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Recent advances in fertility preservation

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Abstract

Background: Most cancer treatments like chemotherapy, radiotherapy or a combination of both are highly toxic to the gonads, putting girls and young women at risk of premature ovarian insufficiency and subsequent infertility. Non-oncological conditions may also require therapies that put women's reproductive potential at risk. Fertility preservation counseling should therefore be offered to all patients requiring gonadotoxic treatments, and to those who wish to postpone motherhood for social/personal reasons. Among the most effective fertility preservation options available today, oocyte and embryo cryopreservation, and ovarian tissue cryopreservation have emerged as the front-runners.

Aim: This review focuses on the currently available and most widely accepted fertility preservation and restoration strategies, with a special focus on recent advances in ovarian tissue cryopreservation and transplantation.

Conclusions: To manage cancer patients satisfactorily and offer proper counsel on the most appropriate option available to them, different parameters need to be taken into account, including pubertal status, partner status and urgency of treatment for the underlying pathology. When fertility preservation is carried out for non-oncological indications or personal reasons, oocyte cryopreservation by vitrification is clearly the highest-yield clinical strategy. Ovarian tissue cryopreservation followed by transplantation is rapidly gaining ground as a fertility preservation and restoration strategy, and will hopefully soon have its 'experimental' label removed to allow practitioners to move on to open clinical application. Techniques to improve grafted ovarian tissue life span and quality as well as to avoid transmission of malignant cells have been developed, showing promise as a way to expand this procedure.

Key words: embryo cryopreservation, fertility preservation, oocyte cryopreservation, ovarian tissue cryopreservation, ovarian tissue transplantation.

Introduction

Cancer incidence in children, adolescents and young adults has seen a slight increase since the 1970s,[1] but death rates in patients aged 0–19 years have continued to fall. Current 5-year overall survival estimates for childhood cancer exceed 83% (around 90% for most hematological malignancies), which translates into a growing population of adult survivors.[2] It is widely known that most cancer treatments like chemotherapy, radiotherapy or a combination of both are highly toxic to the gonads, putting girls and women of reproductive age at risk of premature ovarian insufficiency (POI) and subsequent infertility.[3,4] Moreover, non-oncological hematological diseases (thalassemia, aplastic anemia), autoimmune disorders (rheumatoid arthritis, systemic lupus erythematosus)[5–7] and other ovarian pathologies,[4] often require treatment that may impair future fertility, exponentially increasing the number of women likely to suffer from iatrogenic menopause or POI.

Indeed, anticancer treatments are known to diminish the primordial follicle pool and cause ovarian

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atrophy.[8,9] However, the extent of anticancer drug-induced damage is variable and the effects of chemotherapy and radiation are not considered to be 'all or nothing'. Therapy can be sterilizing in some instances, while in other cases, it may only cause partial ovarian injury.[10] As very recently reviewed by Donnez and Dolmans,[4] the gonadotoxicity of these treatments depends on multiple factors, such as the ovarian reserve, patient age at the time of treatment, administered therapies (chemo- and/or radiotherapy) and their doses and duration. This is why fertility preservation counseling should be offered to all patients requiring gonadotoxic treatments. This review focuses on the currently available fertility preservation and restoration strategies, with a special focus on ovarian tissue cryopreservation (OTC) and transplantation.

Indications for Fertility Preservation

Ultimately, the probability of POI after cancer treatment is related to the ovarian reserve. This reserve (population of primordial follicles) can vary enormously from one woman to the other,[11] so patients need to be evaluated and counseled in a personalized manner. A classification of the risk of POI was established based on permanent amenorrhea rates,[12] since some therapies may compromise the follicular reserve without causing cessation of menstrual periods (Table 1). Hence, fertility may already be severely affected before the onset of amenorrhea.[13,14] All women and parents of underage girls requiring gonadotoxic therapy need to be fully informed of the risk of fertility impairment. There are a number of indications for fertility preservation, divided into oncological and non-oncological (benign) conditions, as well as personal reasons (Table 2).

Oncological conditions

Fertility preservation remains a challenge, particularly in case of hematological cancers (Hodgkin's lymphoma, non-Hodgkin's lymphoma and leukemia) and breast cancer.[15,16] These pathologies constitute the most common oncological indications for fertility preservation since chemotherapy, radiotherapy, surgery (or a combination thereof) and bone marrow transplantation can induce POI. Other indications include sarcoma, colorectal cancer, borderline ovarian tumors and central nervous system malignancies among others.[15,17]

Non-oncological medical conditions

Fertility preservation should also be offered to women suffering from benign conditions that may put them at risk of POI. Certain autoimmune and hematological disorders require chemotherapy, radiotherapy or both, and sometimes even bone marrow transplantation (Table 2). Other conditions can also impair future fertility, such as the presence of bilateral ovarian tumors, severe or recurrent ovarian endometriosis[18] and recurrent ovarian torsion. Ovarian endometriomas diminish the ovarian reserve by triggering follicle 'burn-out'.[19] Moreover, there is evidence that surgery itself cause different degrees of damage to the ovarian reserve.[20–22] Turner syndrome and a family history of POI are also indications for fertility preservation[4,23] (Table 2).

In patients receiving treatments for gender dysphoria, data on reproductive health issues are scarce. There are no established techniques at present for preserving gonadal function in prepubertal or pubertal

Table 1 Risk of premature ovarian insufficiency in women†

High risk	Stem cell transplantation, external beam irradiation to fields including the ovaries, breast cancer adjuvant combination chemotherapy regimens containing cyclophosphamide, methotrexate, fluorouracil, doxorubicin and epirubicin in women aged >40 years
Intermediate risk	Breast cancer adjuvant chemotherapy regimens containing cyclophosphamide in women aged 30–39 years, or doxorubicin/cyclophosphamide in women aged >40 years, bevacizumab
Low risk (<20%)	Combination chemotherapy regimens for non-Hodgkin's lymphoma, acute lymphoblastic or myeloid leukemia, breast cancer adjuvant chemotherapy regimens containing cyclophosphamide in women aged <30 years, or doxorubicin/Cy in women aged <40 years
Very low risk or no risk	Vincristine, methotrexate, fluorouracil
Unknown risk	Paclitaxel, taxotere, oxaliplatin, irinotecan, trastuzumab, cetuximab, erlotinib, imatinib

†Risk assessment is based on amenorrhea rates. Because some therapies compromise the follicular reserve without causing amenorrhea, fertility may be impacted before the cessation of menstrual periods. Adapted from Lee *et al.*,[12] and Loren *et al.*,[13] with permission.

Table 2 Indications for fertility preservation (Adapted from Donnez and Dolmans,[4] with permission.)

Malignant diseases requiring gonadotoxic chemotherapy, radiotherapy or bone marrow transplantation
Hematological diseases (leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma)
Breast cancer
Sarcoma
Some pelvic cancers
Benign conditions
Systemic diseases requiring chemotherapy, radiotherapy, or bone marrow transplantation
Ovarian diseases
Bilateral benign ovarian tumors
Severe and recurrent ovarian endometriosis
Possible ovarian torsion
Risk of premature ovarian insufficiency
Family history
Turner syndrome
Personal reasons
Age
Delayed childbearing

adolescents who will never develop reproductive function in their natal sex due to blockers or cross-gender hormones.[24] However, the most recent Standards of Care of the World Professional Association for Transgender Health recommend clearly informing patients regarding their future reproductive options prior to initiation of treatment. Indeed, while genital reconstructive surgery results in definitive sterility, hormone therapy has a considerable but partially reversible impact on fertility.[25]

Personal reasons

Elective fertility preservation for personal reasons or age-related fertility decline has emerged as an indication for fertility preservation over recent decades, as the age at which women now attempt their first pregnancy has been steadily rising.[4,26,27] The largest group of women seeking fertility preservation includes those who wish to postpone childbearing for various personal reasons, with age becoming the biggest threat to their fertility. Lack of a stable partner, financial reasons, self-realization and career status are some of the factors that influence a woman's decision to delay having children. Indeed, a recent study showed that career-focused women place more value on intentional pregnancy planning to postpone childbearing, and have more confidence in assisted reproductive technology to counteract age-related fertility decline, than those who do not place as much importance on career success.[28] Female fertility decreases gradually at first, but significantly after the age of 35 years, which inevitably compromises fertility over time. The term 'AGE banking' (oocyte banking for anticipated gamete exhaustion) has also been proposed for oocyte cryopreservation in such cases.[24]

Current, Most Widely Accepted Strategies for Fertility Preservation

After their cancer diagnosis and POI risk assessment based on planned treatments, women need to be informed of the different fertility preservation alternatives and strategies, either by their oncologist or by an infertility specialist. To manage patients satisfactorily and offer proper counsel on the most appropriate option available to them, different parameters need to be taken into account, including pubertal status, partner status and urgency of treatment for the underlying pathology.[4] Table 3 summarizes the current best options for fertility preservation in women.

Embryo and oocyte cryopreservation

Embryo and oocyte cryopreservation are the most widely accepted and well established fertility preservation techniques,[13] with sufficient evidence to support their feasibility and efficacy.[29] However, both require women to undergo at least one cycle of ovarian stimulation in order to proceed to oocyte pick-up, so can only be proposed to postpubertal patients with time for ovarian stimulation.

Ovarian stimulation protocols typically require around 2 weeks, with daily follicle stimulating hormone injections from the onset of menses.[12] Although it is best to stimulate the ovaries within 3 days of the start of the menstrual cycle, random-start stimulation

Table 3 Current (most widely accepted) cryopreservation options for fertility preservation in women

	Embryos	Oocytes	Ovarian tissue
Age restriction	Postpubertal	Postpubertal	No restriction
Cancer treatment	Delayed (2–6 weeks)	Delayed (2–6 weeks)	No delay
Need for sperm	Yes	No	No
Freezing protocol	V	V	SF (>130 LB); V (three LB)
LB likelihood	5–8 embryos	20 oocytes	One in three women after one transplantation
Method	IVF	IVF	Spontaneous conception possible
Advantages	Well established	Reproductive autonomy	Reproductive autonomy; large number of PF; endocrine function restored
Risks	Poor quality embryos	Small number of retrieved oocytes	Risk of disease retransmission
Status	Clinical	Clinical	Experimental†

†Evaluation ongoing. IVF, *in vitro* fertilization; LB, live birth; PF, primordial follicles; SF, slow-freezing; V, vitrification.

protocols have also proved effective.[30] Indeed, random-start controlled ovarian stimulation (COS) yielded similar outcomes in terms of numbers of retrieved oocytes and early developmental competence to conventional early follicular phase-start COS.[31] A recently described protocol introduced the 'back-to-back' concept, better known as 'the Shanghai protocol', which consisted of a random-start double COS during both follicular and luteal phases yielding higher oocyte pick-up rates in less time.[32] Advances in COS protocols have not only helped overcome timing limitations for cancer treatment, but can also benefit patients with hormone-sensitive tumors like breast cancer, as specific protocols that reduce estrogen exposure have also been shown to be effective.[33]

Follicle development during COS is monitored by ultrasound and blood tests. At the appropriate time, an injection of human chorionic gonadotropin or gonadotropin-releasing hormone antagonist is administered to initiate the ovulatory cascade, and pick-up is performed by ultrasound-guided transvaginal needle aspiration under sedation. Once oocytes have been recovered, *in vitro* fertilization is undertaken for embryo cryopreservation if sperm is available, or freezing if oocyte cryopreservation is the selected approach.

Embryo cryopreservation

In vitro fertilization of retrieved oocytes precedes embryo cryopreservation, which is currently the most effective procedure offered as first-line fertility preservation approach to postpubertal patients with time for COS and with sperm available.[34,35]

Embryos at the cleavage stage can be successfully cryopreserved by slow-freezing or vitrification. Evidence in favor of vitrifying day-3 embryos and blastocysts has been steadily building over the years, and it has now become the method of choice for embryo cryopreservation,[36] yielding superior survival, pregnancy and live birth rates (LBR) compared to slow-freezing.[29] Furthermore, transferring vitrified-warmed embryos has been shown to be at the very least as efficient as fresh embryo transfer, if not better.[4,29] Indeed, the 2015 US national report on assisted reproductive technology revealed a LBR of 48% per frozen embryo transfer compared to 46.5% per fresh embryo transfer.[37]

In a study by Dolmans *et al*.[38] regarding pregnancy and LBR in women affected by cancer seeking to preserve their fertility, embryos were obtained from 52 of 54 patients undergoing COS for embryo cryopreservation. The cumulative pregnancy rate achieved was 66% and the LBR per patient was 44%,[38] similar to rates obtained with fresh embryos in noncancer patients. However, fewer good-quality embryos were found in cancer survivors than in the normal infertile population.[38]

Oocyte cryopreservation

Cryopreservation of unfertilized oocytes is another approach that has gained ground recently due to better cryopreservation protocols. It is especially suitable for women who do not have a male partner, do not wish to use donor sperm, or have religious or ethical objections to embryo freezing, hence giving them more reproductive autonomy.

The initial promise of this approach was limited by the fragility of metaphase-II oocytes related to their large size, water content and chromosomal arrangement. The meiotic spindle apparatus of these mature oocytes, typically retrieved after superovulation, was found to be damaged by intracellular ice crystal

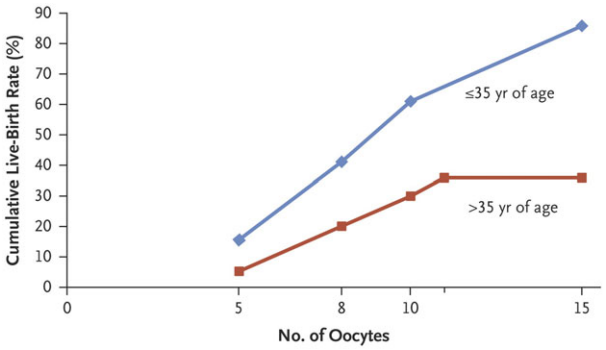

Figure 1 Cumulative live birth rates according to age. The cumulative live birth rate increases with the number of oocytes and is higher among younger women (≤35 years of age) than among older women (>35 years of age). Data are from Cobo *et al*. Adapted from Donnez and Dolmans,[4] with permission.

formation during the freezing or thawing process.[39] The introduction of oocyte vitrification to replace slow-freezing protocols was associated with progressive improvements in oocyte cryosurvival. Moreover, data from a recent review suggest that the strategy of oocyte vitrification and warming is superior to slow-freezing and thawing in terms of clinical outcomes.[29]

The pregnancy rate per cryopreserved oocyte is 4.5–12% according to the American Society for Reproductive Medicine and the Society of Assisted Reproductive Technology Practice Committee,[40] indicating that one live birth requires at least 10 cryopreserved oocytes (Fig. 1). This estimate is supported by recently reported results showing that cumulative LBR issuing from 10 and 15 cryopreserved oocytes for nononcological reasons were 60.7% and 85.2% respectively, even in young women aged 35 years or younger.[26]

Nevertheless, these optimized oocyte/embryo/blastocyst cryosurvival rates and clinical outcomes achieved with use of vitrification have far-reaching clinical implications, which enable a personalized approach in the care of different patient populations.[29]

Ovarian tissue cryopreservation

Ovarian tissue cryopreservation for the purposes of autotransplantation does not require ovarian stimulation and can therefore be performed immediately, with no delay in cancer treatment. Furthermore, it does not require sexual maturity, which makes it the only fertility preservation method available to prepubertal girls at risk of treatment-induced POI.[4,34,35] It also restores general ovarian endocrine function.[4,41]

The most frequent indications for OTC in most centers are hematological malignancies, especially Hodgkin's lymphoma and leukemia, followed by breast cancer (Fig. 2).[15–17,43,44] However, benign indications for OTC are also on the increase, with nonmalignant conditions requiring treatment that may result in POI, such as systemic disorders, autoimmune disease, bilateral benign gynecological diseases,[45] borderline ovarian tumors,[46] endometriosis[47] and genetic disorders such as Turner's syndrome.[23,48]

Certain selection criteria clearly need to be applied before contemplating OTC, as first stressed by Wallace *et al*.[49] and very recently emphasized by Donnez and Dolmans,[4] the most important being age less than 35 years (when the ovarian reserve is still relatively high), a realistic chance of surviving for 5 years, and at least a 50% risk of POI.[49,50]

The amount of ovarian tissue to be harvested for cryopreservation purposes varies according to the risk of POI and existing ovarian volume. Oophorectomy is usually indicated in case of pelvic radiotherapy or total body irradiation and in very young girls due to the small size of the ovaries. Otherwise, four to five fragments of tissue (10 × 5 × 1 mm) are typically recovered by laparoscopy and then processed for freezing.[51] With regard to biopsy thickness, based on the results of earlier studies,[52] it is recommended that a 1–1.5-mm-thick piece of ovarian cortex be taken.[53] This is considered to be of paramount importance, since superficial or very thin biopsies may not contain follicles in the removed cortex, as primordial follicles are generally found at a distance of 0.8 mm from the mesothelium.

Methods for OTC include vitrification and slow-freezing, with the latter currently the most widely accepted and implemented.[54] Indeed, all but two live births to date have been achieved using slow freezing for OTC.[55] However, since there are fewer reports on OTC with vitrification, only an approximate comparison can be made. Studies must now establish whether OTC would benefit from switching to vitrification, as oocyte cryopreservation, or if slow-freezing should remain the 'gold standard' thanks to numerous successful clinical outcomes obtained with its use.[4]

With the exception of some countries such as Israel,[56] Denmark and Norway,[43] OTC remains

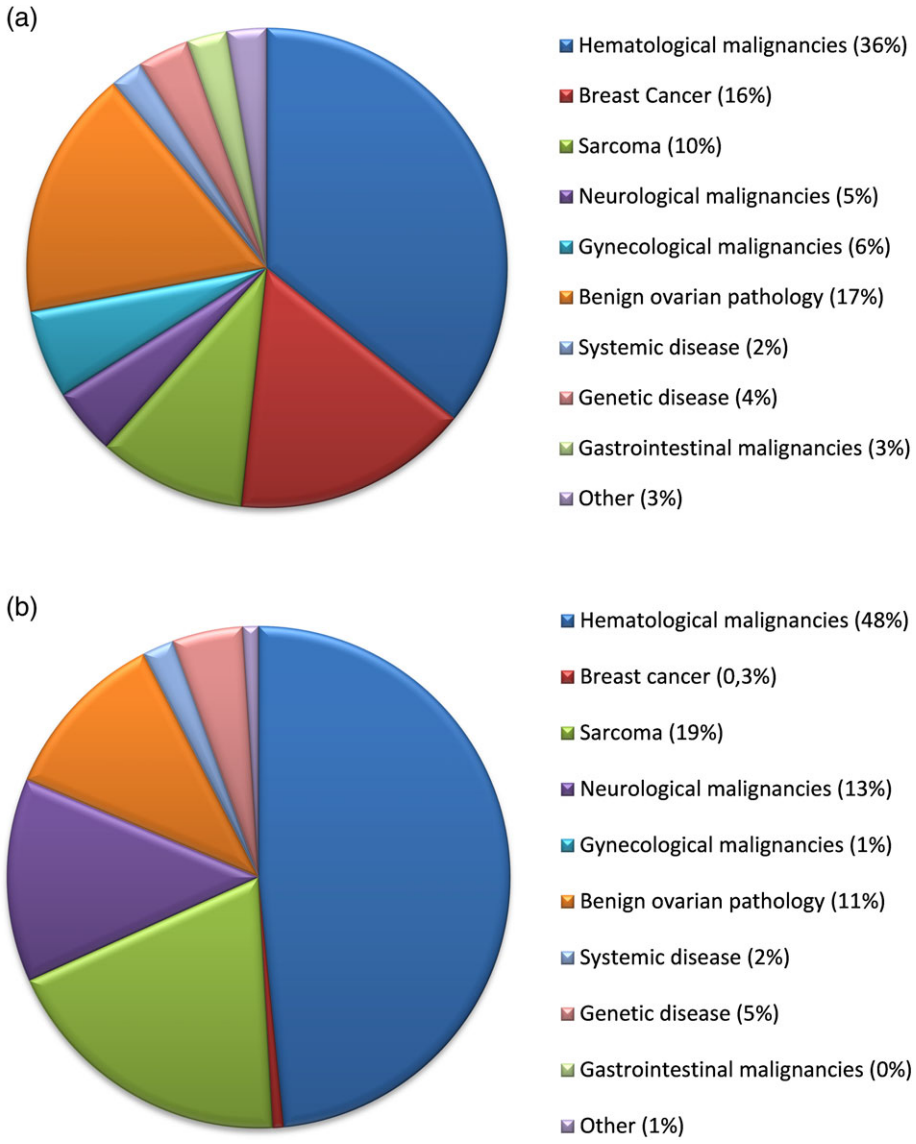

Figure 2 Indications for ovarian tissue cryopreservation. Indications for ovarian tissue cryopreservation in adult women (a) and prepubertal and adolescent girls (b) in our center. Adapted from Dolmans and Donnez,[42] with permission.

experimental, but encouraging new data may prompt reconsideration of this designation in the future. Its experimental status is currently undergoing evaluation in the United States.[35]

The combined technique

One way of improving fertility preservation outcomes would be to combine OTC and oocyte vitrification. Ovarian tissue retrieval followed immediately by ovarian stimulation and oocyte pick-up (with a view to vitrifying mature oocytes) does not impair oocyte number or quality, and could actually increase the efficacy of the fertility preservation procedure by giving young patients with cancer greater chances of success.[57]

Fertility Restoration Strategies with a Focus on Ovarian Tissue Transplantation

Ovarian tissue transplantation techniques

Ovarian tissue transplantation (OTT) is a procedure that involves thawing of previously recovered ovarian cortical pieces and surgically grafting the fragments back to patients. Described techniques include both orthotopic (pelvic cavity) and heterotopic (outside the pelvic cavity) sites.[58]

Orthotopic OTT

Orthotopic transplantation involves grafting ovarian cortical fragments to the exposed medulla of the denuded ovary or a specially created peritoneal site.[45] Orthotopic transplantation takes advantage of the anatomical position of the ovaries for possible natural conception if other conditions are met: permeable fallopian tubes, no male factor issues and restoration of ovarian function after transplantation.

The majority of reported orthotopic transplantations are carried out by minimally invasive surgery. The choice of grafting site and decision to graft in one or more locations depend on whether or not the patient previously underwent complete unilateral or bilateral oophorectomy.[45] Indeed, if at least one ovary is present, ovarian tissue may be transplanted both to the ovary after decortication and to a newly created peritoneal window. On the other hand, if no ovaries remain or what is left is severely atrophic or nonfunctional (due to the effects of high doses of radiotherapy), the only alternative for orthotopic transplantation is use of a peritoneal window.[45]

The first live birth after orthotopic transplantation of cryopreserved ovarian tissue was reported in a patient with iatrogenic POI was reported by our team in 2004.[59] Our technique applied a two-step laparoscopic approach. The first laparoscopy was performed 7 days before reimplantation in order to induce neovascularization by creating a peritoneal window just beneath the right ovarian hilus, followed by bipolar coagulation of the edges. The second laparoscopy was performed 7 days after and fragments of thawed human ovarian tissue were pushed into the previously created furrow, very close to the ovarian vessels and fimbria.

There are currently different approaches that may be applied depending on the presence or not of the ovaries, and the amount of tissue available for thawing and reimplantation:

1. If at least one ovary is present, decortication of the ovary should be performed first. A relatively large piece of ovarian cortex (1–2 cm) is removed by means of scissors to have access to the medulla and its vascular network. Strictly adhering to microsurgical techniques and principles, ovarian cortical pieces are then simply placed on the medulla and covered by Interceed, the edges of which are fixed with stitches or fibrin glue.
2. If both ovaries are absent, a peritoneal window may be created in two steps to induce angiogenesis before the grafting procedure, as in the case described above,[59] or in one step.[45] The incision for this peritoneal window is made on the anterior leaf of the broad ligament in an area where a vascular network is visible (retroperitoneal vessels). Vessels may be easily localized by transillumination using the optical light of the instrument. Fragments are placed in the window and subsequently covered with Interceed, the edges of which are fixed with fibrin glue.
3. A third option for patients with one or two ovaries still in place is grafting the tissue to both orthotopic sites simultaneously (if there is enough ovarian tissue), namely to the denuded ovary and the peritoneal window.[60] With this type of transplantation, it is of utmost importance to be circumspect with amounts of tissue used, in case further reimplantations are needed in the same patient. It is recommended that only on third of a patient's cryopreserved tissue be thawed and grafted for each transplant.

Figure 3 illustrates the procedure used by our team.

Fixing ovarian tissue fragments to any of the grafting sites using direct stitches is not advised. Indeed, potential induction of inflammatory reactions leading to fibrosis could negatively impact the quality of follicles present at the time of grafting. In addition, considerably more handling and manipulation of thawed ovarian fragments are required when stitches are placed in such small pieces of tissue, causing further mechanical damage to follicles.

Heterotopic OTT

Common sites for heterotopic transplantation are the abdominal wall, forearm and rectus muscle, among others. While heterotopic ovarian transplantation has debatable clinical value, as it may not provide an optimal environment for follicle development,[4] restoration of endocrine function and embryo development have been demonstrated consistently after this procedure.[61,62] However, its effectiveness for restoration of fertility remains questionable, as the success rate in terms of live births is extremely low.[63]

OTT outcomes

Since the first live birth after frozen-thawed OTT in 2004,[59] the number of children born worldwide using this technique has shown a slow but steady increase, becoming exponential over the past couple of years (Fig. 4).[4] Success rates of OTT are now similar to those achieved by other assisted reproductive technologies.

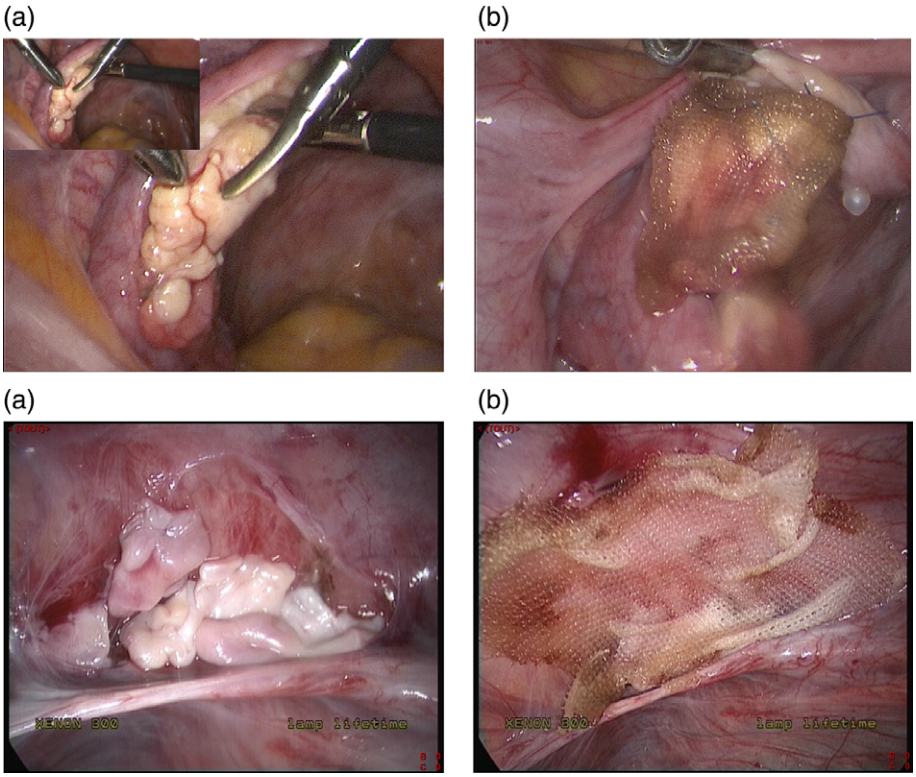

Figure 3 Techniques for ovarian tissue reimplantation. Frozen-thawed ovarian tissue fragments placed in a decorticated ovary (a) and subsequently covered with Interceed and fibrin glue (b). Peritoneal window created by means of scissors and ovarian cortex pieces (c), covered after with Interceed and fibrin glue. Medulla of each fragment has to be grafted facing and in contact with the denuded ovary (a) or the peritoneum (c) accordingly.

Restoration of endocrine function

Restoration of ovarian endocrine function is measured by either follicle growth or recurrence of menstruation. Several teams have found that around 95% of transplanted women experiences return of menses and back-to-normal hormone levels.[4,15,41] The period from the first OTT to observed follicular function is on average 4 months and ovarian activity usually persists for more than 5 years. Moreover, by repeating the procedure, ovarian activity could be restored for longer depending on the amount of stored tissue and patient age at the time of OTC.[53] These encouraging results on ovarian function restoration lead us to speculate that in the future, OTC at a young age, followed by reimplantation at menopause, could well provide an alternative to hormone replacement therapy.[42]

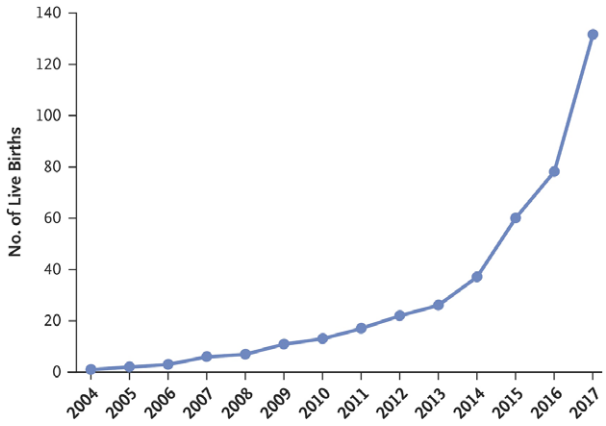

Figure 4 Live births after ovarian tissue transplantation in an orthotopic site. Since the publication of the first pregnancy after reimplantation of human ovarian tissue in an orthotopic site (pelvic cavity), the number of live births has reached more than 130, showing a logarithmic increase during the past 2 years and highlighting the need to move from experimental studies to widespread clinical application. Adapted from Donnez and Dolmans,[4] with permission.

Pregnancy and live birth rates

To date, more than 130 live births have been reported,[4] 93 in peer-reviewed journals.[41] Patients who achieved a live birth were found to be significantly younger at OTC than those who failed to conceive despite trying.[41] Furthermore, of all the reported children born after OTT, around half were conceived spontaneously.[4,41]

Since the denominator (the number of reimplantations performed worldwide) is difficult to estimate,

calculation of LBR could be based on patients from major centers with available published data. Data from 5 centers (a series of 111 women) yielded a pregnancy rate of 29% and a LBR of 23%.[54] These rates were subsequently confirmed in 2016 in a case series of 74 women, with pregnancy and LBR of 33% and 25%, respectively,[64] and in another series of Meirow's team with a LBR of 30%.[44] In our series of 22 women who underwent OTT, pregnancy and LBR were 41% (9 of 22) and 36% (8 of 22), respectively, with a total of 15 live births.

A woman in our institution had her ovarian tissue cryopreserved at the age of 17 due to a neuroectodermal tumor, and gave birth three times after one orthotopic OTT procedure making her one of the three women in the world to have delivered more than two babies with a single graft.[53]

The Future

Challenges of the early postgrafting period

Despite increasing success and better clinical outcomes of OTT, there is still room for improvement. Since it is an avascular grafting procedure, one of the biggest challenges in OTT is the early postgrafting period.[58]

The size of fragments typically cryobanked excludes the presence of any distinguishable blood vessels for further reanastomosis during grafting. Hence, graft function depends on reestablishment of vascularization, with implants exposed to ischemic damage during the initial posttransplantation phase until they become fully vascularized.

Using *in vivo* electron paramagnetic resonance oximetry to measure partial pressure of oxygen (pO_2) in a human ovarian tissue xenografting model, our group was the first to demonstrate that the period of hypoxia lasts at least 5–7 days before optimal pO_2 levels are reached in a peritoneal grafting site by postgrafting day 10.[65] These findings were further supported by results from a study in which graft vascularization and perfusion were analyzed, revealing that both host and graft vessels contributed sequentially to graft revascularization. Murine angiogenesis initiated reperfusion from day 5 onwards, while by day 10, human angiogenesis was found to participate in graft revascularization.[66]

Furthermore, studies by our group have shown that grafted ovarian tissue perfusion occurs progressively, from the periphery to the center[66] and that anaerobic metabolism prevails for the first 9–10 days after transplantation.[67] Other teams have also reported that functional blood vessels are found only 7–10 days after transplantation.[68]

Strategies to improve the quality and life span of grafted ovarian tissue

Early posttransplantation hypoxia remains an issue because of its negative impact on follicle survival, with follicle loss of >60% often observed during the first few days postgrafting,[69,70] leading to massive follicular activation and 'burn-out'.[71,72] Increasing vascularization in grafted tissue is therefore crucial and efforts are being made to improve follicle survival rates with a view to increasing the efficiency of OTT.

One approach involves enhancement of graft revascularization by delivering both angiogenic and antiapoptotic factors,[73–76] while others use antioxidants to decrease oxidative stress.[77–79] Although some improvements have been seen in ovarian tissue outcomes, no clinically applicable protocol has yet had any discernible positive impact on follicle survival rates or graft vascularization.

Two-step OTT using adipose tissue-derived stem cells

A novel two-step OTT procedure aiming to increase follicle survival by boosting revascularization in xenografted human ovarian tissue with adipose tissue-derived stem cells (ASCs) was very recently proposed by Manavella *et al*.[80,81] Using this approach, higher primordial follicle survival rates were found 7 days after transplantation, reaching a mean survival rate of 62%. Moreover, significantly lower rates of follicle growth and apoptosis were evidenced. This reduced follicle loss and other observed differences may be explained by the early concomitant increase in oxygenation and vascularization detected in the group grafted with the addition of ASCs, which served to shorten tissue exposure to hypoxia.

The proangiogenic potential of ASCs in the context of this two-step OTT procedure was very clearly demonstrated. Through secretion of proangiogenic factors like vascular endothelial growth factor and fibroblast growth factor, and differentiation into endothelial cell lineages, ASCs were shown to improve graft vascularization in the early postgrafting period.[81,82] Enhanced angiogenesis contributed to increased follicle survival rates, while at the same time decreasing apoptosis and follicular activation, thereby better preserving the

Table 4 Risk of ovarian involvement (Adapted from Dolmans and Masciangelo,[83] with permission.)

Low (0–2%)	Intermediate (2–10%)	High (>10%)
Breast cancer: Stage I, II; infiltrating ductal subtype	Breast cancer: stage III, IV; infiltrating lobular subtype	Leukemia
Squamous cell cervical carcinoma	Adenocarcinoma of the cervix	Neuroblastoma
Hodgkin's lymphoma	Non-Hodgkin's lymphoma	Burkitt's lymphoma
Osteogenic carcinoma	Ewing sarcoma	
Nongenital rhabdomyosarcoma	Colon cancer	
Wilms's tumor	Borderline ovarian tumors	

primordial follicle pool compared to currently used transplantation procedures.

These results showed that the life span and quality of ovarian tissue grafts may be increased and improved, potentially extending patient fertility and ovarian endocrine activity for longer periods post-transplantation. These findings highlight a growing interest in further investigating the long-term effects of using ASCs in terms of safety, restoration of endocrine function, and ultimately pregnancy and LBR with a view to future clinical application.

Challenges of eliminating malignant cells for OTT

The artificial ovary

For women with certain types of cancer, especially with acute leukemia, the risk of reimplantation of malignant cells along with grafted tissue is high (Table 4).[83] Although Shapira *et al*., reported a case of successful transplantation in an acute myeloid leukemia survivor,[56] alternative approaches are needed. One promising option is the so-called transplantable artificial ovary. Isolating primordial follicles eliminates the risk of transmission of malignant cells, and transferring them onto a scaffold allows creation of this artificial organ.[84] Recent developments in the isolation technique, involving washing the follicles three times, have proved successful.[85] Growing antral follicles were observed after autografting primordial follicles inside a fibrin scaffold in a mouse model and after xenografting human primordial follicles to severe combined immunodeficient mice.[86,87] Moreover, a very recent study attempted to develop a three-dimension-printed artificial ovary prototype, demonstrating restoration of both endocrine and reproductive function in ovariectomized mice.[88]

In vitro development of primordial follicles

A dynamic multistep culture system is needed to support each of the transitional stages of follicles. This multistep approach to *in vitro* follicle growth must meet the changing requirements of the developing oocyte and its surrounding somatic (granulosa) cells in order to maintain interactions between these cells.[89] The challenges are numerous, involving acquisition of meiotic and developmental competence and genome imprinting.

In a very recent paper, development of human oocytes from primordial/unilaminar stages to resumption of meiosis (metaphase II) and emission of a polar body were described by Telfer's group.[90] As stressed by McLaughlin *et al.*,[90] further optimization with fertilization potential of *in vitro*-grown oocytes is required to determine whether they are normal.

In conclusion, when fertility preservation is carried out for nononcological indications or personal reasons, oocyte cryopreservation by vitrification is clearly the highest-yield clinical strategy. For women of advancing childbearing age who do not yet wish to conceive, this technique may be used to extend their fertility potential. For cancer patients, on the other hand, there are a number of points to be taken into account before choosing the best option. It is important to clarify that (i) for oocyte/embryo vitrification, chemotherapy needs to be delayed by at least 10 to 12 days and one cycle of COS may not be sufficient to achieve good clinical outcomes, as the number of obtained oocytes/embryos cannot be predicted; (ii) patient have to be postpubertal; and (iii) cancer steroid sensitivity must be assessed in order to implement safe and adapted COS protocols.

OTC followed by transplantation is rapidly gaining ground as a fertility preservation and restoration strategy, and will hopefully soon have its 'experimental' label removed to allow practitioners to move on to open clinical application. Techniques to improve grafted ovarian tissue life span and quality as well as to avoid transmission of malignant cells have been developed, showing promise as a way to expand this procedure.

Disclosure

None declared.

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