

Cryopreservation of human ovarian tissue and oocyte banking: **An eye** to the future

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A cancer diagnosis no longer necessarily ends a woman's life. Although there is still no "cure" for many cancers, steady improvements in early detection and treatment have greatly increased survival after a cancer diagnosis. Even victims of advanced ovarian cancer can now expect to survive an average of 5 years. Improved cancer treatment and the social phenomenon of later childbearing present us with an enviable problem. More and more women with cancer are now considering fertility preservation as part of their treatment planning.

Premature ovarian failure may occur in women with genetic diseases (such as Turner's syndrome), after chemotherapy or radiotherapy for malignant disease, in severe or recurrent ovarian disease (such as cysts, benign tumors or endometriomas), or after removal of the ovaries to treat endometriosis, ovarian pain or genital cancer. For women at risk of premature ovarian failure, there are now three possibilities for preserving their fertility: cryopreservation of their oocytes, embryos, and, most recently, ovarian tissue. This article provides an overview of these procedures, highlighting the recent developments in ovarian tissue cryopreservation.

Background

Several diseases and their treatments threaten to destroy all the follicles in a woman's ovaries. Diseases rarely have a direct effect on the oocytes in the ovary. Exceptions include genetic disorders such as women with a single X chromosome (Turner Syndrome) or women missing a specific piece(s) of an X chromosome, in which case the oocytes die quickly and the women have premature menopause. Chemotherapy or radiation used to treat cancer or some non-cancerous disorders have the unfortunate side effect of destroying the follicles in the ovary as well as the diseased cells.

Unlike sperm production in men which is continuous, women are born with all their primordial follicles and they do not produce any more. The natural process of each menstrual cycle consumes approximately 500-1000 oocytes until the supply is exhausted (about age 51, menopause). Any treatment that accelerates the loss of oocytes threatens to decrease fertility and will cause menopause at an earlier age than expected. Surgery to remove all or a portion of one or both ovaries, some chemotherapy (cyclophosphamide, doxorubicin, vinblastine, etc.), and radiation therapy all have known toxic effects on oocytes. The number of

oocytes destroyed from these treatments depends primarily on the agent(s), the dose, and the age of the woman when treated. The higher the dose and the older the woman, the more likely that most oocytes will be lost and that menopause will occur.

Men have been able to cryopreserve (freeze) their sperm for decades. However, in the past, women have not been able to freeze their oocytes reliably because the oocytes are hard to retrieve and unfertilized oocytes have not generally a high survival freezing or thawing rate.[1] With the advent of IVF, egg or embryo freezing became possible. IVF has several significant limitations. IVF takes time to complete since the hormones used to stimulate the ovary are administered at specific times of the menstrual cycle and then are administered for 2-4 weeks.[2] Many cancer patients must begin their cancer treatment before IVF can be completed. Many cancer patients are young and unmarried; therefore, they do not have a partner to provide sperm to fertilize the oocytes. Cryopreservation of unfertilized oocytes is an alternative that has gained interest with newer freezing techniques [3,4,5] and there are successful pregnancies. Further, IVF is medically inappropriate for many women with hormonally responsive tumors such as breast cancer. The ovaries can be surgically moved from the field of radiation in selected cases but this technique does not help women with chemotherapy.

Oocyte cryopreservation

It seems obviously that female gamete cryopreservation lags behind frozen sperm technology. Male gamete cryopreservation has been a reality since the early 1970s, when cryopreserved sperm first came into routine use[6]. Advances since then include intracytoplasmic injection using cryopreserved sperm [7].

Oocytes can be collected when either mature or immature. Cryopreservation of mature oocytes, although successful

in the mouse, has met with little success in most other species, including human. All cryopreservation procedures in humans have led to impaired oocyte survival and subsequent reduced rates of fertilisation and embryo cleavage, with the result that fewer than 2% of oocytes are capable of producing a term pregnancy [8].

Oocytes are more difficult to preserve because they're the largest cells in the body and contain a complex intracellular architecture that is critical to biologic function. Research continues, however, because oocyte freezing is an attractive

Intracytoplasmic sperm injection (ICSI) seemed to have partly obviated initial problems with fertilization

alternative to embryo preservation. Preserving oocytes raises fewer ethical and legal red flags than does use of residual frozen embryos. This alternative is especially crucial for patients at risk of premature ovarian failure due to antineoplastic treatments [9]. Techniques such as slow freezing and rapid thawing can minimize intracellular ice formation and thus subsequent structural damage. Selecting the right cryoprotectant is also critical and requires variation of the freezing protocol [10].

To circumvent the difficulties encountered in oocyte cryopreservation, some research facilities have incorporated vitrification. This process uses high concentrations of carbohydrate-containing cryoprotectants to solidify the cell in a glass-like state and minimize ice damage. Superior results in animals have been achieved with vitrified cryopreserved oocytes, and although rare, there have been some human births [3,4,5]. Oocyte cryopreservation without vitrification has been performed since the

1980s, but with limited success [11].

Intracytoplasmic sperm injection (ICSI) seemed to have partly obviated initial problems with fertilization [12]. However, the first report of a live birth using cryopreserved oocytes and ICSI did not appear until late in the 1990. [13] Although ICSI may improve the success rate with cryopreserved oocyte fertilization, it may also add another reason for concern over the long-term health consequences in the children conceived [14,15].

Perhaps these concerns will give way to completely new alternatives to cryopreservation as gamete reconstruction research produces astonishing alternatives and hopefully, results. For instance, it is possible to cryopreserve immature human oocytes [16,17]. This offers no advantage over cryopreserving mature oocytes at present, because of the difficulty of obtaining viable oocytes after in-vitro maturation. However, considerable research is being directed to cryopreserving oocytes of domestic animal species, and this may ultimately benefit human oocyte freezing.

Another example is the reconstitution of oocytes from bone marrow-derived stem cells may soon be an option [18] Another alternative includes first activating the oocytes to allow them to complete the final meiotic division and then freezing the haploid zygotes as we have been freezing embryos. This obviates the large cytoplasm and temperature-sensitive spindles that make oocyte freezing difficult. However, drawbacks with this technique include the need for artificial activation before freezing. Second, fertilization requires nuclear transfer techniques that currently are not routine in any IVF lab.

Embryo cryopreservation

Cryopreservation of embryos is reasonably successful [19-22]. The pregnancy rate after transfer of two to three cryopreserved embryos is around 15%-25%, and the probability of pregnancy accumulates with repeated transfers. However, there are associated problems:

obtaining oocytes for fertilization is technically difficult, limiting the number that can be used; there is a need for a male partner; unmarried couples may be excluded by government regulations or legal restrictions; and there may be significant ethical problems in disposing

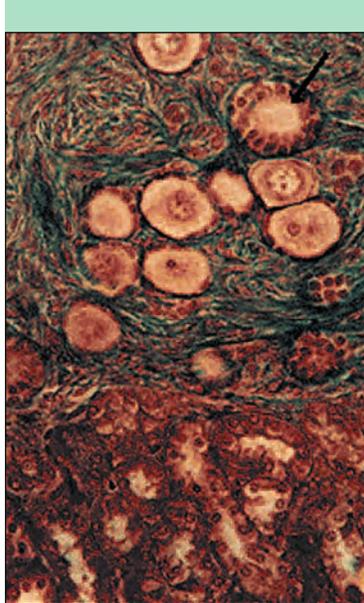
In addition, in women with cancer there is a risk of transferring cancer cells together with the frozen thawed embryos; the embryos should be denuded of all other cells before transfer to the uterus

any unwanted embryos. Unforeseen circumstances, such as death, divorce or other family problems, may prevent embryos being implanted into the natural mother. Further, recovering oocytes to generate embryos usually requires ultrasound-guided needle aspiration of the leading follicle(s) in several successive menstrual cycles and ovarian stimulation to induce superovulation. Ovarian stimulation involves raising blood levels of oestrogen up to 20-fold above normal and may not be appropriate in women with oestrogen-sensitive cancers of the breast, ovarian epithelial tumors or severe endometriosis. In addition, in women with cancer there is a risk of transferring cancer cells together with the frozen thawed embryos; the embryos should be denuded of all other cells before transfer to the uterus.

Ovarian tissue cryopreservation

Although still in its infancy, ovarian tissue cryopreservation is a viable alternative to cryopreservation of oocytes or embryos. The ovarian cortex of young women contains several hundred thou-

sand primordial follicles [23]. Even small pieces (1 mm³) may contain several hundred follicles. Although it has not yet been demonstrated that cryopreserved ovarian tissues can restore fertility in humans, live young have been obtained from fresh and frozen ovarian tissue grafts in mice [24] and sheep [25] but not in human.



Cryopreserved ovarian tissue (top section) a month after thawing and grafting into the kidney (bottom section) of an immunodeficient mouse, showing growth of ovarian follicles (arrow). (Masson's trichrome stain, x450) Picture courtesy: Carl E Wood, Jillian M Shaw and Alan O Trounson (MJA 1997; 166: 366-369)

Cryopreservation of ovarian tissue is a technique to bank oocytes in situations where the woman may lose all her oocytes from a medical treatment, disease process or even the natural loss from natural aging. Potential uses for this technique include restoring fertility and normal ovarian hormone production without the use of medications. The technique of ovarian tissue cryopreservation and transplantation of the thawed tissue is experimental. Since the first significant publication on ovarian tissue cryopreservation and transplantation in

1994 [2], over 100 publications attest to the scientific interest in this technique or treatment strategy.

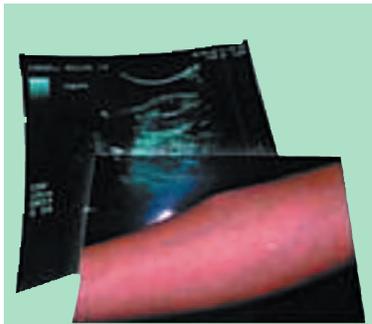
Gosden et al demonstrated in 1994 the ability to cryopreserve ovarian tissue, transplant it after thawing and obtain functioning ovarian tissue that led to successful births of healthy animals [25]. His team also demonstrated long-term functioning of transplanted cryopreserved ovarian tissue for about two years in sheep [26]. The fertility rate after ovarian cryopreservation in mice was approximately 50 - 75% after ovarian tissue autotransplantation [27-28].

Technique

Ovarian tissue cryopreservation begins with laparoscopy or mini-laparotomy. Ordinarily, the surgeon removes only one ovary to allow normal ovarian hormone production from the other ovary during treatment and because the woman may have ovarian function after treatment of her disease. The laboratory staff portion sections the ovarian cortex (which contains the oocytes) into thin tissue slices. The tissue slices are cryopreserved at -196°C using specialized cryoprotectants and controlled-rate freezing equipment. When the woman and her physicians feel fertility is appropriate, transplantation of the ovarian tissue strips can be attempted. In animals and humans, frozen ovarian slices have a high survival rate after thawing.

The location for tissue transplantation could be in the abdomen near the fallopian tube to allow natural ovulation and conception. Natural conception occurred in all animal studies. The ovarian tissue began to function within several months after transplantation. The disadvantage of transplantation into the abdominal cavity is limited access, potentially lower viable tissue since ingrowth of blood vessels occurs primarily from only one side of the tissue, and adhesion formation (scar tissue) during the recovery period after surgery. Recently, transplantation of human

ovarian tissue into the forearm resulted in follicle formation and egg retrieval with a needle [29]. Parathyroid tissue transplanted into the forearm routinely functions normally in patients with other medical disorders. The forearm is well-vascularized, easy to access, and is a site with little surgical risk. Consequently, because the ovarian tissue is surrounded with vascularized tissues, it may have a greater probability of short and long-term function. Transplantation of tissue into the forearm precludes natural conception. Assisted reproduction with IVF using oocytes retrieved from the arm would necessarily be applied.



Ultrasound image shows ovarian follicle development beneath the forearm skin after transplantation of ovarian cortical strips to a woman's forearm. The lump seen in photo of forearm itself represents developing dominant ovarian follicle. (Both photos courtesy of Kutluk Oktay, MD © Kutluk Oktay.)

A very interesting avenue of research involves the growth of ovarian tissue from one species in another species (xenograft) [30-32]. If this technique works and proves safe, several problems become less of an issue.

- ◆ First, tissue grown in another animal prevents cancer in the human recipient. Even patients with ovarian cancers may be able to use this technique to recover normal oocytes without any associated cancer cells.
- ◆ Second, the ovarian tissue contains a limited number of oocytes. A xenograft may allow more efficient

maturation and retrieval of oocytes for IVF.

- ◆ Third, the limited tissue strips might be used more gradually over time for additional children.
- ◆ Fourth, if only small amounts of tissue prove adequate for IVF, then a small biopsy of tissue in a young woman may be a means of banking oocytes if she later finds herself infertile

Advantages of ovarian cryopreservation

Ovarian cryo preservation has the advantage that wedges of ovarian tissue (about 2 cm² in area) can be collected by laparoscopy from each ovary at any stage of the menstrual cycle without compromising a woman's health or fertility. Unlike embryo and oocyte cryopreservation, collection of ovarian cortical material does not delay hysterectomy and oophorectomy for severe endometriosis and does not involve use of "fertility drugs". The amount of ovarian tissue needed to restore regular menses and fertility after ovarian failure or removal is not currently known, but ovulation can occur with as few as 100 oocytes (in women and mice) to 400 oocytes (in cattle) remaining in the ovary [33,34].

Risks

The value of ovarian tissue grafting for cancer patients is debatable. Viruses, including HIV [35-37] and hepatitis virus, [38,39]and cancers [40]can be transmitted by grafts, and cancers have been known to recur in patients in remission after the replacement of autologous cryopreserved bone marrow. [41-43] It is therefore possible that blood-borne cancers such as leukaemia, systemic cancers such as lymphoma and metastasising cancers could be transmitted by ovarian tissue grafts. Shaw et al.[44]have shown that when small pieces (around 1 mm³) of ovarian tissue obtained from donor mice with lymphoma were grafted into normal healthy

recipient mice, 13 of 14 recipients developed the lymphoma. This occurred with both fresh and cryopreserved ovarian grafts, highlighting a significant risk for clinical applications of the procedure.

Separating primordial follicles from stromal tissue or cancer cells is currently not possible. Polymerase chain reaction (PCR) techniques have increased the sensitivity of tests to detect the presence of remnant cancer cells,[45,46]and it may be possible to culture tissue in vitro under conditions aimed at eliminating cancer cells, however, it is unlikely that cancer cells can be flushed out of an ovary or removed from the graft by cytotoxic drugs at the time of replacement, because of the deleterious effect on the graft.

The alternative of removing primordial follicles from the graft and using matured oocytes for in-vitro fertilisation, or replacing follicles in the patients, is currently not feasible as the enzymatic procedure used to obtain human primordial follicles [47]results in separation of the oocyte and its surrounding support cells in culture[48] and also because of the long time needed for in vitro growth and culture that may reach 120 days which till this moment not possible.

Future advances in collection and culture procedures for primordial follicles may allow in-vitro maturation of follicles in humans, as a live birth has recently been reported for mouse primary follicles matured and inseminated in vitro and returned to the recipient as an embryo [49].

Future directions

A number of questions remain unanswered, but of critical importance, to bring this technique into mainstream use. For example:

- ◆ How long can the tissue remain frozen and still function after thawing?,
- ◆ Where is the best location to transplant the tissue strips?,
- ◆ How much ovarian tissue is required to provide enough oocytes for successful pregnancy?,

- ◆ How long will the tissue function after transplantation?, and many more.

Ethical issues

Transplantation of cryopreserved ovarian tissue may raise new ethical issues. It could be transplanted into women beyond the age of the menopause as a form of hormone replacement therapy. It could also facilitate older women having access to their own "younger oocytes", which have been stored for 10 or more years. At present, IVF units in Australia limit the use of donor oocytes to women within the reproductive age

span, a generous upper limit being healthy women up to 52 years of age. Storage of the woman's own ovary from a younger age may avoid the use of donor oocytes at an older age.

Conclusion

Despite reassuring data on the questionable risk of cancer associated with ART, women and clinicians continue to be apprehensive about fertility issues in cancer patients.

In the best of circumstances, pursuing the goal of having a family demands difficult decision-making. A regimen of fertility impairing-cancer therapy adds an

extra measure of anxiety to family planning for all concerned. Fortunately, there are some new options and others on the horizon for preserving fertility. Maintaining her fertility potential and keeping her options open may greatly comfort a cancer patient and her family. At the same time, trying to deal with two of life's greatest challenges, family planning and the life-and-death struggle of cancer, can elicit tremendous anxiety in a woman, her family, and clinician. We as physicians should urge family planning and raise fertility issues with our patients who have, or have had cancer.

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