# Transplantation of cryopreserved ovarian tissue

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The lifesaving treatment endured by cancer survivors provokes, in many cases, early menopause and subsequent infertility. In clinical situations where there is often a pressing need to start chemotherapy, ovarian tissue cryopreservation looks to be a promising option to restore fertility.

It has been estimated that, by 2010, one in 250 people in the adult population will actually be childhood cancer survivors (1).

The treatment of childhood malignancy is becoming increasingly effective. Aggressive chemotherapy and radiotherapy, as well as bone marrow transplantation, can cure more than 90 percent of girls and young women affected by such malignancies. However, the ovaries are very sensitive to cytotoxic treatment, especially to alkylating agents and ionizing radiation, generally resulting in the loss of both endocrine and reproductive function (2). Moreover, it is known that uterine irradiation at a young age reduces adult uterine volume (3).

There are several potential options available to preserve fertility in patients facing premature ovarian failure, including immature and mature oocyte cryopreservation, embryo cryopreservation and cryopreservation of ovarian tissue(4, 5).

For those patients who require immediate chemotherapy, cryopreservation of ovarian tissue is a possible alternative (4, 6, 7).

## Hystory of experimental and clinical studies

To date, ovarian tissue has been successfully cryopreserved and transplanted in mice and rodents, as well as large animals like sheep and marmoset monkeys (8-10). The first successful fertilization and pregnancy following egg collection from fresh transplanted ovarian tissue in a primate was recently described (11). The grafted tissue functioned without any surgical connection to major blood vessels. Experimental studies have indicated that the decrease in the number of primordial follicles in grafted tissue is due to hypoxia and the delay before reimplanted cortical tissue becomes revascularized. The loss of primordial follicles in cryopreserved ovarian tissue after transplantation is estimated to be 50 to 65 percent in some experimental studies (7,8,12). In one experimental study, in which ovarian cortex was grafted onto the uterine horn and under the skin, the loss was found to be more than 90 percent (13).

Oktay et al have reported laparoscopic transplantation of frozen-thawed ovarian tissue to the pelvic side wall (14), to the forearm (15) and, more recently, beneath the skin of the abdomen. A four-cell embryo was obtained from 20 oocytes retrieved from tissue transplanted to the latter site, but no pregnancy occurred after transfer (16). Radford et al. reported on a patient with a history of Hodgkin's disease treated by chemotherapy, in whom ovarian tissue had been biopsied and cryopreserved four years after chemotherapy and later reimplanted (17). In this case, histological section of ovarian cortical tissue revealed only a few primordial follicles because of the previous chemotherapy. After reimplantation, the patient had only one menstrual period.

Very recently, the first livebirth after a fresh ovarian tissue transplant in a primate was reported (11).

We have described a livebirth after orthotopic autotransplantation of cryopreserved ovarian tissue. Our findings suggest that cryopreservation of ovarian tissue should be offered to all young women diagnosed with cancer (18).

## Freeze-thawing

Freezing of ovarian tissue was carried out according to the protocol described by Gosden et al [6]. The biopsies were immediately transferred to the

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laboratory in Leibovitz L-15 medium supplemented with Glutamax (GIBCO, Paisley, Scotland). There, the remaining stromal tissue was gently removed.

Four biopsies of the cortex were then cut into 70 small cubes of 2x2mm. One strip of 12x4mm was left whole.

Fragments (cubes and a strip) of ovarian tissue were suspended in the cryoprotective medium. All the fragments were placed into precooled 2ml cryogenic vials (Simport, Quebec, Canada) filled with Leibovitz medium supplemented with 4mg/ml of human serum albumin (Red Cross, Brussels, Belgium) and 1.5mM DMSO (Sigma, St. Louis, MO). The cryotubes were cooled in a programmable freezer (Kryo 10, Series III; Planer, Sunbury-on-Thames, United Kingdom) using the following program: (1) cooled from 0°C to –8°C at –2°C/min; (2) seeded manually by touching the cryotubes with forceps prechilled in liquid nitrogen; (3) cooled to – 40°C at – 0.3°C/min; (4) cooled to –150°C at –30°C/min, and (5) transferred to liquid nitrogen (-196°C) immediately for storage.

## Reimplantation of ovarian cortical tissue

In 1997, a 25-year-old woman presented with clinical stage IV Hodgkin's lymphoma. Ovarian tissue cryopreservation was carried out before chemotherapy. Written informed consent was obtained. Using laparoscopy, 5 biopsies, about 1.2-1.5cm long and 5mm wide, were taken from the left ovary. Removing the whole ovary was not an option, as one can never completely exclude recovery of ovarian function after chemotherapy. Indeed, premature ovarian failure after chemotherapy is age-, drug- and dose-dependent and does not occur in 100% of cases.

After laparoscopy, the patient received MOPP/ABV hybrid chemotherapy (mechlorethamine, vincristine, procarbazine, prednisone, adriamycin, bleomycin, vinblastine) from August 1997 to February 1998, followed by radiotherapy (38 grays).

She became amenorrheic shortly after the initiation of chemotherapy. The levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH) and estradiol ($E_2$) following chemo- and radiotherapy were, respectively, 91.1 mIU/ml, 85 mIU/ml and 17 pg/ml, confirming castration. This ovarian failure profile was confirmed three months later. Hormone replacement therapy (HRT) was started in June 1998 and then stopped in January 2001, as the patient wished to become pregnant. A thorough evaluation by oncologists demonstrated that she was disease-free.

After cessation of HRT, FSH, LH and 17 b-estra-diol levels returned to levels consistent with ovarian failure. From January 2001 to December 2002, the patient experienced only one ovulatory cycle? proved by a progesterone level of 10 nmg/ml and the presence of a corpus luteum on the left ovary, diagnosed by vaginal echography. The decision to reimplant was therefore taken.

A first laparoscopy was carried out 7 days before reimplantation in order to create a peritoneal window by means of a large incision just beneath the right ovarian hilus, followed by coagulation of the edges of the window. The goal was to induce angiogenesis and neovascularization in this area. Both ovaries looked atrophic. Nevertheless, a small corpus luteum was visible on the left ovary. A decrease in LH and FSH was observed and the concentrations then returned to castrated levels. A second laparoscopy was carried out seven days after the creation of the peritoneal window. A biopsy of 4-5mm in size was taken from each of the atrophic ovaries in order to check for the presence or absence of primordial follicles.

The thawed ovarian cortical tissue was then placed in sterile medium and immediately transferred to the operating theatre. The large strip and 35 small cubes of frozen-thawed ovarian tissue were pushed into the furrow created by the peritoneal window very close to the ovarian vessels and fimbria on the right side. No suture was used. An extensive neovascular network was clearly visible in this space

A third laparoscopy was carried out four and a half months after reimplantation to evaluate the survival of the graft. A follicle was visible at the site of reimplantation. Biopsy was performed.

The grafted tissue was biopsied and histology and fluorescent probe staining revealed the presence of viable primordial follicles and a follicular structure with inhibin A-marked cells. Follicles at an early growth stage require more than 85 days to reach the antral stage (19). Primordial follicles obviously require even more. The appearance of the first follicle in the grafted tissue five months after reimplantation is totally consistent with the expected time course. This time interval observed in our study between implantation of cortical tissue and the first estradiol peak (5 months) is also consistent with data obtained from sheep and human beings (8, 17).

From five to nine months after reimplantation, ultrasonography revealed the development of a follicle followed by corpus luteum formation with each cycle, at the site of reimplantation. This corresponded to an estradiol level of more than 100 pg/ml and a progesterone level ranging from 12 to 37 ng/ml. The LH and FSH levels were significantly ($p<0.05$) lower than those observed before reimplantation. This led to the restoration of consecutive menstrual bleeding each month.

At nine and a half months, FSH levels increased to 78.7 mIU/ml and returned to normal values seven days later. Three weeks later, a follicle of 2.6cm in size had developed on the right side, clearly outside the right ovary. Both native ovaries were well visualized and found to be obviously atrophic. Eighteen days after ovulation, calculated by basal body temperature, the hCG level was 2,853 mIU/ml.

We cannot explain this sudden and temporary surge in FSH. It is possible that it was associated with a decline in inhibin secretion, as suggested in the sheep model (20,21) or with slower follicular growth from a poor follicular reserve in the graft. Indeed, due to the loss of primordial follicles in the transplant, the follicular density per mm3 was low but, in any case, the total amount of cortical tissue transplanted is relatively unimportant. After transplantation, the patient would have been considered a poor responder as, of the 500 to 1000 primordial follicles that would have been transplanted, more than 50 percent would have been lost due to hypoxia (12).

## Is it risky?

Unfortunately, in the majority of cases, aggressive chemotherapy and radiotherapy lead to ovarian failure. The restoration of ovarian function after chemotherapy or radiotherapy has two main goals: to improve quality of life and restore reproductive function. For those patients who require immediate chemotherapy, ovarian tissue cryopreservation, performed before cancer treatment is begun, may be a means of preserving fertility without delaying the initiation of chemotherapy. However, one major concern surrounding the use of ovarian cortical strips for orthotopic autotransplantation is the potential risk that the frozen-thawed ovarian cortex might harbour malignant cells which could induce a recurrence of the disease after reimplantation. Shaw et al reported that ovarian grafts from AKR mice could transfer lymphoma to recipient animals (22). Nevertheless, more recent studies have suggested that ovarian tissue transplantation in Hodgkin's disease is safe (23-25).

In our study, histological evaluation of ovarian cortex before and after reimplantation demonstrated the absence of disease. But confirming the absence of malignant cells by light microscopy may not be sufficient, especially in other types of cancer (especially hematogenous or systemic neoplasms)(9). Screening methods to detect minimal residual disease must be developed to eliminate the risk of cancer cell transmission with reimplantation (5).

## Lines of evidence

1. The patient experienced, in total, 3 ovulatory cycles over a period of more than two years. All of them originated from the left native ovary. This was proved by laparoscopy and/or echography.
2. The native right ovary never demonstrated any ovarian activity at all (no follicles, no corpus luteum).
3. Even if we cannot absolutely exclude the presence of isolated follicles in the atrophic ovary, their density must be very low since serial sections of 4 large biopsies of atrophic ovaries failed to detect any.
4. Laparoscopy proved, by direct visualization, the development of a follicle from the grafted tissue five months after reimplantation.
5. Biopsy proved, by histologic examination, not only the survival of primordial follicles in the grafted tissue, but also the maturation of a follicle (granula-cells marked by inhibin-A). This is the first time that survival of primordial follicles has been histologically proved after cryopreserved ovarian tissue transplantation.
6. After follicular development was proved by laparoscopy and histology, the patient experienced regular menstrual bleeding. The progesterone level was systematically more than 10ng/ml in the mid-luteal phase, calculated on the basis of BBT. During each ovulatory cycle (from 5 to 9 months), vaginal echography demonstrated a corpus luteum on the grafted tissue outside the right atrophic ovary, which had demonstrated no ovarian activity for almost 3 years.
7. Finally, vaginal echography revealed the presence of a preovulatory follicle at the reimplantation site during the cycle leading to the pregnancy, but no follicles were seen on either of the native ovaries. This argument is a crucial one.

## Conclusion

This is the first report of the birth of a healthy infant, obtained after orthotopic autotransplantation of cryopreserved ovarian tissue. It opens new perspectives for young cancer patients facing premature ovarian failure. Ovarian tissue cryopreservation should be an option offered to all young women diagnosed with cancer, in conjunction with other existing options for fertility preservation such as immature oocyte retrieval, in vitro maturation of oocytes, oocyte vitrification or embryo cryopreservation.

Even if more and more papers are now describing the restoration of ovarian function after orthotopic

transplantation of fresh and frozen ovarian tissue and the first livebirth has recently been reported, we should still bear in mind that many questions remain unanswered. In our department, research is presently under way on freezing an entire ovary. A recent paper by our group described not only the technique, but also the high rate of survival of primordial follicles after freeze-thawing an entire ovary [26,27]. This could lead to the transplantation of an intact ovary,

with microvascular anastomosis carried out to restore immediate vascularization and minimize post-transplantation ischemia responsible for the reduction in follicular density.

A major limitation of intact organ transplantation, which needs to be investigated, is the problem of storage of an intact ovary with its vascular tissue.

Other issues, like the question of the optimum number of grafts and hence oocytes, how long the

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