Advances in Fertility Preservation in Young Female Cancer Patients

a report by

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Advances in the diagnosis and treatment of childhood, adolescent and adult cancer have greatly enhanced the life expectancy of pre-menopausal women with the disease. As a result, there is a growing population of adolescent and adult long-term survivors of childhood malignancies.[1] For the majority of women, ovarian damage caused by radiotherapy and/or chemotherapy will result in premature menopause. Indeed, the ovaries are sensitive to cytotoxic treatment, especially radiation and alkylating agents. Follicular destruction generally results in the loss of both endocrine and reproductive functions. Therefore, procedures to preserve fertility have to be undertaken as an integral part of treatment of patients at risk of such side effects. The American Association of Clinical Oncologists (ASCO) recommends that all cancer patients of reproductive age be informed about the possibility of treatment-related infertility.[2]

The different fertility preservation options that may be offered to patients before the initiation of chemo- and/or radiotherapy include cryopreservation of embryos, oocytes or ovarian cortical tissue.[3,4] Decision-making in this area is particularly difficult because of the experimental nature of some of the techniques involved. With the continued development and optimisation of these techniques, however, it may one day be possible to offer an individualised approach to management.[5,6] The choice of the most suitable strategy for preserving fertility depends on different parameters: the type and timing of chemotherapy, the type of cancer, the patient's age and the partner status. According to the Ethics Committee of the American Society for Reproductive Medicine (ASRM),[7] the only established method of fertility preservation is embryo cryopreservation, but this option requires the patient to be of pubertal age, have a partner or use donor sperm and be able to undergo a cycle of ovarian stimulation.

Most female cancer patients of reproductive age do not have the option of utilising *in vitro* fertilisation (IVF) and embryo cryopreservation to safeguard their fertility. Indeed, in many cancers chemotherapy is initiated soon after diagnosis. For patients who require immediate chemotherapy, or those with hormone-sensitive cancer or who are still pre-pubertal, the practice of oocyte and ovarian tissue cryopreservation has rapidly become the technique of choice, although it remains experimental. Cryopreservation of oocytes can be performed in single women who are able to undergo a stimulation cycle, but the effectiveness of this technique is still low, with delivery rates of 1–5% per frozen–thawed oocyte.[8,9] Nevertheless, since the recent introduction of oocyte vitrification, the delivery rates are about two-fold per thawed oocyte. Cryopreservation of ovarian tissue is the only option available for pre-pubertal girls and for woman who cannot delay the start of chemotherapy. Ovarian cryopreservation and transplantation procedures have so far yielded six live births in humans after autologous transplantation.[10–14]

First, we will describe the technique of embryo cryopreservation, and then investigate the more experimental techniques of cryopreservation of oocytes and ovarian tissue.[15]

Embryo Cryopreservation

Since the report of the first human live birth from frozen–thawed embryos in 1983,[16] embryo cryopreservation has become routine practice in all IVF centres, and has proved its efficacy in terms of pregnancy and 'take-home-baby' rates. It yields a survival rate of up to 90% per thawed embryo and a pregnancy rate of 10–50% per transfer.[17,18] In our department, we have a pregnancy rate of 38% per transfer with frozen–thawed embryos. However, there are some drawbacks, i.e. ovarian stimulation, oocyte retrieval and IVF delay the initiation of chemotherapy or radiotherapy, which may not be acceptable in some cases. Also, high oestrogen concentrations associated with ovarian stimulation may be contraindicated in women with oestrogen-sensitive malignancies. Even if IVF can theoretically be undertaken on the basis of a spontaneous ovarian cycle,[19] the small number of obtainable oocytes (and subsequently viable embryos for transfer) makes it extremely unlikely that any live births will be achieved. Recently, tamoxifen and letrozole were employed to stimulate the ovaries for IVF and embryo cryopreservation with some success, possibly providing a safer alternative to traditional ovarian stimulation methods in these patients.[20,21] Finally, this technique is inappropriate for patients without a partner who do not wish to use donor sperm and for children who have not reached puberty.

For now, it appears that embryo freezing is the only established option for fertility preservation, according to the ASRM guidelines;[22] freezing of

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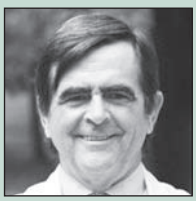


Jacques Donnez is Head and Chairman of the Department of Gynaecology at the Catholic University of Louvain, where he founded the Infertility Research Unit. He has focused his research activities on three main topics: tubal infertility, endometriosis and, more recently, ovarian cryopreservation and transplantation. He has made a major contribution to these fields by examining and interpreting clinical data in the light of cellular and molecular observations and developing original experimental models and innovative microsurgical approaches.

early-stage embryos yields better survival and pregnancy rates than blastocyst freezing and thawing; and the duration of freezing does not affect embryo survival after thawing or implantation potential.[23]

In conclusion, embryo cryopreservation is an efficient technique, but an option only for post-pubertal patients who have time for ovarian stimulation prior to cancer treatment and who have a partner (or agree to use donor sperm). It should be borne in mind that IVF with embryo cryopreservation should be proposed only before starting chemotherapy. Indeed, we have shown that when ovarian stimulation is performed in the interval between two chemotherapy regimens, the efficacy of IVF is dramatically reduced.[24]

Oocyte Cryopreservation

Oocyte cryopreservation can be used to preserve the fertility of female cancer survivors, but it is still considered experimental. It is an alternative to embryo storage for patients who do not have a male partner or refuse to use donor sperm. Human oocytes can be cryopreserved at a mature (metaphase II) stage or immature (germinal vesicle [GV]) stage.

Mature Oocyte Cryopreservation

Mature oocyte freezing appears to be the most logical way of storing female germ cells, comparable to routine sperm banking. It is an attractive option for post-pubertal patients without a partner if they have time to complete ovarian stimulation before cancer therapy.

Clinical research has been slow in the area of oocyte cryopreservation, as freezing mature oocytes is a technical challenge. They are extremely sensitive to temperature changes and have limited capacity for repair of cytoplasmic damage.[25] Indeed, the metaphase II oocyte is a large and highly specialised cell that is extremely fragile. The zona pellucida hardens during the freezing process, probably as a consequence of premature exocytosis of the cortical granules. It could then act as a fence, impairing spermatozoan penetration and normal fertilisation, although micromanipulation techniques – such as intracytoplasmic sperm injection (ICSI) – can to a certain extent bypass this problem.[26–29] Moreover, in the mature oocyte the metaphase chromosomes are lined up by the meiotic spindle along the equatorial plate, but the spindle apparatus is easily damaged by intracellular ice formation during the freezing or thawing process. Cellular cooling can induce depolymerisation of the meiotic spindle and, consequently, aneuploidy.[30,31]

Since the first report of a live birth from a frozen oocyte,[32] the worldwide results of oocyte slow freezing have been variable, with a reported success rate of 1–5% per frozen–thawed oocyte.[8,9] Data on frozen–thawed mature oocytes from 21 studies in peer-reviewed journals were examined by Sonmezer et al.,[5] who found a mean survival rate of 47% and a mean fertilisation rate of 52.5%. Newly developed techniques in slow freezing for oocyte cryopreservation include the use of elevated dehydrating sucrose concentrations,[28,33,34] longer pre-equilibration and thawing time[35] and the utilisation of sodium-depleted media.[36]

Vitrification, a novel freezing technique with high cryoprotectant concentrations and ultra-rapid cooling, has been developed for oocyte freezing, resulting in better survival without the need for sophisticated equipment.[37–39] Indeed, ongoing advances in vitrification methods[39–42] have yielded oocyte survival rates over 85%, and Cobo et al. reported an implantation rate of 8.6% per thawed oocyte.[39] Currently, the clinical effectiveness and safety of vitrified oocytes cannot be adequately assessed because of a lack of well-controlled clinical trials.[43] However, a recent study by Chian et al.[44] reported a 2.5% incidence of congenital anomalies in a cohort of 165 pregnancies and 200 infants conceived following oocyte vitrification, which is comparable to the normal population.[44] A recent report describes the first twin live birth achieved with autologous cryopreserved oocytes in an ovariectomised borderline cancer patient.[45]

Immature Oocyte Cryopreservation from Antral Follicles and In Vitro *Maturation*

An alternative approach is to freeze immature oocytes at the GV stage (GV oocytes) and mature them *in vitro*. Oocyte maturation is considered as the re-initiation and completion of the first meiotic division from the GV stage (prophase I) to metaphase II, and the accompanying cytoplasmic maturation phase for fertilisation and early embryonic development.[46]

At the GV stage, oocyte chromatin is diffused and the cell is still at the diplotene stage of prophase I, when no polymerised tubules are present. This strategy offers some practical advantages because hormonal stimulation is not needed,[47] and freezing GV oocytes avoids the risk of meiotic spindle damage and cytogenetic abnormalities during subsequent cellular divisions.[48] After thawing, GV oocytes need to undergo nuclear and cytoplasmic maturation to become fertilisable, but techniques to achieve this *in vitro* are still suboptimal. Indeed, even though GV oocytes have a superior post-thaw survival rate, the continued inefficiency of *in vitro* maturation (IVM) protocols results in a final yield of mature oocytes that is similar to that obtained with cryopreserved metaphase II oocytes.[49] Although recent studies have reported a marked improvement in pregnancy outcomes after IVM of fresh GV oocytes,[50] only one live birth has resulted from a GV oocyte cryopreserved with a slow-freezing protocol and matured *in vitro*.[51] Recently, Chian et al. reported a live birth from immature oocytes after IVM and cryopreservation of the oocytes by vitrification.[52] In summary, the future of GV-stage oocyte cryobanking depends on the optimisation of cryopreservation protocols and the development of reliable IVM procedures.[25,53]

Ovarian Tissue Cryopreservation

For pre-pubertal girls and woman who cannot delay the start of chemotherapy, cryopreservation of ovarian tissue is the only option available.[54–57] Theoretically, ovarian tissue can be frozen using three approaches: as fragments of ovarian cortex, as the entire ovary with its vascular pedicle or as isolated follicles. The indications for cryopreservation of ovarian tissue in cases of malignant and non-malignant disease are summarised in a recent review.[6]

Fragments of Cortical Ovarian Tissue

Cryopreservation of ovarian tissue requires a surgical procedure under general anaesthesia, but laparoscopy allows relatively simple retrieval of ovarian tissue by either oophorectomy or multiple ovarian biopsies. The number of biopsies taken varies according to the estimated risk of premature ovarian failure (POF) after chemotherapy, which is dependent on age, drug used and dose given. This technique has been performed in children as young as 2.7 years of age.[58] In our

department, the youngest patient who had her ovarian tissue retrieved by laparoscopy for cryopreservation was three years old. Such a procedure can be easily carried out before the start of cytotoxic chemotherapy, with the aim of re-implanting tissue into the pelvic cavity (orthotopic site)[10–12,14] or a heterotopic site[59,60] such as the forearm once treatment is completed and the patient is disease-free. The majority of follicles in the ovarian cortex are at the primordial (70–90%) or primary (20–30%) stages, which are the most resistant stages to cryoinjury and ischaemic damage. However, designing a cryopreservation protocol for ovarian tissue is challenging because of its cellular heterogeneity. The choice of appropriate cryoprotectant and freeze–thawing rates thus entails a compromise between oocytes, follicular cells and stromal cells. Experimental animal studies on ovarian tissue cryopreservation, resulting in live-born offspring, preceded the currently used freezing systems in humans. On the basis of current knowledge, the standard method for human ovarian cryopreservation is slow-programmed freezing using human serum albumin-containing medium and propanediol, dimethylsulphoxide (DMSO) or ethylene glycol as a cryoprotectant, with or without sucrose.[53]

Human ovarian tissue can be successfully cryopreserved, with good survival and function after thawing. To date, ovarian tissue has been successfully cryopreserved and transplanted into rodents,[61] sheep[54] and marmoset monkeys.[62] Experimental studies have indicated a fall in the number of primordial follicles in grafted tissue that could be due to hypoxia and the delay before re-implanted cortical tissue becomes revascularised. The loss of primordial follicles in cryopreserved ovarian tissue after transplantation is estimated to be at least 50–65% in some studies.[63–65]

In 2004, we reported in *The Lancet* the first live birth after orthotopic autotransplantation of human cryopreserved ovarian tissue.[10] So far in our department, six patients have undergone frozen–thawed ovarian tissue autotransplantation after POF due to chemotherapy. Thawed fragments were grafted to an orthotopic site. The first signs of ovarian function restoration (oestradiol peak, decrease in follicle-stimulating hormone [FSH] and ultrasound showing follicular development) occurred between 16 and 26 weeks after re-implantation. There were no signs of disease recurrence in any of the patients with malignant disease and restoration of ovarian endocrine function was observed in all cases.[66] To date, six successful pregnancies have been achieved in cancer survivors after auto- transplantation of cryopreserved ovarian tissue.[10–14] They were all from orthotopic transplants.

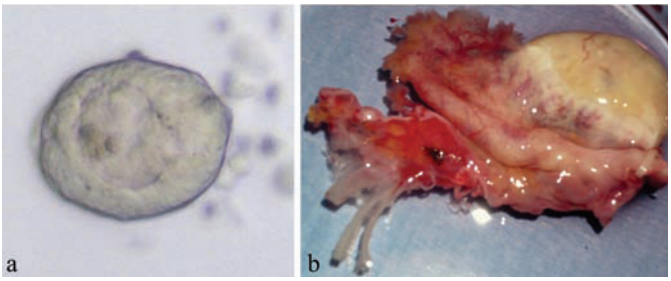
Safety

One major concern raised by the use of ovarian cortical strips is the potential risk that the frozen–thawed ovarian cortex may harbour malignant cells. This could induce a recurrence of the disease after re-implantation if the biopsy is taken before chemotherapy. Shaw et al. reported that ovarian grafts from aldo-keto reductase (AKR) mice could transfer lymphoma to recipient animals.[67] More recent studies suggested that human ovarian tissue transplantation in Hodgkin's disease was safe.[68–70] In cases of leukaemia, however, malignant cells may be present in the bloodstream, with the risk of transferring leukaemic cells.[71] For these patients, safer approaches need to be developed.

Isolated Preantral Follicles

To avoid transferring malignant cells, ovarian tissue culture and *in vitro* follicle maturation may be performed. Culturing isolated follicles from the primordial stage is a particularly attractive proposition, since they represent >90% of the total follicular reserve and show high cryotolerance.[72] Nevertheless, this *in vitro* approach is challenging in humans due to the prolonged duration of folliculogenesis. Human isolated primordial follicles do not grow properly in culture.[73,74] Indeed, despite the encouraging results achieved by Hovatta,[75] it has not yet been possible to grow human isolated primordial follicles to the mature oocyte stage. This led us to consider an alternative strategy, which involves grafting isolated ovarian follicles. Transplantation of frozen–thawed isolated primordial follicles has been successfully achieved in mice, yielding normal offspring.[76] This method has theoretical advantages for diseases that carry a high risk of ovarian metastasis, as the follicular basal lamina encapsulating the membrana granulosa excludes capillaries, white blood cells and nerve processes from the granulosa compartment.[77]

Figure 1: Isolated Human Follicle After Enzymatic Digestion (A) and Whole Human Ovary After Vascular Pedicle Dissection (B)



A: This follicle is 40µm in size. B. Ovary shown prior to perfusion with heparinised and cryoprotectant solution. The ovary, fallopian tube and ovarian vessels are visible.

However, for human primordial follicles mechanical isolation is not possible due to their size (30–40µm) and their fibrous and dense ovarian stroma. Currently, enzymatic tissue digestion using collagenase is used for ovarian follicle isolation. We developed a protocol to digest human ovarian cortex using the Liberase enzyme blend to isolate primordial and primary follicles from ovarian cortical tissue.[78] These isolated human follicles can survive and grow after transplantation, and some are even able to reach the antral stage.[79,80] As this approach has successfully restored fertility in mice, our optimisation of follicle isolation and recovery protocols now allows us to consider its development for humans. Furthermore, our experimental study has highlighted certain areas that need to be addressed in order to progress in this field.

Whole Ovary

One future strategy to preserve fertility is transplantation of whole intact ovary by vascular anastomosis. With this strategy, immediate revascularisation of the transplant can be expected, thus minimising ischaemic injury and the related massive follicle loss.

Successful vascular transplantation of intact frozen–thawed ovaries has been reported in rats,[81] rabbits[82] and sheep.[83–85] However, cryopreserving a large-sized intact human ovary is problematic because of the difficulty of adequate diffusion of cryoprotective agents into large tissue masses and the risk of vascular injury caused by intravascular ice formation. Nevertheless, we showed that it was possible by perfusing the ovarian vessels with a cryoprotectant solution before freezing the ovary with a passive cooling device.[86] The protocol we applied ensured a high survival

rate of follicles (75.9%), small vessels and stromal cells after thawing, as well as a well-preserved ultrastructure and no signs of apoptosis.[87] Our results have led us to seriously consider proposing this option to women[88] when there is no risk of transmitting malignant cells via the graft after transplantation. Research and development of technology to cryopreserve whole organs, as well as surgical techniques for the autotransplantation of an ovary with its vascular pedicle, should be encouraged. Indeed, thrombotic events in the re-anastomosed vascular pedicle are described in animals.

Conclusion

All patients who may become infertile have the right to receive proper consideration of their interests for future possibilities in the field of ovarian function preservation. The selection of cases should be carried out following a multidisciplinary staff discussion including oncologists, gynaecologists, biologists, psychologists and paediatricians. Counselling should be given and informed consent obtained from the patient. We believe that it is preferable to remove only one ovary if possible, to avoid the psychological stress of surgical castration in a young patient, as cases of spontaneous pregnancy have been described after total body irradiation[89–92] and because, in many cases, the remaining ovary will be able to resume all endocrine function after some years. The idea of 'oocyte banking' is attractive, but it requires a sustained effort to achieve better results with ovarian tissue cryopreservation techniques and *in vitro* oocyte maturation procedures.

Live births obtained after transplantation of frozen–thawed ovarian tissue give hope to young cancer patients, but great efforts are still required in research programmes in order to determine whether active angiogenesis can be induced to accelerate the process of neovascularisation in grafted tissue, if isolated human follicles can be grafted or indeed if grafting an entire ovary with its vascular pedicle is a valuable option. In order to propose the most suitable transplantation procedure in each clinical situation (type of cancer, age of patients and risk of transmission), we believe that the approach to fertility preservation should be individualised. Also, just like ovarian cryopreservation, oocyte and embryo cryopreservation for cancer patients should be studied only in the context of Institutional Review Board/Ethics Committee-approved protocols. ■

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