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Auto transplantation of cryopreserved ovarian tissue to the right forearm 4 1/2 years after autologous stem cell transplantation

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Running headline: Cryopreserved ovarian tissue auto-graft

Premature ovarian failure (POF) may occur as a consequence of chemotherapy and of total body irradiation (1). Alkylating agents such as cyclophosphamide and chlorambucil are particularly prone to cause ovarian damage (2). Less than 2% of children, who had been treated with total body irradiation, could later reproduce (3). Cryopreservation of ovarian tissue harvested prior to ovariotoxic treatment is one approach to try to preserve a patient's fertility. Thus, investigators have reported auto transplantation of fresh ovarian tissue to the forearm (4) and of cryopreserved ovarian tissue heterotopically to the retroperitoneum (5), the rectus abdominis muscle (6) or orthotopically to the remaining ovary (7). Generally, in reported cases, the transplants have survived transplantation, but have survived for relatively short time and have not resulted in fertilization. However, recently, Oktay et al. reported on a woman from whom a mature oocyte obtained from autotransplanted cryopreserved ovarian tissue beneath the skin of her abdomen was fertilized and developed into a four-cell embryo (9). Lee et al. succeeded in fertilizing a macaque monkey with an oocyte obtained from cryopreserved ovarian tissue transplanted to subcutaneous pockets on the animal´s abdomen, resulting in the birth of a healthy female infant end of 2003 (10). This is the first reported auto transplantation of cryopreserved ovarian tissue to the forearm.

Case-report

The patient was a 30-year old, nulliparous woman who in 1991 was diagnosed with Sjogren´s syndrome. In 1993, ~~the~~ she was diagnosed with Pure Red Cell Aplasia (PRCA), requiring numerous blood transfusions. Recurrent, major venous thrombotic events and a pulmonary embolus necessitated long-term anticoagulation with warfarin (Waran^{R}). From 1995, attempts to treat the PRCA were made with antithymocyte globulin (ATG), anti-CD52 and anti CD4 antibodies, desferrioxamine (Desferal^{R}), azatioprin (Imurel^{R}), cyclosporine, extracorporal photophoresis, and immunoglobuline infusions (Gammagard^{R}). In February 1998, stem cell mobilisation was attempted with cyclophosphamide and granulocyte-colony stimulating factor (G-CSF). Later in 1998, autologous stem cell transplantation was performed. Prior to treatment, conservation of her fertility was considered. At that time, the patient did not have a partner ruling out cryopreservation of fertilized eggs. To cryopreserve ovarian tissue, one ovary was removed laparoscopically, cut into small pieces which were put in 37^{o}C Gamete-100 medium (Vitrolife Sweden AB, Gothenburg, Sweden), and immediately transported to the laboratory at IVF-Öresund. The tissue was further cut into 1-2 x 1-2 x 1 mm pieces and transferred to a cryosolution containing 1,5 M propanediol and 0.1 M sucrose in PBS (Cryosolution nr. 2, Vitrolife Sweden AB, Gothenburg, Sweden) at ambient temperature. Fifteen straws, prefilled with cryosolution, were loaded with the pieces of ovarian tissue and put immediately for cryopreservation in a programmable freezing device, CTE 920 (Cryo-Technik-Erlangen GmbH, Erlangen, Germany). This device has a self-seeding system which results in crystallization at an optimal temperature. A slow freezing protocol was used and the straws were moved for further storage in liquid $N_{2.}$ After laparoscopy, the patient received a combination of cyclophosphamide and fractionated total body irradiation (Cy-fTBI) with a total of 8400 mg of cyclophosphamide, and 8 Gy of total body irradiation. Then she was successfully infused with 4.0 x 10^{6}/kilo

body weight of CD34 stem cells. A few months later, she had climacteric symptoms and amenorrhoea with a FSH-level of 31 IU/litre, LH of 16 IU/litre, and oestradiol < 70 pmol/litre, suggesting primary ovarian failure (POF). She was then put on hormone replacement therapy (HRT).

Early in 2003, she attended for infertility. HRT-treatment was intermittently discharged, rendering the patient climacteric with hot flushes and a FSH-level of 25 IE two weeks later suggesting continuing POF. She gave informed consent for autotransplantation of her cryopreserved ovarian tissue On April 29, 2003, straws with cryopreserved ovarian tissue were transported in liquid N_2 from IVF-Öresund in Malmö to the University Hospital of Lund. The thawing procedure was similar to the one described by Oktay et al.[6] In short, two of the straws containing ovarian pieces were thawed in room air for 30 seconds, then placed in a 37^{o}C water bath for 2 minutes. The contents of each straw were expelled in a solution containing 1.5 M propanediol, 0.1 M sucrose and 20% of fresh autologous serum in phenolred-free Minimal Essential Medium, MEM, (with L-glutamine, ribonucleosides and deoxyribonucleosides, Gibco, cat. no. 41061-029). After washing stepwise in decreasing concentrations of cryoprotectant, the tissue was finally transferred to and kept in a transport medium consisting of 10 μg/mL cefuroxim, 10 IU of FSH (Serono S.A.) and 20% fresh autologous serum in phenol-free MEM for 5 minutes before transplantation. All dishes were kept on ice and after completion of the procedure the temperature of the media was 8^{o}C.

The transplantation was largely performed according to Oktay et al. (5,6). On the right arm, a 12 mm transverse incision was made approximately 8 cm distally of the antecubital fossa above the brachioradial muscle. Using blunt dissection, a 2 cm deep pocket was created distally between the subcutaneous tissue and the muscle fascia. Lifting the skin above the pocket with small hooks to keep it expanded, 10 pieces of tissue were put into the pocket and distributed with a micro pick-up. The pieces were of uneven sizes and shapes, i.e.,

1-2 mm x 1-2 mm x 0.5-1 mm. The incision was sutured with a continuous, intradermal, 4-0 polyglycolic suture. The forearm was loosely bandaged and put in a mitella. The patient was asked to keep her right arm in the mitella for the next four days.

For the next 7 days, 75 IU/day of FSH was injected and spread out into the subcutaneous tissue above the pocket to improve follicular growth (9**SCID**). Hormone treatment with a patch supplying estradiol 100μg/24 hours was continued. Power doppler ultrasound using a 8 MHz crystal was performed regularly the following weeks to look for monitor vascularisation and follicle development. Follicle development was first observed after 18 weeks. The oestradiol patch treatment was then discontinued. A week later, estradiol levels had strongly increased to a maximum of 6861 pmol/L (Figure 1). To further stimulate follicle development, 50 IU of FSH increasing to 100 IU was given subcutaneously every day from day 135 to day 145. Despite this supporting therapy, the oestradiol levels again went down and remained on lower levels until day 209 where after the levels remained at 70 pmol/L or lower. The follicle increased slowly in size to a maximum 12.6 mm in average diameter (figure 2). A second follicle was noted on day 162, reaching its maximum of 6,7 mm in average diameter on day 216. A new attempt to stimulate the ovarian tissue with FSH eight months after transplantation failed, suggesting the tissue was exhausted.

Discussion

To our knowledge, this is the first reported auto transplantation of cryopreserved ovarian tissue to the forearm. Nevertheless, the tissue survived too short and no oocytes were obtained. Several issues should be addressed to further develop the method:

Auto transplantation has two main goals: to restore hormone production and follicular development in the patient in order to achieve a pregnancy. The amount and

duration of oestradiol production are probably intimately connected to the total number of intact follicles in the tissue at the time of adequate revascularisation. At the outset, this number will be dependent on age of the patient and amount of tissue transplanted. However, follicles are lost during the procedures from oophorectomy to complete revascularisation. One factor affecting the follicular loss is the cryopreservation procedure. Different cryoprotectant are associated with more or less follicle damage. For example, better survival was reported with propanediol than with DMSO (10). Also, exposure- time to the cryoprotectant before freezing may be critical for follicle survival (11). Another important factor causing loss of follicles is ischaemia. Ischaemia starts at the removal of the ovary form the patient. However, Jeremias et al. showed that warm ischaemia in up to 30 minutes before cryopreservation did little damage to the follicles (12). After cryopreservation and thawing procedures, on the other hand, the numbers of primordial follicles in small cortical strips (1-2 mm), but not in larger strips (5 mm), were significantly reduced (12). After placing the tissue back into the body, ischaemia will persist until revascularisation. It has been suggested that 50 % of the follicular population is damaged after transplantation of fresh ovarian tissue in mice (13). After transplantation of cryopreserved human ovarian cortical strips under the kidney capsules of SCID mice, 10% to 84% of the follicles survived, depending on which cryoprotectant that was used (14). To reduce follicular loss during revascularisation, it is necessary to find ways of speeding up the revascularisation process. Dissen et al showed that vascular endothelial growth factor (VEGF) is a potentially important endogenous angiogenic stimulus for subsequent revascularisation of autotransplanted ovaries in young rats (15). Vascular corrosion casting followed by scanning electron microscopy revealed that the transplanted ovary became profusely revascularised within 48 h after transplantation. Vascular in-growth was accompanied by a 40- to 60-fold increase in expression of the genes

encoding VEGF and transforming growth factor-beta 1 (TGF beta 1), and it seemed that FSH-secretion contributed to this up-regulation (15).

Regarding the refertilisation issue, several questions are unresolved, including when and how to stimulate the growing follicles, and when to harvest oocytes. The reason we did not harvest the small follicles observed in the forearm, was an expectation of further growth beyond the 12 mm. However, Lee et al. in their experiment with macaques, aspirated follicles with only 4 mm diameter achieving metaphase-II oocytes from one of ten.

Another issue is where to place the frozen-thawed ovarian tissue at auto transplantation. There are several principles that should be paid attention to: revascularisation should occur rapidly, the transplantation should be possible with minimal surgery, and the graft easily accessible to examination and manipulations including aspiration of the follicle. Whether revascularisation is site-dependent is unknown. Risks of transplant exposure to suboptimal temperatures or to mechanical stress depend on transplantation site. For example, tissue transplanted under the skin of the forearm, as in the present case, will probably be exposed to both higher and lower temperatures than ovaries in there normal location. Thus, the possible thermal injury the oocytes are exposed to might go beyond the freezing and thawing-procedures. Finally, convenience and aesthetics should not be forgotten. Even when our patient was right-handed, it was decided to transplant to that arm because her work required her to carry heavy loads on her left forearm. For aesthetical reason, transplantation to the side of the throat, as was recently suggested (16), seems less attractive. Lee et al. achieved the most metaphase-II oocytes from transplants on the abdomen (X), a site Oktay also had used for the first successful fertilization of an oocyte from cryopreserved human ovarian tissue (Y)

In conclusion, auto transplantation of cryopreserved ovarian tissue to the forearm is feasible, but might not be the optimal site for such transplantation. Further research

is needed to elucidate how to increase the number of surviving follicles and to prolong tissue survival, and how to achieve mature oocytes which can be fertilized.

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