 patients) and continuous treatment with estroprogestinic after transplant (1 patient).

Twenty-five out of 27 evaluable patients (93%) developed hypergonadotropic hypogonadism at a median of 23.30 months (range: 1.13-123.37 months) after the end of treatment. Twenty-four patients (96%) are in estroprogestinic therapy.

The median age at diagnosis was 10.59 years (range: 0-16.86 years). The median age at cryopreservation was 13.6 years (range: 4.39-20.31 years). Fourteen patients (56%) who developed hypogonadism were pubertal at time of cryopreservation.

The therapy before cryostorage consisted of polichemotherapy in 20 patients (80%), tyrosine kinase inhibitor treatment in 2 patients (8%), iron chelation in 2 patients (8%) and no therapy in 1 patient (4%).

Conditioning regimen was TBI-based in 13 patients (52%) and Busulfan-based in 11 patients (44%).

Two patients have not developed, to date, hypogonadism, but no evaluation could be done on subsequent development of premature ovarian failure.

One patient is affected by Chronic Myeloid Leukemia, diagnosed at the age of 13.74 years, treated with oncocarbide before HSCT, and with a TBI-based HSCT. She is now 23.78 years old, in treatment with tyrosine kinase inhibitors, due to a molecular relapse of the disease.

The second patient is affected by thalassemia and underwent a Busulfan-based HSCT at the age of 7.66 years. She is now 15.9 years old with regular menses.

Follicles

The median of collected follicles was 25/mm^2 (range: 0-120/mm^2).

Evaluating the pubertal status at time of ovarian tissue collection the median of collected follicles was 20/mm^2 (range: 4-45/mm^2) and 35/mm^2 (range: 0-90/mm^2) in pubertal and pre-pubertal patients, respectively.

The median of collected follicles was 25/mm^2 (range: 3-120/mm^2) in patients that underwent only chelation treatment or tyrosine kinase inhibitor treatment and 26/mm^2 (range: 0-90/mm^2) in patients that underwent gonadotoxic treatment before collection.

The median in patients who subsequently developed hypogonadism is 25/mm^2 (range: 0-85/mm^2).

Pregnancies

One patient requested autologous orthotopic transplantation of ovarian cortical tissue that resulted in one live birth (25).

Conclusions

Fertility after childhood cancer has become a topic of major concern in the last few years. Wallace at al. have defined the risk of subfertility related to the type of malignant disease

and its associated treatment (12). According to these criteria the conditioning regimen (TBI and chemotherapy) before HSCT presents a high risk (>80%) of subsequent infertility (7, 9, 12).

TBI and older age at treatment are well known risk factors for subsequent hypogonadism as well as busulfan administration but no prediction can be made of the real risk for developing infertility (7, 12, 26).

Since 2000, in our Centre, we proposed an experimental protocol for ovarian tissue cryostorage to female patients undergoing HSCT, to preserve future fertility.

Different series of ovarian tissue cryopreservation in female children have been recently reported (27-32). The number of patients ranged between 23 and 58 for each study. The surgical technique varied from whole ovary collection to multiple biopsies of the cortical tissue. The main goal was to evaluate the feasibility of the procedure.

To date almost 30 live births have been reported worldwide after orthotopic autologous ovarian transplant (24, 26, 33-48) whereas heterotopic graft has led to one twin pregnancy (49), a biochemical pregnancy (50) and four spontaneous pregnancies with three live births were described as a result of a reactivation of the native ovary (51).

The analysis of the recovery of ovarian function is difficult because of the lack of reports in the literature which indicate how many patients in the world have been subjected to transplantation of ovarian tissue, anyway the recovery of ovarian function has been described in all published cases of ovarian transplantation, both orthotopic and heterotopic. Donnez et al. describes an average time of approximately 3-4 months from graft to the recovery of ovarian function, in agreement with the timing of folliculogenesis (34).

At our Centre all the patients addressed to ovarian tissue cryopreservation presented a high risk to develop future infertility. The results showed a very high percentage of hypergonadotropic hypogonadism (93%) occurrence on the evaluable patients, and no

current evaluation of POF could be done. This is a confirmation that the patients we selected were actually good candidates for ovarian tissue cryostorage thus balancing possible surgical complications. Nonetheless, in our series we reported no surgical complications and the patients underwent subsequent HSCT only after a median time of 25 days from surgery.

Some of the previous reports also included patients with a low-medium risk of subsequent infertility, who went through spontaneous pregnancies after treatments. In our opinion ovarian tissue cryopreservation should not be offered to patients with a moderate risk of gonadotoxicity as surgical risk is not compensated with a real advantage in this group of patients.

No evaluation could be done on the follicle density of the collected tissue and any correlation to previous therapy because of the low number of patients with the same characteristics.

The report of a live birth among our patients gave us a confirmation of the effectiveness of our technique of ovarian tissue retrieval, cryopreservation and transplantation (25).

Two problems should be argued: the possibility to restore hormonal ovarian function and the tumor contamination of the cryopreserved tissue.

It has been reported that there are individual variations in the duration of endocrine function after transplantation; ovarian function has been demonstrated to persist up to 7 years after transplantation with a mean duration of 4-5 years if follicular density is well preserved (24). Ovarian tissue transplantation can represent an option to induce spontaneous puberty in pre-pubertal patients undergoing primitive hypogonadism, as it has been recently reported (52-53), but this approach should be validated in the future.

Many authors recently debate about the risk of reimplanting ovarian tissue with tumor cells contamination (54). Sommerzer at al have previously defined the risk of ovarian

metastases according to cancer type with the higher risk for leukemia, neuroblastoma and Burkitt Lymphoma (55).

In particular ovarian metastases have been found in up to 30% of ALL patients at autopsy, even if they are rarely clinically detected (56). Recently, using disease specific PCR techniques, Dolmans et al found contamination of ovarian tissue in 33% of CML patients and in 70% of ALL patients (57). At the same time, Greve et al. reported that ovaries from leukemia patients in complete remission do not appear to contain viable malignant cells, in contrast to ovarian tissue retrieved before chemotherapy (58-59).

As far as our series of patients, no disease could be detected by histological examination of the tissue before cryostorage. Furthermore, we experimentally evaluated by RT-PCR the expression of molecular translocations, when present at the diagnosis of the disease (n=7), and no positivity has been found (data not shown).

However it is now recommended that ovarian tissue autotransplantation should be discouraged when there is any risk of reimplanting malignant cells (51). Other techniques such as isolated follicles transplantation (60), *in vitro* follicular culture (61-63) and isolation of primordial follicles followed by transplantation of an alginate matrigel matrix containing isolated ovarian cells (58) require additional research before becoming available for humans.

In conclusion even if ovarian tissue cryopreservation is still to be considered investigational, it offers very encouraging results and represents the only option to preserve fertility in pre-pubertal girls.

Conflict of interests*:*

The authors declare no conflicts of interest.

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