Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: a review of 60 cases of reimplantation

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Aggressive chemotherapy/radiotherapy and bone marrow transplantation can cure >90% of girls and young women affected by disorders requiring such treatment. However, the ovaries are very sensitive to cytotoxic drugs, especially to alkylating agents. Several options are currently available to preserve fertility in cancer patients. The present review reports the results of 60 orthotopic reimplantations of cryopreserved ovarian tissue performed by three teams, as well as 24 live births reported in the literature to date. Restoration of ovarian activity occurred in almost all cases in the three series. Among the 60 patients, eleven conceived and six of those had already delivered twelve healthy babies. In the future, we are looking to: 1) improve freezing techniques; and 2) enhance the “vascular bed” before reimplantation to increase pregnancy rates. On the other hand, 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. (Fertil Steril® 2013;99:1503–13. ©2013 by American Society for Reproductive Medicine.)

Key Words: Cryopreservation, ovarian tissue, chemotherapy

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Aggressive chemotherapy/radiotherapy and bone marrow transplantation (BMT) can cure >90% of girls and young women affected by disorders requiring such treatment. However, the ovaries are very sensitive to cytotoxic drugs, especially to alkylating agents, which are classified as having high risk for gonadal dysfunction (1–6). Cyclophosphamide is the agent most commonly implicated in causing damage to oocytes and granulosa cells in a dose-dependent manner (4–6). Abdominal ionizing radiation associated with alkylating agents often induces premature ovarian failure (POF), rendering patients infertile in almost 100% of cases. Indeed, for radiotherapy, it has been reported that a dose of 5–20 Gy administered to the ovary is sufficient to completely impair gonadal function (4–7), whatever the age of the patient. Intensive chemotherapy and/or total body irradiation (TBI) required before BMT constitute the treatment combination presenting the greatest risk of POF. The risk of POF was estimated to be 92% in a study by Metrow and Nugent (8), and Teinturier et al. (9) actually reported 0% ovarian recovery after treatment with the alkylating substance busulfan before BMT. Recently, Jadoul et al. (10) reported that ovarian function is
impaired in the majority of women after BMT and that this impairment is mostly related to older age at the time of treatment and conditioning treatment with TBI (10).

**FERTILITY PRESERVATION IN CANCER PATIENTS: DIFFERENT CRYOPRESERVATION OPTIONS**

Several options are currently available to preserve fertility in cancer patients and give them the opportunity to become mothers when they have overcome their disease: embryo cryopreservation, immature or mature oocyte cryopreservation, and ovarian tissue cryopreservation. Oocyte and embryo cryopreservation will be discussed elsewhere in this issue by Cobo et al. and Bedoschi and Oktay (11, 12).

Cryopreservation of ovarian tissue is the only option available for prepubertal girls and for women who cannot delay the start of chemotherapy (1, 3). Cryopreservation of ovarian tissue should be seriously considered for any patient undergoing treatment likely to impair future fertility, the indications being pelvic, extrapelvic, and/or systemic malignant diseases, as well as nonmalignant diseases (1, 3). The age of the patient should be taken into account, because the follicular reserve of the ovary is age dependent (10). It is well documented that fertility becomes compromised during the mid–30s, and the procedure should probably not be offered to women after the age of 38 years, but restricted to patients below this limit (1, 3, 13). In many departments, the limit is fixed at 35 years (1, 3, 13). In the Valencia fertility preservation program, antimüllerian hormone (AMH) and antral follicle count (AFC) are used as selection criteria.

**OVARIAN CRYOPRESERVATION IN CHILDREN: A SPECIFIC ISSUE**

There is currently no consensus or directive on the age at which reproductive potential is actually reached, making it unclear how recommendations can be effectively applied to cancer patients under the age of 18 years.

The most common reasons given for not discussing fertility preservation options in a pediatric cancer population include “not at significant risk” in 29% of cases, “too young” in 27%, “techniques unproven” in 22%, “no facilities” in 10%, and “no funding” in 8% (6, 10, 13).

A number of authors have addressed the question of ovarian tissue cryopreservation during childhood (4–6, 14–21), but only a few series performing this procedure in children have been documented (20) (Table 1). Jadoul et al. reported the largest series of ovarian tissue cryopreservation in girls <16 years old. When classified according to Wallace et al.’s list of risk factors for fertility impairment (7), Jadoul et al. found that 14 patients were at low risk, 32 at medium risk, and 4 at high risk of POF, but they demonstrated that it is difficult to give the patient or her parents an accurate assessment of the risk to fertility, because disease evolution is never completely predictable (20). Indeed, ~20% of girls changed categories from low/medium to high risk because BMT was needed or because further gonadotoxic drugs were required for disease recurrence. Moreover, persistent ovarian function does not exclude the risk of subsequent POF (10, 20).

An important issue is the quantity of ovarian cortex to be harvested for cryopreservation. The decision as to how much ovarian cortex to remove is influenced mainly by the estimated risk of ovarian failure related to the planned treatment and existing ovarian volume. For cases with pelvic irradiation, TBI, and high doses of alkylating agents, oophorectomy should be performed. It should also be performed in very young girls owing to the small size of the ovaries (10, 20).

In Poirot et al.’s series (16) a whole ovary was removed in all cases, whereas in Anderson et al.’s series (21) the procedure involved biopsies of cortical tissue. In Jadoul et al.’s series (20), 20 girls (mean age 6.5 years) underwent unilateral oophorectomy and 38 girls (mean age 12.4 years) had cortical biopsies taken without oophorectomy.

**ORTHOTOPIC AUTOTRANSPLANTATION OF CRYOPRESERVED HUMAN OVARIAN TISSUE**

The main aim of ovarian tissue cryopreservation is to restore fertility. The method involves reimplantation of cortical ovarian tissue into the pelvic cavity (orthotopic site) or a heterotopic site like the forearm or the abdominal wall once treatment is completed and the patient is disease-free. First, we should be very clear about the terms we use. Orthotopic means in the pelvic cavity on the remaining ovary or close to it or in the uterine environment. Heterotopic means clearly outside the peritoneal cavity. Natural pregnancy may be achieved via orthotopic tissue transplantation if the fallopian tubes remain intact.

**TECHNIQUES OF ORTHOTOPIC AUTOTRANSPLANTATION**

There are essentially two techniques that may be used depending on the presence or not of at least one remaining ovary.

1. If at least one ovary is present, the technique starts with decortication of the ovary. A large piece of ovarian cortex is removed to have access to the medulla (Fig. 1) and its vascular network. Even if this area shows some signs of fibrosis, a vascular network is still present (albeit significantly reduced) (1, 26). Ovarian cortical pieces are then fixed with the use of 7-0 or 8-0 propylene stitches according to previously described microsurgical techniques (1, 3, 27, 28), or simply placed on the medulla and fixed with Interceed or fibrin glue (26, 28). This procedure can be performed by laparoscopy or laparotomy. Half of the patients in Pellicer’s group series underwent this procedure under microsurgical assistance, as described by Silber et al. in his series of fresh tissue reimplantation (27). In Andersen’s group (29), ovarian tissue is deposited on the medulla after making an incision in the cortex; the tissue is thus placed in a subcortical area, as previously described.
2. If both ovaries are absent, a peritoneal window may be created in two steps, as in the case published in 2004 (30), to induce angiogenesis before the grafting procedure or in one step as recently described (31). 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; Fig. 2). The fragments are placed in the window and subsequently covered with Interced, the edges of which are fixed with fibrin glue. The peritoneal window method may also be performed if a nonfunctional ovary is still in place.

**REVIEW OF THREE CENTERS (BELGIUM, DENMARK, SPAIN) (TABLE 2)**

We analyzed data from Donnez’s group, Andersen’s group, and Pellicer’s group to evaluate 60 orthotopic transplantations. All of the ovarian biopsies were frozen with the slow-freezing procedure and in the majority of cases (73%) the ovarian cortex was removed before any chemotherapy. The indications were hematologic diseases in 35% of cases, malignant pathology (other than hematologic diseases) in 45%, and benign pathology (Turner syndrome, family history of POF, endometriosis, recurrent ovarian cysts, etc.) in 20%.

**Restoration of Ovarian Activity**

Restoration of ovarian activity was observed in at least 52 cases among the 56 (93%; Table 2). In the reported series, the peritoneal window created close to the ovarian hilus and the ovarian medulla both proved to be equally efficient sites of reimplantation, at least for restoration of ovarian activity (as evidenced by follicular development). Large strips (8–10 mm × 5 mm) and small cubes (2 mm³) of tissue were both shown to effectively restore ovarian endocrine function.

In Donnez’s series, restoration of ovarian function was observed in all cases but three, where no follicles were present in the reimplanted frozen-thawed ovarian tissue, explaining the absence of ovarian activity restoration. Among these three women, two had undergone ovarian tissue cryopreservation in another country and one in another hospital. This highlights the importance of evaluating follicular density before making the decision to reimplant tissue.

### TABLE 1

<table>
<thead>
<tr>
<th>Authors</th>
<th>Patients</th>
<th>Age (y)</th>
<th>Patients &lt;16 y</th>
<th>Patients &lt;10 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feigin et al. (22)</td>
<td>23</td>
<td>5–17.5</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Poirot et al. (16)</td>
<td>47</td>
<td>0.8–15</td>
<td>47</td>
<td>38</td>
</tr>
<tr>
<td>Anderson et al. (21)</td>
<td>36</td>
<td>5–35</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Revel et al. (23)</td>
<td>19</td>
<td>5–20</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Oktay and Oktem (24)</td>
<td>26</td>
<td>4–21</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Borgstöm et al. (25)</td>
<td>57</td>
<td>8–19.8</td>
<td>40</td>
<td>4</td>
</tr>
<tr>
<td>Jadoul et al. (20)</td>
<td>58</td>
<td>0.8–15.8</td>
<td>58</td>
<td>21</td>
</tr>
</tbody>
</table>

*Note: Adapted from Jadoul et al. (20).* 


**FIGURE 1**

Ovarian cortex reimplantation on the ovarian medulla. Large pieces of thawed ovarian cortex are sutured to the medulla. From Donnez et al., 2006 (1).


**FIGURE 2**

Ovarian cortex reimplantation in a peritoneal pocket. Large pieces of ovarian cortex are placed in a peritoneal pocket. From Donnez et al., 2012 (31).

In Pellicer’s series, four patients underwent transplantation only three months before the writing of the present paper. None of them had resumed ovarian function by then, and a longer follow-up will be required to correctly assess their outcomes. Seventeen out of 18 patients were shown to have recovered ovarian function.

In Andersen’s series, all 25 patients regained ovarian function. However, in one patient, only three pieces of cortical tissue were originally retrieved and transplanted, and in another the woman was 36 years old at the time of retrieval. In these two cases, ovarian activity did not exceed 8 months (32).

In all instances, it took 3.5–6.5 months after reimplantation before a rise in E2 and a decrease in FSH were detected (mean 4.5 months). The time interval between implantation of cortical tissue and the first E2 peak is consistent with data obtained from sheep and humans, although some variation may be observed (1, 3, 26, 30). Such variation may be explained by a difference in follicular reserve at the time of cryopreservation, some women having already received a first regimen of chemotherapy before ovarian biopsy and cryopreservation (26). Indeed, the time to the E2 peak, concomitant with the FSH decrease, was longer (5.5–6.5 months) in patients who had received chemotherapy before cryopreservation than in those who had not (3.5–4.5 months) (26).

Pregnancies (Tables 2 and 3)

Table 2 summarizes data from the three groups, including a total of 60 patients who underwent orthotopic transplantation. Eleven patients became pregnant (including two ongoing pregnancies at 24 and 30 weeks), and six of these women have already delivered 12 healthy babies.

Table 3 shows the worldwide series of 24 live births obtained to date. The slow-freezing procedure was used in all cases. The fact that >50% of women were able to conceive naturally constitutes a good argument in favor of orthotopic reimplantation.

The age of patients at the time of cryopreservation is a predictive factor, as previously reported (26). Indeed, the majority of pregnant women were <30 years old. In Donnez’s group, a woman whose tissue was cryopreserved at the age of 17 years and reimplanted at the age of 25 years has already given birth to three healthy babies in <3 years (Table 3). In Andersen’s group also, a woman has delivered three healthy babies after cryopreservation and reimplantation.

The peritoneal window created close to the ovarian hilus proved to be effective in the cases described here (1, 26, 33–35). In other cases, tissue was grafted to the medulla after decortication or placed under the cortex (1, 3, 32, 36, 37). Although it has been shown that chemotherapy causes damage to ovarian blood vessels by hyalinization and lumen narrowing, neovascularization with CD34, proliferation has been concomitantly demonstrated (38). The angiogenic potential of the ovary probably explains why the ovarian medulla is a good grafting site, even after several years of atrophy (3).

It is important to stress that all babies born were healthy and that no recurrence of the initial disease was observed.

| Team       | Patients  | Freezing technique | Cryopreservation before chemotherapy | Pathology | Average time for ovarian activity (no. of patients) | Outcome | Ovarian medulla | Total no. of pregnancies | Natural IVF patients | Spontaneous IVF patients | No. of live births | No. of live births | No. of live births | No. of live births | No. of live births | No. of live births |
|------------|-----------|-------------------|-------------------------------------|-----------|-----------------------------------------------|---------|----------------|------------------------|-------------------|------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Belgium    | 13        | Slow-freezing     | 77% (9/13)                          | Hematologic malignancies             | 4.5 mo                                           | 6       | Ovarian medulla | 95.5% (21/22)         | 84% (17/20)       | 79% (17/22)            | 61.5% (14/23) | 54% (13/24) | 36% (8/22) | 17% (3/18) | 21% (4/19) | 16% (3/19) | 16% (3/19) |
| Denmark    | 25        | Slow-freezing     | 88% (22/25)                         | Hematologic malignancies             | 4.5 mo                                           | 8       | Ovarian medulla | 100% (25/25)          | 72% (18/25)       | 68% (17/25)            | 61.5% (16/26) | 45% (11/24) | 36% (9/25) | 25% (6/24) | 25% (6/24) | 25% (6/24) | 25% (6/24) |
| Spain      | 22        | Slow-freezing     | 55% (12/22)                         | Hematologic malignancies             | 4.4 mo                                           | 4 d     | Ovarian medulla | 100% (22/22)          | 77% (17/22)       | 73% (16/22)            | 61.5% (14/23) | 45% (10/22) | 36% (8/22) | 18% (4/22) | 18% (4/22) | 18% (4/22) | 18% (4/22) |

* For the three patients without restoration of ovarian activity, cryopreservation was performed in other centers. No follicles were observed in frozen-thawed ovarian fragments used for histologic analysis in any of those three cases.

** Including four patients with ongoing pregnancies at 24 and 30 wk.

LESSONS: WHAT CAN WE LEARN FROM THE PRESENT STUDY AND THE EXISTING LITERATURE?

Thickness of the Biopsy

Based on the results of earlier studies (1, 3, 13, 39), we recommend taking a 1–1.5-mm-thick biopsy of ovarian cortex. Indeed, primordial follicles can be found at a distance of 0.8 mm from the mesothelium (Fig. 3). We consider this recommendation to be of paramount importance, because superficial or very thin biopsies can result in an absence of primordial follicles in the removed cortex. As previously mentioned, no follicles were found in such cases in Donnez’s series. In two cases, biopsies performed in another country were too thin and therefore inadequate.

Transport of the Biopsy

Even if most authors recommend that ovarian tissue be transported in optimal conditions to the laboratory for freezing, and that transport times be reduced as much as possible, the Danish experience (40) has proved that ovarian tissue may be successfully frozen after 4–5 hours of transport. Indeed, pregnancies were reported in this group after autologous transplantation of ovarian tissue that had been transported for this length of time before freezing (32, 40, 41).

Slow Freezing Versus Vitrification

A major development currently under investigation in an experimental model is vitrification of ovarian tissue fragments. The debate is very relevant, because all pregnancies obtained after human ovarian tissue reimplantation have so far been from tissue frozen according to the slow-freezing technique (Table 3).

However, vitrification results are increasingly promising. In an extensive review, Amorim et al. (42) summarized the principles of vitrification and stressed the need to understand how vitrification solutions and protocols can affect ovarian tissue. Both slow freezing and vitrification were found to preserve the morphology of primordial and primary follicles (42), but secondary follicles and stroma were consistently better preserved with vitrification, based on gross histologic observations (43). Although stromal integrity has been suggested to be maintained after vitrification (43, 44, 45) and damaged after slow freezing (42, 46, 47) in earlier studies, Zelinski’s group was the first to show morphologically intact secondary follicles in macaque ovarian tissue after vitrification (43). Ting et al. (43) recently demonstrated that granulosa cell

### TABLE 3

Series of 24 live births after transplantation of frozen-thawed ovarian cortex.

<table>
<thead>
<tr>
<th>References</th>
<th>Cryopreservation procedure</th>
<th>Graft site</th>
<th>Live birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donnez et al. (1, 3, 26, 30, 31)</td>
<td>SF</td>
<td>Peritoneal window (2 steps)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peritoneal window (1 step)</td>
<td>++</td>
</tr>
<tr>
<td>Meirow et al. (58)</td>
<td>SF</td>
<td>Ovarian medulla</td>
<td>+++</td>
</tr>
<tr>
<td>Demeestere et al. (33)</td>
<td>SF</td>
<td>Beneath the ovarian cortex</td>
<td>–</td>
</tr>
<tr>
<td>Andersen et al. (40, 41, 76, 77)</td>
<td>SF</td>
<td>Ovarian and peritoneal windows (2 steps)</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Subcortical ovarian pocket</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ovarian medulla</td>
<td>+</td>
</tr>
<tr>
<td>Silber et al. (37, 75)</td>
<td>SF</td>
<td>Ovarian medulla</td>
<td>++</td>
</tr>
<tr>
<td>Piver et al. (24)</td>
<td>SF</td>
<td>Ovarian medulla</td>
<td>+</td>
</tr>
<tr>
<td>Roux et al. (35)</td>
<td>SF</td>
<td>Ovarian medulla</td>
<td>+</td>
</tr>
<tr>
<td>Sanchez et al. (28)</td>
<td>SF</td>
<td>Peritoneal window (slice)</td>
<td>–</td>
</tr>
<tr>
<td>Revet et al. (78)‡</td>
<td>SF</td>
<td>Peritoneal window (slice)</td>
<td>++ (twins)</td>
</tr>
<tr>
<td>Dittrich et al. (79)</td>
<td>SF</td>
<td>Peritoneal window</td>
<td>+</td>
</tr>
<tr>
<td>Revelli et al. (80)</td>
<td>SF</td>
<td>Ovarian medulla</td>
<td>+</td>
</tr>
<tr>
<td>Garcia Rada (81)</td>
<td>SF</td>
<td>Peritoneal medulla</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peritoneal window (2 steps)</td>
<td>(+ twins)</td>
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<td></td>
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<td>Peritoneal window (1 step)</td>
<td>(+ twins)</td>
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<td></td>
<td></td>
<td>Subcortical ovarian pocket</td>
<td>(+ twins)</td>
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<td></td>
<td></td>
<td>Ovarian medulla</td>
<td>(+ twins)</td>
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<td></td>
<td></td>
<td>Ovarian medulla</td>
<td>(+ twins)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peritoneal medulla</td>
<td>(+ twins)</td>
</tr>
</tbody>
</table>

Note: Four ongoing pregnancies at the present time: two in Spain, one in South Africa, and one in Australia.

‡ Personal communication, 2012.


FIGURE 3

Ovarian cortical biopsy demonstrating the distance between the mesothelium and primordial follicles, ranging from 414 μm to 617 μm.

damage in secondary follicles was observed more frequently after slow freezing than after vitrifaction. This damage to granulosa cells was also observed by Nottola et al. after slow freezing (48), as was dysynchrony between granulosa cell proliferation and oocyte maturation.

Morphology and growth of preantral follicles from cryopreserved human ovarian tissue has been examined only in frozen, not vitrified, tissues and tested for up to 14 days in culture (49, 50). Ting et al. were the first to demonstrate long-term secondary follicle viability and function in vitro after tissue vitrifaction with the use of encapsulated three-dimensional culture (43).

With slow freezing, extracellular ice formation can sometimes cause damage, especially when cell density is high and cell-to-cell connections are extensive (as in ovarian tissue) (42, 49–51). In contrast, vitrifaction was designed to prevent all forms of ice crystals; therefore, it should benefit tissue cryopreservation (51, 52). As stressed by Amorim et al. in a review (42), vitrifaction depends on the balance between cryoprotective agent toxicity and concentrations of cryoprotectant needed for successful vitrifaction and optimal cooling and warming rates. Factors that can affect the outcome of vitrifaction include tissue size, cryoprotectant type, exposure time, and temperature (42, 43).

Nonhuman primates will continue to be an important model that can be used to systematically compare and optimize vitrifaction protocols, as well as test novel cryoprotective agents and various closed-system devices for eventual clinical use (42, 43). Donnez’s group very recently obtained encouraging results after vitrification of cryopreserved human ovarian tissue in baboons. Indeed, normal follicular density and viability, as well as development of antral follicles and corpus luteum, were observed (53). However, these encouraging results are not sufficient to allow us to propose vitrifaction of human ovarian tissue as yet, and only live births in primates obtained after reimplantation of vitrified tissue would represent a strong enough argument in its favor. Indeed, pregnancy and live birth constitute the strongest proof that the quality of both the granulosa cells and oocyte are well preserved.

Orthotopic Versus Heterotopic (Table 4)

In theory, ovarian tissue can be transplanted orthotopically (into the pelvic cavity or onto the ovarian medulla or peritoneal window) or heterotopically (to the abdominal wall, forearm, rectus muscle, etc.) (54, 55). Nevertheless, the clinical value of heterotopic reimplantation remains questionable owing to the lack of pregnancy with this approach compared with the 24 live births obtained after orthotopic reimplantation (Table 3). Moreover, heterotopic sites may not provide an optimal environment for follicular development, possibly because of differences in temperature, pressure, paracrine factors, and blood supply. The pelvic cavity with its intraperitoneal environment (peritoneal fluid and specific oxygenation) is likely to be a better area for follicular development. (1, 3, 32, 36). However, restoration of endocrine function has been demonstrated consistently after heterotopic ovarian transplantation (56). In a very recent study by Kim (56), all five patients recovered endocrine function 3–5 months after grafting of frozen-thawed ovarian tissue to the space between the rectus muscle and rectus sheath.

According to Oktay et al. (54, 55) and Kim (56), the advantages of transplanting ovarian tissue to heterotopic sites include: 1) avoidance of invasive procedures; 2) easy recovery of oocytes; 3) cost-effective technology when repeated transplantation is required; and 4) feasibility even in case of severe pelvic adhesions that preclude orthotopic transplantation (Table 4).

Quality of Eggs

Some patients required IVF to become pregnant, but it should be pointed out that in series published by Andersen et al. (13, 32), Dolmans et al. (57), and Meirow et al. (58), an empty follicle rate as high as 29%–35% was observed during the IVF procedure after stimulation by gonadotropins. This dysfunctional folliculogenesis could be due to asynchrony between granulosa cells and oocyte maturation, as reported in human ovarian xenotransplants, the granulosa cells being mature while the oocytes are immature (48, 59). The oocyte itself could be damaged by freezing and thawing procedures, leading to a higher rate of empty follicles or oocyte alterations, whereas granulosa cells may be more resistant, as demonstrated by several studies (13, 32, 57, 58) where a rate of >30% empty follicles was observed during IVF attempts after ovarian cortex reimplantation. Altered folliculogenesis may also be due to markedly reduced ovarian reserve after grafting. Grafting of cortical tissue with sporadically low follicular density could be the cause of dysfunctional folliculogenesis.

<table>
<thead>
<tr>
<th>TABLE 4</th>
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<tbody>
<tr>
<td>Advantages and disadvantages of heterotopic and orthotopic sites for ovarian tissue reimplantation.</td>
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<tr>
<td><strong>Heterotopic (subcutaneous)</strong></td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
</tr>
<tr>
<td>No limitation of number of fragments transplanted.</td>
</tr>
<tr>
<td>Easy transplantation procedure.</td>
</tr>
<tr>
<td>Easy access for follicular monitoring and oocyte collection.</td>
</tr>
<tr>
<td><strong>Disadvantages</strong></td>
</tr>
<tr>
<td>Restoration of fertility not yet demonstrated.</td>
</tr>
<tr>
<td>IVF procedure required.</td>
</tr>
<tr>
<td>Effect of the local environment on oocyte quality is unknown.</td>
</tr>
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</table>

Another cause of damage to oocytes is the delay that occurs before efficient revascularization of the graft is achieved after transplantation (60–62). Other factors, such as the existing vascular network in the ovarian medulla, also influence the revascularization rate, highlighting the crucial role of the preexisting vascular network and giving rise to the concept of the “vascular bed” that was recently proposed (63).

**Concept of the Vascular Bed**

After reimplantation, >50% of primordial follicles are lost, mainly due to tissue ischemia after transplantation while awaiting angiogenesis. Two mechanisms are responsible for this follicular loss: 1) ischemic injury and the delay in reoxygenation (61, 62); and 2) follicular activation (64, 65).

The first mechanism to provoke loss of follicles is ischemia. It was clearly demonstrated that reoxygenation of ovarian grafts occurs from day 5 onward, as proved by the significant increase in pO2 values assessed by electron paramagnetic resonance from day 5 after grafting. It was also demonstrated, by revascularization studies, that initiation of reperfusion of grafts also takes place on day 5 and, very importantly, that chimeric vessels are present, evidencing the essential role of graft vessels as well as host vessels (61, 62). Prevention of ischemic tissue damage is therefore essential to reduce follicular loss. Angiogenesis may be stimulated by delivery of angiogenic factors (vascular endothelial growth factor (VEGF), gonadotropins, etc.), and administration of antiapoptotic factors (Kit ligand, c-Kit, insulin–like growth factor I and II, 8br–cyclic guanosine monophosphate, growth and differentiation factor 9, etc.) could also play a role in the survival of follicles (60). However, most studies were performed on the basis of in vitro culture experiments and there is no robust evidence that these agents would work in vivo in human beings (63).

The second mechanism to provoke loss of follicles is follicular activation (64, 65). This activation should be considered as accelerated growth without brakes (Fig. 4). However, this accelerated growth is not physiologic, as discrepancies in granulosa cell maturation and oocyte maturation have been detected (48). Moreover, freezing and thawing procedures affect morphology, particularly the thickness of the theca cell layer (65). Since theca cells are implicated in follicular development, providing structural support for follicles, the concept of vascular bed preparation prior to grafting should be further explored in order to significantly decrease follicular loss. Recent studies in the baboon and rhesus macaque (unpublished data) have demonstrated that grafting to a “fresh” ovarian hilus (24 hours after oophorectomy) yields better follicular survival than to a fibrotic area (1 month after oophorectomy). In practice, the site of reimplantation could be prepared by encapsulated VEGF (66) and/or stromal cells enriched in CD34 cells (67).

Very recent publications (68) suggest that host treatment with melatonin or graft incubation with hyaluronic acid–rich biologic glue (hyaluronan), especially combined with VEGF-A and vitamin E, can improve graft survival. It is nevertheless unclear whether increasing vascularization in grafted cortex is beneficial or not to maintaining ovarian reserve after grafting. Indeed, Delgado-Rosas et al. (69) and Meirow et al. (38) reported changes in microvessel density in aged ovarian cortex, as well as in cortical tissue exposed to chemotherapy. These two reports may indicate, although it is unclear whether it is the cause or result of a decreased ovarian reserve, that increased cortical vascularity may be beneficial to growth of advanced-stage follicles but that it may also be harmful to dormant primordial follicles.

All these recent data stress 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.
Ovarian Function Restoration and Duration of Activity

In the majority of cases, follicular development occurs within 4–5 months of transplantation, but great variability has been observed. Indeed, even if it is generally accepted that a period of ≥120 days is needed to initiate follicular growth, restoration of ovarian activity has been observed to occur in a wide window, ranging from 8 to 26 weeks. Some authors have reported very quick restoration (2 months) (5, 27, 70). It is not excluded that follicular activation observed in experimental models (64) or survival of antral follicles (which is infrequent, because they are not cryoresistant) may be responsible for the rapid restoration of ovarian activity observed in some, albeit few, cases. Indeed, in the initial period following the grafting procedure (in an experimental model), significantly higher numbers of primary and secondary follicles were observed in grafted fragments than in fresh tissue (64). Hypoxic stress and lack of AMH in ovarian grafts at the time of grafting provoke follicular activation and growth, and it is only later that AMH produced by secondary follicles exerts an inhibitory effect on follicular activation (65) (Fig. 4).

There are individual variations in the duration of endocrine function after transplantation. Nevertheless, it appears that the lifespan of ovarian grafts is longer than expected. It has been reported that ovarian function can persist for 7 years after transplantation of fresh or cryopreserved ovarian tissue, but we should consider that the mean duration is ~4–5 years if follicular density is well preserved (1, 3, 26, 32, 55, 56, 71). Ovarian function markers are of limited value following transplantation (72). Indeed, AMH remains undetectable and inhibin-β levels were found to be low and to vary between individuals. It was demonstrated that AMH and inhibin-β values were not associated with the duration of ovarian graft function or probability of achieving pregnancy (72).

Several factors can affect ovarian graft longevity, including: 1) ovarian reserve (follicular density, which is age dependent); 2) chemotherapy before cryopreservation; 3) graft size and method of ovarian tissue preparation (freezing-thawing techniques); 4) inhomogeneous distribution of follicles in grafted cortical pieces; and 5) angiogenic potential of the graft site influencing the degree of ischemia after transplantation.

Interestingly, Poirot et al. (73) and Andersen’s group (74) were able to induce puberty by reimplanting frozen-thawed prepubertal ovarian tissue in two young girls, proving that ovarian tissue biopsied and cryopreserved before puberty is functionally normal after reimplantation, at least in terms of endogenous production of steroids. This new development will nevertheless need to be validated in the future.

THE FUTURE

Despite the fact that there have been “only” 24 babies born using this method in the last decade, frozen-thawed ovarian tissue reimplantation should no longer be considered an experimental procedure (Table 3). It is difficult to perform statistical analysis on the efficiency of the technique, because a limited number of women have undergone ovarian tissue autotransplantation compared with the number of patients who have had their tissue cryopreserved. In the present review, analyzing the results of Andersen’s, Pellicer’s, and Donnez’s series, 11 patients out of 60 conceived, and 6 of them have already delivered 12 healthy babies.

Ovary harvesting could also lead to the development of additional techniques for fertility preservation (82). If chemotherapy can be delayed, vitrification of mature oocytes or embryo freezing (if sperm is available) could be initiated first after ovarian tissue cryopreservation (Fig. 5). If there is a risk of transmission of malignant cells, as in the case of leukemia (83–86), isolation of primordial follicles followed by transplantation of an alginate matrigel matrix containing isolated ovarian cells could be envisaged in the light of ongoing research in the field, one of the main lines of investigation for the future (87).

FIGURE 5

Fertility preservation methods in women at risk of POF depending on patient age and possible delay before starting chemotherapy.

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Indeed, in the coming years, we are looking essentially to:
1) improve freezing techniques; and 2) enhance the vascular bed before reimplantation to increase pregnancy rates. On the other hand, 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, including those present in the dissection medium when ovary fragments are generated and preantral follicles are present in the medulla, where they may be highly abundant (78, 88, 89).

In conclusion, since the first live birth after orthotopic transplantation of frozen–thawed ovarian tissue (30), 24 babies have been born (Table 3). It is time to consider fertility preservation in women as one of the foremost challenges of the next decade and to offer women facing the risk of induced or iatrogenic premature menopause the best chances of becoming mothers (Fig. 5).

REFERENCES