Micro-organ ovarian transplantation enables pregnancy: a case report

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ABSTRACT: A 19-year-old thalassemic woman had tissue from one of her ovaries cryopreserved prior to bone marrow transplantation, total body irradiation and sterilizing chemotherapy. As expected, premature ovarian failure resulted from this treatment. Transplantation of her thawed ovarian tissue resulted in return of menstrual cycling and the patient then underwent several IVF cycles. The patient, however, had poor ovarian response to hyperstimulation. We thus considered an alternative approach based on the observation that very thin ovarian fragments that preserve the basic ovarian structure [ovarian micro-organs (MOs)] induce angiogenesis and remained viable after autologous transplantation in animals. We report that preparation of autologous tiny ovarian fragments (MOs) and reimplantation into our patient resulted in IVF pregnancy and delivery of a healthy baby.

Key words: ovarian transplantation / thalassemia / micro-organs / avascular transplantation / cryopreservation

Introduction

Stem cell transplantation (SCT) has greatly enhanced life expectancy in malignant and nonmalignant disorders. However, chemoradiotherapy to ablate the bone marrow and prevent rejection is gonadotoxic (Sanders et al., 1988); in one retrospective study, only 0.6% of patients successfully conceived after SCT (Salooja et al., 2001). Ideally, patients should be referred to a fertility preservation specialist before gonadotoxic treatment (Letourneau et al., 2011).

Embryo cryopreservation is the established method of female fertility preservation (Lee et al., 2006). Ovarian cryopreservation is the only alternative for young patients without a male partner, when treatment entails a significant risk of premature ovarian failure (POF) (Donnez et al., 2006; Jeruss and Woodruff, 2009). Subsequently, ovarian fragments are transplanted under the pelvic peritoneum to restore fertility. Whole ovary transplantation, reported in rat (Wang et al., 2002) and sheep (Revel et al., 2004), provides longer term function (Arav et al., 2010); however, it remains experimental in human beings.

The ovary is amenable to avascular transplantation (i.e. the transference of an organ without attaching blood vessels) since the abundant primordial follicles, present on the periphery, benefit rapidly from neo-vascularization. Moreover, ovarian follicles normally develop in the avascular epithelium and thus tolerate ischemic conditions. Nevertheless, it is estimated that more than half of the follicles are lost due to ischemic damage (Nisolle et al., 2000) caused by the delay before the reimplanted ovarian cortical strips become revascularized.

A dozen human pregnancies have been reported following autotransplantation of frozen-thawed ovaries (Donnez et al., 2004; Meirow et al., 2005; Silber and Gosden, 2007; Rosendahl et al., 2008; Ernst et al., 2010; Roux et al., 2010; Demeestere et al., 2010). The success rate of avascular transplantation is however unclear (Kim et al., 2009). Following repeated absence of follicular response using more conventional ovarian strip transplantation methods (see below), we developed an alternative technique based on the observation that fragments, derived from various organs which preserve the natural architecture and whose thickness is about 300–350 μM thickness, remain viable and transcribe specific genes for long periods both in culture (Hasson et al., 2005; Mitrani et al., 2005) and when implanted into hosts (Hasson et al., 2006). Such fragments have been termed micro-organs (MOs) to emphasize the fact that they are of microscopic thickness while at the same time preserve the basic structure of the organ of origin. Of particular interest is that MOs up-regulate a whole repertoire of angiogenic factors, which induce a very rapid formation of an extensive capillary network within 3–4 days when implanted into syngeneic hosts (Hasson et al., 2005, 2006).

We here report a case in which autologous transplantation of human ovarian fragments, which preserve the ovary’s main cortex structure and whose thickness are no more than 350 μM (MOs), lead to successful pregnancy after failure of conventional ovarian strip transplantation.
Case Report

Patient

A 19-year-old unmarried, splenectomized patient with transfusion-dependent but well-chelated thalassemia major was offered allogenic peripheral blood (PB) SCT. Following menarche (16 years old), the patient had regular menstruation until the PBSCT for which preparation protocol was deemed to put her at high risk of POF. Following informed consent and ethics committee approval, we recommended ovarian cryopreservation for fertility preservation.

Ovarian cryopreservation and PBSCT

Following laparoscopic right oophorectomy, the ovary was cryopreserved as previously described (Newton et al., 1996; Revel et al., 2009). In brief, most of the ovarian medulla was removed and the resulting ovarian cortex tissue was manually cut into slivers varying in size from 0.8 to 1.4 cm in length, about 2–4 mm in depth and about 1.5–2.0 mm in width. The slivers were then cryopreserved as described previously. Seven weeks after laparoscopy, the patient was admitted for PBSCT. After conditioning with IV fludarabine (30 mg/m² × 6 days), PO busulfan (4 mg/kg × 4 days) and IV anti-thymocytic globulin (ATG Fresenius 5 mg/kg × 4 days), the patient underwent PBSCT from a HLA matched female donor. Cyclosporine served as graft versus host disease (GVHD) prophylaxis from Day 4. She developed grade 4 GVHD that responded fully to treatment.

Complications of transplantation included: pulmonary aspergillosis, cytomegalovirus antigenemia and GVHD of the gut. Moreover, the patient developed POF soon after PBSCT. This presented as amenorrhea, hot flushes and elevated FSH (Fig. 1). The patient was treated using hormonal replacement therapy.

Following her marriage at age 23, she requested fertility treatment. Five immature oocytes, cryopreserved at the time of ovarian cryopreservation, were thawed but did not mature. The couple declined oocyte donation for religious reasons. Following clinical ethics committee approval and signed informed consent, we undertook ovarian tissue transplantation to restore fertility.

First ovarian transplantation

Laparotomy revealed a small left ovary. Histological evaluation of ovarian biopsy demonstrated complete absence of follicles. After thawing at 37°C, eight slivers of ovarian cortex were transplanted into a pocket we created in the right broad ligament. Then nine pieces were sutured onto the atrophic left ovary. A summary of ovarian transplants and IVF treatments is detailed in Table I. Following ovarian transplantation, we observed a decrease in FSH levels as well as an increase in estradiol (Fig. 1). Following three IVF cycles obtaining three poor quality embryos, the transplanted ovarian tissues stopped responding as ovulation induction failed to induce follicle development. On the basis of our recommendation, the patient agreed to undergo a second ovarian tissue transplantation.

Second ovarian transplantation

A laparoscopic approach revealed an atrophic left ovary with no follicles. The neo-ovarian tissue in the right broad ligament appeared to be vascularized yet no follicular structure was visible in the area where the tissue had been reimplanted. Biopsies of both sides were sent to histology, which showed the presence of fibrotic tissue and no follicles. We created a peritoneal pocket, inserted 15 thawed ovarian pieces into the right broad ligament and sutured the pocket. Small cryopreserved cubes were also thawed. A vicril 3/0 suture was passed through five cubes and was then sutured to the left ovary. Following the second ovarian transplantation, we again observed a decrease in FSH levels as well as an increase in estradiol (Fig. 1).

Ovulation induction with maximal doses of human menopausal gonadotrophin (Menogon) (Ferring, Caesarea, Israel) or recombinant

![Figure 1](image-url) Measurement of FSH and of Estradiol before and after each ovarian cortex transplantation. FSH decrease is compatible with resumption of ovarian activity.
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FSH (Gonal F) supplemented with recombinant LH (Luveris) (Merck Serono, Herzlia, Israel) was not helpful. Four IVF cycles produced a total of six oocytes with the transfer of two poor quality embryos that did not implant. Following repeat ovarian failure and no follicular response despite administration of maximal FSH doses, we decided to change the technique before offering the patient a third ovarian transplantation.

Third ovarian transplantation

Prior to application of the MO technique in humans, we confirmed the capacity of rat-derived ovarian MOs to induce angiogenesis in syngeneic hosts (data not shown). We then also compared the histology of human ovarian slivers prepared by manual cutting with precision-cut human ovarian MOs (Fig. 2). Analysis of fresh and frozen-thawed ovary reassured us that preparation of MOs preserved the ovarian architecture (Fig. 2).

We thus proceeded to the third ovarian transplant in this patient. Thawed ovarian slivers ($n = 13$) from the remaining patient’s ovary were cut with a specially designed apparatus parallel to the long axis into 350-µm thick MOs, which basically preserved both the length and the depth of the original sliver (Fig. 3). Under general anaesthesia and laparotomy, we opened the right peritoneal pocket and transplanted the MOs close to the vascularized atrophic neo-ovary created from previous transplanted ovarian slivers. The MOs were gently placed into the peritoneal pocket one next to the other as in paving. The pocket was then sutured close using a vicril 3/0 running suture. Pelvic ultrasound, performed 2 days post-surgery, revealed a right neo-ovary $30 \times 22 \times 21$ mm. The left ovary post-grafting measured $22 \times 29$ mm.

IVF and pregnancy

Ovulation induction enabled development of 1–2 follicles per cycle (Table I). Nevertheless, about one-third of aspirated follicles were empty and yielded no oocyte. On the fourth IVF cycle, two mature oocytes were retrieved from the right transplanted tissue, yielding two good quality embryos, which were transferred and resulted in a
pregnancy. Gestation was normal except for admission at the 30th week of pregnancy due to cholecystolithiasis. The patient delivered (20 September 2010) a healthy 3026 g boy by Caesarean section.

Discussion

Although transfusions and regular iron chelation extend life expectancy, PBSCT is the only curative therapy available for thalassemia major and sickle cell disease patients (Rund and Rachmilewitz, 2005). A live birth after ovarian tissue autograft in a patient with sickle cell disease treated by allogeneic bone marrow transplantation was recently reported (Roux et al., 2010).

We report a successful human delivery, which was achieved when thinner ovarian MO fragments were prepared prior to transplantation, when conventional ovarian strips failed to achieve pregnancy.

The ovary develops as a result of complex interactions between an epithelium and a mesenchymal stroma. Tissues and organs are not only formed as a result of embryonic inductions, but continued growth and differentiation of cells depends on repeated inductions provided by the micro-environment prevailing in adult tissues.

Ex vivo handling and culturing of whole organs suffers from the lack of suitable circulation required for gas and nutrient exchange. Several attempts have been proposed to develop a vessel-like network that would allow the survival of engineered organ constructs in vitro followed by their integration in vivo due to improved vascularization after implantation (Kaully et al., 2009).

We have chosen an alternative approach that recognizes the importance of the micro-environment, but eliminates the need for vascularization. Paradoxically, MOs, which can function in the absence of a blood supply because of their small size, up-regulate a whole repertoire of angiogenic factors, which induce the formation of an extensive capillary network when implanted into an appropriate host. The result is that after several days, blood flow is restored to the MO implant (Hasson et al., 2005, 2006).

The ovarian MOs were prepared from previously cryopreserved autologous ovarian strips. A preferable approach may be to prepare the ovarian MOs immediately after oophorectomy. The small dimensions of MOs are likely to be advantageous during the cryopreservation process as well. It has been shown in the past that viability of cryopreserved MOs derived from other organs (e.g. liver) is much higher than those of larger organ fragments (Gershonowitz et al., 2004). This comes as no surprise as it is important to obtain an even distribution of the cryoprotectants during cryopreservation.

More critical, we believe, is the need to get a homogeneous temperature as freezing takes place. The small MO size allows for more uniform freezing and avoids local, intra-ovarian water pockets, which could freeze locally, increasing the salt concentration and thus causing harm to the cell’s organelles and cytoskeleton.

Because MOs preserve the basic epithelial–mesenchymal interactions, they allow for highly complex ex vivo function of epithelial cells (Hasson et al., 2006). These considerations are however currently hypothetical as at the time of ovarian cryopreservation for our patient, MOs were not prepared.

Clearly, oocyte maturation is a highly complex process requiring not only theca support but also essential interactions with the surrounding granulosa cells in the primary and maturing follicles. In fact, the ovary can be seen as a continuously developing organ where primordial follicles appear during the third month of fetal development. In the mature ovary, these follicles are located in the stroma of the cortex with a single layer of follicular cells surrounding the oocyte. As the primordial follicle makes the transition to a growing follicle, a series of developmental processes take place. It is therefore essential to preserve these delicate microstructures as intact as possible. In addition, making the fragments only a few hundred microns thick ensures appropriate diffusion of gases and nutrients. Viability is preserved and as a result, angiogenic factors are secreted ensuring in its turn that a proper vascular network is formed facilitating delicate sensing of hormonal signals required for oocyte maturation. Similar to

Figure 3 Comparison of manual preparation of human ovarian cortex slivers (A) with very thin precision cut ovarian fragments (B) that were prepared by cutting the ovarian slivers to 350-μM thick fragments parallel to the longitudinal axis using a specially designed apparatus. Two fragments (one from a sliver and one precision cut) are shown from a top view (arrows A and B, respectively) to illustrate difference in thickness (about 5-fold) (bar = 2mm).
previous reports (Dolmans et al., 2009), we found that some follicles developing in the transplanted ovary are empty. Indeed, though oocytes were obtained in the early cycles after the first and second transplantations, it appears that ovarian MOs assure high oocyte and embryo quality, yielding clinical pregnancy and delivery.

The right broad ligament was chosen as the transplantation site. As right oophorectomy was performed, it was easy to follow blood flow and follicular development in the transplanted ovarian tissue. Moreover, follicular aspiration could be performed vaginally, in the routine fashion. Creating a new ovary by MOs transplant is therefore feasible, and clearly, oocytes aspired from this ovary could have originated only from the graft.

The first ovarian transplantation may have served a vascular function for the subsequent grafts (Donnez et al., 2004). Thus, when we inserted our MOs to the broad ligament, we may have been taking advantage of the neovascularization previously formed in this location.

A two-step orthotopic autotransplantation of ovarian tissue is an efficient procedure for reestablishing fertility in women with POF after bone marrow transplantation (Roux et al., 2010). Similar to that paper, in diseases in which the ovaries may be a sanctuary for malignant cells, minimal residual disease should be assessed in stored ovarian tissue before reimplantation, but in nonmalignant diseases, such as thalassemia, there is no risk of reintroducing malignant cells.

This report, therefore, opens up new perspectives for patients with nonmalignant disease requiring gonadotoxic therapy. Precision cutting may be important in order to preserve ovarian cortex architecture. The fact that the resulting fragments are of uniform thickness and only a few hundred microns thick ensures reproducibility as well as appropriate and homogeneous diffusion of gases and nutrients to all cell components. Furthermore, very thin (350 μm) ovarian fragments secrete a whole range of angiogenic factors, which ensure that a vascular network is rapidly formed. It should be noted that in this report, very thin fragments were prepared from previously cryopreserved slivers. For future cases, we plan to examine as well the effect of preparing such fragments prior to cryopreservation in order to reduce the formation of high salt concentration pockets and further minimize cell death during the freezing process. On the basis of our results, we propose to prepare MOs directly from the harvest of fresh ovarian tissue and to obtain enough pieces to enable repetitive transplants. Nevertheless, comparative research is needed in order to propose the better technique to cryopreserve ovarian cortex. We conclude that transplant of MOs, following failure of two prior ovarian sliver transplants and numerous IVF cycles, enabled pregnancy and delivery in a patient with thalassemia major.

**Authors’ roles**

A.R. planned the project, removed and transplanted the ovarian cortex and drafted the manuscript. N.L. approved and participated in the planning and performance of ovarian transplantation. A.B.M. carried out the of FSH and Estradiol measurements before and after each ovarian cortex transplantation. M.L. cut, cryopreserved and thawed the ovarian cortex. E.M. has developed the micro-organ technology and has adapted it to ovarian tissue.

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