—Review—

The safety of autotransplantation of cryopreserved ovarian tissue in cancer patients

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Abstract: Aggressive chemotherapy and radiotherapy can cure cancer in young female patients, but they can also result in the loss of ovarian function. For these young survivors, both recovery of ovarian function and reproductive potential after treatment have become important quality of life issues. Ovarian tissue cryopreservation (OTC), followed by transplantation after cancer remission is the most commonly applied fertility restoration approach in prepubertal females and women who require immediate cancer therapy. A major concern of frozen-thawed ovarian tissues (FTOT) autotransplantation in cancer survivors is the reintroduction of malignant cells that may have metastasized to the graft. There are several detection methods for minimal residual diseases (MRD) in ovarian cortex tissues. The aim of this paper is to review the available data describing the safety of transplantation of FTOT from cancer patients, focusing on the methods used to detect tumor cells in ovarian tissues and future perspectives in this field.

Key words: Ovarian tissue cryopreservation, Ovarian metastasis, Minimal residual disease, Malignant cell detection, Fertility preservation

Introduction

Ovarian tissue cryopreservation (OTC) is one option for fertility preservation in cancer patients of reproductive age, and the only choice for prepubertal patients [1]. *In vitro* fertilization (IVF) with embryo or oocyte cryopreservation is a potential option for cancer patients if ovarian stimulation can be done between surgery and the initiation of adjuvant chemotherapy. When there is no time or the patient is prepubertal, cryopreservation and transplantation of ovarian tissue has proven to be a promising approach for restoring fertility. Today, transplantation of frozen-thawed ovarian tissue (FTOT) has been performed worldwide, and the number of live births achieved through this procedure had exceeded 130 as of June 2017 [2–7]. However, minimal residual disease (MRD) is a significant problem in transplants of FTOT, since there is a risk that FTOT might harbor malignant cells that could induce disease recurrence following transplantation. Previous studies have indicated that hematological malignancies, breast cancer, and ovarian tissue implantation following cryopreservation carry the risk of harboring malignant cells [8–11]. A variety of methods must be used to detect MRD. However, current methods do not guarantee that MRD will not be reimplanted and further analytical techniques need to be developed. The aim of this paper is to review the available data describing the safety of transplantation of ovarian tissue from cancer patients. It focuses on the several methods used to detect MRD, and the future challenges in this field.

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Relapse after FTOT Transplantation

According to worldwide FTOT transplantation data, at present more than 350 FTOT transplantations have been performed for diagnoses such as leukemia, sarcoma, and genetic conditions [2]. Among these transplantations, one relapse that could be directly related to grafting of FTOT was reported in a patient with a granulosa cell tumor [12]. Histological assessment of a sample of FTOT was performed before transplantation in this case and there was no evidence of tumor cells. There are two other case reports. One case of breast cancer [13] and another of Ewing sarcoma [14] exhibited tumor recurrence after FTOT transplants. A histological examination was performed for MRD in the case of Ewing sarcoma.

On the other hand, in the case of breast cancer no examination was performed to detect MRD. However, it is unlikely that the recurrences in these two cases were related to the transplantation [15].

Analysis of Ovarian Cortex Tissue to Detect MRD

The analysis of ovarian cortex tissue to detect the presence of cancer cells is usually performed by standard histological examination, immunohistochemistry (IHC), polymerase chain reaction (PCR) or by xenografting into severe combined immunodeficiency (SCID) mice. A review of MRD in cryopreserved ovarian tissue from cancer patients reported the discovery of MRD in 7% (31/422) of OTC from cancer patients: 2/58 were detected by imaging, 1/367 by histology, 1/220 by IHC, 21/43 by PCR, and 5/101 by xenotransplantation of ovarian tissue into SCID mice [16] . Although these methods detected MRD, no recurrence had been reported at the time of this publication.

Histological Examination, IHC

The cortex is isolated and cut into fragments and cryopreserved using freezing methods. Usually, both the ovarian medulla and one small biopsy are taken for histological evaluation. Finally, clinical re-transplantations are performed for cancer survivors with and without pretransplant histology.

Histological and IHC evaluations by expert pathologists for the detection of malignant cells are needed. If the cancer has a specific IHC marker, an IHC evaluation can be completed. The IHC markers should be selected based on anatomical pathology analysis of the primary tumor. Research in this field indicates that histology alone is insufficient for the detection of MRD [10, 11, 16, 17].

Molecular Marker Examinations Using Primary Cancer Tissues

Finding molecular markers is important for assessing MRD. For instance, we can use fusion genes as molecular markers and perform PCR and/or xenotransplantation if the primary cancer tissues exhibit characterized fusion genes. Usually, detailed genetic characterization of the primary tumor is tested by karyotyping and FISH using the frozen-thawed primary tumor stored within a bio bank and/or formalin-fixed paraffin-embedded (FFPE) tissue blocks [18]. When a cancer patient is referred from another medical institution, genetic characterization of the primary tumor is required, with the patient's permission. Genetic counseling should be considered, according to patient's needs.

Polymerase Chain Reaction (PCR)

If molecular markers are available, we can use PCR to assess MRD. In leukemia patients, no malignant cells were detected by histology in the ovarian tissues of 6 patients with chronic myelocytic leukemia (CML) and 12 patients with acute lymphoblastic leukemia (ALL). On the other hand, ovarian tissues of 33% of CML and 70% of ALL patients were found to be positive using quantitative RT-PCR. Moreover, xenograft experiments showed leukemia invasion of grafts originating from 5/12 ALL patients [10]. These results show that IHC may not be sensitive or specific enough to identify micro-invasion, and that other techniques such as PCR and xenografting should be applied.

Xenotransplantation of Ovarian Tissue into SCID Mice

A large number of studies have shown that xenografting ovarian tissue into SCID mice is a useful model for assessing the risk of reimplanting malignant cells. The recommended observation period is more than 20 weeks and different graft sites under the kidney capsule, intraperitoneally, the back muscle, the ovarian bursa, and subcutaneously, have been used with success [16]. Dolmans *et al.* reported that after long-term xenografting (6 months) of FTOT from patients with CML and ALL into SCID mice, one third of the mice grafted with tissues from ALL patients showed massive macroscopic peritoneal invasion [10]. No malignant cells were microscopically identified in grafts retrieved from SCID mice transplanted with ovarian tissue from CML patients; however, obvious invasion of lymphoblasts was observed in 5 of 12 SCID mice grafted with ovarian tissue from ALL patients. In another study where ovarian tissue was taken from leukemia patients in complete remission, the PCR results were positive in some cases, but FTOT did not appear to contain sufficient numbers of viable malignant cells to transmit the disease when xenografted to mice for 20 weeks [17]. These results show that positive PCR results do not reveal the viability or malignant potential of cells when they are transplanted. However, it is unclear how many malignant cells are actually necessary to cause a relapse. The detection of MRD in the ovarian tissues of advanced breast cancer patients has also reported. The results of PCR and MGB2-gene sequencing were posi-

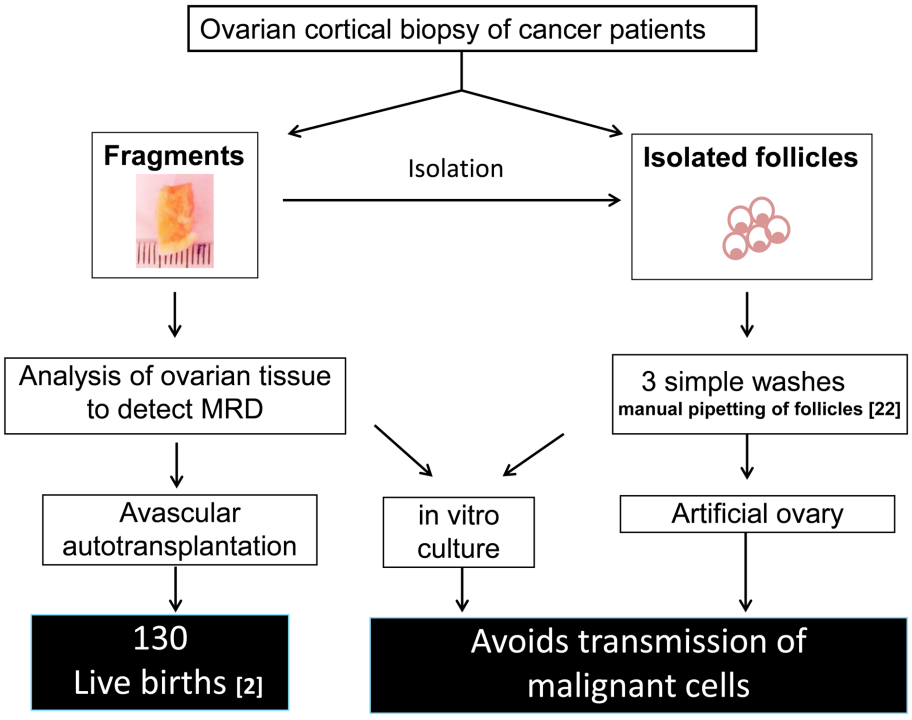

Fig. 1. Options for the safety of autotransplantation of cryopreserved ovarian tissue. Modified according to Donnez and Marie-Madeleine Dolmans [19].

tive for the ovarian tissue of 5 out of 10 patients, but none of the xenografted mice developed tumor masses during the 6-month grafting period [11].

Analytical Methods Used to Prevent Reimplantation of Tumor Cells

There are several methods for detecting MRD; however, current methods do not guarantee that tumor cells will not be reimplanted and further analytical techniques need to be developed. At the moment, it is recommended that performing histological analyses including IHC, PCR (if markers are available) and xenotransplantation to SCID mice (if no markers are available), are performed before any tissue is reimplanted. Only if all the available analyses prove negative and show reasonable sensitivity should re-implantation be done. Using a battery of tests, the risk of reseeding cancer cells is minimized.

Importance of Appropriate Counseling

Appropriate counseling and support given by specialists are required to secure patients' fertility and survivorship. Kyono *et al.* [15] stated the importance of informed consent for cancer patients. The actual transplanted tissue cannot be examined. Even when all malignancy tests are negative there is still a risk of malignant cells being present in the transplanted pieces, because the amount of resected ovarian tissue analyzed for MRD is very small and MRD detection methods vary in sensitivity. Additionally, there are few clinicians who are familiar with the current options for OTC. Therefore, training for fertility preservation counseling professionals is important. Not only gynecologists, but also pediatricians and oncologists, need to know when to refer patients for possible fertility preservation [2].

Future Perspectives

An alternative approach consists of the elimination of malignant cells from ovarian cortex tissue and the prevention of reimplantation of tumor cells in patients who are at high risk. For women with acute leukemia, the risk of reimplantation of cancer cells along with the grafted tissue is high [10]. Several attempts have been made to obtain mature oocytes without grafting whole tissue samples, such as in vitro maturation of primordial follicles and an artificial ovary (AO) (Fig. 1).

In Vitro Culture of Preantral Follicles

Researchers around the world have been trying to design a culture system for follicle development from the primordial to the pre-ovulatory stage. In spite of many years of efforts, the optimal conditions for culture systems for the development of primordial follicles have not yet been elucidated. Moreover, a long period of in vitro culture may influence epigenetic mechanisms, in particular, genomic imprinting [20, 21].

Artificial Ovary

As follicles are enclosed in a basement membrane that prevents direct contact between follicular cells and capillaries, white blood cells, and nerve processes, their isolations ensure that no malignant cells are returned to the patient. That is to say, if malignant cells were found in ovarian stromal tissue, isolated follicles could provide an alternative method, avoiding the transplantation of malignant cells [20, 21]. Conceptually, an AO would require the removal and cryopreservation of ovarian tissue before cancer treatment. After the patient has been treated, the fragments of the cryopreserved ovarian tissue would be thawed and mechanically and enzymatically digested in order to isolate preantral follicles. Then, these follicles would be encapsulated in a three-dimensional scaffold with isolated ovarian cells from fresh ovarian biopsies, and micro-/nanoparticles with bioactive factors [20, 21]. A research group working in this area has focused on isolation methods and the development of matrices for supporting isolated follicles [22]. A recent study showed that artificial ovaries made with a 3D printer enabled sterilized mice to have pups and release hormones [23]. It is not clear if the same approach would work in larger animals, including humans, because of the physiological differences that exist between rodent and primate ovaries [22], and human follicles are much larger and grow rapidly until they are grossly visible.

Artificial Ovaries for Leukemia Patients

For women with a high risk of reimplantation of cancer cells, the possibility that some malignant cells could contaminate isolated follicle suspensions obtained from patients with leukemia cannot be denied. Soares *et al.* [24, 25] performed two studies of AO especially for leukemia patients. The first study evaluated a follicle isolation technique for obtaining disease-free follicle suspensions when malignant cells are present in ovarian cell suspensions. They found that in cases of contamination of ovarian tissue by malignant cells, there is a real risk of picking up cancer cells along with isolated follicles during follicle retrieval. However, this risk is reduced when 3 simple washes involving manual pipetting of follicles are performed with transfer to fresh medium droplets in order to remove unwanted contaminating cells. The second study investigated whether or not transplantation of a few leukemic cells inside an AO induced leukemia in a xenografting model. It is currently unknown how many cancer cells are necessary to cause a relapse. Understanding the minimum number of cancer cells capable of causing a relapse would be helpful in discussions about the safe transplantation of ovarian tissues. Leukemic cells were embedded in a fibrin matrix along with ovarian cells in order to evaluate leukemic cell survival and proliferation in conditions resembling the AO environment. After xenotransplantation, it was found that mice grafts with a fibrin matrix containing 10 or 100 leukemic cells did not develop any signs of leukemia after 20 weeks [23], meaning that transplantation of small numbers of leukemic cells appears to be insufficient to induce the disease. Furthermore, follicle suspensions must be completely purged of leukemic cells prior to grafting in clinical applications, as even the slightest risk must be avoided [24–26].

Full Field Optical Coherence Tomography

Optical coherence tomography (OCT) utilizes interferometric selection of singly backscattered photons using superposition of waves to decipher their characteristics. It is a powerful technique for imaging biological tissues. Full field (FF)-OCT, in contrast with most OCT approaches (time domain- or Fourier domain OCT, for example), directly takes 2D “en face” images using megapixel cameras [27]. FF-OCT rapidly generates high-resolution histology-like images without the need for tissue fixation, freezing, or staining. Peters *et al.* [28] found that FF-OCT can be used to visualize metastasis as well as micrometastases in human cortical ovarian tissue (using deparaffinized FFPE tissue samples). The maximum depth of FF-OCT imaging that provided high-resolution images in the cortical ovarian strips was approximately 100 μm, which is considerably shallower than the imaging depth of up to 500 mm reported for other tissues [29]. Nevertheless, the imaging depth can be increased by imaging the cortical ovarian tissue fragments from both sides, effectively doubling the amount of tissue that is imaged. Moreover, rapid advances in the field of optical imaging will likely enable clinicians to use this non-invasive approach to visualize even deeper structures in the near future. The OCT technique may be able to screen for

cancer metastases present in ovarian tissue prior to transplantation. In addition, FF-OCT may advance basic research in the area of OTC, because it allows researchers to count the follicles in the ovary before and after ovarian tissue transplantation. Therefore, FF-OCT will promote the development of methodologies for OTC and transplantation [30].

A New Model System to Detect Cancer Cells

Peek *et al.* [31] described an *in vitro* culture method for creating a model system that mimics ovarian metastatic disease in order to detect cancer cells and prevent cancer cell transmission via ovarian tissue autotransplantation. They assessed the ability of injected human cancer cell lines (leukemia, lymphoma, Ewing's sarcoma, and breast cancer cells) to proliferate and form tumor-like structures in bovine and human ovarian cortex tissues *in vitro*. After 4 days of culture, some tissue fragments were harvested for standard histological staining and IHC staining of tumor cell specific antigens and the Ki67 proliferation marker. It was found that human ovarian cortex tissue could be cultured for up to 7 days without any loss of viability. These preliminary findings show that the presented model system, in which cells from different types of cancers are present as small tumors within the ovarian tissue, might significantly contribute to the development of methods for the efficient detection of contaminating cancer cells, the safe purification of ovarian components, and the elimination of malignant cells from intact ovarian cortex tissue.

Conclusion

There are three main methods detecting MRD: IHC, histology, and PCR and analysis of xenografts. At present, histology and IHC need to be carried out with IHC markers expressed by primary tumor cells. Additionally, PCR and/or analysis of xenografts should be performed in order to minimize the risk of reseeding cancer cells.

Patients should be informed that transplanted tissue cannot be examined and although all the malignancy test results are negative there is still a risk of malignant cells in the transplanted pieces. Training for professionals in fertility preservation counseling is also needed in order to give up-to-date and correct information about OTC and fertility retention options. Currently, research is being performed on the in vitro growth of follicles to mature oocytes, and on the isolation of follicles. Both methods hold the potential of eliminating the risk of introducing a cancer to the patient, but are not available as treatments for humans yet. New methods to find MRD have seen remarkable development. Nevertheless, future prospective studies are still needed.

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