

Duration of fertility after fresh and frozen ovary transplantation

Sherman Silber, M.D.,^a Nori Kagawa, Ph.D.,^b Masashige Kuwayama, Ph.D.,^b and Roger Gosden, Ph.D., D.Sc.^c

^a Infertility Center of St. Louis, St. Luke's Hospital, St. Louis, Missouri; ^b Kato Ladies Clinic, Tokyo, Japan; and ^c Center for Reproductive Medicine and Infertility, Weill Medical College of Cornell University, New York, New York

Objective: To evaluate the function of human ovarian transplants.

Design: Follow a series of fresh ovarian transplants for up to 5 years, and compare fresh and frozen ovarian tissue transplantation.

Setting: Tertiary referral community hospital.

Patient(s): Nine women with premature ovarian failure who received an ovary donated from a monozygotic twin sibling, and 16 young cancer patients undergoing ovarian cryopreservation. Two of the transplant recipients were cancer survivors rendered sterile by their therapy.

Intervention(s): Fresh ovary transplantation between monozygotic twin sisters, as well as transplantation of previously frozen ovarian tissue, and study of cryopreserved tissue in cancer patients.

Main Outcome Measure(s): Return of normal menstrual cycling, hormone levels, pregnancy, healthy babies, duration of transplant function, and ovarian tissue evaluation.

Result(s): Normal serum FSH and regular menstrual cycles returned by 5 months after surgery in all cases, both fresh and frozen. Fourteen spontaneous pregnancies were established leading to eight healthy live births and two healthy ongoing conceptions. All three frozen tissue transplants conceived spontaneously, one delivered, and two were ongoing. Oocyte survival with slow freezing was 42% and after vitrification 89%.

Conclusion(s): Ovarian transplantation in humans is a robust procedure, even after cryopreservation, and vitrification might prove to be more effective than slow freezing. (*Fertil Steril*® 2010;94:2191–6. ©2010 by American Society for Reproductive Medicine.)

Key Words: Ovarian transplant, vitrification, cryopreservation, premature ovarian failure, oncofertility

A successful case of human ovarian tissue transplantation between monozygotic (MZ) twin sisters discordant for premature ovarian failure (POF) was first reported in 2005 (1). Menstrual cycles resumed after 4 months, and spontaneous pregnancy occurred after the second ovulation, leading to the birth of a healthy child. Subsequently, a consecutive series of seven more successful cases was reported for a total of eight, all demonstrating ovulatory cycles with normalized serum FSH levels (2, 3). Spare ovarian cortical tissue from the donor ovary was cryopreserved for future grafting as a backup in case the first transplant became depleted of follicles and ceased to function. In a ninth case a different technique was used, microvascular transplantation of a whole ovary, and this too led to a prompt return of normal cycles, pregnancy by natural conception, and the delivery of a healthy child (4). Thus far, 12 pregnancies and eight healthy babies have resulted from these cases, none of whom required immunosuppression.

Despite this apparent success, there has been concern whether ovarian tissue grafts, either fresh or cryopreserved, have only transient function (5–7). So far, there have been a few successful cases reported of thawed autotransplanted ovarian tissue in former cancer patients (8–13), but information about graft longevity is sparse, and successes were only sporadic case reports. The present report represents a long-term follow-up of the duration of function of fresh and

frozen human ovarian grafts in a large series so as to estimate the degree of follicle loss from ischemia and from cryopreservation.

One solution to ischemic loss, microsurgical transplantation of an intact whole ovary is technically much more difficult and risky than cortical grafting (4, 14–16). It would, however, be preferable and simpler if a cortical grafting technique could minimize loss.

A long-term follow-up of our series of MZ twins offered an unusual opportunity to study the duration of function of fresh ovarian cortical grafts to evaluate oocyte loss from the transplant itself without the confusion created by cryopreservation, and to try to improve results with cryopreservation by vitrification (17). We now have evidence of long-term ovarian function in the current twin series, suggesting that a substantial reserve of follicles survives in fresh cortical grafts despite being subjected to lengthy ischemia compared with vascular transplantation. We have also found that slow freezing causes significant loss of oocyte viability compared with the vitrification technique.

MATERIALS AND METHODS

Patient Population and Surgery

Monozygotic twin pairs Nine pairs of female MZ twins, in which one sister had either idiopathic (8) or iatrogenic (1) POF and the other had normal reproductive function, underwent fresh ovarian transplantation. Each donor underwent unilateral oophorectomy by laparoscopy or minilaparotomy under general anesthesia. The ovary was transferred immediately to a Petri dish with Leibovitz L-15 medium cooled over a dish of saline ice slush. The following surgical principles were strictly adhered to: 1) the cortex was trimmed down to a bare 0.75–1.0 mm thickness to promote rapid revascularization; 2) perfect haemostasis of the graft bed was assured using microbipolar forceps; 3) 9-0 nylon interrupted sutures were used to prevent microhematoma

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Reprint requests: Sherman Silber, M.D. (FAX: 314-576-1442; E-mail: silber@infertile.com).

formation under the graft; 4) the grafts were applied to the highly vascular ovarian medulla; and 5) continual pulsatile irrigation with heparinized saline was applied to the graft surface to prevent adhesion formation (Fig. 1). One-fourth to one-third of the donor ovary was transplanted as a cortical slice, the remaining tissue being cryopreserved as a backup for replacing the primary graft if necessary. Because the ovaries and tubal ampullae were congenitally absent in case #3, donor tissue was grafted onto the peritoneum of the denuded fallopian tube isthmus, without any expectation of natural fertility.

In addition to these nine fresh transplants, two patients underwent a transplantation of frozen cortical tissue. In both frozen cases, the tissue had been cryopreserved by the slow freezing protocol. One of these frozen tissue transplants was for a 31-year-old woman who, 11 years earlier, had her tissue frozen for Hodgkin lymphoma and subsequently underwent several cycles of chemotherapy and radiation before having a bone marrow transplant. After >8 years she was considered to be cancer free, though menopausal, had married, and had gained approval from her oncologist for her frozen ovarian tissue to be transplanted back. The other frozen tissue recipient was from the identical twin series (case #1) who had undergone a successful fresh ovary transplant in 2004 leading to two spontaneous pregnancies and the delivery of a healthy child. After >3 years, she became menopausal again and had a second transplant of spare donor sibling tissue which had been cryopreserved at the time of the original fresh transplant. She became pregnant and delivered her second baby from this frozen transplant, became menopausal again 1.5 years later, and then underwent a second frozen tissue transplant.

Cancer patients tissue study A total of 16 cancer patients requesting fertility preservation by ovarian banking consented to an oocyte viability test and histologic review of a small sample (<10%) of their fresh or pre-

served tissue. In eight cases, the tissue had been preserved by vitrification, six by a slow freezing protocol, and in two only fresh tissue was analyzed. The goal of this section was to determine which method produced a higher cell survival rate.

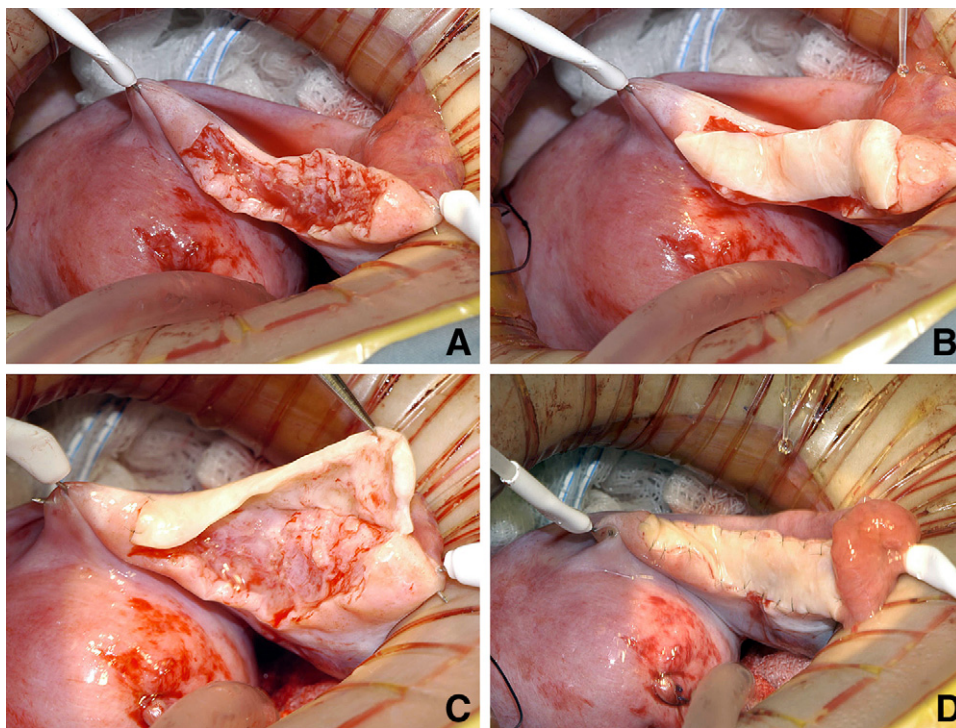
Cryopreservation

For slow freezing, after enucleating medullary tissue with sharp scalpel dissection, the cortex was pared down manually to an ultrathin translucent shell with a thickness of ≤ 1 mm. Tissue for cryopreservation was divided into multiple strips and transferred to 1.5 mL cryovials after equilibration in 1.5 mol/L 1,2-propanediol and 0.1 mol/L sucrose at 37 °C for 30 minutes, followed by 1.5 mol/L 1,2-propanediol and 0.2 mol/L sucrose for 5 minutes, and then cooled at a controlled rate, as described previously (18–20). Thawing was achieved rapidly by agitating the vials in a warmed water bath. If tissue had thickened by contraction after thawing, it was pared down again to <1 mm under an operating microscope with microsurgical scissors before transplantation. For vitrification, details have been described elsewhere (17).

Tissue analyses Small samples of cortical tissue from donor organs were assessed: 1) on a semiquantitative scale of relative follicle density in histology slides (0, +, ++, or +++, in order of increasing abundance); and 2) by testing viability after enzymatic disaggregation. Also, all original cortical tissue from transplant recipients who were in ovarian failure was removed and prepared histologically after excision to verify that total follicular depletion had occurred. For viability testing, tissues were incubated and pipetted in type I collagenase (1 mg/mL) for 10 minutes to isolate the small follicles and visualize their oocytes. The cells were briefly incubated in Hoechst 33342 and propidium iodide, then washed before viewing by fluorescence microscopy, for a total of 2,301 oocytes from 16 patients. Transmission electron

FIGURE 1

Transplantation of strips of cortical ovarian tissue involves: (A) resecting the sterile recipient ovary down to the vascular medullary bed; (B) trimming the thin donor tissue to approximate the exposed recipient surface; and (C and D) suturing the tissue to the recipient to achieve tight apposition and avoid hematoma formation.



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TABLE 1

Restitution of normal serum FSH, menstrual cycles, and fertility in all recipients of ovarian transplants donated by their monozygotic sibling.

Case no. ^a	Age (y)	Pre-op FSH, mIU/mL	Post-op FSH, mIU/mL	Initial post-op menses intervals (d)	Pregnancies	Live births
1	24	75	7.1	80, 62	3	2
2	38	96	5.2	93, 42, 24, 27, 25	2	2
3	25	112	6.8	76, 23, 30, 26, 25, 26, 21, 24, 27, 34, 25, 27, 51, 30, 27, 26, 28, 19	0 ^b	0 ^b
4	34	58	9.4	81, 22, 47, 26, 21, 20, 27, 26	2	1
5	40	60	6.8	86, 29, 38, 34, 28, 28, 31, 35, 34, 28, 33, 35, 30	0	0
6	26	101	7.5	64, 20, 39, 40, 32, 26, 29, 26, 26, 41	1	1
7	34	86	4.4	83, 22, 29, 29	2	1
8	37	86	7.4	100, 17, 39, 29, 27, 22, 23, 20, 34, 25, 26, 29	1	1
9	35	54	4.2	128, 42, 18, 25	1	0

^a In chronologic order of surgery.

^b Agenesis of fallopian tube ampullae.

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microscopy was also used to analyze ovarian tissue that had been either cryopreserved by slow freezing or vitrified by ultrarapid freezing (21).

Ethical Review

The surgical procedures and analyses of spare biopsy tissue were approved by the Ethics Review Committee and the Institutional Review Board of St. Luke's Hospital, and both donors and recipients gave written informed consents.

Statistics

Differences in oocyte survival between (fresh and preserved) tissues were compared using the chi-square test.

RESULTS

Transplant Function

The pregnancy and endocrine data of all nine cases of fresh ovarian transplantation are summarized in Table 1 and Figure 2. All nine returned to regular menses and ovulatory cycles by 60–130 days after surgery. Six of the eight who had normal fallopian tubes delivered eight healthy babies after natural conception, and cases #1, #4, #7, and #9 had one early miscarriage each. Case #5 had not become pregnant yet but continued to cycle for >4 years. Serum FSH levels on day 3 of the cycles had begun to decline by 3 months after surgery in all cases and reached normal baseline levels in every recipient by 5 months (Fig. 2). There was no difference in the rate of decline of FSH between fresh and frozen grafts.

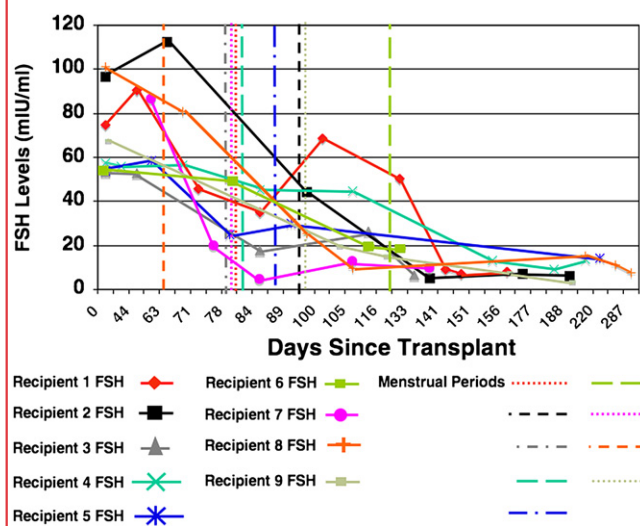
Case #1 became menopausal again at a little over 3 years after surgery. A retransplantation of frozen-thawed donor tissue then led to a prompt decline in FSH levels again, and she became pregnant again and delivered another healthy child. She then became menopausal once more 1.5 years later, had a second frozen graft, and then once more conceived and was carrying her third child. The other case involving a frozen transplant (who had undergone unilateral oophorectomy and ovarian cortex freezing for Hodgkin lymphoma many years earlier) followed a similar course after transplantation, with serum FSH falling to normal levels by 4 months and becoming pregnant without the need for further medical assistance. She had a healthy ongoing pregnancy (20 weeks) at the time of writing. Case #2, who had a transplant at age 38 years, became pregnant at 5 months and delivered a healthy girl the following year. She then became pregnant again 4 years later from the same fresh graft and delivered a healthy boy at age 42 years. Case #7 miscarried the first pregnancy after

transplantation, but 3 years later became pregnant again from the same graft and delivered a healthy baby. Two other healthy babies were born to women who had undergone pelvic irradiation.

A longer duration of transplant function was associated with a larger ovarian reserve expressed either by the antral follicle count (AFC) or the relative density of primordial follicles. Six of the eight fresh cortical grafts functioned for 3 or 4 years or more, one lasted for only 2 years and another transplant was still functioning at the time of writing <2 years after surgery. The six with ≥ 3 years of ovarian function had an AFC per ovary of nine follicles or greater, and histology revealed abundant primordial follicles (+++). The two with <3 years of cycling had an AFC of less than five and few small follicles (+ or ++).

FIGURE 2

Serum FSH levels were elevated at the time of ovarian transplantation but fell to basal levels within 90–150 days, where they remained long term. All recipients resumed menses in 65–130 days.



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Cryo-injury

Most of the stroma cells in the slow freeze–cryopreserved specimen were lysed and their nuclei compressed between dense bundles of extracellular fibers. The same cells were generally intact after vitrification. Small follicles were found in each specimen, all of which were intact within their basement membranes. The high viability (92%) of oocytes in control (fresh) specimens indicated that disaggregation per se had only caused minimal damage to this cell type. Overall, 2,301 oocytes were examined from 16 specimens. Results within each of the three groups were consistent and revealed no significant difference overall between fresh and vitrified tissue, although the viability of slow freeze–cryopreserved tissue was less than one-half (42%) and highly significant ($P < .01$; Table 2).

DISCUSSION

The chief significance of this long-term study of ovarian tissue transplantation is not so much for MZ twins who are discordant for POF, which will always be rare, but for young cancer patients needing fertility preservation. Ovarian tissue banking for a future transplantation provides another fertility option for these patients, and sometimes the only one available.

At least 1 in 250 women of reproductive age is a cancer survivor, and nowadays 90% of them become long-term survivors depending on the type of disease (22–24). However, their treatment is likely to reduce their fertility or render them completely sterile (25–28). Most such women with cancer are anxious to preserve their potential for having children genetically related to them rather than resorting to eggs from an unrelated donor or adoptions, which are not always available or desirable (29, 30). While pediatric patients may not understand the full implications of cancer treatment for future parenthood, fertility preservation is equally important for them, and they do not have the option of using IVF technology for oocyte or embryo banking. Another indication for fertility preservation is ageing, particularly for women with a family history of POF, but also for those with normal ovaries who are increasingly postponing childbearing (31–37). The uterus does not seem to play a significant role in this age-related decline, as evidenced by the high pregnancy rates in women of advanced reproductive age who use oocytes donated by younger women (38). Whereas the MZ twin series involved histocompatible donor tissue instead of an autograft, and healthy individuals rather than cancer patients, this is the largest series of ovarian transplants to date with the largest number of pregnancies and live births. It provides rare information for guiding fertility preservation practices and counseling patients about the likelihood of success.

It is difficult to draw inferences about the impact of ischemia except from a series of fresh human ovarian transplantations such as this. Ischemia time is reduced from an estimated 2–4 days with cortical tissue slices to only 1–2 hours for restoring perfusion after microvascular surgery, which provides the most physiologic approach (4, 39). Yet data from this MZ twin series provide striking proof of the effectiveness and safety of fresh cortical transplants. Eight healthy babies were born to six women from fresh cortical grafts, and all three frozen grafts also resulted in healthy pregnancies. Assisted reproductive technology was not required, natural conception occurring often within the first year after surgery and in one instance after the first ovulation. Early conception is not a rule, however: One woman conceived after 3 years and another had her second child >4 years after her transplant. It is not surprising that at 40 years old, the oldest recipient (#5) had not become pregnant by the time of writing. Nevertheless, her primordial follicle density and AFC were both relatively high, which probably accounted for the transplant maintaining normal menstrual cycles for >4 years.

Menstrual cycles were reinitiated within 2–4 months in all cases and continued from 1 to ≥ 4 years, with seven of eight women still cycling regularly from their fresh cortical transplant, and without any negative impact reported by the ovary donors. Maximizing the number of ovulatory cycles is obviously desirable for the opportunity that many ovulations provide for conception. The number of cycles to viable conception were one, two, five, seven, eight, eleven, sixteen, and thirty. Nevertheless, if the quality of oocytes is high and coitus is carefully synchronized at midcycle, even short-lived transplants can be effective, as with case #6, who conceived in the first year, gave birth to a child, and then once again became menopausal. Pregnancy results after frozen transplantation were as robust as after fresh, but duration of function was shorter. Ovarian transplantations involve a single operation with a relatively low surgical burden and low cost, with the advantages of no further medicalization of reproduction and thus far only singleton pregnancies. There have been few miscarriages to date nor any birth defects or obstetric complications associated with transplantation.

We surmise that careful preparation of the donor tissue as a very thin wafer, avoiding microhematoma formation between the highly vascular graft bed, and closely apposing the graft all contributed to the success of the present series by promoting rapid revascularization. The orthotopic location may also be important, not only for natural conception but possibly for avoiding pressure from neighboring tissues, which in some heterotopic sites might conceivably distort growing follicles and affect their physiology. Future technical advances may further improve clinical outcomes through significantly reducing ischemia time and accelerating angiogenesis (40, 41).

The long-term function of these fresh ovarian cortical grafts indicates that follicle reserve was well preserved. If a sterile recipient was given a whole ovary transplant from a 40-year-old donor, and no follicles were lost in the process, a simple model of follicle dynamics predicts that function will last for ~ 8 years (42). It was impressive, therefore, to find that both case #2 and case #5, who were 38 and 40 years old, respectively, at the time of their transplantation, were still cycling after >4 years, which is half-way to their theoretic limit. Furthermore, if one expects 20 years of remaining ovarian function in the average 30-year-old with two ovaries, then one-third of one ovary might be anticipated to function perhaps not so much longer than what we have observed in our younger patients whose donor had good ovarian reserve.

Viability markers indicated that <50% of human oocytes survived the slow freezing protocol, which has been the standard method for

TABLE 2

Survival of small oocytes after enzymatic isolation from ovarian tissues following cryopreservation by vitrification or slow freezing.

Group	No. of ovaries	No. of oocytes harvested	No. of surviving oocytes (%)
Fresh	2	358	329 (91.9%) ^a
Vitrified	8	1,122	1,000 (89.1%) ^a
Cryopreserved	6	821	342 (41.7%) ^b

^{a,b}Groups with the same superscripts are not significantly different ($P > .05$), whereas those with different superscripts are significantly different ($P < .01$).

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ovarian tissue since the pioneering studies in sheep (18, 19, 43–50). Our studies have revealed that vitrification may produce results superior to standard cryopreservation (17), although there have been no long-term studies or live offspring. It is too early to report the results of human ovarian tissue transplanted after vitrification. Because most centers, including our own, have mainly used the slow freezing cryopreservation protocol, it is reassuring that three viable pregnancies were obtained in the present study as well as five others for cancer patients in other centers (8, 10–13). The percentage of viable oocytes in vitrified tissue was remarkably similar to that of fresh tissue controls, suggesting that vitrification might provide even better results after transplantation than slow freeze cryopreservation, although this cannot be stated definitively without long-term studies.

What is the future for women whose ovaries were frozen before undergoing treatment for leukemia or breast cancers which might have already metastasized to the ovary (51–60)? Hodgkin disease is the safest cancer for transplanting ovarian tissue back to the patient. But what can be done with frozen ovarian tissue of the leuke-

mia survivor if there happen to have been leukemic cells in that tissue? Culturing that tissue to obtain mature follicles for IVF in these patients is currently the subject of intense research efforts (61–63). Culturing of primordial follicles may still be a distant goal (64–68), but progress is now being made with three-dimensional culturing of secondary follicles, because one mechanism controlling follicle maturation in the ovarian cortex may be physical pressure and tissue rigidity (69–72). Using this concept, remarkable progress has been made in culturing ovarian tissue and maturing secondary follicles in vitro. But for patients in whom there is no significant risk of ovarian metastasis, ovary tissue transplantation may now be ready for clinical use.

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REFERENCES

- Silber SJ, Lenahan KM, Levine DJ, Pineda JA, Gorman KS, Friez MJ, et al. Ovarian transplantation between monozygotic twins discordant for premature ovarian failure. *N Engl J Med* 2005;353:58–63.
- Silber SJ, Gosden RG. Ovarian transplantation in a series of monozygotic twins discordant for ovarian failure. *N Engl J Med* 2007;356:1382–4.
- Silber SJ, DeRosa M, Pineda J, Lenahan K, Grenia D, Gorman K, Gosden RG. A series of monozygotic twins discordant for ovarian failure: ovary transplantation (cortical versus microvascular) and cryopreservation. *Hum Reprod* 2008a;23:1531–7.
- Silber SJ, Grudzinskas G, Gosden RG. Successful pregnancy after microsurgical transplantation of an intact ovary. *N Engl J Med* 2008b;359:2617–8.
- Oktay K, Karlikaya G. Ovarian function after transplantation of frozen, banked autologous ovarian tissue. *N Engl J Med* 2000;342:1919.
- Radford JA, Lieberman BA, Brison DR, Smith AR, Critchlow JD, Russell SA, et al. Orthotopic reimplantation of cryopreserved ovarian cortical strips after high-dose chemotherapy for Hodgkin's lymphoma. *Lancet* 2001;357:1172–5.
- Sanchez M, Alama P, Gadea B, Soares SR, Simon C, Pellicer A. Fresh human orthotopic ovarian cortex transplantation: long-term results. *Hum Reprod* 2007;22:786–91.
- Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, et al. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet* 2004;364:1405–10.
- Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, et al. Restoration of ovarian function after orthotopic (intraovarian and periovarian) transplantation of cryopreserved ovarian tissue in a woman treated by bone marrow transplantation for sickle cell anaemia: case report. *Hum Reprod* 2006;21:183–8.
- Meirow D, Levron J, Eldar-Geva T, Hardan I, Fridman E, Zalel Y, et al. Pregnancy after chemotherapy. *N Engl J Med* 2005;353:318–21.
- Demeestere I, Simon P, Buxant F, Robin V, Fernandez SA, Centner J, et al. Ovarian function and spontaneous pregnancy after combined heterotopic and orthotopic cryopreserved ovarian tissue transplantation: case report. *Hum Reprod* 2006;21:2010–4.
- Demeestere I, Simon P, Emiliani S, Delbaere A, Englert Y. Fertility preservation: successful transplantation of cryopreserved ovarian tissue in a young patient previously treated for Hodgkin's disease. *Oncologist* 2007;12:1437–42.
- Andersen CY, Rosendahl M, Byskov AG, Loft A, Ottosen C, Dueholm M, et al. Two successful pregnancies following autotransplantation of frozen/thawed ovarian tissue. *Hum Reprod* 2008;23:2266–72.
- Baird DT, Webb R, Campbell BK, Harkness LM, Gosden RG. Long-term ovarian function in sheep after ovariectomy and transplantation of autografts stored at -196°C . *Endocrinology* 1999;140:462–71.
- Bedaiwy MA, Falcone T. Harvesting and autotransplantation of vascularized ovarian grafts: approaches and techniques. *Reprod Biomed Online* 2007;14:360–71.
- Arav A, Revel A, Nathan Y, Bor A, Gacitua H, Yavin S, Gavish Z, et al. Oocyte recovery, embryo development and ovarian function after cryopreservation and transplantation of whole sheep ovary. *Hum Reprod* 2005;20:3554–9.
- Kagawa N, Silber S, Kuwayama M. Successful vitrification of bovine and human ovarian tissue. *Reprod Biomed Online* 2009;18:568–77.
- Gosden RG, Baird DT, Wade JC, Webb R. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at -196°C . *Hum Reprod* 1994;9:597–603.
- Newton H, Aubard Y, Rutherford A, Sharma V, Gosden R. Low temperature storage and grafting of human ovarian tissue. *Hum Reprod* 1996;11:1487–91.
- Gook DA, Edgar DH, Stern C. Effect of cooling rate and dehydration regimen on the histological appearance of human ovarian cortex following cryopreservation in 1,2-propanediol. *Hum Reprod* 1999;14:2061–8.
- Keros V, Xella S, Hulthenby K, Pettersson K, Sheikhi M, Volpe A, et al. Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue. *Hum Reprod* 2009;24:1670–83.
- Bleyer WA. The impact of childhood cancer on the United States and the world. *CA Cancer J Clin* 1990;40:355–67.
- Ries LAG. Cancer incidence and survival among children and adolescents: United States SEER program 1975–1995. 1999.
- Jeruss JS, Woodruff TK. Preservation of fertility in patients with cancer. *N Engl J Med* 2009;360:902–11.
- Anderson RA, Themmen AP, Al-Qahtani A, Groome NP, Cameron DA. The effects of chemotherapy and long-term gonadotrophin suppression on the ovarian reserve in premenopausal women with breast cancer. *Hum Reprod* 2006;21:2583–92.
- Anderson RA, Cameron DA. Assessment of the effect of chemotherapy on ovarian function in women with breast cancer. *J Clin Oncol* 2007;25:1630–1. author reply 1632.
- Larsen EC, Muller J, Schmiegelow K, Reznitzer C, Andersen AN. Reduced ovarian function in long-term survivors of radiation and chemotherapy-treated childhood cancer. *J Clin Endocrinol Metab* 2003;88:5307–14.
- Lee SJ, Schover LR, Partridge AH, Patrizio P, Wallace WH, Hagerty K, et al. American Society of Clinical Oncology. American Society of Clinical Oncology recommendations on fertility preservation in cancer patients. *J Clin Oncol* 2006;24:2917–31.
- Schover LR, Rybicki LA, Martin BA, Bringelsen KA. Having children after cancer. A pilot survey of survivors' attitudes and experiences. *Cancer* 1999;86:697–709.
- Schover LR, Brey K, Lichtin A, Lipshultz LI, Jeha S. Knowledge and experience regarding cancer, infertility, and sperm banking in younger male survivors. *J Clin Oncol* 2002;20:1880–9.
- Leridon H. Can assisted reproduction technology compensate for the natural decline in fertility with age? A model assessment. *Hum Reprod* 2004;19:1548–53.
- Lampic C, Svanberg AS, Karlstrom P, Tyden T. Fertility awareness, intentions concerning childbearing, and attitudes toward parenthood among female and male academics. *Hum Reprod* 2006;21:558–64.
- Maheshwari A, Porter M, Shetty A, Bhattacharya S. Women's awareness and perceptions of delay in childbearing. *Fertil Steril* 2008;90:1036–42.
- te Velde ER, Pearson PL. The variability of female reproductive ageing. *Hum Reprod Update* 2002;8:141–54.
- Devroey P. Female age predicts embryonic implantation after ICSI: a case-controlled study. *Hum Reprod* 1996;11:1324–7.
- Silber SJ, Nagy Z, Devroey P, Camus M, Van Steirteghem AC. The effect of female age and ovarian reserve on pregnancy rate in male infertility: treatment of azoospermia with sperm retrieval and

- intracytoplasmic sperm injection. *Hum Reprod* 1997;12:2693–700.
37. Fretts RC. Effect of advanced age on fertility and pregnancy in women. Up To Date Online 2007. Available at: <http://www.uptodate.com>.
 38. SART. Assisted reproductive technology success rates. National summary and fertility clinic reports. Atlanta, GA: Centers for Disease Control and Prevention, 2005.
 39. Yin H, Wang X, Kim SS, Chen H, Tan SL, Gosden RG. Transplantation of intact rat gonads using vascular anastomosis: effects of cryopreservation, ischaemia and genotype. *Hum Reprod* 2003;18:1165–72.
 40. Candy CJ, Wood MJ, Whittingham DG. Restoration of a normal reproductive lifespan after grafting of cryopreserved mouse ovaries. *Hum Reprod* 2000;15:1300–4.
 41. Israely T, Dafni H, Granot D, Nevo N, Tsafiriri A, Neeman M. Vascular remodeling and angiogenesis in ectopic ovarian transplants: a crucial role of pericytes and vascular smooth muscle cells in maintenance of ovarian grafts. *Biol Reprod* 2003;68:2055–64.
 42. Faddy MJ, Gosden RG, Gougeon A, Richardson SJ, Nelson JF. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. *Hum Reprod* 1992;7:1342–6.
 43. Aubard Y, Piver P, Cogni Y, Fermeaux V, Poulin N, Driancourt MA. Orthotopic and heterotopic autografts of frozen-thawed ovarian cortex in sheep. *Hum Reprod* 1999;14:2149–54.
 44. Salle B, Lornage J, Demirci B, Vaudoyer F, Poirel MT, Franck M, et al. Restoration of ovarian steroid secretion and histologic assessment after freezing, thawing and autograft of a hemi-ovary in sheep. *Fertil Steril* 1999;72:366–70.
 45. Lee DM, Yeoman RR, Battaglia DE, Stouffer RL, Zelinski-Wooten MB, Fanton JW, et al. Live birth after ovarian tissue transplant. *Nature* 428;6979:137–8.
 46. Kim S, Battaglia DE, Soules MR. The future of human ovarian cryopreservation and transplantation: fertility and beyond. *Fertil Steril* 2001;75:1049–56.
 47. Kim SS, Yang HW, Kang HG, Lee HH, Lee HC, Ko DS, et al. Quantitative assessment of ischemic tissue damage in ovarian cortical tissue with or without antioxidant (ascorbic acid) treatment. *Fertil Steril* 2004;82:679–87.
 48. Kim SS, Radford J, Harris M, Varley J, Rutherford AJ, Lieberman B, et al. Ovarian tissue harvested from lymphoma patients to preserve fertility may be safe for autotransplantation. *Hum Reprod* 2001;16:2056–60.
 49. Kim SS. DEBATE: Ovarian tissue banking for cancer patients—To do or not to do? *Hum Reprod* 2003;18:1759–61.
 50. Kim SS, Kang HG, Kim NH, Lee HC, Lee HH. Assessment of the integrity of human oocytes retrieved from cryopreserved ovarian tissue after xenotransplantation. *Hum Reprod* 2005;20:2502–8.
 51. Falcone T, Attaran M, Bedaiwy MA, Goldberg JM. Ovarian function preservation in the cancer patient. *Fertil Steril* 2004;81:243–57.
 52. Gadducci A, Cosio S, Genazzani AR. Ovarian function and childbearing issues in breast cancer survivors. *Gynecol Endocrinol* 2007;23:625–31.
 53. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics. *CA Cancer J Clin* 2008;58:71–96.
 54. Lobo RA. Potential options for preservation of fertility in women. *N Engl J Med* 2005;353:64–73.
 55. Colgan TJ, Murphy J, Cole DE, Narod S, Rosen B. Occult carcinoma in prophylactic oophorectomy specimens: prevalence and association with BRCA germline mutation status. *Am J Surg Pathol* 2001;25:1283–9.
 56. Elizur SE. Detection of microscopic metastasis of solid tumors and hematological malignancies in cryopreserved ovaries. *Fertil Steril* 2004;(Suppl):S82–116.
 57. Meirou D, Hardan I, Dor J, Fridman E, Elizur S, Ra'anani H, et al. Searching for evidence of disease and malignant cell contamination in ovarian tissue stored from hematologic cancer patients. *Hum Reprod* 2008;23:1007–13.
 58. Nakayama K. Gonadal failure after treatment of hematologic malignancies: from recognition to management for health-care providers. *Natl Clin Pract Oncol* 2008;5:78–89.
 59. Schroder CP, Timmer-Bosscha H, Wijchman JG, de Liej LF, Hollema H, Heineman MJ, et al. An in vitro model for purging of tumour cells from ovarian tissue. *Hum Reprod* 2004;19:1069–75.
 60. Sklar CA, Mertens AC, Mitby P, Whitton J, Stovall M, Kasper C, et al. Premature menopause in survivors of childhood cancer: a report from the childhood cancer survivor study. *J Natl Cancer Inst* 2006;98:890–6.
 61. Smitz J, Picton H, Platteau P, Rutherford A, Cortvrindt R, Clyde J, et al. Principal findings from a multicenter trial investigating the safety of follicular-fluid meiosis-activating sterol for in vitro maturation of human cumulus-enclosed oocytes. *Fertil Steril* 2007;87:949–64.
 62. Papanikolaou EG, Platteau P, Albano C, Nogueira D, Cortvrindt R, Devroey P, et al. Immature oocyte in-vitro maturation: clinical aspects. *RBM Online* 2005;10:587–92.
 63. Nogueira D, Cortvrindt R, Everaerd B, Smitz J. Effects of long-term in vitro exposure to phosphodiesterase type-3 inhibitors on follicle and oocyte development. *Reproduction* 2005;130:177–86.
 64. Picton HM, Harris SE, Muruvi W, Chambers EL. The in vitro growth and maturation of follicles. *Reprod Rev* 2008;136:703–15.
 65. Sadeu JC, Cortvrindt R, Ron-El R, Kasterstein E, Smitz J. Morphological and ultrastructural evaluation of cultured frozen-thawed human fetal ovarian tissue. *Fertil Steril* 2006;85(Suppl. 1):1130–41.
 66. Sadeu JC, Mazoyer C, Smitz J. Human follicle culture in vitro. In: *Infertility and assisted reproduction*. Rizk B, Garcia-Velasco J, Sallam H, Makrigiannakis A, eds. Cambridge, U.K.: Cambridge University Press, 2008:25–38.
 67. Amorim CA, Van Langendonck A, David A, Dolmans MM, Donne J. Survival of human pre-antral follicles after cryopreservation of ovarian tissue, follicular isolation and in vitro culture in a calcium alginate matrix. *Hum Reprod* 2009;24:92–9.
 68. Telfer EE, McLaughlin M, Ding C, Thong KJ. A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. *Hum Reprod* 2008;23:1151–8.
 69. West ER, Xu M, Woodruff TK, Shea LD. Physical properties of alginate hydrogels and their effects on in vitro follicle development. *Biomaterials* 2007;28:4439–48.
 70. West-Farrell ER, Xu M, Gomberg MA, Chow YH, Woodruff TK, Shea LD. The mouse follicle microenvironment regulates antrum formation and steroid production: alterations in gene expression profiles. *Biol Reprod* 2009;80:432–9.
 71. Xu M, Kreeger PK, Shea LD, Woodruff TK. Tissue-engineered follicles produce live, fertile offspring. *Tissue Eng* 2006;12:2739–46.
 72. Xu M, West E, Shea LD, Woodruff. Identification of a stage-specific permissive in vitro culture environment for follicle growth and oocyte development. *Biol Reprod* 2006b;75:916–23.