Ovarian Tissue Transplantation: Current State and Research Advancement

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Abstract During the past decade, babies born from cryopreservation and transplantation of ovarian tissue have emerged as a promising alternative to restore ovarian function and fertility in cancer patients. Despite successful results obtained with conventional freezing protocols to preserve ovarian tissue, attempt for whole ovary transplantation is still underpropelling in large animal and human studies. Patient selection to avoid reimplanting cancer cells in ovarian grafts as a prerequisite safety concern is as important as advanced transplantation technique. Multidisciplinary strategies for the combined isolation immature follicle for culture or in vitro activation of ovarian follicle with the frozen-thawed ovarian tissue raised the diversity for female fertility preservation along with the egg-embryo freezing or in vitro maturation of immature eggs. The purpose of this updated review of fertility preservation techniques is to develop a network among health care professional interested in this challenging field for those who are threatened the reproductive function.

Keywords Fertility preservation · Ovary · Orthotopic autotransplantation · Heterotopic autotransplantation cryopreservation · Female infertility

Introduction

Advances in cancer therapy have improved the long-term survival of cancer patients to make implementation of

procedures aimed at preserving fertility for young victims. These procedures must not be limited to preserve and restore fertility in patients undergoing therapies but also be applied to any cases where the reproductive function is threatened due to benign ovarian disease or primary ovarian insufficiency (POI).

Experimental and clinical strategies for preserving fertility include ovariostasis, embryo cryopreservation (IVF), cryopreservation of mature and immature oocytes, reimplantation of cryopreserved ovarian cortex, culture for ovarian tissue, preantral follicles and oocyte-cumulus complexes, whole ovary cryopreservation and transplantation, in vitro maturation of immature (IVM) oocytes [1•] (Table 1), and in vitro activation (IVA) of ovarian follicles [2•]. Among the currently available options for fertility preservation, restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue is rapidly gaining found as a valid method alongside cryopreservation of embryos and oocytes.

The best follicular survival rate obtained to date with frozen-thawed human ovarian tissue is approximately 70–80 % [3, 4]; primordial follicles are more resistant to cryoinjury. Earlier work revealed that almost 50 % of primordial follicles are lost as a result of initial ischemia rather than as a result of the freeze–thaw process itself [5–7]. The majority of previous studies related to ovarian tissue cryopreservation and transplantation have focused on short-term viability [8]. Given the current accumulation of experience for ovarian cryopreservation, a slow cooling and rapid rewarming rate for mouse and human ovarian tissue [9, 10] or vitrification for sheep and monkey ovarian tissue effectively preserves primordial follicles [11, 12].

Tissue ischemia without vascular anastomosis remains a problem for implants because the process of revascularization can take 2–7 days to complete, dependent on factors including the size of implanted tissue [13, 14]. In theory, cryopreservation of intact ovary in vitro, followed by restoration of reproductive function after autologous transplantation using microvascular anastomosis, could achieve an immediate blood

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Table 1 Strategy of fertility preservation in women

Fertility preservation in women		
Ovariostasis	Toxicity of chemotherapy and radiotherapy	Embryo cryopreservation (IVF)
Cryopreservation of mature and immature oocytes	Cryopreservation of ovarian cortex technical aspects	Whole ovary cryopreservation
Culture procedures for ovarian tissue, preantral follicles and oocyte cumulus complexes	Cryopreservation of ovarian cortex <i>Reimplantation of cryopreserved ovarian cortex: technique and results</i>	Whole ovary transplantation <i>Lesson learned from experimental studies</i>
<i>Progress in cryopreservation</i>		

Note: Adapted from <http://www.isfp-fertility.org> [1•]

supply in vivo to maximize graft survival. The success is subjected to improve freezing and implantation technique, graft function and lifespan, patient selection, identification of tumor involvement in the ovaries, and detection of small number of cancer cells in ovarian tissue.

To address these challenges, we aimed at the current state of basic and research regarding to transplantation of frozen-thawed ovarian tissue, influence of the site of ovarian transplantation, environmental factor with possible effects on transplantation, patient criteria before transplantation, method and monitoring of transplanted grafts, safety issue after transplantation.

Patient Selection

Criteria for ovarian tissue banking (by S. Samuel Kim)

- 1) Age: younger than 37 years (may be individualized based on the status of ovarian reserve)
- 2) Ovarian function: premenopausal by FSH, antral follicle count (AFC), or AMH
- 3) Communication with oncologists: cancer treatment plan, prognosis
- 4) When embryo freezing or oocyte freezing is not indicated: delaying cancer treatment is not acceptable, hormonal stimulation is not permitted, ART is not allowed.
- 5) Prepubertal girls who do not have any other options
- 6) High risk for POF (when significant loss of ovarian follicles is anticipated with cancer therapy)
- 7) Informed consent from adult patients
- 8) Informed consent from parents/guardians as well as informed assent from minors, if the patient is younger than age 18 years
- 9) Physically and mentally healthy enough for surgery
- 10) Desires to have a child in the future (preferably before age 50 years)
- 11) Thorough patient counseling: currently available fertility preservation options, including embryo and oocyte cryopreservation, how to use cryobanked ovarian tissue for fertility restoration
- 12) Should understand experimental nature and potential risks of cancer cell transmission [15•].

Live Birth After Transplantation of Frozen-Thawed Ovarian Tissue

Transplantation of frozen-thawed ovarian tissue for fertility restoration and regaining of menstrual cycles is rapidly gaining ground as a valid method. A relative constant period elapses from transplantation until the tissue become active, also when a second transplant is performed. A total of 24 live births after orthotopic autotransplantation of slow-freezing technique have been reported between 2004 and 2013 [16•]. Only a few groups overwhelmed the majority of limited successes, including by Donnez's group in Belgium and Andersen's group in Denmark [16•].

Silber backs up the promising technique for a series of fresh ovarian isografts between genetically identical monozygotic twins to yield live birth [17]. Despite good evidence to support that the transplanted tissue is active, myth or reality for ovarian tissue transplantation remains a matter of concern. Progress in improving freezing techniques and enhance the avascular reimplantation to increase pregnancy rates or combined culture isolated immature oocytes is still challenging [16•].

Orthotopic autotransplantation of ovarian cortex has advantages, such as easy collection of ova and the possibility of spontaneous pregnancy. Although children have been born after successful orthotopic autotransplantation into the residual ovaries, some patients cannot undergo this procedure such as those who need bilateral ovariectomy of pelvic radiation therapy; therefore, it is still necessary to investigate suitable heterotopic autotransplantation sites in cynomolgus monkey [18]. A surgical technique for heterotopic transplantation of ovarian tissue to the forearm was reported by Oktay, of the eight oocytes suitable for in vitro fertilisation, one fertilized normally and developed into a four-cell embryo without embryo transfer [19, 20].

When ovarian tissue to a subcutaneous site and orthotopically to the pelvic peritoneum or to the ovaries, follicles developed more often in ovarian sites than at the peritoneal or subcutaneous sites. Furthermore, follicle development was limited at the heterotopic site (<15 mm) and oocyte recovery rate and fertilization rate obtained from heterotopic sites were low [21, 22].

As high as >50 % of primordial follicle loss after reimplantation due to tissue ischemia after transplantation while awaiting angiogenesis. Donnez emphasized the importance of preparing the vascular bed to favor faster revascularization of the graft, because enhanced vascularization of the vascular bed is likely to increase vascularization in the grafted cortex [16•]. Ovarian cortex cryopreservation can be performed at the time of surgery for benign diseases when fertility is impaired. The first pregnancy to occur after ovarian tissue cryopreservation for benign ovarian pathology after bilateral oophorectomy was reported [23•].

Monitor Ovarian Restoration

Clinical workup before ovarian tissue harvesting also should include investigation of patient's medical status, investigation of possible risk of ovarian involvement, and consultation with an anesthesiologist. The main points for clinical evaluation of patients before ovarian tissue harvesting are presented in the list given below:

- (1) Diagnosis, staging;
- (2) Sterilization risk: disease, treatment protocol, age;
- (3) Previous exposure to chemotherapy: time of treatment, protocol, menstrual history;
- (4) Ovarian reserve:
 - (a) Hormones: gonadotrophins, E2, P, anti-Müllerian hormone (AMH);
 - (b) Sonography: total AFC ovarian volume;
- (5) Evaluation of medical status;
 - (a) Leukemia: anemia, thrombocytopenia;
 - (b) Lymphoma: mass, pressure, effusion;
 - (c) Previous exposure to chemotherapy;
 - (ii) Anemia, thrombocytopenia;
 - (iii) Liver function;
 - (iii) Renal function;
 - (iv) Heart evaluation – echo;
- (6) Risk of ovarian involvement sonography, computed tomography (CT) [24].

Ovarian function recovery was evaluated by hormone concentration measurement, follicular development on ultrasound, and menstruation recovery. There are individual variations in the duration of endocrine function after transplantation. The mean duration is ~4–5 years if follicular density is well preserved [16•].

The first signs of ovarian function restoration (oestradiol peak, decrease in FSH, ultrasound showing follicular development) occurred between 16 and 26 weeks after reimplantation [25]. There were three case reports describe the long-term duration of function of ovarian cortical tissue grafts among patients in a university fertility preservation

programme in Europe and in a private practice programme in the United States. One woman underwent sterilizing cancer treatment and had frozen ovarian tissue transplanted, and two women underwent fresh ovarian tissue transplants. The function of ovarian cortical strips has continued for more than 7 years in these three women, with the birth of eight healthy babies following a single graft per patient [26•].

Large numbers of primordial follicles were present in the harvested tissue. During the first months after transplantation, gonadotropins were high, AMH and inhibin B were low, and in three IVF cycles, eggs were not found. After recovery of endocrine activity 9 months after transplantation, a mature oocyte was retrieved. Embryo transfer resulted in a normal pregnancy and delivery of a healthy baby. Although spontaneous menstruation resumed after delivery, endocrine profile 22 months after transplantation indicated low reserve [27]. The recovery of endocrine function after transplantation correlated with the result of oocyte recovery. Fertility preservation using ovarian tissue is effective also in cases when the ovaries are injured after chemotherapy. However, transplant life span is limited [27]. Low AMH and inhibin B concentrations may suggest decreased ovarian reserve in women after ovarian transplantation. AMH and inhibin B levels may not be associated with the duration of ovarian graft function or probability to achieve a pregnancy [28•].

Vitrification and Human Ovarian Tissue

Vitrification procedures appeared to preserve not only the morphology and survival of preantral follicles after 1 week of xenografting, but also their ability to resume folliculogenesis. In addition, vitrification protocol had a positive impact on the quiescent state of primordial follicles after xenografting [29•]. Despite the encouraging results obtained with vitrification of ovarian tissue from humans and different animal species, it is necessary to understand how vitrification solutions and protocols can affect ovarian tissue, notably preantral follicles. In addition, it is important to bear in mind that the utilization of different approaches to assess tissue functionality and oocyte quality is essential in order to validate the promising results already obtained with vitrification procedures [30•]. However, so far there have been no human live births after ovarian vitrification.

Whole Ovary Transplantation

Spontaneous pregnancy after transplantation of frozen-thawed rat upper genital tract and intact ovary with microvascular anastomosis shows that this technique has promise [31]. Subsequently, transplantation of whole fresh ovaries has been successfully performed in rats [31, 32], rabbit [33], sheep [34, 35], monkey [36], and human [17, 37, 38], Leporrier et al. [39] and Hilders et al. [40], and Chen et al. reported that autologous

heterotopic transplantation of intact rabbit ovary after frozen banking at -196°C [41]. Between 2003 and 2009, four groups reported whole ovary cryopreservation in sheep, and only one pregnancy was achieved [42]. There have been a total of four experiments of whole ovary cryopreservation without transplantation in humans [42]. Large animal studies have been encouraging, and it is likely that this option for fertility preservation will be a viable treatment option in the future.

Current research to determine the optimal technique for whole-ovary cryopreservation remains a challenge compared with tissue and cell cryopreservation; techniques are still in relative infancy. Combined microvascular manipulation with perfusion of cryoprotectant agents through intact organ vessels is a prerequisite for whole-ovarian cryopreservation and microvascular anastomosis. Long-term longevity and functional performance of whole cryopreserved ovary still is unclear [43]. If current obstacles to successful cryopreservation of ovarian tissue were overcome, transplantation could provide an abundant source of oocytes and hormone secretion appropriate for prolonged reproductive function. The choice of the recipient pedicle has to fulfill several criteria [44•].

- Easy surgical accessibility of the pedicle
- Feasible orthotopic transplantation into the pelvic cavity
- Optimal size match between the recipient pedicle and the gonadic vessels
- Possible postoperative ultrasound monitoring of the ovarian graft
- Easy puncture of the transplant for IVF

Given the significant improvement over the ischemic loss of follicles that is seen with cortical strip cryopreservation and retransplantation, cryopreservation of the whole ovary could potentially provide a superior alternative to patients requiring this method of fertility preservation. Large animal studies have been performed [45].

Ten monozygotic (MZ) twin pairs requested ovarian transplantation and nine have undergone transplantation with cryopreservation of spare tissue. Eight had a fresh cortical tissue transplant, one of whom received a second frozen-thawed transplant after the first ceased functioning at 3 years. One had a fresh microvascular transplant. All recipients reinitiated ovulatory menstrual cycles and normal day 3 serum follicle-stimulating hormone levels by 77 to 142 days. Seven have already conceived naturally (three twice). Currently, seven healthy infants have been delivered out of ten pregnancies. The oldest transplant ceased functioning by 3 years, but then she conceived again after a frozen-thawed secondary transplant. There was no apparent difference in return of ovarian function between the nine fresh ovarian grafts and the one frozen graft. Ovarian transplantation appears to restore ovulatory function robustly. Ten pregnancies, leading to seven healthy infants, including one after cryopreservation, bode well for application to fertility preservation [46]. Fertility

preservation, from cancer to benign disease to social reasons: the challenge of the present decade is proposed [47•].

Xenograft

Host and graft vessels contributed sequentially to graft revascularization: murine angiogenesis initiated reperfusion from day 5, and by day 10, human angiogenesis was shown to participate in graft revascularization. Host and graft angiogenesis are potential targets to reduce the avascular period after grafting [48]. The results demonstrate, for the first time, that isolated human follicles are able to survive and grow after xenografting. This study also shows massive *in vivo* follicular activation after transplantation of grafted fragments and isolated follicles. One week after grafting, well-structured stroma-like tissue of human origin was observed around the isolated follicles [49]. The study demonstrates, for the first time, that cryopreservation and xenotransplantation do not appear to greatly affect human primordial/primary follicle ultrastructure.

Interestingly, in frozen-thawed xenografts, secondary human ovarian follicles presented a well-preserved ultrastructure, but asynchrony between oocyte and granulosa cell development was detected [50]. Impact of freezing and thawing of human ovarian tissue on follicular growth after long-term xenotransplantation is nevertheless warranted to confirm the identity of theca cells and assess if they retain the ability to respond to luteinizing hormone and produce androgens [51•].

Iso- and Allografts

Ovarian transplantation between monozygotic twins was discordant for premature ovarian failure [52]. Allotransplantation might be a remedy. Silber and colleagues reported a series of monozygotic twins who underwent ovarian isograft transplantation to rescue the sterile sister [46, 52, 53].

Furthermore, orthotopic ovarian allotransplantation has been performed on patients diagnosed with Turner syndrome. This restored regular menstruation and ovulation raised hormonal concentrations and led to the development of secondary sexual characters [38, 54]. This manuscript reports the first live birth after allografting between monozygotic twins with 45,XO/46,XX mosaicism and discordant growth pattern and ovarian function [55•].

The present state of transplant biology, as both a basic and a clinical science, is unlikely to have been achieved without lessons learned from rodent transplant models. Progress in this area has occurred in several ways during the past few decades, including organ preservation, overcoming ischaemia/reperfusion injury and immune rejection, and the development and testing of new immunosuppressive regimens. Many efforts have been made to evaluate possible rejection and survival models of solid organ transplantation and to generate monitoring systems for tissues, such as the pancreatic islets, kidney,

liver, and heart [56–59]. However, few studies have addressed the pathophysiology of ovarian allografts, which are indispensable in the development of ovarian transplantation [60].

In an *in vivo* rodent model to evaluate the rejection of ovarian allografts while mice were treated with or without cocktail immunosuppressant [61], luciferin bioluminescence imaging (BLI) was used to follow graft survival, and viable follicle numbers were counted as a measure of success. Bioluminescence intensity fluctuated but was consistent with the endpoint counts of viable follicle numbers. Graft survival and measurable bioluminescence for up to 30 days in NOD-SCID mice and allografts treated with immunosuppressive therapy. Without immunosuppressant, the ovarian grafts were rejected completely as early as 10 days following ovarian engraftment.

On the contrary, even in the absence of immunosuppressive therapy, ovarian allografting between genetically nonidentical sisters allowed restoration of ovarian function in cases where previous bone marrow transplantation from the HLA-compatible sister resulted in full chimerism, avoiding the threat of rejection in a case report [62].

In human model, ten MZ twin pairs requested ovarian transplantation and eight have undergone transplantation with cryopreservation of spare tissue. Seven had a fresh cortical tissue transplant, one of whom received a second frozen-thawed transplant after the first ceased functioning at 3 years. One had a fresh microvascular transplant. All recipients reinitiated ovulatory menstrual cycles and normal Day 3 serum FSH levels by 77–142 days. Six have already conceived naturally (one twice). Currently, two healthy babies have been delivered, and another three pregnancies are ongoing. The oldest transplant functioned for 36 months, resulting in one child and one miscarriage [17].

In Vitro Activation of Ovarian Follicles

In patients with primary ovarian insufficiency, also known as premature ovarian failure (POF), early exhaustion of ovarian follicles is characterized by high serum FSH along with amenorrhea before 40 years of age. Earlier studies demonstrated that oocyte-specific deletion of the phosphatase and tensin homolog (PTEN) gene led to the activation of dormant primordial follicles. After IVA of primordial follicles by incubating ovaries from neonatal mice with PTEN inhibitors and a phosphatidylinositol 3-kinase PI3K stimulating peptide, ovaries were transplanted under kidney capsules of ovariectomized hosts. Primordial follicles in ovarian grafts developed to the preovulatory stage containing mature eggs. After *in vitro* fertilization and embryo transfer, healthy progeny were delivered [63].

Kawamura applied PTEN inhibitor and PI3K stimulators to activate dormant primordial follicles *in vitro*, following by xenografting of human cortical fragments into immunodeficient mice, mature human oocytes were generated from

dormant primordial follicles after 6 months. Based on the success to obtain human oocytes by the IVA procedure, this application for POI patients for fertility preservation turns out to be possible after ovarian grafts transplanted in the mesosalpinx to yield mature oocytes, followed by *in vitro* fertilization and embryo transfer [2•].

Facilitate Angiogenesis and Protect Avascular Ovarian Grafts

In contrast to cryopreservation, which seems to have only a small deleterious effect on the primordial follicles, there is some evidence that the key factor responsible for follicle loss is postgrafting ischemia with hypoxia. This ischemia causes a proportion of cortical cells to undergo apoptosis after grafting. The ovary is a three-dimensional structure composed of gametes and somatic cells. During the transplantation process, complete revascularization takes time, and the transplantation site selected is important. Therefore, reducing the damage caused by reperfusion–ischemia is an issue requiring urgent resolution. Yang et al. demonstrated that the majority of apoptotic processes occur within the first week after transplantation, before well-organized blood vessels are established between the graft and the host [64].

The survival of transplanted human ovarian tissue may be improved by treatment of both the host (with vitamin E and gonadotropins) and the graft (with vascular endothelial growth factor A [VEGF-A] and vitamins) [65•]. Five young patients with cancer donated ovarian tissues that were transplanted into immunodeficient mice. Graft survival improved after the host was treated with melatonin, or the graft was incubated with hemagglutinin-rich biological glue, especially when combined with VEGF-A and vitamin E [66•]. Tsai et al. [67•] reported that the protective effects of the antiapoptotic agent sphingosine-1-phosphate (S1P) on vitrified ovarian grafts to prove the S1P protects vitrified ovarian grafts from ischemic reperfusion injury rather than from vitrification-associated process.

Live birth in a woman without ovaries after autograft of frozen-thawed ovarian tissue combined with growth factors plasma rich in platelets (PRP) to promote neoangiogenesis [68•].

Coadministration of an immunomodulator, trichloro (dioxoethylene-O,O') tellurate (AS101), reduced follicle activation, thereby increasing follicle reserve and rescuing fertility after cyclophosphamide (Cy), and also increased the efficacy of Cy against breast cancer cell lines. These findings suggest that the mechanism in Cy-induced loss of ovarian reserve is accelerated primordial follicle activation, which results in a "burnout" effect and follicle depletion. By preventing this activation, AS101 shows potential as an ovarian-protective agent, which may be able to preserve fertility in female cancer patients [69•].

Chorioallantoic membrane (CAM) model provides a new approach to study human ovarian tissue transplantation in its

Fig. 1 Risk of ovarian metastasis according to cancer type

first ischemic stages, yielding information on the timing of tissue changes before the establishment of neovascularization [70].

Among the large series of 60 patients who underwent reimplantation of cryopreserved ovarian tissue, 11 conceived and 6 of those had already delivered 12 healthy babies. Donnez pointed out in the future to look to: 1) improve freezing techniques; and 2) enhance the “vascular bed” before reimplantation to increase pregnancy rates [16•]. Cryopreservation of ovarian tissue may be combined with removal, via puncture, of small antral follicles, making it possible to freeze both ovarian tissue and isolated immature oocytes for in vitro maturation [16•].

An alginate-based matrix is a promising proposition to graft isolated ovarian cells. After transplantation, this matrix was able to degrade, allowed vascularization and elicited a low inflammatory response [71•].

Safety Issue

Pregnancy and live birth after autotransplantation of frozen-thawed ovarian tissue in a patient with metastatic disease underwent chemotherapy and hematopoietic stem cell transplantation [72]. By quantitative RT-PCR, two of six CML patients were positive for *BCR-ABL* in their ovarian tissue. Among the 12 ALL patients, 7 of the 10 with available molecular markers showed positive leukemic markers in their

ovarian tissue (translocations or rearrangement genes). Four mice grafted with ovarian tissue from ALL patients developed intraperitoneal leukemic masses. This study demonstrates, by quantitative RT-PCR, ovarian contamination by malignant cells in acute as well as chronic leukemia, whereas histology fails to do so. Moreover, chemotherapy before ovarian cryopreservation does not exclude malignant contamination. Finally, reimplantation of cryopreserved ovarian tissue from ALL and CML patients puts them at risk of disease recurrence [73, 74•] (Fig. 1).

Conclusions

Fertility preservation by the cryopreservation of ovarian tissue is a new but increasingly successful clinical option. The extraction of ovarian tissue can be performed easily by laparoscopy, and the risk of transplantation of cancer cells remains very low in a number of diseases. This technique therefore should be considered in women in the mid-thirties or younger receiving highly gonadotoxic chemotherapy or pelvic radiation. The cryopreservation of ovarian tissue requires the establishment of centralised and highly specialist cryobanks as well as sophisticated networks to integrate oncologists and reproductive specialists in order to provide this procedure to all patients in need [75].

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Compliance with Ethics Guidelines

Conflict of Interest Chi-Huang Chen and Chii-Ruey Tzeng declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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