REVIEW

Ovarian tissue freezing: current status

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Purpose of review
This article aims to carefully evaluate a number of critical points related to ovarian tissue freezing and presents factual data in terms of live birth rates and risks.

Recent findings
Reimplantation of frozen-thawed ovarian tissue remains an experimental procedure according to the American Society of Reproductive Medicine, despite almost 40 live births reported in the literature. Recent literature on the topic has focused on the risk of reimplanting malignant cells, so the present review assesses the risks according to the disease.

Summary
This manuscript emphasizes the crucial importance of not only preserving fertility in young women but also clearly explaining to patients the different available options and their respective success rates. Some previously published reviews have reported inaccurate reimplantation success rates. In this review, we report the true picture, with a live birth rate of 25%. Ovarian tissue freezing may be combined with pickup of immature oocytes (at the time of ovarian biopsy and tissue removal) or mature oocytes (if chemotherapy can be delayed).

Keywords
freezing, ovarian tissue cryopreservation, ovary, reimplantation

INTRODUCTION

Fertility preservation, whether in the context of treating cancer or benign diseases or for social reasons, will be a major challenge over the next 5 years [1,2▪▪]. As its popularity grows and indications increase, a number of reviews have recently been published to evaluate all available methods [3▪▪,4].

Thanks to advances in cancer therapy over the past 2 decades, overall rates of death attributable to cancer in women have fallen by greater than 1.6% per year during the past 5 years [5▪▪]. Unfortunately, treatments such as chemotherapy, radiotherapy, and/or surgery can induce premature ovarian failure (POF) in some circumstances [6–8]. Indeed, the ovaries are very sensitive to cytotoxic drugs, especially alkylating agents and pelvic radiation (exposure to 5–10 Gy) [9], which are likely to cause gonadal dysfunction [5▪▪,6–13]. Cyclophosphamide is the alkylating agent that causes most damage to oocytes and granulosa cells in a dose-dependent manner [8,14,15].

Giving a patient an accurate assessment of the risk to fertility is very difficult, as how a disease will develop cannot be predicted [16]. Hence, evaluating the likelihood of POF after chemotherapy or radiotherapy in young patients with cancer is often extremely problematic [16,17▪▪].

Benign systemic diseases such as autoimmune and hematological conditions sometimes also require chemotherapy or radiotherapy, associated with bone marrow transplantation [15]. This treatment combination puts patients at the greatest risk of POF, estimated to be 92–100% [14,18], which is mostly related to use of total body irradiation prior to transplantation and increased age at the time of treatment [10].

In addition, women are increasingly postponing childbearing to later in life for social or financial reasons, and the incidence of most cancers rises with age [3▪▪,4]. This review evaluates the current status of ovarian tissue freezing for fertility preservation purposes.

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KEY POINTS

- Women with cancer have several options, such as ovarian transposition, embryo cryopreservation, immature or mature oocyte cryopreservation, and ovarian tissue cryopreservation, to preserve their fertility and enable them to conceive when they have recovered.
- Embryo and mature oocyte cryopreservation following IVF are the only methods endorsed by the ASRM, but they required a delay before starting chemotherapy.
- However, cryopreservation of ovarian tissue is the only fertility preservation option available to prepubertal girls and women who cannot delay the start of chemotherapy.
- There is now enough evidence to support the technique of ovarian tissue cryopreservation and stop considering it an experimental and investigational approach.

DIFFERENT OPTIONS FOR FERTILITY PRESERVATION

Women with cancer have several options, such as ovarian transposition, embryo cryopreservation, immature or mature oocyte cryopreservation, and ovarian tissue cryopreservation, to preserve their fertility and enable them to conceive when they have recovered [3▪▪,4,6,7]. At present, embryo and mature oocyte cryopreservation following IVF are the only methods endorsed by the American Society of Reproductive Medicine (ASRM). Although these are of course very effective means of preserving fertility, several important points should be borne in mind:

(1) If chemotherapy can be delayed, oocyte vitrification should be proposed to patients with cancer, but further studies in patients with cancer are needed to confirm the excellent results obtained in egg donation programs [19].
(2) Patients should be aware that around 20 vitrified oocytes are required to achieve a live birth, as the live birth rate per vitrified oocyte (in egg donation programs) is 5.7% in the most experienced teams in the world [19,20▪▪,21,22]. They should also be informed that the expected number of oocytes retrieved after controlled ovarian stimulation (COS) in the context of malignancy might be lower than that obtained from healthy patients of a similar age [23▪▪].
(3) The choice of COS protocol depends on the disease the patient is being treated for, not only because she often has only a single opportunity due to limited time available until initiation of radiotherapy and/or chemotherapy but also on account of the specificity of some estrogen-sensitive cancers [5▪▪,23▪▪].
(4) Conventional COS for IVF requires 9–14 days of ovarian stimulation with gonadotropins. Fortunately, use of gonadotropin-releasing hormone antagonists shortens this interval [24,25]. Cakmak and Rosen [23▪▪] have published extensively about the possibilities of at-random-start protocols. AQ2

OVARIAN TISSUE CRYOPRESERVATION

Cryopreservation of ovarian tissue is the only fertility preservation option available to prepubertal girls and women who cannot delay the start of chemotherapy. In the opinion of many pioneers, there is now enough evidence to support the technique of ovarian tissue cryopreservation and stop considering it an experimental and investigational approach. As the follicular reserve of the ovary is age dependent, the age of the patient should be considered before offering this treatment. Many teams worldwide have set the upper limit for undergoing this treatment at 35 years [2▪▪,6,12,26–28].

Ovarian tissue cryopreservation in children is a specific issue. A number of investigators have addressed the question of ovarian tissue freezing during childhood and adolescence [8,10,13,16,29–32,33▪▪], but only a few series have been documented [3▪▪,16]. As recently reviewed [33▪▪], research into personalizing the approach to care is critical if we are to meet the needs of this patient population.

How much ovarian cortex should be harvested for cryopreservation?

This decision is influenced mainly by the estimated risk of POF relative to the planned treatment and existing ovarian volume. Oophorectomy should be performed in patients undergoing pelvic irradiation or total body irradiation and in those receiving high doses of alkylating agents. This procedure should also be performed in very young girls (namely prepubertal girls) because of the small size of their ovaries [16]. Otherwise, in adults, four to five ovarian cortical biopsy samples of approximately 1 cm in length, 4–5 mm in width, and 1.0–1.5 mm in depth are taken in most departments around the world [2▪▪,6], although left oophorectomy is carried out almost systematically in some countries [34].

Techniques of orthotopic (in the pelvic cavity) reimplantation

If an ovary is present, pieces of thawed ovarian cortex are fixed to the medulla after decortication

of the ovary [2▪▪,3▪▪,4,35] or beneath the cortex [27,34]. If no ovary is present, the ovarian tissue pieces are placed in a peritoneal window in an area where some small retroperitoneal vessels are visible [2▪▪,26]. In our opinion, the pelvic cavity (orthotopic site) provides the optimal environment for follicular development compared with heterotopic sites, as temperature, pressure, paracrine factors, and blood supply are similar to those observed in a physiological situation. Even if transplanting ovarian tissue to heterotopic sites has some advantages [36–38], only one pregnancy has been reported following this procedure [39], making this approach somewhat questionable [2▪▪].

LIVE BIRTH RATE: THE TRUE PICTURE

The number of reimplantations performed worldwide (the denominator) is not known. In 2013, results from three centers (Denmark, Spain, and Belgium) were collected to evaluate a series of 60 cases of orthotopic reimplantation [2▪▪]. Restoration of ovarian activity was observed in 93% of patients and the pregnancy rate was 23%. The absence of primordial follicles in the reimplanted tissue explains the lack of postgrafting ovarian activity in the remaining 7% [2▪▪]. Restoration of ovarian activity occurred between 3.5 months and 6.5 months after grafting [2▪▪,6,7,40]. This finding is consistent with follicle growth from the primordial to the antral stage. The mean duration of ovarian function after transplantation is normally about 4–5 years [2▪▪,3▪▪,4].

The true picture

In the light of growing interest in fertility preservation, we feel it is time to carefully evaluate all currently available options to preserve fertility in female patients with cancer and women at risk of POF. It is also necessary to amend some misleading information in a very recent article [41], in which data presented in the tables were confused and inaccurate, as numbers of reported cases and pregnancies did not correspond to those published in the literature. Moreover, the exact number of transplants was unknown, yielding erroneous pregnancy and live birth rates. Many of the cases were simply case reports, making any interpretation unreliable.

Only existing data published in peer-reviewed journals, an analysis of which was recently published, should be taken into account [42▪▪]. To date, 37 live births have been reported in peer-reviewed journals (Table 1). It is important to stress that only published live births can be included in this analysis. A hypothetical number resulting from phone calls to different centers is in no way representative of the facts.

Pregnancy and live birth rates cannot be extrapolated from case reports, as the denominator is unknown. For this reason, results from four centers (Denmark, Spain, Belgium, and Germany) [2▪▪,43▪▪,44▪▪], including a very recent series [44▪▪], were combined, totaling 80 cases (Table 2). These results provide a clear and accurate picture of existing pregnancy and live birth rates.

In this series of 80 cases, the pregnancy rate, expressed as the number of women who conceived,

Table 1. Series of 37 live births after transplantation of frozen-thawed ovarian cortex

	Cryopreservation procedure	Live births		
		Spont	IVF	N
Donnez *et al.*, Dolmans *et al.*	SF	++++	++	6
Meirow *et al.*	SF	+	+++	4
Demeestere *et al.*	SF	++	–	2
Andersen's team	SF	++++	++	6
Silber *et al.*	SF	++	–	2
Piver *et al.*, Roux *et al.*	SF	++	–	2
Sanchez *et al.*, Pellicer *et al.*	SF	++	++ (twins)	4
Revel *et al.*	SF	–	++	2
Dittrich *et al.*	SF	+++	+	4
Revelli *et al.*	SF	+		1
Callejo *et al.*	SF		+	1
Stern *et al.*	SF		++	2
Kawamura *et al.*	VF		+	1

SF, slow freezing; VF, vitrification. The complete list of authors can be found in the reference list [2▪▪,4,42▪▪–44▪▪].

Table 2. Results from four centers, allowing evaluation of pregnancy and live birth rates, as the number of transplants is known

Teams	Number of transplanted women	Women who conceived	Women who gave birth	Number of live births	Miscarriages
Donnez, Dolmans team	13	3	3	6 (*)(**)	
Andersen, Macklon team	25	6	4	6(*)(**)	2
Pellicer team	22	4	3	4	1
Dittrich team	20	7	4 (+2***)	4 (+2***)	1
	80	20 (25%)	14	20	

*One woman delivered twice.
**One woman delivered three times.
***Two ongoing pregnancies.

is 25% (20/80) and the number of women who gave birth is 16. As some women delivered twice or three times after transplantation, the number of live births cannot be used as the numerator. Only the number of women who gave birth can be used as the numerator, whereas the number of transplanted women is the denominator. This last review confirms the previous rate obtained in a first series, published in 2013 [2▪▪].

Combined procedure

In the future, cryopreservation of ovarian tissue might be associated with removal of small antral follicles followed by in-vitro maturation (IVM). Immature oocytes can also be collected from antral follicles in ovarian tissue at the time of the cryopreservation procedure, matured in vitro and then cryopreserved [45,46]. Indeed, for half of the patients who underwent ovarian tissue cryopreservation, this combined procedure offered an additional chance of becoming pregnant. The first live birth resulting from a cryopreserved embryo obtained from in-vitro-matured oocytes collected after oophorectomy was recently reported [47▪▪], as was the second clinical pregnancy [48].

On the contrary, as recently published by Dolmans *et al.* [49▪▪], COS for pickup of mature oocytes could be performed immediately after removal of ovarian tissue for freezing, without any adverse effects on the number of oocytes or embryos. Indeed, cryopreservation of bilateral ovarian cortex followed by IVF treatment is a feasible and well tolerated approach to preserve fertility before oncological treatment. The number of cryopreserved embryos obtained was not statistically different from the control group and not affected by the previous bilateral biopsy carried out for cryopreservation purposes. Moreover, this combined technique does not delay further oncological treatment. It may even be proposed to patients without a male partner in association with oocyte vitrification.

Variable outcomes of transplantation

Dysfunctional folliculogenesis has been described [50] and attributed to a synchrony between granulosa cells and oocyte maturation [51]; a reduced ovarian reserve after grafting; the delay that occurs before efficient revascularization of the graft; and specific postgrafting activation.

Ischemia is responsible for loss of follicles, as the graft needs 4–5 days to be reoxygenated [52]. Ischemia and oxidative stress could be reduced by using drugs to stimulate revascularization of the graft [such as vascular endothelial growth factor or sphingosine-1-phosphate (S1P)] or some inhibitory hormones [e.g., anti-Müllerian hormone (AMH)] that normally operate in an intact ovary [3▪▪].

Another mechanism by which remaining follicles are overactivated due to lack of AMH may also be implicated [53,54]. AMH present in growing follicles after transplantation is able to act as a 'brake' on initial follicular activation (occurring immediately after transplantation) [53], thereby protecting the pool of residual primordial follicles [54].

The concept of the vascular bed is therefore important in this regard [3▪▪]. It involves preparing the host vascular bed prior to grafting by addition of encapsulated vascular endothelial growth factor [55] or stromal cells enriched in CD34, for example, and represents one principal means of improving graft revascularization [56].

Cryopreservation of ovarian tissue: vitrification or slow freezing?

Vitrification of ovarian tissue might be one way of improving outcomes after freezing and reimplantation [57–63,64▪▪–66▪▪].

During the past decade, vitrification has gradually replaced slow-programmed freezing for the cryopreservation of embryos and oocytes. Although all live births (but one) to date have resulted from slow-frozen ovarian cortex [66▪▪], vitrification is an emerging and increasingly pertinent focus of research [58,59,65▪▪,66▪▪].

Compared with slow freezing, vitrification may be associated with improved maintenance of ovarian follicular and stromal structures [57], as well as increased follicle survival rates [58,62,64▪▪–66▪▪], which should lead to improved tissue function after transplantation, even if some teams have obtained similarly good results in terms of follicle survival after slow freezing [2▪▪]. It should nevertheless be pointed out that the high concentrative of cryoprotectant chemicals and ultrarapid cooling rates used for vitrification require direct contact with liquid nitrogen, raising questions about safety. Comparative data on the efficiency of vitrification and slow freezing of ovarian cortex must now be validated in prospective randomized studies, with healthy live birth rates as the primary endpoint [4,64▪▪,67].

Risk of reimplanting malignant cells

One serious concern that remains is the risk of reimplanting malignant cells together with the grafted tissue. A review published in 2013 examined all available evidence of this risk, particularly in patients with leukemia, which is the most common hematological cancer in women below 20 years of age, followed by Hodgkin's lymphoma and non-Hodgkin's lymphoma (Table 3) [68▪▪].

Leukemia

Molecular biology has been used to evaluate the presence of leukemic cells in ovarian tissue from patients with leukemia. PCR showed ovarian tissue from patients with acute leukemia to be positive for malignant cells in more than 50% of patients, leading the researchers to conclude that reimplantation in these patients would be unsafe [69].

Ovaries from leukemia patients in complete remission, on the other hand, were not found to contain malignant cells [70]. These results, even if reassuring, need to be interpreted with caution at this stage and remain to be confirmed [71]. By contrast, to avoid compromising the pool of primordial follicles by exposure to chemotherapy before cryopreservation, alternative methods such as IVM [72▪▪] or isolated follicle transplantation (artificial ovary) should be further investigated and could be proposed to patients with leukemia [71,73].

In conclusion, each type of leukemia might represent a different risk scenario depending on how the first course of chemotherapy is performed, how long the patient has been in remission, and other factors, such as the number of viable malignant cells present in the tissue that could cause relapse [68▪▪].

The particular case of breast cancer

As breast cancer is the most frequently encountered type of cancer in women, a number of studies have investigated the possible presence of malignant cells in cryopreserved ovarian cortex from patients with breast cancer.

Two studies analyzed frozen-thawed ovarian biopsy samples from women with breast cancer [74,75], but neither found any evidence of malignant cell infiltration of the cryopreserved ovarian tissue by histology or immunohistochemistry. Autotransplantation of frozen-thawed ovarian fragments also appears to be well tolerated in patients with early-stage breast cancers [2▪▪].

A third study reported a series of 13 cases of advanced-stage breast cancer [76▪]. *MGB2* gene expression was detected in five cases by quantitative PCR, but histology and immunohistochemistry failed to identify malignant cells in the ovarian

Table 3. Risk of ovarian metastasis according to cancer

High risk (>11%)	Moderate risk (0.2–11%)	Low risk (<0.2%)
Leukemia	Breast cancer	Breast cancer
	Stage IV	Stages I–II
	Infiltrating lobular subtype	Infiltrating ductal subtype
Neuroblastoma	Colon cancer	Squamous cell carcinoma of the cervix
Burkitt lymphoma	Adenocarcinoma of the cervix	Hodgkin's lymphoma
	Non-Hodgkin's lymphoma	Osteogenic carcinoma
	Ewing's sarcoma	Nongenital rhabdomyosarcoma
		Wilm's tumor

tissue. The xenografted mice did not become ill [76▪].

In conclusion, although these studies are encouraging so far, reassuring, they nevertheless demonstrate that further procedures, such as PCR and long-term xenografting, are required to prove the safety of frozen-thawed ovarian tissue transplantation in women with advanced-stage breast cancer [76▪].

Future directions

For the future, some new directions should be investigated and optimized.

Mitigating the effects of gonadotoxic treatment

As suggested by De Vos *et al.* [64▪▪], mitigation strategies to reduce the undesired effects of chemotherapy and/or radiotherapy on the ovaries need to be developed and evaluated. One approach involves encapsulating the chemotherapeutic agent together with a nanoparticle, whereas another, still controversial, technique looks to inhibit the immediate early apoptotic effects of chemotherapy and radiation on primordial follicles. Primordial follicles are very susceptible to gonadotoxic drugs because of the process of programmed cell death, as indicated by high p63 expression levels [77,78].

Modulation of apoptosis-related pathways may thus be considered a possible new therapeutic approach to chemoprevention, with sphingolipids such as S1P known to reduce death of primordial follicles and block ovarian apoptosis in cultured human ovaries.

Clinical application of S1P therapy is, however, still problematic. First, S1P has both proangiogenic and antiapoptotic effects, and systemic S1P administration may increase the risk of disease progression [79,80]. Second, because women do not have an ovarian bursa as mice, an alternative route for delivery is needed. Use of a drug-delivering scaffold applying tissue engineering technology or injection into the ovary for local treatment may be potential solutions for women in the future. Nevertheless, the feasibility of S1P therapy in humans remains to be determined, and additional trials need to be designed to address its clinical suitability.

Follicle culture and in-vitro development

Initiation of primordial follicle development and early growth, and optimization of development from the preantral to the antral stage, with completion of oocyte growth, are critical steps in folliculogenesis [2▪▪,79,80].

A dynamic multistep culture system is therefore required to support each of the transitional stages of follicles [72▪▪,81]. Such a multistep approach for follicles grown *in vitro* needs to meet the changing requirements of the developing oocyte. The ultimate objective is to obtain developmentally competent oocytes capable of yielding endpoints.

The artificial ovary

The aim of this strategy is to obtain mature oocytes with the help of the so-called transplantable artificial ovary, currently under development. Isolation of primordial follicles and their transfer onto a specially created scaffold will, of course, eliminate the risk of transmission of malignant cells [73].

Whole-ovary cryopreservation and transplantation

The first reports on human whole-ovary retrieval and freezing published by Martinez-Madrid and Donnez [82] and Jadoul *et al.* [83] were not followed by active research in this area. Thus, the technique has not so far enjoyed the same success as ovarian cortical tissue freezing and transplantation. Some studies have nevertheless been conducted. In an experimental study, Nichols-Burns *et al.* [84] encountered loss of four out of 10 ovaries because of anastomotic thrombosis. Very recently, Campbell *et al.* [85] demonstrated that full restoration of ovarian function and high rates of fertility can be obtained after whole-ovary cryopreservation and transplantation by optimizing cryoprotectant penetration during perfusion, along with use of antithrombotic agents to prevent postoperative clot formation in the ovarian vasculature.

CONCLUSION

Aggressive chemotherapy and/or radiotherapy and bone marrow transplantation can cure more than 90% of girls and young women affected by diseases requiring such treatment. However, the risk of impairing gonadal function is high, particularly when gonadotoxic drugs (especially alkylating agents) are used.

Embryo and oocyte cryopreservation are the options of choice (endorsed by the ASRM) if chemotherapy can be delayed, giving patients with cancer hope of a successful pregnancy when they have overcome their disease. In prepubertal girls and patients who require immediate treatment, ovarian tissue cryopreservation is the only available alternative [2▪▪].

In the future, cryopreservation of ovarian tissue might be combined with removal of small antral follicles (by puncture), making it possible to freeze both ovarian tissue and isolated immature oocytes, including those present in the dissection medium. Indeed, they may well be highly abundant in the medulla, especially in very young patients.

In July 2013, the American Society of Clinical Oncology (ASCO) [86▪▪] added some clarifications to the 2006 ASCO recommendations, with the goal of updating guidance for healthcare providers. Healthcare providers (including gynecologic oncologists, and pediatric oncologists and hematologists) should address the possibility of infertility with patients requiring treatment with gonadotoxic drugs and/or irradiation as part of the informed consent procedure, and advise them as to available methods of fertility preservation, referring them to appropriate reproductive specialists. In the ASCO recommendations, it is stressed that no patients should be excluded from discussion of fertility preservation options for any reason (such as age, prognosis, or parity), even if financial or insurance barriers might be present [86▪▪].

Optimizing techniques and minimizing the risks of fertility preservation strategies represent key challenges of the next decade. In the near future, indications will expand to include benign diseases (such as recurrent endometriosis) and social reasons, even if the most immediate concern is helping women affected by cancer.

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Conflicts of interest

There are no conflicts of interest.

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