Restoration of ovarian function after orthotopic (intraovarian and periovarian) transplantation of cryopreserved ovarian tissue in a woman treated by bone marrow transplantation for sickle cell anaemia: Case report

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Ovarian function after orthotopic transplantation of cryopreserved ovarian tissue has been restored in women with malignant disease. Here the techniques are adapted for a non-cancer patient. In 1999, right oophorectomy was performed in a 21 year old woman before chemotherapy, prior to bone marrow transplantation. Ovarian cortex was frozen, according to a strict protocol. After thawing, ovarian cortex was reimplanted into the ovary and in a peritoneal window close to the ovary in 2004. Four-and-a-half months after reimplantation, LH, FSH, 17β-estradiol and progesterone levels, as well as ultrasonography, demonstrated the presence of an ovulatory cycle. After this cycle, the patient experienced two other ovulatory cycles, evidenced by FSH and 17β-estradiol concentrations, as well as ultrasound demonstration of a follicle. Follicular development was clearly observed in both the intraovarian site (1st and 2nd cycle) and the peritoneal window (3rd cycle). Restoration of endocrine ovarian function occurred after ovarian cortical strips, biopsied and cryopreserved before chemotherapy, were reimplanted into the ovary itself and a periovarian peritoneal window.

Key words: chemotherapy/cryopreservation/ovarian cortex/orthotopic transplantation/reimplantation

Introduction

In most female cancer patients, aggressive chemotherapy and radiotherapy lead to ovarian failure. Restoration of ovarian function after chemotherapy or radiotherapy has two main goals: to improve quality of life and restore reproductive function. For patients who need immediate chemotherapy, ovarian tissue cryopreservation, undertaken before cancer treatment starts, could be a means of preserving fertility without delaying the initiation of chemotherapy.

In 2004, the first live birth after orthotopic transplantation of cryopreserved ovarian tissue was reported in The Lancet (Donnez et al., 2004). Laparoscopy performed 4½ months after reimplantation demonstrated, by direct visualization, the development of a follicle from the grafted tissue. Furthermore, on histological examination, the biopsy samples demonstrated not only the survival of primordial follicles in the grafted tissue, but also the maturation of a follicle (granulosa cells marked by inhibin A) (Donnez et al., 2004).

These findings have opened up new perspectives for young cancer patients facing premature ovarian failure. But cryopreservation should not be reserved solely for women with malignant disease as suggested in our recent review (Donnez et al., 2005). Indeed, bone marrow transplantation (BMT) is increasingly used for non-cancerous diseases. Unfortunately, the high doses of chemo- and/or radiotherapy given prior to BMT result in ovarian failure in almost all cases (Meirow et al., 2004).

Here, we describe the first case of orthotopic transplantation (into the ovary and peritoneal pocket) of cryopreserved ovarian tissue in a non-cancer patient, using tissue obtained and frozen before chemotherapy prior to BMT.

Case report

Patient

In 1999, a 21 year old woman presented with complications (spleen abscess and cerebral thrombosis) due to homozygous sickle cell anaemia. BMT was proposed to the patient, her sister being HLA compatible.

Ovarian tissue cryopreservation was undertaken before chemotherapy. The protocol for cryopreservation of ovarian tissue from women treated by high doses of chemotherapy was
approved by the Catholic University of Louvain in 1995. We obtained written informed consent.

By laparoscopy, we performed a right oophorectomy. Removal of the whole ovary was decided upon in the present case because ovarian failure is induced almost always after chemotherapy given prior to BMT (Meirow and Nugent, 2001; Wallace et al., 2005).

After laparoscopy, the patient received two alkylating agents (busulfan 16 mg/kg; cyclophosphamide 120 mg/kg). On July 15, BMT was carried out, the sister of the patient being HLA compatible.

The patient became amenorrhoeic immediately after initiation of chemotherapy. Concentrations of FSH were 48.2 mIU/ml, LH 18.5 mIU/ml and estradiol <10 pg/ml, confirming castration. This ovarian failure profile was confirmed 3 and 5 months later and HRT was started in December 1999 and stopped in December 2002.

After cessation of HRT, bimonthly measurements of FSH, LH and 17β-estradiol concentrations proved the absence of ovulatory cycles from December 2002 to August 2004. The decision to reimplant the cryopreserved tissue was therefore taken.

**Procedures**

Freezing of ovarian tissue was undertaken according to the protocol described by Gosden et al. (1994). This protocol was absolutely identical to the one described in our previous publication (Donnez et al., 2004).

A first laparoscopy was performed 7 days before reimplantation to create not only a peritoneal window just beneath the left ovarian hilus, as previously described, but also an ovarian incision along the longitudinal ovarian axis. Coagulation of the edges of the window and the ovarian incision was performed in order to induce neovascularization in this area.

Knowing from experimental data that the ovary itself, even if atrophic, may be an ideal site for reimplantation, we decided to simultaneously prepare two sites for reimplantation (Almodin et al., 2004).

A biopsy measuring 0.5 cm in size was taken from the left atrophic ovary (1.5×1 cm in size).

A second laparoscopy was carried out 8 days later. It was decided to thaw only part of the cryopreserved tissue. Forty cubes were thawed according to the previously described technique, and immediately transferred to the operating theatre. We placed 15 cubes in the peritoneal window and 24 cubes in the intraovarian area (Figure 1A–C). One cube was sent for histological analysis and fluorescent calcein AM and ethidium homodimer-III staining confirmed the survival of primordial follicles.

**Results**

After cessation of HRT between December 2002 and August 2004, bimonthly measurements of FSH and 17β-estradiol systematically revealed values of >40 mIU/ml for FSH and <10 pg/ml for 17β-estradiol (13 pg/ml on one occasion).

Measurement of ovarian volume by ultrasound revealed a volume of 1.4×1×1 cm. No remaining follicles were visible by ultrasound during this 20 month period.

On the day of reimplantation, the left ovary was atrophic but the intraovarian area, which had been incised and slightly coagulated, demonstrated an extensive vascular network. Angiogenesis was less pronounced in the area of the peritoneal window. Vital fluorescent staining confirmed survival of all the primordial follicles after freeze–thawing. The follicular density was between three and four follicles per microlitre.

By contrast, no primordial follicles were found in serial sections of the biopsy of 0.5 cm in size taken from the left atrophic ovary. This biopsy from an atrophic ovary (1×1.5 cm in size) must be considered representative, since it represents ~10% of the residual value.

From the day of reimplantation to 4 months later, FSH, LH and 17β-estradiol levels ranged from 32 to 45 mIU/ml (FSH), from 15 to 22 mIU/ml (LH) and from 10 to 14 pg/ml (17β-estradiol) (Figure 2).

At 4½ months, FSH and LH concentrations decreased to 20.8 and 10.2 mIU/ml respectively, while the 17β-estradiol level rose to 58 pg/ml. Ultrasonography demonstrated the presence of an intraovarian follicle of 9.2 mm, which grew to 14 mm (Figure 3A). Three days later, sequential serum concentrations of LH demonstrated an LH peak, leading to the development of a corpus luteum of 21.2 mm in size (Figure 3B). The luteal phase was confirmed by a progesterone level of 6.5 ng/ml. During the luteal phase, FSH and LH concentrations decreased to 15 and 10 mIU/ml respectively. The patient menstruated 14 days after the LH peak.

After this first cycle, FSH levels rose to 34 mIU/ml for 6 weeks, concomitant with 17β-estradiol levels of ≤15 pg/ml. Thereafter, 17β-estradiol concentrations rose from 15 to 42 pg/ml, concomitant with a decrease in FSH, which nevertheless remained at ~20 mIU/ml. Ultrasonography revealed the presence of two small intraovarian follicles which achieved a maximum size of 11 mm. The patient experienced menstrual bleeding.

Following this cycle, FSH and 17β-estradiol values returned to castrated levels, 35.2 mIU/ml and 11 pg/ml respectively for another 4–5 weeks, before hormone measurements and ultrasound proved the presence of follicular maturation. Indeed, the
17β-estradiol level increased to 53 pg/ml and the FSH concentration decreased to 18.2 mIU/ml. Ultrasound revealed a follicle of 16 mm in size emerging from the tissue grafted into the peritoneal window, close to the ovary, but clearly separated from it. After an ovulatory cycle, demonstrated by progesterone measurement, the patient experienced a menstrual bleed.

**Discussion**

Very recently, the first human live birth after transplantation of cryopreserved ovarian tissue was reported in a woman who had undergone ovarian tissue cryopreservation before chemotherapy for Hodgkin’s disease (Donnez et al., 2004).

Ovarian cryopreservation was initially designed to protect and restore reproductive function in female cancer patients receiving chemotherapy and/or radiotherapy. In the USA, it has been estimated that >4000 female children are exposed to potentially sterilizing chemo- and/or radiotherapy annually (Meirow et al., 2004). Ovaries are very sensitive to cytotoxic treatment, especially to alkylating agents (e.g. busulfan, carboplatin, chlorambucil, cisplatin, cyclophosphamide, dacarbazine, ifosfamide, thiopeta), generally resulting in loss of both endocrine and reproductive function depending on the dose administered and the age of the patient.

Cryopreservation should not, however, be reserved solely for women with malignant disease. Indeed, BMT has been increasingly used for non-cancerous diseases in recent decades, but the high doses of chemo- and/or radiotherapy given prior to BMT lead to ovarian failure in almost all cases, children and adults alike (Meirow et al., 2004). Indeed, the risk of premature ovarian failure has been estimated at 92% in a study by Meirow and Nugent (2001), and 100% in a study by Teinturier et al. (1998).

A common non-total body irradiation conditioning protocol for BMT is the busulfan-cyclophosphamide (Bu-Cy) regimen. Our patient received this regimen, which was shown to induce premature ovarian failure in 98.6% of cases in a series of women treated with the ‘big’ Bu-Cy protocol (200 mg/kg Cy) and in 100% of cases in a series of women treated with decreasing doses (120 mg/kg) (Saunders et al., 1996; Grigg et al., 2000). This very high known risk of premature ovarian failure led us to remove an entire ovary instead of just cortical strips. As recently reviewed by Wallace et al. (2005), when the risk is low or medium, we recommend removal of cortical strips, as fertility outcome is not predictable. Indeed, in these women, the chance of recovery of ovarian function is much higher.

Thus, in the present case, we decided to remove a whole ovary, even if generally we do not consider this procedure to be the best option, as one can never completely exclude recovery of ovarian function after chemotherapy. Here, removal of an entire ovary was justified because premature ovarian failure was considered as an inevitable consequence of the patient receiving high doses of two alkylating agents before bone marrow transplantation. The risk of premature ovarian failure was therefore estimated to be almost 100%.

We now report restoration of ovarian endocrine function after orthotopic intravarian transplantation of cryopreserved ovarian tissue in a non-cancer patient suffering from sickle cell anaemia.

Ovarian tissue has already been successfully cryopreserved and transplanted into rodents, sheep and marmoset monkeys (Candy et al., 1995, 2000; Meirow et al., 1998). Oktay and colleagues reported transplantation of frozen–thawed ovarian tissue to the pelvic site, forearm and beneath the skin of the
abdomen (Oktay and Karlikaya, 2000; Oktay et al., 2001). A 4-cell embryo was obtained from 20 oocytes retrieved from tissue transplanted to the abdomen (Oktay et al., 2004). However, oocyte quality might be compromised by transplantation to a heterotopic site, such as beneath the skin of the abdomen. Indeed, as suggested by the authors, oocyte quality could be altered because of differences in temperature and blood flow in the subcutaneous environment compared to a pelvic (orthotopic) location (Oktay et al., 2004).

In the case we previously described, we created a peritoneal window with the aim of inducing angiogenesis and neovascularization (Donnez et al., 2004). Indeed, we demonstrated, in an earlier experimental study, that peritoneal tissue is superior to subcutaneous tissue as a site of transplantation (Nisolle et al., 2000). The fall in the number of primordial follicles found in cryopreserved tissue ranged from 50 to 65% in some studies (Baird et al., 1999; Meirow et al., 1999; Nisolle et al., 2000), to >90% in one study (Aubard et al., 1999). The loss of primordial follicles in grafted tissue is due to hypoxia and the delay that occurs before the grafted tissue becomes revascularized. Although primordial follicles are more resistant to ischaemia than stromal cells, there is a correlation between ischaemic tissue damage and the duration of ischaemia (Kim et al., 2004).

In an experimental model, Israely et al. (2004) analysed angiogenic events following ovary xenotransplantation. Characterization of neovascularization by dynamic contrast-enhanced MRI revealed that functional vessels within the graft could be detected from day 7 onwards.

In order to reduce the time needed for a graft to become revascularized, we created an intraovarian incision and a peritoneal window with the aim of inducing neovascularization, as we previously described (Donnez et al., 2004).

To our knowledge, only Radford et al. (2001) have reported a patient treated by chemotherapy whose cryopreserved ovarian tissue was reimplanted by laparotomy into the ovary. But in this case the ovarian tissue had been biopsied and cryopreserved 4 years after a first course of chemotherapy, and histological section of the cryopreserved tissue revealed only a few primordial follicles. Not surprisingly, the patient menstruated only once, 9 months after transplantation. This delay of 9 months in their study compared to the 4 months observed in our study is probably due to the very poor ovarian reserve in the cryopreserved tissue, the patient having received a full course of chemotherapy 4 years before.

Here, we describe the first orthotopic (intraovarian and paroovarian) transplantation of ovarian tissue, biopsied and cryopreserved before chemotherapy. Histological analysis of a small cube proved that the follicular density was normal after freeze–thawing. We should nevertheless bear in mind that follicle distribution is not homogeneous (Qu et al., 2000). Indeed, we and others (Qu et al., 2000; Schmidt et al., 2003; Lass, 2004) have demonstrated large variations in follicle density from ovary to ovary, as well as within the same ovary, when multiple samples are examined. Primordial follicles are located in clusters and not equally distributed in the ovarian cortex.

By extrapolation, in the present case, ~500 primordial follicles would have been transplanted, taking into account a loss of >50% owing to hypoxia (Donnez et al., 2000; Nisolle et al., 2000).

In the case we describe, vaginal echography and sequential serum concentrations of FSH, LH, 17β-estradiol and progesterone revealed the onset of an ovulatory cycle 4½ months after reimplantation of ovarian tissue.

Large biopsies of the native left ovary contained no primordial follicles and, as the patient maintained castrated levels for

Figure 3. Four-and-a-half months after reimplantation, ultrasonography demonstrated the presence of a follicle of 9.2 mm in size, which reached 14 mm 3 days before the LH peak. At 4½ months, a corpus luteum of 21.2 mm was clearly visible, concomitant with the presence of a serum progesterone level of 7 mg/ml.
almost 2 years, we could practically exclude the possibility of the development of an isolated follicle originating from the native left ovary. Indeed, FSH, LH and 17β-estradiol levels were measured every 2 weeks from December 2002 to August 2004. In this 20 month period, FSH concentrations were systematically found to be >40 mIU/ml, while 17β-estradiol levels were <10 pg/ml (13 pg/ml on one occasion). This supports our assertion that the origin of the ovulatory cycle was the autotransplanted tissue.

The time interval seen between the implantation of cortical tissue and the first estradiol peak and first signs of follicular development (4½ months) is consistent with data obtained from sheep and human beings (Gougeon, 1986; Baird et al., 1999, Donnez et al., 2004). Indeed, follicles at an early growth stage need >85 days to reach the antral stage (Gougeon, 1986). Primordial follicles obviously need even more.

Another very interesting finding is the persistence of relatively high FSH levels during the follicular phase. FSH levels remained as high as 22 mIU/ml during the follicular phase and decreased to 18 mIU/ml during the luteal phase. As previously explained in the present paper, the number of surviving primordial follicles is relatively low and the patient should be considered a poor responder. Recently, Baird et al. (2004) also observed raised basal levels of FSH in the sheep model and suggested that it was the consequence of a reduction in antral follicles and the secretion of inhibin A in the transplanted strip of cryopreserved ovarian cortex. In his opinion, the rate of recruitment may be accelerated after transplantation. Indeed, he observed massive recruitment of primordial follicles, demonstrated by a higher proportion of growing follicles, in the first 2 months following grafting.

The return to an FSH level of >35 mIU/ml immediately after each menstrual bleed is one argument supporting the theory that some inhibitory mechanisms, such as inhibin A or anti-Müllerian hormones (AMH) normally produced by developing follicles in an intact ovary, are probably almost non-existent in transplanted tissue.

In conclusion, this is the first time that cryopreserved ovarian cortical strips biopsied before chemotherapy have been reimplanted into the ovary and peritoneal window of a woman suffering from a non-cancerous disease (sickle cell anaemia), who received aggressive chemotherapy before bone marrow transplantation. This reimplantation allowed restoration of ovarian function as demonstrated by the presence of ovulatory cycles, although each of them was preceded by a rise in FSH to >35 mIU/ml. Interestingly, development of follicles was demonstrated by ultrasound in both sites of reimplantation.

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Ovarian function after reimplantation of frozen tissue


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