Orthotopic and heterotopic ovarian tissue transplantation

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BACKGROUND: Transplantation of ovarian tissue is, at present, the only clinical option available to restore fertility using cryopreserved ovarian tissue. More than 30 transplantations of cryopreserved tissue have been reported, and six babies have been born, worldwide, following this procedure. Despite these encouraging results, it is essential to optimize the procedure by improving the follicular survival, confirming safety and developing alternatives. Here, we review the different factors affecting follicular survival and growth after grafting.

METHODS: Relevant studies were identified by searching Pubmed up to January 2009 with English language limitation. The following key words were used: (ovarian tissue or whole ovary) AND (transplantation) AND (cryopreservation or pregnancy). Using the literature and personal experience, we examined relevant data on the different exogenous and clinical factors affecting follicular development after grafting.

RESULTS: Clinical factors such as the patient’s age and the transplantation sites influenced the lifespan of the graft. A heterotopic transplantation site is not optimal but offers some advantages and it may also promote the hormonal environment after a combined heterotopic and orthotopic transplantation. Exogenous factors such as antioxidants, growth factors or hormones were tested to improve follicular survival; however, their efficiency regarding further follicular development and fertility potential remains to be established.
CONCLUSION: Additional evidence is required to define optimal conditions for ovarian tissue transplantation. Alternatives such as whole ovary or isolated follicles transplantations require further investigation but are likely to be successful in humans in the future.

Key words: ovarian tissue / transplantation / pregnancy / sites / vascularization

Introduction

Major advances in oncological treatments and diagnosis have resulted in a marked improvement in the survival of children and young adults with cancer over the last decade. Chemotherapy treatments including alkylating agents as well as radiotherapy may unfortunately compromise future fertility (Meirow, 2000; Lobo, 2005). The risk of premature ovarian failure depends on various factors such as the age of the patient, the type and the dose of cytotoxic therapy. Alkylating agents, imposing the highest risk in causing ovarian failure, induce follicular depletion in an exponential proportion to increasing doses. Among premenopausal women treated with alkylating agents for breast cancer, it has been estimated that up to 68% of them faced premature ovarian failure after treatment. Aggressive treatment with cytotoxic chemotherapy and radiotherapy for lymphoma results in ovarian failure in 38–57% of the patients (Meirow and Nugent, 2001). Conditioning regimen for bone marrow transplantation represents the most gonadotoxic regimen, with an ovarian failure rate after treatment exceeding 90% (Meirow, 2000). Radiotherapy is also recognized to cause destruction of the follicular pool, with an LD50 of human oocyte <2 Gy (Wallace et al., 2003). The effective sterilizing dose at which ovarian failure occurs immediately after treatment in almost all the patients is estimated at <20 Gy, when pelvic radiotherapy doses for intra-abdominal tumour, including gynaecological cancer, ranged from 25 to 50 Gy (Meirow and Nugent, 2001; Chemoradiotherapy for cervical cancer Meta-Analysis Collaboration, 2008; Wo and Viswanathan, 2009).

On the other hand, restoration of the ovarian function does not always ensure normal fertility after oncological treatments. The chance of spontaneous pregnancy in women treated after 25 years of age has been estimated to be only 5% (Lobo, 2005). Because fertility preservation is of great concern for young women diagnosed with cancer, the possibility of treatment-related infertility should systematically be brought up by physicians in collaboration with gynaecologists at the time of diagnosis (Langeveld et al., 2004; Thewes et al., 2005; Lee et al., 2006). According to the type of the disease and the health state of the patient, various options to preserve fertility have been proposed (Donnez et al., 2006; Demeestere et al., 2007). Many young cancer patients desiring fertility preservation may not have access to the technologies used for Assisted Medical Procreation such as embryo or oocytes cryopreservation because of their oncological context and/or personal situation. These options impose a 2–4 week delay before oocyte collection that is often not compatible with the urgency to treat the cancer, and this procedure must be performed before beginning chemotherapy. Oocyte retrieval after even one round of chemotherapy is not practicable because of the dramatic reduction of IVF efficiency, as well as the increased risk of aneuploidy due to this treatment (Dolmans et al., 2005).

Cryopreservation of ovarian tissue is a promising experimental technology, presenting with several advantages. It allows the storage of a large number of primordial and primary follicles, can be rapidly performed at any time of the menstrual cycle and is the only available option to preserve fertility in children (Nugent et al., 1997; Donnez and Bassil, 1998; Oktay, 2001; Poirot et al., 2002, 2007; Demeestere et al., 2003; Dudzinski, 2004; Oktay and Sonmez, 2004; Oktay et al., 2004; Donnez et al., 2006; Lee et al., 2006; Meirow et al., 2007a; Moffa et al., 2007; Weintraub et al., 2007). The procedure is proposed as a fertility preservation option for various indications in a growing number of centres around the world. In our institution, breast and haematological cancers represent two-thirds of the indications (Fig. 1, unpublished data). The ovarian tissue cryopreservation procedure benefits those not only with oncological diseases, but also

Figure 1  Indications for ovarian tissue cryopreservation in Erasme Hospital from 1999 to 2008 ($n = 133$).
with benign diseases such as drepanocytosis or thalassaemia that require conditioning regimen for bone marrow transplantation (Sonmezer et al., 2005). Patients affected by autoimmune diseases such as lupus nephritis, genetic disorders associated with premature ovarian failure, or benign ovarian diseases requiring oophorectomy may also be concerned with fertility preservation (Hreinsson et al., 2002; Demeestere et al., 2003; Gidoni et al., 2008; Huang et al., 2008a; Oktay and Oktrem, 2008). These non-oncological conditions represent nearly 20% of the indications in our population of patients requiring fertility preservation (Fig. 1). Finally, healthy women who chose to delay childbearing for professional or personal reasons may also wish to retain their fertility (Tao and Del Valle, 2008).

Although the freezing-thawing procedure of ovarian tissue is now relatively well established, the use of the cryopreserved ovarian cortex in order to restore fertility remains a challenge. Orthotopic or heterotopic transplantation is currently the only available option to restore fertility using cryopreserved ovarian tissue. Alternatives such as in vitro follicle culture require additional research before becoming available for humans (Hovatta, 2000; Picton et al., 2008). To date, 43 women who underwent cryopreserved or fresh ovarian tissue transplantsations have been reported in the literature, leading to the restoration of spontaneous cycles for several months in almost all cases (Bedaiwy et al., 2008). Restoring fertility after autotransplantation of cryopreserved ovarian tissue has been recently achieved, and five healthy babies were born following this procedure (Donnez et al., 2004; Meirow et al., 2005; Demeestere et al., 2007; Andersen et al., 2008). Pregnancies have also been obtained after heterologous transplantation of fresh or cryopreserved cortical tissue between twins discordant for premature ovarian failure (Silber et al., 2005, 2008a; Silber and Gosden, 2007). Despite these encouraging results, some important concerns still limit the application of the procedure and its success. Besides the age of the patient at tissue collection, a key factor is the ischemic injury occurring during the time necessary for the revascularization of the transplanted tissue from the support vessels. This affects follicular survival as well as the life span of the ovarian tissue after transplantation, which are both correlated with the fertility restoration potential.

This review will provide insight into these different factors that affect follicular development and fertility restoration after ovarian tissue transplantation.

**Methods**

A MEDLINE search was performed to identify articles published in the English language dealing with ovarian tissue and whole ovary transplantation in both animals and humans. Relevant articles up to January 2009 were selected and checked for previously unidentified articles. The following keywords were used: (ovarian tissue or whole ovary) AND (transplantation) AND (cryopreservation or pregnancy).

Selection criteria and outcomes of interest: We reviewed all literature focused on ovarian tissue and whole ovary transplantation and selected relevant articles based on their originality and innovative characteristics. Articles and recent reviews were classified by human and animal experiments. Outcomes of interest were pregnancies, vascularization and factors affecting further follicular development after transplantation.

**Ischemic injuries after ovarian tissue transplantation without vascular anastomosis**

Because transplantation of fragments of ovarian cortex is performed without vascular reanastomosis, perfusion of the tissue depends on the growth invasion of new blood vessels. The time needed to achieve an adequate perfusion of the transplanted tissue is critical for the follicular survival and the functional longevity of the graft. In mice, initial perfusion of the autograft (revealed with Evan’s blue dye injection) is observed 3 days post-transplantation (Nugent et al., 1998). The first stage of neovascularization is detected within 48 h in autologous immature transplanted rat ovaries and the tissue is revascularized and functional after 1 week (Dissen et al., 1994). Using MRI and histology, functional vessels have been detected within ectopic xenotransplanted rat ovarian tissue after only 7 days (Israely et al., 2004). In humans, the neovascularization process was observed after only 3 days following ovarian tissue transplantation onto a chick chorioallantoic membrane (Martinez-Madrid et al., 2009).

The integrity of the stroma is also essential for the neovascularization process and follicular survival after graft. Primordial follicles can tolerate ischemia for at least 4 h during tissue transport (Schmidt et al., 2003), whereas stromal cells surrounding the follicles appeared to be more sensitive to ischemia compared with primordial follicles (Kim et al., 2004a).

**Consequences on the follicular pool**

The ischemic injury occurring directly after transplantation without vascular anastomosis is involved in the dramatic follicular depletion observed in grafted ovarian tissue. At least 25% of the primordial follicles are lost as a result of cryopreserved xenografts of human ovarian tissue into mice (Newton et al., 1996; Nisolle et al., 2000). Others estimated that ischemic injury during autograft processes induces the depletion of 60–95% of the follicular reserve, including the loss of virtually the entire population of growing follicles (Candy et al., 1997; Aubard et al., 1999; Baird et al., 1999; Aubard, 2003; Liu et al., 2008). This phenomenon is associated with a dramatic reduction of the graft size and a significant fibrosis in most grafts (Kim et al., 2002). This follicular depletion observed after ovarian tissue transplantation is a main concern, especially in humans and large animal species that have a dense ovarian cortex, as it may affect the follicular growth dynamic, the hormonal environment and the fertility restoration potential.

**Consequences on graft function**

In sheep, oestrus cycles were maintained until 22 months after ovarian tissue transplantation (Baird et al., 1999). Salle et al. (2003) observed gestation for more than 2 years after hemi-ovary autograft in ewes. Experiments in sheep, however, showed that the autograft resulted in a 2- to 4-fold increase in FSHI during the oestrus cycle, possibly due to a deficiency in inhibit A production by the growing follicles (Campbell et al., 2000). An inhibit deficiency, associated with an elevation of FSH level, could explain the granulosa cell hyperplasia observed in the grafted tissue during the re-establishment of follicular development (Callejo et al., 2003). Low anti-Mullerian hormone levels, normally produced by the pool of developing follicles in intact ovaries,
also promote massive follicular recruitment after ovarian tissue transplanta-
tion (Visser and Themmen, 2005).

This hormonal environment reflects a poor ovarian reserve that could affect the natural fertility capacity and the response to gonado-
trophin stimulation. In humans, follicular depletion and cortical injury lead to a ‘poor responder’ status after transplantation. Both hormonal profiles and follicular dynamics observed after transplantation in humans are indeed in agreement with experiments in large mammals. It is established that an optimal hormonal environment is associated with a higher response rate during the IVF cycle (Broekmans et al., 2006).

Follicular development and restoration of ovarian function usually occurs 4–5 months after a transplantation procedure (Donnez et al., 2006), as more than 120 days are necessary to initiate follicular growth and approximately 85 days to reach final maturation stage from a pre-antral follicle (Gougeon, 1996). Ovarian function after transplantation remains for a few months to more than 5 years (Oktay and Karlikaya, 2000; Callejo et al., 2001; Radford et al., 2001; Schmidt et al., 2005; Donnez et al., 2006; Demeestere et al., 2007; Oktay and Oktem, 2008). Despite the restoration of regular menstruation cycles, high basal FSH levels are usually observed after ovarian tissue transplantation in women, reflecting the poor ovarian reserve (Donnez et al., 2005). Persistence of high FSH concentrations most likely contributes to poor oocyte quality and an inadequate maturation stage (Tryde Schmidt et al., 2004). Recently, a large prospective study showed that a basal FSH level greater than 8 IU/l was a strong negative predictor of spontaneous pregnancy in a general subfertile population, even after taking into consideration the age and the cycle length (Van der Steeg et al., 2007). Scarce pregnancies described after ovarian transplantation were obtained during an adequate menstrual cycle (Donnez et al., 2004; Meirow et al., 2005; Demeestere et al., 2006, 2007; Silber et al., 2008b). These case reports well illustrate the importance of achieving an optimal hormonal environment by improving the vascularization process and by grafting sufficient amounts of ovarian tissue.

Consequences on fertility restoration

Although restoration of long-term fertility after ovarian tissue grafts and normal reproductive performance have been reported in mice (Candy et al., 2000), most authors describe a lower fertility rate after ovarian transplantation compared with non-grafted animals (Gunasena et al., 1997; Aubard et al., 1999; Almodin et al., 2004a; Liu et al., 2008; Sauvat et al., 2008). Caution should be taken concerning studies on mice, as ovariectomy procedures can result in incomplete removal of the host ovary. Some authors evaluate that 3–36% of the litter obtained from grafted animals could be derived from the remaining ovarian host fragments (Sztein et al., 1998; Candy et al., 2000) and a suitable (non-graft) control should be always employed to validate studies on mice.

Decreases in the fertility rate after transplantation is actually directly correlated with follicular depletion induced by the ischemic processes, however, others factors may be involved. The reduction in litter size may be linked to abnormal epigenetic status. In mice, methylation status of H19 and Lit1 genes, both sensitive to external conditions, were not modified after ovarian transplantation (Sauvat et al., 2008).

Despite the correct imprinting of at least two genes, the reduction in the litter size observed in most studies could also reflect spontaneous miscarriages due to malformations linked with imprinting genes.

Factors affecting graft function after ovarian tissue transplantation

The cryopreservation procedure

The tolerance of human ovarian tissue to the freezing-thawing pro-
cedure has been now well studied, with a follicular survival rate reach-
ing 70–80% after slow freezing with appropriate cryoprotectants (Hovatta et al., 1996; Gook et al., 2000; Fabbrini et al., 2003; Hreinnsson et al., 2003; Maltaris et al., 2006a). The slow-freezing cryopreservation procedure may, however, influence the reproductive outcome after graft.

Although some authors did not observe differences in the litter size between cryopreserved and fresh mouse ovarian grafts (Gunasena et al., 1997; Candy et al., 2000; Shaw et al., 2000), others have suggested that cryopreservation procedure before grafting reduced litter size (Szein et al., 1998). Immature follicles can be cryopreserved without subsequent DNA fragmentation (Demirci et al., 2002), but the integrity of the granulosa cell structure and function after this process has been questioned (Siebzehnrubl et al., 2000; Navarro-Costa et al., 2005). Using microarray technologies, abnormal gene expression in the granulosa cells has been reported after cryo-

preserved tissue transplantation compared with normal unmanipu-

lated tissue (Lee et al., 2008). Whether the higher rate of apoptosis and the abnormal gene expression observed in this study can be attributed directly to the cryopreservation procedure or to the transplant-

ation remains to be seen.

Others also describe a decrease in the number of growing follicles after 5 days of culture of frozen-thawed 1-day-old mouse ovaries com-
pared with fresh cultured tissue (Choi et al., 2007). This may be caused by the apoptosis and necrosis phenomenon observed after cryopreservation. Despite the lower development rate of primordial follicles, no significant difference was observed between the level of mRNA expression of markers such as growth differentiation factor GDF-9, inhibin-α or ZP3 for the developing follicle in fresh and frozen-thawed ovaries cultured for 5 days (Choi et al., 2007).

The xenograft model, frequently used as an experimental model to evaluate follicular viability and oocyte competence after transplanta-
tion (Newton et al., 1996; Oktay et al., 1998, 2000; Nisolle et al., 2000; Gook et al., 2001, 2005; Van den Broecke et al., 2001; Kim et al., 2002, 2005; Maltaris et al., 2006b), has also been described in the study of factors affecting the neovascularization process. Active angiogenesis was demonstrated 24 days after a human tissue xenograft into nude mice (Nisolle et al., 2000), but fibrosis relative to the surface area was significantly higher after xenotransplantation of cryopre-
served tissue compared with fresh tissue xenotransplantation. This difference did not, however, affect follicular depletion rate or the vascu-

larization process. In conclusion, the effect of the cryopreservation of ovarian tissue on the follicular developmental ability and oocyte competence requires further elucidation.
Vitrification procedure has been newly applied to ovarian tissue cryopreservation as an alternative approach to the slow-freezing method in various species such as in mice (Chen et al., 2006b; Aerts et al., 2008), sheep (Bordes et al., 2005; Wang et al., 2008), dogs (Ishijima et al., 2006), bovines, pigs (Gandolfi et al., 2006) and humans (Huang et al., 2008b; Wang et al., 2008). This promising technique may have the advantage of preserving the stromal cells, the collagen bundles, the intercellular space as well as the primordial follicles (Chen et al., 2006b; Wang et al., 2008), but the efficiency and the safety of this procedure should be proved before clinical use.

The clinical factors

The life span of the heterotopic or orthotopic graft is likely to be influenced by several clinical factors such as the age of the patient at the time of cryopreservation, the previous gonadotoxic treatment and the volume of ovarian tissue transplanted. A correlation between these factors and the life span of the graft is not always easy to establish. Previous chemotherapy before the cryopreservation procedure and the localization of the ovarian graft could interact with the revascularization process of the transplanted tissue. Blood vessel injuries and cortical fibrosis have both been implicated in the follicular loss phenomenon induced by chemotherapy (Meirow et al., 2007b). These cortical injuries could also influence the neovascularization processes after ovarian tissue transplantation for patients receiving chemotherapy prior to the cryopreservation procedure.

Through cortical injury, both the ovarian tissue cryopreservation procedure itself and previous chemotherapy may interfere with neovascularization process after transplantation, inducing higher fibrosis rates in the graft.

Role of exogenous factors

Multiple attempts have been reported to shorten the ischemic period and increase the viability and fertility potential after ovarian graft (Table I).

Antioxidants factors

During ischemia-reperfusion processes, oxygen free radicals constitute the most important component that induces damage of the cell membrane proteins and decreases mitochondrial function and lipid peroxidation (Kupiec-Weglinski and Busuttil, 2005). Endogenous antioxidant molecules are able to neutralize these oxygen free radicals produced in excess during the ischemic process. This system, however, can be rapidly overwhelmed. During solid organ transplantation, exogen antioxidants are used to quench free radicals and preserve organs. Both ascorbic acid and mannitol have been shown to be effective in reducing surgically-induced ovarian ischemic injury in a rat model (Sagsoz et al., 2002). A potential benefit of antioxidants administration was also tested during ovarian tissue transplantation. Local antioxidant injection of vitamin E before graft could improve follicular survival rate (Nugent et al., 1998), but these results were not confirmed by others (Weissman et al., 1999). Other antioxidants such as melatonin and oxytetracyclines locally administered during intraperitoneal rat ovarian graft were effective to reduce ovarian necrosis (Sapmaz et al., 2003). Kim et al. (2004a) evaluated the efficiency of ascorbic acid to reduce apoptosis of primordial follicles and stromal cells after deprivation of bovine ovarian cortex blood supply for up to 48 h. They showed that stromal cells were more sensitive to ischemic injury than primordial follicles, and that apoptosis was reduced when the tissue was incubated with ascorbic acid up for to 24 h, but not later.

No beneficial effect of antioxidant agents on the follicular survival rate after ovarian transplantation has been yet demonstrated. Moreover, the use of these agents should be further investigated in vitro and in vivo to guarantee their safety.

Growth factors

Multiple growth factors such as fibroblast growth factor, transforming-growth factor (TGFβ-α) or vascular endothelial growth factor (VEGF) are involved in the invasion of the tissue by new vessels. The invasion of the rat cortex by vessels 48 h after a graft is associated with a 5- and 10-fold increase in the expression of mRNA in the outer cortex for TGFβ1 and VEGF, respectively (Dissen et al., 1994). Surprisingly, angiogenic factors such as VEGF failed to have beneficial effects on primate graft function (Schnorr et al., 2002). In contrast, erythropoietin (EPO) may enhance the survival of transplanted tissue, as it promotes the differentiation and proliferation of erythroid progenitor cells as well as preventing apoptosis (Suzuki et al., 2008). The effect of growth factors is still controversial but recent results are encouraging. Their beneficial effect on further follicular development or fertility restoration should be confirmed.

Hormonal factors

Gonadotrophin administration, starting immediately after ovarian tissue transplantation for 3–4 days with the aim of up-regulating VEGF mRNA levels, did not improve the primordial or growing follicles survival rate in the grafts compared with untreated recipients (Nugent et al., 1998; Imthurn et al., 2000). Imthurn et al. (2000) evaluated the effect of 4 days of intraperitoneal administration of gonadotrophins (recombinant human FSH and LH, 3 IU) beginning 2 or 4 days prior to, or on the day of the ovarian tissue graft at a poorly vascularised site (the abdominal wall). They showed that gonadotrophins stimulation 2 days before and 2 days after grafting increased the total number of growing follicles in the graft. Wang et al. (2002a) showed an increase in the growing follicular population when gonadotrophins (human menopausal gonadotrophin, hMG, 5 IU/d) were administered to recipients 4 days before the graft compared with untreated grafted recipients. Angiogenic factors as VEGF, up-regulated by gonadotrophins, may be required to be present in effective amounts before transplantation to be efficient.

Furthermore, hormonal pretreatment of the donor before ovary removal appears to also have a beneficial effect on the growing viable population after grafting into recipients (Imthurn et al., 2000). It was suggested that gonadotrophins stimulation (hMG or urofollitrophin) of the recipients 1 or 2 weeks after human tissue xenograft promotes follicular development, however, it seems to contemporaneously deplete primordial follicles pool (Van den Broecke et al., 2001; Maltaris et al., 2007a). In a porcine tissue xenograft model, others showed that gonadotrophin administration (FSH) improves the meiotic competence of the oocytes collected by supporting oocyte growth (Kaneko et al., 2006).
## Table 1  Different options investigated in order to reduce ischemic injuries during ovarian tissue transplantation without vascular anastomosis

| Donor/recipient | Graft site | Effect | References |
|----------------|------------|--------|------------|
| Vitamin E      | Human/mice, Mice/mice | Kidney caps. | Improve survival rate, reduction of lipid peroxide and malondialdehyde | Nugent et al. (1998) |
|                | Mice/mice | Not precise | No beneficial effect | Weissman et al. (1999) |
| Melatonin Oxytetracyclin | Rat/rat | ip | Reduce ovarian necrosis | Sapmaz et al. (2003) |
| VEGF           | Monkey/monkey | sc | Decrease graft viability | Schnorr et al. (2002) |
|                | Human/mice | ip | No vascularization improvement | Donnez et al. (2006a) |
| Androgen (male or testosterone treated hosts) | Human/mice | sc | Increase follicular development after stimulation | Weissman et al. (1999) |
|                | Hamster/hamster | Kidney caps. | Increase follicular population | Arrau et al. (1983) |
|                | Mice/rat | Kidney caps. | Increase oocyte yield after stimulation | Snow et al. (2002) |
|                | Mice/mice | Kidney caps. | Implantation rate, fetal development unaffected | Waterhouse et al. (2004) |
| Gonadotrophins after graft (recipient) | Mice/mice | Kidney caps. | No difference in follicular survival | Nugent et al. (1998) |
|                | Mice/mice | abd. wall | No difference compared with untreated recipients | Imthurn et al. (2000) |
|                | Human/mice | sc/kidney caps. | Earlier initiation of follicular development | Van den Broecke et al. (2001) |
|                | Human/mice | Neck muscle | Depletion of primordial follicles | Maltaris et al. (2007a) |
|                | Human/mice | Kidney caps. | Promote follicular growth | Oktay et al. (1998) |
| Gonadotrophins before graft (recipient) | Mice/mice | sc | Increase of the growing follicles survival | Wang et al. (2002a) |
|                | Mice/mice | abd. wall | Increase of the growing follicles survival | Imthurn et al. (2000) |
| GnRHa (± GnRH) | Human/mice | im | No or detrimental effect on follicular loss prevention | Maltaris et al. (2007b) |
| GnRHa + estradiol | Sheep/sheep | Ovarian pedicle | No difference in the number of primordial follicle, reduce follicular growth | Campbell et al. (2000) |
| Graft into granulation tissue | Rat/mice | im | Improve graft perfusion and follicular survival | Israely et al. (2006) |
| EPO            | Dog/mice | Ov. bursa | Enhanced follicular survival | Suzuki et al. (2008) |

sc: subcutaneous; ip: intraperitoneal; abd: abdominal; ov: ovarian; caps.: capsule; VEGF: vascular endothelial growth factor; EPO: erythropoietin; GnRH: gonadotrophin releasing hormone.
Stimulation with FSH was favourable and required after human tissue xenograft to sustain long-term follicular development beyond the two layers stage in a model using hypogonadic SCID mice (Oktay et al., 1998, 2000). Using another mice strain, however, Gook et al. (2001) reported follicular development up to the antral stage after xenograft into non-hypogonadic SCID mice without exogenous gonadotrophin stimulation.

Elevated endogen gonadotrophin secretion, due to the ovarian failure status before the graft, could also increase the growing follicular proportion but may have a direct toxic effect (Flaws et al., 1997), depleting the primordial follicular pool of the grafted tissue. GnRH agonist, administered to reduce endogenous gonadotrophin levels, surprisingly failed to prevent follicular depletion (Maltaris et al., 2007b) and even severely retarded the re-establishment of normal follicle development (Campbell et al., 2000). The effect of endogenous and exogenous gonadotrophins thus appears to differ.

The results from animal studies lead to options for different approaches in humans. The injection of FSH directly into the subcutaneous site along with an aspirin regimen for 7 days after a heterotopic transplantation procedure was attempted in humans to improve the revascularization process (Oktay et al., 2003). In contrast, Donnez et al. (2006, 2007) suggested the administration of oestro-progesterone tablets before the transplantation along with a GnRH antagonist at the time of the procedure in order to reduce endogenous gonadotrophins levels. Meirow et al. (2005) proposed to administer oestro-progesterone tablets during the first post-transplantation month. Another option was to avoid any hormonal treatment after the transplantation procedure (Tryde Schmidt et al., 2004; Schmidt et al., 2005; Demeestere et al., 2006, 2007).

In conclusion, animal experiments show that gonadotrophin stimulation of a recipient or donor initiated at a reasonable time before and continued to suboptimal sites after grafting could have a positive effect on the viable growth follicle rate, but the impact on the long-term ovarian function and fertility of such treatment must be further investigated. The position regarding hormonal treatment before and after ovarian tissue transplantation in human is variable and not yet standardized.

### Mechanical factors

Angiogenesis can also be mechanically stimulated by triggering endogenous processes of new vessel formation. After injury, the inflammatory phase allows collagen deposits to occur although angiogenesis helps to sustain new tissue formation. This physiological phenomenon was used by Donnez et al. (2004) and later by ourselves (Demeestere et al., 2006), inducing neovascularization by creating a peritoneal pocket or longitudinally opening the ovary at the ovarian tissue transplantation sites 1 week before the transplantation procedure (two steps laparoscopy) (Fig. 2). Animal experiments confirmed that the ovarian grafts transplanted into granulation tissue were already perfused at least 24 h prior the intact control grafts (Israely et al., 2006).

To date, most experiments evaluating different treatments using animal models failed to clearly prevent follicular loss during the

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**Figure 2** Diagram illustrating the different steps of the cryopreserved ovarian tissue transplantation procedure in human by two-step laparoscopy.
ischemic period and increase fertility potential. In humans, the limited number of patients as well as the heterogeneity of the procedure led to the difficult evaluation of the efficiency of the different treatments. The optimal environment before and after transplantation in humans needed to achieve a high follicular survival in the transplanted ovarian tissue remains unclear and needs to be further investigated.

**Ovarian transplantation sites: heterotopic or orthotopic**

The choice of the transplantation sites constitutes an essential factor involved in future graft viability and in the subsequent oocyte competence. Ovarian tissue can be transplanted back to the original site (orthotopic) or to alternative sites (heterotopic). For each site, clinical considerations such as the possibility of natural conception, ease of the procedure, convenient access for oocyte collection and the volume of tissue transplanted must be taken into consideration (Table II).

**Animal experiments**

Animal experiments allow comparison of the follicular development potential at the different orthotopic and heterotopic sites after ovarian tissue autograft or xenograft. Using the xenograft model, Israely et al. (2003) showed that subcutaneous transplantation of rat ovaries into mice is followed by pericyte loss associated with tissue damage, whereas i.m. transplantation allows vascular maintenance and better follicular preservation. In rabbit, histology and ultrastructure of grafted fresh and cryopreserved ovarian tissue into the mesometrium, the ovarian bursa, or the ovary are comparable (Deng et al., 2007).

Other studies concluded that ovarian bursa or kidney capsule sites were more favourable than subcutaneous or intraperitoneal sites (Imthurn et al., 2000; Callejo et al., 2002; Risvanli et al., 2006; Yang et al., 2006). In rat, the subcutaneous site displays fewer primary follicles and corpus luteum than the subperitoneal site (Risvanli et al., 2006). In mice, the grafts placed in subperitoneal pockets contained significantly fewer growing follicles (12%) than non-grafted ovaries and ovaries grafted under the kidney capsule (70%), showing that the transplantation of an ovary to the untreated inner side of the lateral abdominal wall was suboptimal (Imthurn et al., 2000). Subcutaneous grafted ovaries also have a lower oocyte yield compared with those placed under the kidney capsule or in the bursal cavity (orthotopic site) in this species (Yang et al., 2006). Compared with the kidney capsule site, ovarian tissue graft in the back muscle in mice has recently been shown to have a better follicular survival rate (Soleimani et al., 2008).

Considering the endocrine function, no differences in estradiol or FSH levels were observed after 6 months follow-up of rat transplantation at the subcutaneous or intraperitoneal site (Callejo et al., 1999). As the primary indication for the ovarian tissue transplantation is to restore fertility of women and children facing premature ovarian failure as a result of cancer treatments, the evaluation of the oocyte competence and the normal embryo development after ovarian tissue grafts in various sites constitutes an essential prerequisite for human application.

To date, animal experiments have clearly shown that, depending on the graft site, oocytes collected from graft ovarian tissue have a lower embryo developmental potential than controls (Gunasena et al., 1997; Aubard et al., 1999; Snow et al., 2002; Waterhouse et al., 2004; Yang et al., 2006). The 2-cell cleavage rate from the in vitro matured oocytes was higher when oocytes were derived from graft in the bursal cavity compared with other heterotopic sites. The implantation rate did not differ regarding the graft sites (Yang et al., 2006). Orthotopic as well as heterotopic sites (kidney capsule) led to the birth of normal live young (Table III). One malformed mouse fetus born after fresh ovarian tissue transplantation was reported (Shaw et al., 2000). Vitrification has been also used as an ovarian tissue cryopreservation method and young have been obtained after grafting in different species (Bordes et al., 2005; Chen et al., 2006a; Hasegawa et al., 2006; Bagis et al., 2008). Bordes et al. (2005) reported four lambs born following ovarian tissue vitrification and graft, from which one had a malformation of the leg and oesophagus.

A few reports of live young, obtained after in vitro fertilization of oocytes derived from ovarian tissue grafted subcutaneously, were described in monkey (Lee et al., 2004) and mice (Yang et al., 2006). Embryos were obtained after in vitro fertilization of oocytes collected from ovarian tissue were transplanted subcutaneously in sheep, however, they failed to reach the blastocyst stage (Aubard et al., 1999).

**Human experiments**

Ovarian xenografts into mice provide a valuable experimental model to study the follicular development potential of tissue samples taken from various large mammals including humans (Aubard, 2003). This technique could also be useful to evaluate the gonadotoxicity of various drugs (Oktem and Oktay, 2007) or for the conservation of rare and endangered species (Paris et al., 2004). Most experiments using xenotransplantation of human ovarian tissue into mice also show a difference in the number of resting follicles when grafts are located

| Table II Advantages and disadvantages of heterotopic and orthotopic sites for ovarian tissue transplantation |
|-----------------------------------------------|
| **Heterotopic site (subcutaneous)** | **Orthotopic site** |
| Advantages | Possibility of natural conception |
| No limitation of the number of fragments transplanted | Restoration of fertility demonstrated |
| Easy transplantation procedure | Favourable environment for follicular development |
| Easy access for follicular monitoring and oocytes collection | Number of fragments transplanted limited by the ovarian size |
| Disadvantages | Invasive transplantation procedure |
| Restoration of fertility not yet demonstrated | |
Table III  Pregnancies and young obtained since 1990 after transplantation of fresh and cryopreserved ovarian tissue in animal models

| Species | Graft site | Tissue transplanted | Pregnancy rate | Total number of pregnancies | Live birth | References |
|---------|------------|---------------------|----------------|-----------------------------|------------|------------|
| Mice    | Ov. bursa  | SF Suspend tissue in fibrin clot | 80% (4/5) | 5 pups 6 implants | Normal | Carroll and Gosden (1993) |
| Mice    | Ov. bursa  | SF                    | 86%           | –                          | Normal     | Cox et al. (1996) |
| Mice    | Ov. bursa  | Fresh/SF             | 100%/72%      | >50 litters                | Normal     | Gunasena et al. (1997) |
| Mice    | Ov. bursa  | Fresh/SF             | 70%/57%       | 41 pups                    | Normal     | Szein et al. (1998) |
| Mice    | Ov. bursa  | Fresh/SF             | 92%/83%       | –                          | Normal     | Candy et al. (2000) |
| Mice    | Ov. bursa  | Fresh/SF             | 57%/57%       | 4 litters/4 litters        | 1 malformation | Shaw et al. (2000) |
| Mice    | Kidney caps. sc | Fresh | 33–66% (IR) | 19 pups                      | Normal     | Waterhouse et al. (2004) |
| Mice    | Ov. bursa  | Fresh/SF             | 65–100% (IR)  | 8 F (day 15) + 14 pups | Normal | Yang et al. (2006) |
| Mice    | Kidney caps. | Fresh | 53–100% (IR) | 9 F (day 15) + 3 pups | Normal | Chen et al. (2006b) |
| Mice    | Ov. bursa  | DCV/CSV/F/fresh | 83/33/60/93% | >100 pups                  | – | Liu et al. (2008) |
| Rat     | Ov. bursa  | SF | 72% (13/18) | – | Normal | Aubard et al. (1998) |
| Rabbit  | Ov. bursa  | Fresh (allo- or autograft) | 53% (9/17) | 16 litters                  | – | Petroianu et al. (2002) |
| Rabbit  | Ov. bursa  | Fresh (allo- or autograft) | 100% (5/5) | 7 gestations (22 young) | Normal | Almodin et al. (2004a) |
| Mice/rat| Kidney caps. | Fresh (xenograft) | 24.2–37.5% (IR) | 5 pups | Normal | Snow et al. (2002) |
| Sheep   | Ov. pedicle | Fresh/SF             | – | 1 lamb/1 lamb          | Normal | Gosden et al. (1994) |
| Sheep   | Ov. pedicle | SF                    | – | Triplet                  | Normal | Baird et al. (1999) |
| Sheep   | Ov. pedicle | Fresh/SF             | 100% (2/2)    | 4 lambs (1twin)            | Normal | Almodin et al. (2004b) |
| Sheep   | Ov. pedicle | SF                    | 66% (4/6)     | 11 lambs                  | 5 neonatal death, no congenital abnormalities | Salle et al. (2002, 2003) |
| Monkey  | Ov. pedicle | Vitrified | 50% (3/6) | 4 lambs | 1 malformed | Bordes et al. (2005) |
| Monkey  | sc | Fresh | 100% (1/1) | 1 young | Normal | Lee et al. (2004) |

Ov.: Ovarian; caps.: capsule; sc: subcutaneous; IR: implantation rate; FW: fetus weight; SF: slow freezing; CV: conventional vitrification; DCV: direct cover vitrification; F: fetuses.
subcutaneously or under the kidney capsule (Abir et al., 2003; Hernandez-Fonseca et al., 2004), with some exceptions (Van den Broecke et al., 2001). After an average of 24 days, the degree of fibrosis and the relative surface of the capillaries do not differ when intra-peritoneal and subcutaneous human ovarian xenografts into mice were compared (Nisolle et al., 2000).

Concerning autotransplantation, the first orthotopic transplantation of cryopreserved ovarian tissue was reported by Oktay (Oktay and Karlikaya, 2000). Since that time, different sites have been investigated in humans to restore ovarian function and fertility. Orthotopic sites included ovarian tissue transplantation in the peritoneum of the ovarian fossa and/or to the remaining ovary (Fig. 2, personal data). Because of the low invasive surgical aspect and its easy access, the subcutaneous site (the abdominal wall or forearm) is regularly chosen as the heterotopic site and is sometimes associated with transplantation at the orthotopic site (Callejo et al., 2001; Oktay et al., 2001, 2003; Wolner-Hanssen et al., 2005; Demeestere et al., 2006; Oktay, 2006) (Fig. 2, personal data). Other heterotopic sites were also tested in humans, such as the uterus, rectus abdominal muscle (Callejo et al., 2001; Kim et al., 2004b), the space between the breast tissue and superficial fascia of the pectoralis muscle (Kim et al., 2004b) as well as the subperitoneal tissue beneath the abdominal fascia between the umbilicus and the pubic bone (Rosendahl et al., 2006). Heterotopic sites were shown to be effective to restore ovarian function but no clinical pregnancy has been reported from oocyte collected, despite the fact that embryos were obtained and transferred (Oktay et al., 2001, 2004; Demeestere et al., 2006). Nevertheless, Rosendahl et al. (2006) recently showed that an ovarian graft at a heterotopic site could result in the production of mature fertilizable oocytes capable of initiating pregnancy (biochemical pregnancy) (Rosendahl et al., 2006).

In all the cases of birth reported after transplantation of ovarian tissue, the fertilized oocytes originated from tissue transplanted at the orthotopic site: to the peritoneum in the ovarian fossa (Donnez et al., 2004) or to the remaining ovary (Meirow et al., 2005; Demeestere et al., 2007; Andersen et al., 2008; Silber et al., 2008a).

Regarding the influence of the ovarian site in humans, additional interesting observations can be drawn from previously published reports as a result of the ability to compare long-term follicular activities at different sites (subcutaneous, peritoneal and ovary) in the same patient (Demeestere et al., 2006, 2007). Over the 14 documented post-transplantation cycles, follicles $\geq 15$ mm diameter at the time of ovulation were observed in 7, 29 and 64% of the cycles at the peritoneal, subcutaneous and ovarian sites, respectively, although the volume of the tissue transplanted at the ovarian site was 2- to 3-fold smaller than at the other sites (personal data). The follicular development is also delayed at the subcutaneous site compared with the ovarian site in the case of concomitant transplantation (Table IV). In contrast, when subcutaneous ovarian tissue transplantation was performed alone, the time necessary to obtain ovarian function recovery was reported to vary from 10 to 15 weeks, which is even shorter than expected (Kim et al., 2004b; Oktay et al., 2004b). Follicular development could therefore occur preferentially at the ovarian site when heterotopic and orthotopic ovarian tissue transplantations are simultaneously performed. Considering the oocyte competence, a total of three oocytes out of seven punctured follicles (four natural cycles) have been collected from the subcutaneous site, however, two of them were degenerated. One 3-cell embryo was transferred after IVF but no pregnancy was observed (Table IV). After subcutaneous ovarian tissue transplantation, Oktay et al. (2004) obtained 20 oocytes from eight consecutive percutaneous oocyte retrievals and six after ovarian stimulation (Oktay et al., 2004). Eight of them were suitable for IVF, five after in vitro maturation, but only two fertilized. One 4-cell embryo was transferred but failed to implant. Finally, it is interesting to note that follicular development seems to be limited at the heterotopic site as most of the follicles failed to grow more than 15 mm in size. The poor oocyte recovery rate and the low fertilization rate obtained suggest that other factors such as temperature, local pressure and environment at the subcutaneous site might contribute to the poor quality of the oocytes.

These results are consistent with those obtained in animal studies, showing that follicular development is influenced by the site of transplantation and that the heterotopic site is probably suboptimal compared with the ovarian site.

Despite these considerations, the small size of the atrophic organ (range 0.3 – 1.3 cm$^3$) limits the volume of ovarian tissue transplantable in the remaining native ovary (Schmidt et al., 2005; Demeestere et al., 2006). Considering the massive loss of the primordial follicle population by an ischemic process, the pool of functional resting follicles of the small amount of ovarian tissue transplantable at the orthotopic site is likely to be limited. Although peritoneal and subcutaneous sites do not appear to be optimal, the graft of a larger amount of ovarian tissue using a combination of heterotopic and orthotopic ovarian tissue transplantation may have a beneficial effect on endocrine function and fertility restoration potential.

**Ovarian tissue transplantation versus whole ovarian transplantation with vascular anastomosis**

Ovarian tissue transplantation with vascular anastomosis permits an immediate revascularization of the ovarian cortex, significantly reducing the ischemic injury previously described (Bedaiwy and Falcone, 2004). Conversely, the procedure cannot be repeated, and because it is more complex, it requires particular surgical skill. Whole ovary specimens have been transplanted in animal models as well as in humans. Vascular anastomosis of fresh ovary was successfully performed using the ovarian artery, inferior epigastric vessels, carotids vessels or iliac artery in various species (Goding, 1966; Paldi et al., 1975; Scott et al., 1981; Denjean et al., 1982; Wang et al., 2002b). In sheep, the revascularization process was compromised in around 50% of the cases (Jeremias et al., 2002). In humans, ovarian transplantation in the upper arm was performed with success before pelvic irradiation (Leporrier et al., 1987; Hilders et al., 2004). In the first case, a testicular prosthesis was inserted in the forearm of the patients 3 months before the transplantation in order to create a cavity for the transplanted ovary. Over a follow-up period of 16 years, the ovary remained functional (Leporrier et al., 2002). In the second case, the transplantation was performed during the radical hysterectomy for cervical carcinoma and the ovarian cycles remained regular for more
| Days post-transplantation | Cycle          | Foll. phase length | bFSH | Follicles size at ovulation (mm) | Post-ovulation decision | Results            |
|--------------------------|----------------|-------------------|------|---------------------------------|------------------------|--------------------|
|                          |                |                   |      | Ovary                       | Peritoneal | SC right | SC left | Oocytes collected | Fertilization (IVF) | Embryo transfer |
| Nov 2004 (0)             | First transplantation (ovary-SC right- peritoneal sites) |                   |      |                               |                        |                    |
| 148                      | Spontaneous    | 22                | 12–12.5 | 21 | 13.5                      | Timing intercourse    | No pregnancy       |
| 165                      | Spontaneous    | 7                 | 16.5–10–8 | 8 | –                        | Timing intercourse    | No pregnancy       |
| 190                      | Spontaneous    | 5                 | ND    | ND | ND                        | Timing intercourse    | No pregnancy       |
| 213                      | Spontaneous    | 6                 | 16–14–13 | – | –                        | Timing intercourse    | No pregnancy       |
| 237                      | Spontaneous    | 6                 | 15–11.5 | – | 11                       | Timing intercourse    | No pregnancy       |
| 261                      | Spontaneous    | 9                 | 19.5–10 | – | 10                       | Timing intercourse    | Miscarriage         |
| 372                      | Spontaneous    | 21                | 18–14–12 | – | 12                       | Timing intercourse    | No pregnancy       |
| 389–409                  | Stimulation    | –                 | 41    | OC 34 | –                        | –                    | No pregnancy       |
| 434                      | Stimulation    | –                 | –     | 18 | –                        | Timing intercourse    | No pregnancy       |
| 518                      | Spontaneous    | 28                | 17.5–11.5 | – | 16–11                    | 2                    | 1 (SC site) No pregnancy |
| May 2006                 | Second transplantation (ovary-SC left sites) |                   |      |                               |                        |                    |
| 583                      | Spontaneous    | 17                | 25    | – | – 16.5–10.5 | 1 | 0 (deg) | 0 | No pregnancy       |
| 608                      | Spontaneous    | 11                | 24    | – | – 15 | – | 1 | 0 (deg) | 0 | No pregnancy       |
| 635                      | Spontaneous    | 11                | 6     | 11 | – 17.5–13 | 1 | 0 (deg) | 0 | No pregnancy       |
| 650                      | Spontaneous    | 7                 | 6     | 22.5 | – | – | – | Timing intercourse | No pregnancy       |
| 669                      | Spontaneous    | 9                 | 9     | 15–15 | – | – | – | Timing intercourse | Pregnancy           |
| June 2007                | Delivery of healthy girl |                   |      |                               |                        |                    |
| PP-3 months              | Spontaneous    | 2 follicles       | –     | – | – | – | – | – | – | – | – |
| PP-4 months              | Spontaneous    | ND                | ND    | – | – | – | – | – | – | – | – |
| PP-5 months              | Spontaneous    | 6                 | 17.5 | – | 19–14.5 | 12.5 | 0 | 0 | 0 | – | – |
| PP-8 months              | Spontaneous    | 26                | ND    | ND | – | – | – | – | – | – | – |
| PP-15 months             | Spontaneous    | 17.3              | ND    | ND | – | – | – | – | – | – | – |
| PP-17 months             | Spontaneous    | 45                | ND    | ND | – | – | – | – | – | – | – |

Transplantation procedure has been performed twice in November 2004 and in May 2006 at different sites: ovarian, sub-cutaneous and/or peritoneal. Follicular phase length, basal FSH levels (bFSH), the follicular site at the time of ovulation and the outcomes of each cycle are reported.

deg = degenerated; ND = not done; OC = ovarian cyst; SC = sub-cutaneous; PP = post-partum.
Considerable advances in the field of fertility preservation have been obtained in the last decade, leading to the introduction of a new dimension of quality of life in many oncological centres. Consequences include an important increase in the request for fertility preservation procedures such as cryopreservation of ovarian tissue. Recent pregnancies published and the birth of healthy babies after cryopreserved ovarian tissue transplantation represent a great hope for these patients. Despite the evidence available for the efficacy of cryopreserved ovarian tissue transplantation to restore fertility, the success rate of the procedure is still limited. Follicular depletion after tissue transplantation without vascular anastomosis is a major concern, limiting the life-span of the transplanted tissue and influencing the hormonal environment after the procedure. Many attempts have already been made to increase the viability of the graft. Most of the exogenous factors used however, have not been efficient or are not applicable in humans.

The transplantation site plays a key role in the neovascularization process and could also influence the subsequent follicular development and oocyte competence through other mechanisms. Based on animal and human experiences, we show that heterotopic sites are suboptimal compared with the orthotopic site. This, however, presents some interesting advantages, justifying further investigation to improve these results.

To avoid ischemic injury, transplantation of a whole cryopreserved ovary may be the better option. Recent data on the viability of the human whole ovary after cryopreservation are encouraging and further research should allow the utilization of this option in the future.

Finally, transplantation of ovarian tissue cannot be proposed for all patients, due to the risk of tumour cell retransmission during the procedure (Kim et al., 2001; Oktay, 2001; Radford, 2004; Sonmez et al., 2005). Research programmes are needed to develop alternatives for these patients such as isolated follicles transplantation (Dolmans et al., 2007), in vitro follicular culture (Smitz and Cortvriendt, 1999), or pharmacological protection (Paris et al., 2002; Blumenfeld, 2007; Oktay et al., 2007). Recently, the success of 3D culture systems simulating physiological conditions provides a new possibility for the development of in vitro maturation of ovarian follicles in human (Xu et al., 2006).

**Author’s Role**

I.D. is responsible for the fertility preservation project. P.S. is responsible for the Gynaecologic Department and performed surgical procedures (ovarian tissue removal and transplantation). S.E. is the Director of the IVF Laboratory, where the embryo from the oocyte collected at the heterotopic site was obtained. A.D. is responsible for the Fertility Clinic, supported the project and revised the manuscript. Y.E., Director of the Research Laboratory of Human Reproduction and of the Department of Gynaecology and Obstetrics of Erasme Hospital, supported the project and revised the manuscript.

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