

Section 6

Technology and clinical medicine

Chapter

37

Transplantation of ovarian tissue or immature oocytes to preserve and restore fertility in humans

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Introduction

It is now possible to preserve and restore fertility, using ovary and egg freezing and ovary transplantation, in young women with cancer who are undergoing otherwise sterilizing chemotherapy and radiation. This approach can also be used for any woman who wishes to prolong her reproductive lifespan. This chapter is limited to the clinically proven therapeutic applications of this technology. Our clinical results with these new therapeutic approaches are adding to our understanding of the basic science of reproduction, and may eventually obviate the growing worldwide epidemic of female age-related decline in fertility.

The developed world is in the midst of a widespread infertility epidemic. Economies in Japan, the United States, southern Europe, and even China are threatened by a decreasing population of young people having to support an increasing population of elderly and retirees [1]. The most common reason to see a doctor in countries such as India and China, seemingly plagued with overpopulation, is for infertility. Infertility clinics are popping up throughout the world in huge numbers [2].

It is clear to all that the major reason for the world's growing infertility epidemic is that as women put off childbearing, their oocytes die off and those that survive are of poor quality [3–6]. In her teen years a woman has a 0.2% chance of being infertile, and by her early twenties it is up to 2%. By her early thirties, it is up to 20% [2, 7]. Many modern women today do not think of having a baby until their mid thirties, and by then over 25% are infertile, simply because of the aging and the decline in number of their oocytes. This is clearly demonstrated by the high pregnancy rate using donor oocytes from young women placed into the uterus of older women [2, 3, 7]. Yet fertility physicians struggle to make a pathological entity diagnosis to explain the

infertility, which in truth in most cases is just a normal physiological response to oocyte aging [2, 3, 7].

Preserving fertility for women who wish to put off childbearing or who are about to undergo treatment with gonadotoxic drugs: using ovarian freezing and transplantation

Until recently oocyte freezing had very poor to no success, and so ovary tissue slow freezing was the only method we could rely upon for preserving. Of course, now we also have a favorable option of preserving oocytes after ovarian stimulation and egg retrieval, using vitrification instead of slow freezing for cryopreservation [4, 8, 9] (see Chapter 36). Nonetheless, ovarian tissue freezing and transplantation still has great advantages over oocyte freezing. There does not need to be a prior delaying stimulation cycle, so ovarian tissue freezing will delay the cancer treatment by only a few days. Furthermore, one cycle of ovarian stimulation and egg freezing does not assure successful pregnancy as much as an entire ovary would, and finally, transplanting ovarian tissue back not only restores fertility but also restores endocrine function.

Fresh series of identical twins with premature ovarian failure

Let us take the clinical evolution of this technology in logical order. The first successful fresh human ovary transplantation was reported between a pair of remarkable monozygotic twins discordant for premature ovarian failure (POF), using a cortical grafting

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technique [10]. This key event allowed us to assess the results of fresh transplantation unclouded by confusion that might have been caused by freezing. The first successful human frozen ovary auto-grafts were reported around the same time with tissue cryopreserved for cancer patients prior to their sterilizing bone marrow transplants [11, 12]. These followed similar results described in the sheep over a decade earlier [8]. The transplantation technique has subsequently been refined over a larger series of nine consecutive successful fresh ovary transplants in identical twins (plus two fresh allotransplants to be treated separately), with resumption of normal hormonal cycling and menstruation in all cases, eventually leading to 14 pregnancies and 11 healthy babies born from the nine fresh identical twin recipients [5, 13–15]. This unusual consecutive series of fresh ovary cortical transplants helped us also refine the techniques necessary for successful preservation of fertility for cancer patients using ovarian tissue freezing, with three additional successful pregnancies from three frozen transplants. This unusual series also helped to establish a method for distinguishing between the oocyte loss from transplant ischemia and the oocyte loss from cryopreservation. We now can report long-term follow-up (up to 8 years) of this original series of fresh transplants, and add to it our more recent experience with cryopreserved ovarian tissue. The results appear to be remarkably more robust than had originally been contemplated [6, 16].

The first such twin case inquired about the possibility of fresh ovary transplantation originally from researching an earlier testis transplant we had reported for anorchia [10, 17, 18]. From that point forward, patients in similar situations sought this treatment. All of them found the possibility of natural conception more attractive than *in vitro* fertilization (IVF) or oocyte donation. In most cases, the twins lived far apart (even in different countries) and the donors preferred to make a single visit for a one-time ovary donation, rather than go through multiple cycles of ovarian hyperstimulation. We knew when we began this series that there would be few clinical cases in the world like these that would warrant fresh ovarian transplantation. However, this series would allow us to learn how to more effectively freeze and transplant human ovarian tissue, which would have far reaching consequences and widespread application for preservation of fertility in cancer patients and for women who just need to delay childbearing for social reasons.

Despite risks, the evidence does not support a deleterious effect of unilateral oophorectomy either on fertility or on age of menopause [19, 20].

One entire ovary of the donor was therefore removed and the cortex dissected away from the medulla. The cortex of the non-functioning recipient ovary was removed in entirety, and the donor cortex slices were transplanted onto the exposed recipient medulla using 9–0 nylon interrupted sutures. A tiny piece of spare tissue of the donor, as well as the entire resected atrophied ovarian cortex of the recipient were examined histologically in all cases (Figures 37.1A and 37.1B).

Micro-hematoma formation under the graft was avoided by micro-bipolar cautery and micropressure stitches of 9–0 nylon. Constant pulsatile irrigation with heparinized saline prevented adhesions (Figure 37.2A–D).

Only one-third of the ovarian cortex was grafted fresh and two-thirds were frozen.

Ovarian cryopreservation

All of our fresh clinical transplant studies involved cryopreservation of spare tissue for future thawed transplants. We also have frozen the ovarian tissue of 68 cancer patients and 7 patients who simply wanted to delay childbearing, all with Institutional Review Board (IRB) approval. All of the frozen cases thus far transplanted back to the patient have utilized the slow freeze approach [8, 21, 22]. However, we now use vitrification exclusively for cryopreservation in humans because of the results of *in vitro* viability analysis in humans as well as *in vivo* transplant studies in the bovine [5, 23].

The goal of the *in vitro* study was to determine which method produced a higher cell survival rate: slow freezing, or vitrification. The high viability (92%) of oocytes in control (fresh) specimens indicated that disaggregation *per se* had caused only minimal damage to the oocytes [5]. Overall 2301 oocytes were examined from 16 specimens. Results within each of the three groups revealed no significant difference between fresh and vitrified tissue, but the viability of slow freeze-cryopreserved tissue was less than one-half that of vitrified tissue or controls (42%) ($P < 0.01$). Transmission electron microscopy also has been used to analyze ovarian tissue that had been either cryopreserved by slow freezing or vitrified by ultra-rapid freezing, showing vitrification to be superior [24].

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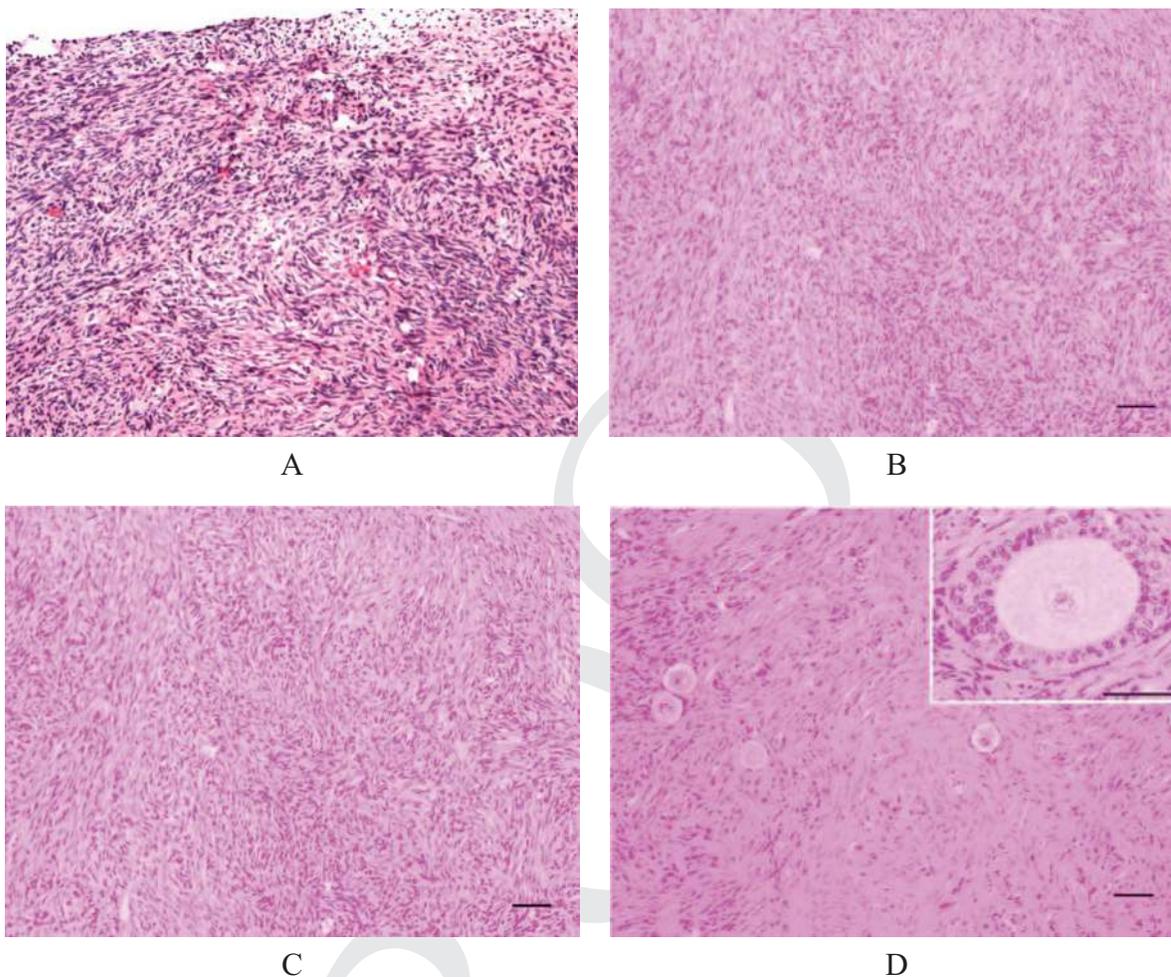


Figure 37.1 (A, B) Showing the absence of primordial or preantral follicles in ovarian biopsies of this candidate for ovarian transplantation compared with (C, D) that in her fertile sister.

Standard hematoxylin and eosin (H&E) histology showed no difference between pre-freeze ovarian tissue and post-vitrification ovarian tissue (Figure 37.3A and 37.3B).

Finally, quantitative histological study of primordial follicles in the bovine after vitrification and transplantation back to the cow two months later remarkably showed no follicle loss.

The basic science concept of vitrification, whether for eggs, embryos, or tissue, is to avoid completely any ice crystal formation by using a very high concentration of cryoprotectant and a very rapid rate (virtually “instant”) of cooling. This is quite different from classic slow-freeze cooling, which relies on a partial and very gradual removal of water from the cell by

encouraging ice crystal formation preferentially on the outside of the cell, drawing water out.

Using the vitrification technique, cortex tissue of each ovary is cut into slices $1 \times 10 \times 10$ mm. The ultra-thinness of the tissue is crucial, not only for the cryopreservation, but also for rapid revascularization after grafting. Ovarian tissues are initially equilibrated in 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) in handling medium (HM: HEPES-buffered TCM-199 solution supplemented with 20% [v/v] synthetic serum substitute [SSS; Irvine Scientific, Santa Ana, CA, USA]) for 25 minutes followed by a second equilibration in 20% EG and 20% DMSO with 0.5 M sucrose for 15 minutes. Ovarian tissues are then placed in a minimum volume of solution

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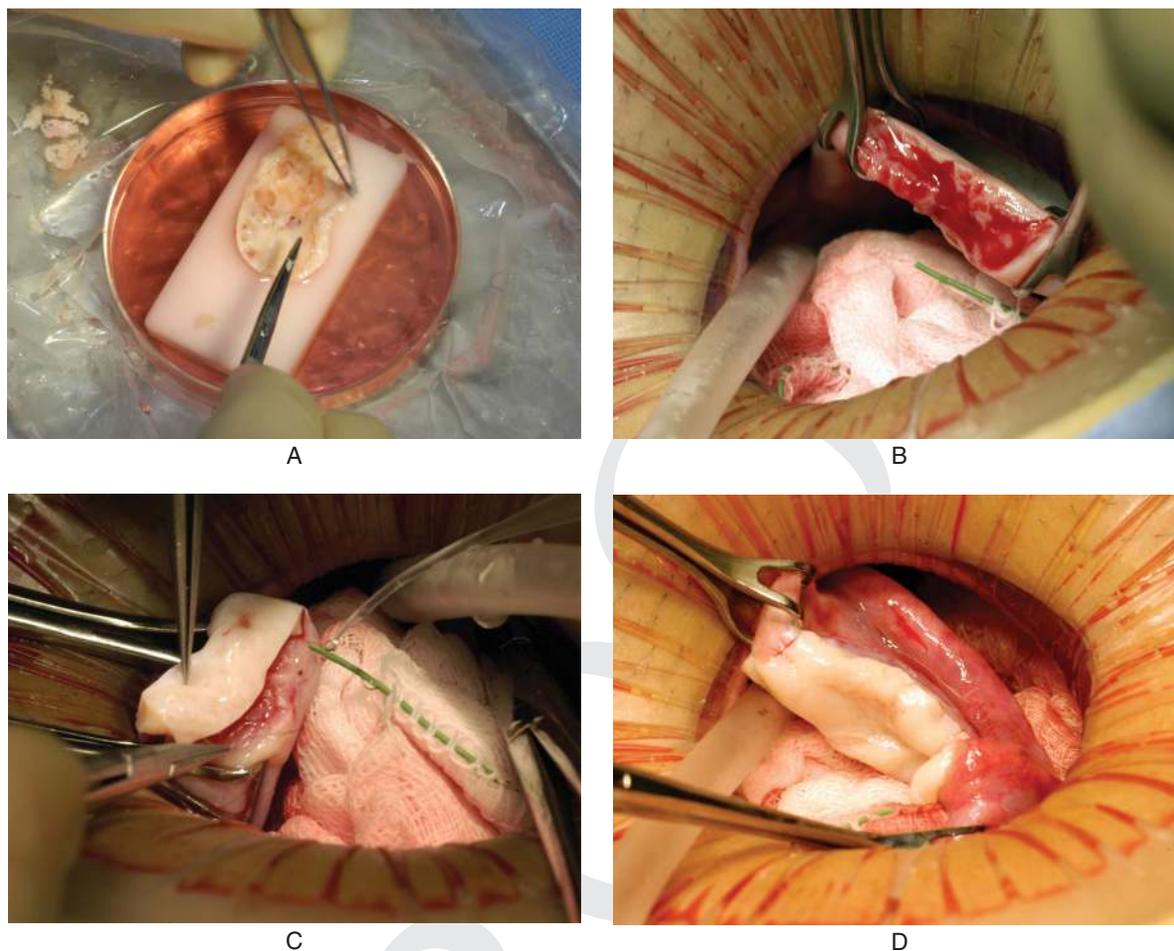


Figure 37.2 (A–D) Steps in the procedure of ovarian transplantation between monozygotic twin sisters: (A) preparation of donor ovarian cortex by dissection in a Petri dish on ice; (B) preparation of recipient ovarian medulla; (C) attaching donor cortical tissue to recipient ovarian medulla; (D) attaching thawed donor cortical tissue for re-transplant to the recipient medulla.

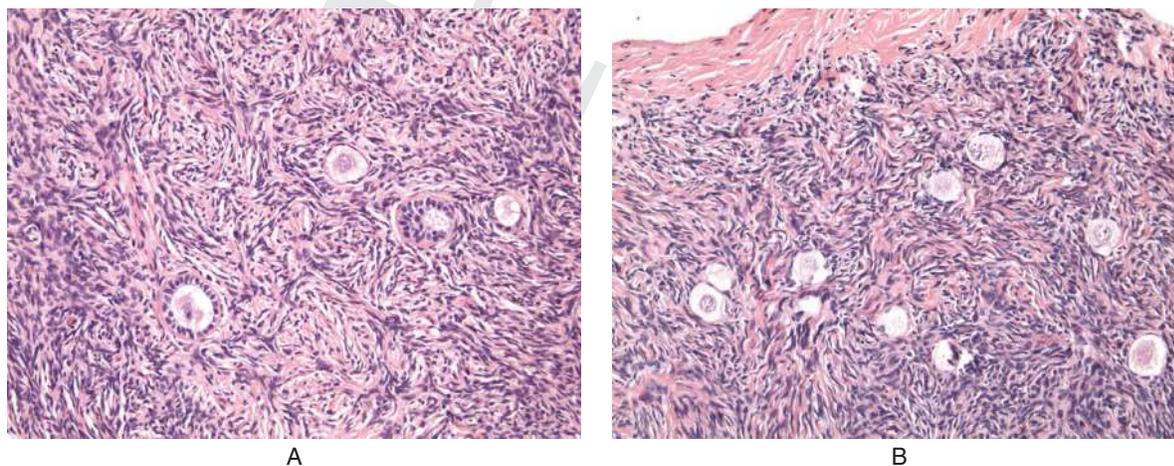


Figure 37.3 Histology pre (A) and post (B) vitrification of ovarian tissue.

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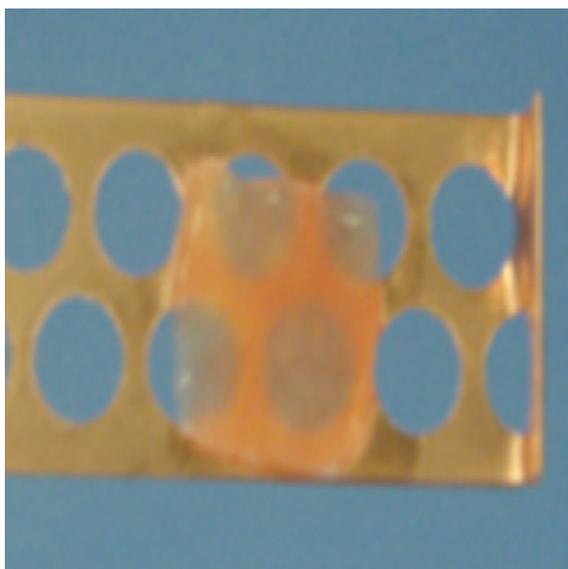


Figure 37.4 Ovarian tissue slice.

(virtually “dry”) onto a thin metal strip (Cryotissue: Kitazato BioPharma, Fujinomiya, Japan), and submerged directly into sterile liquid nitrogen [25], following which the strip is inserted into a protective container and placed into a liquid nitrogen storage tank (Figure 37.4).

For thawing, the protective cover is removed and the Cryotissue metal strip is immersed directly into 40 ml of 37 °C HM solution supplemented with 1.0 M sucrose for 1 minute. Then, ovary tissues are transferred into 15 ml of 0.5 M sucrose HM solution for 5 minutes at room temperature, and washed twice in HM solution for 10 minutes before viability analysis, or transplantation. No ice crystal formation occurs during any of these vitrification procedures [23].

Intact whole ovary transplantation

Before we had long-term follow-up of the cortical tissue grafts, we had postulated, incorrectly we now believe, that we could lengthen graft survival and avoid ischemic loss of follicles by instead doing a whole intact ovary microvascular transplantation.

To transplant an intact whole ovary, the donor ovary is removed by clamping the infundibular pelvic ligament at its base in order to obtain maximum length. The veins (3–5 mm) are easily identified, but the ovarian artery (0.3 mm) is often not grossly visible. The entire specimen is placed in Leibovitz medium at 4 °C and two veins and one artery are dissected

and isolated under the operating microscope. The recipient’s infundibular pelvic ligament is clamped at the base and transected close to her non-functioning ovary. The donor’s ovarian veins are then anastomosed to the recipient’s with 9–0 nylon interrupted sutures, and the ovarian arteries are anastomosed with 10–0 nylon interrupted sutures (Figure 37.5A–C).

When the microvascular clamps are removed, blood flow is confirmed by fresh bleeding from the surface of the ovary where a cortical slice had been taken for cryopreservation as a backup.

This patient is recipient number 8 in Table 37.1, and Figure 37.6. Her recovery of ovulatory menstrual cycling was similar to all of the cortical tissue slice transplants. She conceived spontaneously a healthy baby girl, delivered at term, and her graft functioned for greater than 4 years. However, we found that this intact whole ovary approach did not produce results superior to cortical tissue grafts, which were equally robust. The original concept behind whole intact ovary microvascular transplantation was to avoid the supposed ischemic damage that was incorrectly attributed to cortical grafting [26]. Current results seem to eliminate the need for whole ovary transplantation, as these fresh cortical ovarian tissue grafts have now also been shown to have a very long duration of function.

Results of fresh and frozen ovarian transplantation

Results are summarized in Table 37.1 and in Figures 37.6 and 37.7.

All nine identical twin pairs underwent their orthotopic ovarian is transplantation between April 2004 and April 2008.

The recipients, for the most part, continued to cycle from 2 years in two patients whose donor had low ovarian reserve to over 7 years in most cases. The two whose donor had low antral follicle counts (AFC) of less than ten, only functioned for 2 years. However, even these two cases had spare frozen cortical tissue that remains available for future transplants. Menstrual cycles began within 3 months in all patients, and day 3 follicle-stimulating hormone (FSH) levels returned to normal by 4.5 months and ovulation then resumed in all cases (Figure 37.6).

A total of 14 healthy babies resulted from the 12 ovary transplants, 11 from the 9 fresh transplants, and 3 from the 3 frozen transplants (Table 37.1).

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Table 37.1. Ovarian transplant results 14 babies (11 from 9 fresh; 3 from 3 frozen)

Patient no.	Age	Pre-op FSH	Post-op FSH	Initial post-op menses intervals	Preg	Babies delivered	Years of graft function
1	24	75	7.1	2nd baby: FROZEN	3	2	4
2	38	96	5.2	93, 42, 24, 27, 25 ...	3	3	>7
3	25	112	6.8	76, 23, 30, 26, 25, 26, 21, 24, 27, 34, 25, 27, 51, 30, 27, 26, 28, 19 ...	1	2	>6
4	34	58	9.4	81, 22, 47, 26, 21, 20, 27, 26 ...	2	1	4
5	40	60	6.8	86, 29, 38, 34, 28, 28, 31, 35, 34, 28, 33, 35, 30 ...	0	0	4
6	26	101	7.5	64, 20, 39, 40, 32, 26, 29, 26, 26, 41 ...	1	1	2
7	34	86	4.4	83, 22, 29, 29 ...	3	2	>6
8	37	86	7.4	100, 17, 39, 29, 27, 22, 23, 20, 34, 25, 26, 29 ...	1	1	>4
9	35	54	4.2	128, 42, 18, 25 ...	1	0	2
10	31	78	3.4	FROZEN	1	1	2
11	33	85	8.6	FROZEN	1	1	>1

Note: each graft represents only 1/3 of one ovary or 1/6 of entire ovarian reserve.

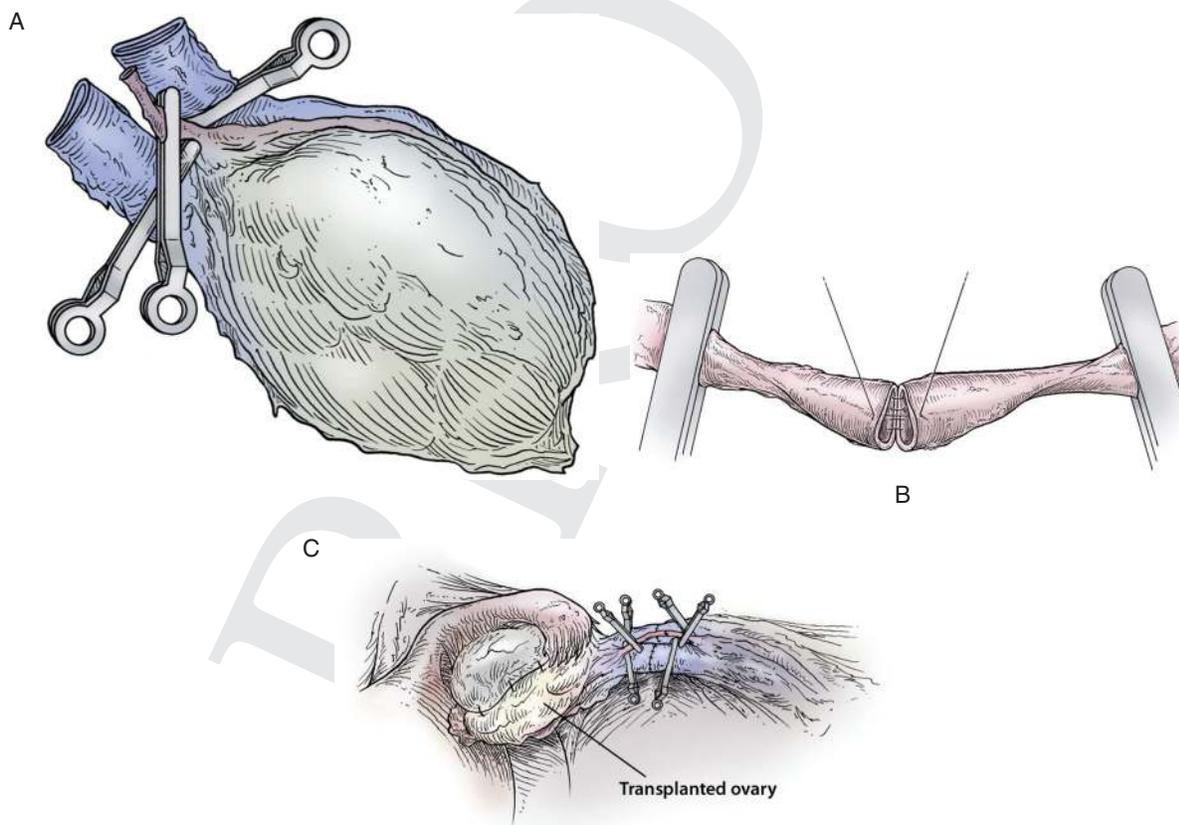


Figure 37.5 (A–C) Steps in the procedure of intact ovary microvascular transplantation: (A) microsurgical isolation of donor ovary blood supply; (B) end-to-end anastomosis of ovarian blood vessel; (C) completed anastomosis of ovarian artery and veins.

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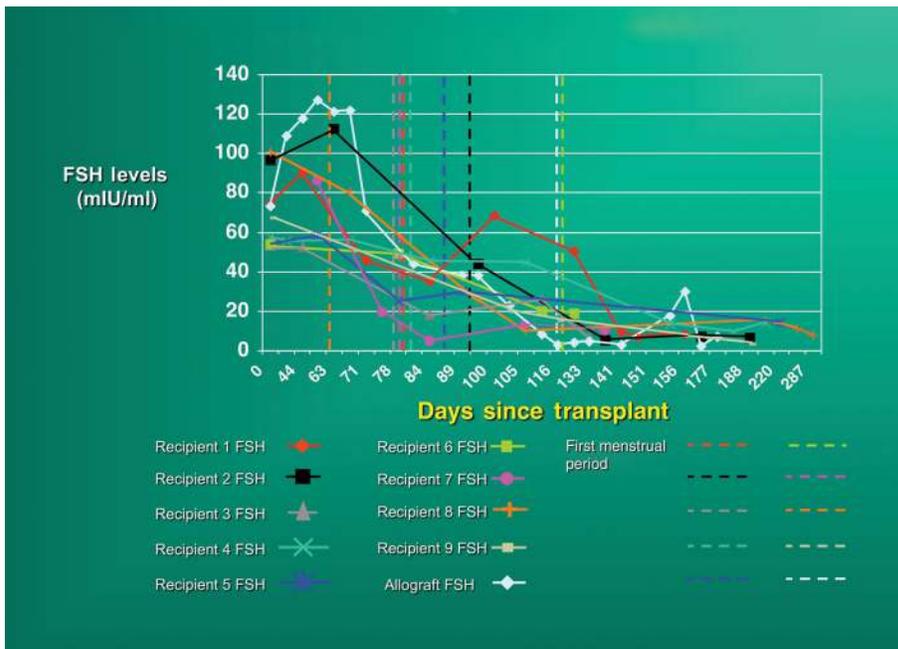


Figure 37.6 Recovery of ovarian function. The eight fresh transplant cases showed a dramatic decline in day 3 serum follicle-stimulating hormone (FSH) by 80–140 days post-operatively corresponding approximately to the resumption of menses. The results of the microvascular whole ovary transplant and the cortical tissue allotransplant are not significantly different from cortical isografts.

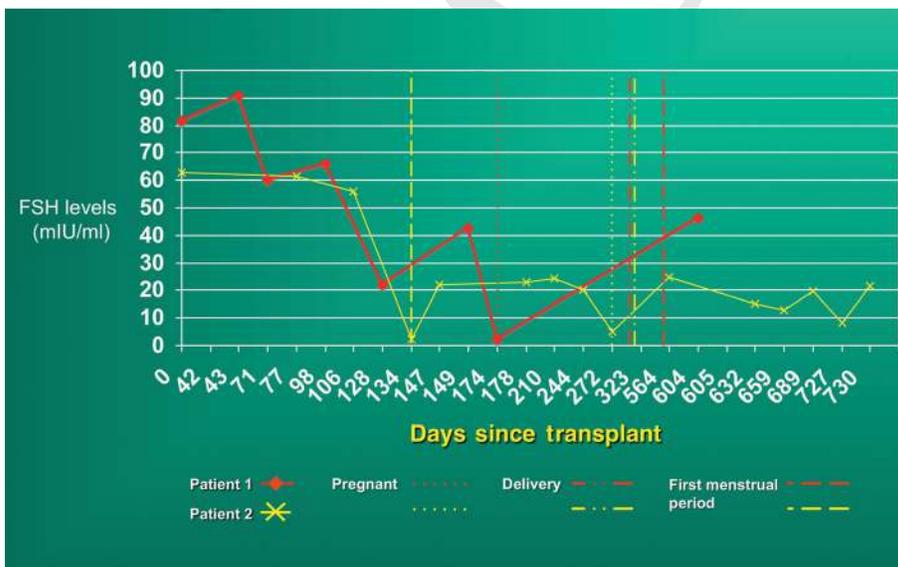


Figure 37.7 Transplant of thawed ovarian tissue. After a frozen cortical re-transplant, serum FSH declined again to normal levels, similar to those of fresh transplants.

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One of our twin recipients became pregnant at 39 years of age without medical assistance after her fifth menses, 8 months after transplantation. She delivered a healthy baby girl at full-term and then conceived again at age 42, and delivered a healthy baby boy, again at full-term, 4 years after her transplant. Her ovary is still functioning to date after 7 years, and she conceived again at age 45 with another healthy boy, more than 7 years after her transplant.

One case of ovary transplant was an identical twin whose POF was caused by a bone marrow transplant with pelvic irradiation for leukemia, with her identical twin sister being the donor. She became spontaneously pregnant 5 months after her fresh ovary transplant from her sister, but miscarried. Then at 1.5 years she became pregnant again and had a healthy baby, and at 5 years after the transplant, she became pregnant again, and had a second healthy baby. Over 6 years later, her original transplant is still functioning, and she still has two-thirds of an ovary that remains frozen. It does not appear from this or from the frozen cases that pelvic radiation is incompatible with a healthy pregnancy, and in fact, it appears (contrary to expectations) that transplantation of ovarian cortical tissue using this technique is a very robust procedure.

This newly favorable experience with ovarian cortex grafting is not limited just to our center [27]. Equally robust results are being experienced in Brussels, Paris, Spain, Denmark, and Israel. Frozen ovarian grafts (even with the slow-freeze technique) in Denmark are lasting over 5 years and many spontaneous pregnancies have been reported, with no need for IVF or other ancillary treatment. At the time of this writing, 28 healthy babies have been born from ovarian tissue grafting fresh and frozen, and most involved no IVF, and resulted from just regular intercourse with no special treatment (Table 37.2).

Frozen cortical ovarian transplantation

The most common benefit of ovarian transplant is not the unusual cases of fresh grafting in identical twins but rather to protect the fertility and future endocrine function of young women undergoing cancer treatment [5, 23, 28–34]. Since 1996, we have frozen ovary tissue for 68 young women with cancer or at risk for POF, of whom 16 had spare frozen tissue subjected to detailed viability testing before cryopreservation and after thawing. All 68 had histological review by a variety of pathologists. Only one had ovarian metastasis

Table 37.2. Worldwide frozen ovarian cortex tissue transplant pregnancies

Case no.	Diagnosis	Babies	Where
1	Hodgkin's	1	Donnez
2	Neuro tumor	1	Donnez
3	Non-Hodgkin's	1	Meirow
4	Hodgkins	1	Demeestere
5	Ewings	1	Andersen
6	Hodgkin's	1	Andersen
7	POF	1	Silber
8	Hodgkin's	2	Silber
9	Polyangiitis	1	Piver
10	Breast cancer	2	Pellicer
11	Sickle cell	1	Piver
12	Hodgkin's	2	Revel
Totals: 12 patients		17 Babies	8 Centers
Fresh + Frozen		28 Babies	Silber – 14 Babies

(and then only in the medulla), a young woman with widespread breast cancer metastasis throughout her entire body. Otherwise, none of our other 61 cancer cases had any tumor cells in their ovary. Andersen has also noted a complete lack of ovarian metastasis, even in the majority of leukemia cases. The reason for the remarkable absence of ovarian metastasis might possibly be due to the fibrous avascular nature of the ovarian cortex (C. Andersen, personal communication, 2007). In fact, the reason why fetal ovarian cords (which in the fetal male become seminiferous tubules) invade the fibrous cortex and become follicles is that the dense fibrous tissue of the cortex (which in the fetal and adult testis is just tunica albuginea) is needed to suppress the resting follicles from developing all at once prematurely. By analogy, in the male, leukemic cells do indeed routinely lodge in the testis proper, but not ever in the tunica albuginea of the testis. That same phenomenon protects these ovarian slices as well. In addition to these 61 pathological cases, 7 women have had ovarian tissue frozen simply to allow them the possibility of having children at an older age because they had to delay childbearing for strong personal or economic reasons.

Thus far, only three of our frozen cases have had their frozen tissue transplanted back. In all three cases, the tissue was cryopreserved with slow freezing because this was before we adopted vitrification as our standard method in 2009. Of our three cases of frozen

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transplanted tissue thus far, even with slow freeze, all have had a robust return of ovulatory menstrual cycles within 4 months with spontaneous pregnancy eventually; although with slow-freezing cases this return of function was somewhat slower and the transplant duration was briefer (Figure 37.7).

The duration of function for these slow freeze grafts was about one-third or less than that of our fresh grafts. This could be an intrinsic problem with our slow-freezing technique. We hope that with vitrification, because we found no loss of oocyte viability, that the frozen grafts, once transplanted, will last as long as the fresh ones. It is clear from our fresh grafts that with proper microsurgical technique, ischemia time is not a serious problem for cortical grafting.

Genetics of non-cancer premature ovarian failure, and low ovarian reserve

Identical twins discordant for ovarian function present a true genetic puzzle [20]. The great majority of women enter menopause in their fifth or sixth decade of life, at an average of 51 years, but 1% undergo menopause quite prematurely, that is, before 40 years of age [35–37]. POF is assumed to have a genetic etiology (see Chapter 34) and menopausal age normally is strongly heritable judging by the greater concordance between monozygotic than dizygotic twins [38–41]. It was remarkable, therefore, to identify monozygotic twin pairs in which one sister had undergone menopause for unexplained reasons at a very early age from 14 to 22 years, whereas the other was still fertile, with naturally conceived children as well as normal ovulatory cycles and ovarian reserve [10, 13–15].

We have not yet taken advantage of the unique opportunity these twins offered for studying the possible genetic or epigenetic origin of ovarian reserve, but genomic DNA and lymphoblastoid cell lines were prepared and carefully stored for future genetic and epigenetic studies. Details of the obstetric records on the original chorionicity at birth of these identical twin sisters revealed 50% were monochorionic-monoamniotic, which was surprisingly high since the incidence of mono/mono is normally only $\approx 2\%$ ($P < 0.0005$). It is clear that late splitting, for whatever reason, predisposes otherwise identical twins to discordant germ cell deficiency [14, 42, 43].

Thus far the search for genes controlling ovarian reserve in the human has yielded meager results, the only modestly common candidate being the

pre-mutation carrier status for fragile X (*FMR1*). The answer oddly enough may be found in the structural peculiarity of the Y chromosome actually, with its huge concentration of amplicons and palindromes, which are very difficult to sequence [44]. These regions of long sequence identity with many multicopy genes would not have been sequenced with routine methods and most would have been missed in the sequencing without special methodology. Similar regions on the X chromosome that did not undergo the very specialized type of sequencing used for the Y would therefore not have been elicited in the genome sequence yet. We now estimate that 12% of the X chromosome remains unsequenced, and in fact is ampliconic, making the X chromosome a very attractive place to find genes which control ovarian reserve (as well as spermatogenesis). But to sequence these ampliconic regions is a very slow and laborious task compared to sequencing the more conventional regions of the genome.

Future prospects for ovarian tissue transplantation

After ovarian transplantation, all patients were able to attempt natural conception every month without medical assistance, and heterotopic sites have produced no successful pregnancies to date. Our patients preferred the chance of natural conception anyway [45–48]. In fact, the commonly held view that egg freezing is a proven technique and ovary tissue transplantation is “experimental,” is belied by the fact that all of the successful pregnancies resulting from fertility preservation in cancer patients thus far have been from frozen ovary tissue, and none at the date of this writing have come from frozen oocytes [27].

It is generally assumed that premature ovarian failure or low ovarian reserve is related to the number of primordial follicles the woman has at birth and this number is certainly heritable and is most likely genetically determined [43]. All modern women are concerned about what is commonly referred to as their “biological clock” as they worry about the chances of conceiving by the time they have established their career and/or their marriage and their financial stability. Most of our cured cancer patients, who have “young” ovarian tissue frozen, feel almost grateful they had cancer, because otherwise they would share this same fear all modern, liberated women have about their “biological clock.” But it is not only having a child that worries them.

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Figure 37.8 All the primordial follicles of the ovary are located in the outer 0.75 mm of the cortex. Therefore, very thin slices of cortex will contain all these follicles and allow rapid revascularization.

The major application of ovarian tissue cryopreservation and transplantation is obviously for fertility preservation in cancer patients and possibly for women who need to delay childbearing. However, for patients who have already lost ovarian function from bone marrow transplant, allografts may be an option. Allografts might be considered if ovarian tissue is available from a young woman who previously donated bone marrow to the same patient. However, it is very important not to attempt this without immune suppression if the graft recipient has any sign of graft-versus-host disease. Reassuringly, well-matched (HLA) kidney transplant recipients on immunosuppression have favorable obstetric outcomes [49].

At the time of this writing, we are aware of numerous other births after implanting ovarian tissue for a total of 30 live births thus far [11, 12, 27, 50–53]. Thus, despite initial skepticism, this technique is now gaining worldwide acceptance, and is being enthusiastically received by young women of reproductive age with cancer. For pediatric cases, certainly, the only option is ovarian tissue freezing, because ovarian hyperstimulation is contraindicated. Although we are certain that one day *in vitro* maturation of primordial follicles for 3 months will be possible in human work, at the present time, *in vivo* maturation via transplantation is all we can hope for.

For leukemia patients, or any patients in whom transplantation of prior frozen ovarian tissue might create a risk of re-introducing cancer cells, we recommend that before the cortical tissue is dissected and frozen, that all the antral follicles of the removed ovary be aspirated for germinal vesicle (GV) oocyte retrieval.

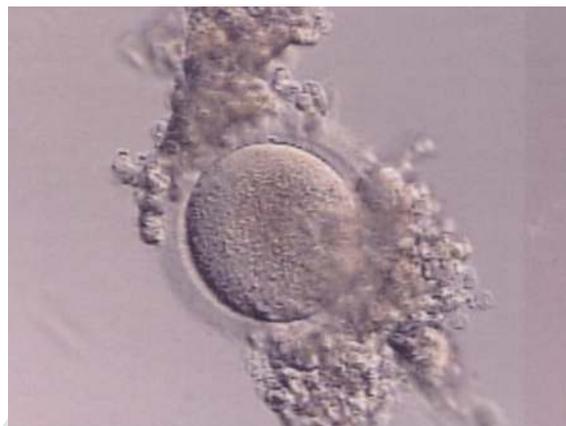


Figure 37.9 Partial cumulus denudation prior to vitrification of GV oocytes.

Of course, as referred to earlier, even most leukemia patients do not have tumor cells in their dense fibrous ovarian cortex, but nonetheless this is still a concern for this particular cancer, which is why we suggest freezing GV oocytes as well. These GVs can then be partially denuded of cumulus cells, and vitrified just as for oocyte freezing (with one minor modification) (See Figures 37.8 and 37.9).

The vitrification media for partially denuded GV oocytes should contain 20% DMSO and 20% EG with 0.5% sucrose, instead of 15% DMSO and 15% EG, similar to the vitrification medium for ovarian tissue. This is because our studies showed poorer results with only partially denuded oocytes if the concentration of vitrification solution was not increased, similar to our results with ovarian tissue vitrification.

Egg and embryo vitrification

Vitrification for freezing eggs or embryos was first suggested in the mid 1980s [54, 55]. However, it was not until 2005 that a highly efficient method was published, which stimulated a huge wave of justified enthusiasm for this approach to egg and embryo freezing [4, 9, 56, 57]. The concept behind vitrification is not just its potential simplicity (such as no freezing machine is required) but that it could eliminate ice crystal formation completely. Instead of clinical IVF programs having to weigh carefully the risks to pregnancy rate posed by embryo freezing, embryos could now be frozen without concern in virtually any case in which there would be a clinical advantage. With the new vitrification methodology, there is no difference

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between fresh and frozen embryos. Furthermore vitrification of eggs and embryos offers an alternative to ovarian tissue freezing for preservation of fertility, giving the patient another option.

For vitrification the cryoprotectant solution is a combination of EG and DMSO (cat. no. 90133; Irvine Scientific, Santa Ana, CA, USA). The embryo is transferred initially into increasing concentrations of equilibration solution (7.5 M EG and 7.5% DMSO in 20% synthetic serum substitute [SSS]) for 10–15 minutes, followed by placement for a minute or longer in vitrification solution (15 M EG and 15 M DMSO in 20% SSS and 0.5 M sucrose). The embryo is not left in a droplet, as that would slow the cooling rate. All excess fluid is removed by pipette from the Cryotop platform so that there is only a thin film of fluid surrounding it, in order to allow for the most rapid temperature drop. The embryo is then directly immersed into liquid nitrogen. The Cryotop containing the embryo is then placed in a canister in the liquid nitrogen tank for storage.

In the warming step, embryos are placed in decreasing concentrations of sucrose solutions to remove the cryoprotectants. The Cryotops are first rapidly plunged into a 37 °C dish containing warming solution (1.0 M sucrose) for one minute. The embryos are then slowly introduced in a stepwise fashion to dilution solutions (0.5 M sucrose). A wash solution (without sucrose) is slowly added to embryos in the dilution solution and the final rinse for the embryos is in 100% wash solution [9]. This protocol was designed to avoid too rapid osmotic shifts that could be caused by such high concentrations of cryoprotectant. The high concentration of cryoprotectant is actually not toxic in itself to eggs or embryos or tissue, in the protocols described. The appearance of toxicity comes only from too rapid an osmotic shift. The ultra-rapid rate of cooling (–23 000 °C per minute) and the high end concentration of cryoprotectant lowers the freezing point dramatically and thus allows the ice crystallization phase to be completely avoided.

With vitrification, mature retrieved oocytes can be successfully frozen with 95% success. For embryos, there really is no difference at all between those fresh or frozen [58].

Conclusion

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New technology in cryopreservation via vitrification allows us to remove ovary tissue and freeze it to protect

it from sterilizing cancer treatment in young women, as well as to freeze individual mature (as well as immature) eggs. It also allows us to stop the aging of the ovary and eggs, which is the major cause of the Current worldwide infertility epidemic. It will protect the future fertility potential of young women with cancer, and will also allow an expansion of the reproductive lifespan in any young woman who wishes to delay childbearing or delay her age of menopause.

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