CHAPTER 28

In-vitro maturation for fertility preservation

Hananel EG Holzer, Ri-Cheng Chian, William M Buckett, and Seang Lin Tan

INTRODUCTION

Fertility preservation for females is a medical issue of the utmost importance that has recently gained attention by researchers, health-care providers, and the public. Fertility preservation should be considered in cases of patients with malignant diseases undergoing potentially gonadotoxic treatments, patients with other diseases undergoing similar treatments, such as some cases of systemic lupus erythematosus (SLE), and practically by all women at risk for premature ovarian failure. Fertility preservation may also be considered for women approaching their fourth decade without a partner to start a family but wishing to preserve their fertility potential.

INDICATIONS FOR FERTILITY PRESERVATION

Patients undergoing potentially gonadotoxic treatments

In the modern era, cancer is considered as a common lethal disease. It was estimated that in 2003, over 650 000 new cases of female cancer were diagnosed in the USA. It is encouraging that during the last three decades there has been a tremendous improvement in the success rates of cancer treatments and a continual rise in the survival rates[1]. It is estimated that, in the foreseeable future, one in 250 people will be a cancer survivor[2].

Unfortunately, the agents used for treatment of many types of cancer, even though successful in up to 95% of patients, carry a considerable risk for the future fertility potential. Patients (and their families) are now seeking more than just cure for their disease – they wish to have other options to try to preserve their fertility potential for a future normal healthy and fulfilling life.

The ovaries, containing a limited number of germ cells, are prone to irreversible damage as a result of chemotherapy[3]. The ovaries may undergo follicular loss that may end up in a complete absence of follicles and ovarian fibrosis, leading to premature ovarian failure, infertility, and a premature menopause[4,5]. The rates of premature ovarian failure and infertility are affected by many factors – particularly the age of the woman at the time of treatment. Young women and prepubescent girls are much more likely to retain some degree of ovarian function compared with women in their late 30s and 40s. Other features which affect the rates of ovarian failure are the type of disease, the duration of

treatment, and the combination of chemotherapeutic agents.

Some drugs present a higher risk for ovarian failure. Cyclophosphamide is the drug probably most associated with ovarian failure and subsequent infertility. Cyclophosphamide causes depletion of primordial follicles even at low doses, but the damage is dependent on both the dose and the cumulative dose given. Cyclophosphamide is used for treating both malignant diseases and non-malignant diseases such as SLE[5–8].

The patient's age correlates with the risk of premature ovarian failure. As age advances the ovarian primordial follicle pool declines and the woman has a shorter duration to ovarian failure after exposure to gonadotoxic drugs[9]. Even if the normal cycling pattern is resumed, patients who have been treated with gonadotoxic drugs are more prone to develop premature ovarian failure later in life[10–12].

Ionizing radiation also causes a reduction in the primordial follicle pool; oocytes are sensitive to radiation and show pyknosis, chromosome condensation, disruption of the nuclear envelope, and cytoplasmic vacuolization. These result in oocyte depletion and may cause ovarian failure and infertility[13,14]. The ovarian damage is dose related and the cut-off dose for ovarian damage depends on the age, extent, and type of radiotherapy. It was calculated that the threshold for destroying 50% of the oocytes is 200 cCy. Less than 150 cCy will have minimal or no deleterious effect in young women, whereas 250–500 cCy will cause infertility in about 60% of young women (aged 15–40 years) and in almost 100% of women over 40 years[13–15]. Oophorectomy (with or without uterine conservation) in women with borderline ovarian cancers will also lead to iatrogenic premature ovarian failure and infertility.

Patients with genetic abnormalities

Young patients with Turner's syndrome mosaics are destined to have premature ovarian failure and infertility. However, adolescent girls with Turner's syndrome mosaics may still have some follicles in their ovarian cortical tissue[16]. The follicles and/or oocytes retrieved may be preserved for future fertility. The issues of whether the oocytes retrieved from girls with Turner's syndrome have a normal chromosomal complement and of their fertility potential still need to be addressed.

The fragile-X premutation is also associated with premature ovarian failure[17], and female carriers should be advised of this risk and may consider fertility preservation. Obviously, any oocytes or embryos cryopreserved from these women would need to undergo some form of karyotypic evaluation (such as preimplantation genetic diagnosis or polar body testing) prior to embryo transfer.

Extending the fertile age span

In the last three decades, women have tended to delay childbirth and the mean age at first delivery continues to rise in most countries. However, advancing female age is associated with declining fertility, increased miscarriage rates, increased congenital and chromosomal abnormalities, and poorer obstetric and neonatal outcome[18]. These are due to decreasing numbers and quality of oocytes. Therefore, women in their thirties without a partner may consider fertility preservation. However, discussion of the psychologic and social issues arising from 'social' fertility preservation, although important, is well beyond the scope of this chapter.

OPTIONS FOR FERTILITY PRESERVATION

Various strategies for fertility preservation have recently been discussed in the medical literature and in the worldwide media. The strategies today could be regarded as either ovarian protection in vivo or extracorporal cryopreservation.

Nevertheless, most of these strategies should currently be regarded as still experimental.

Ovarian protection

Ovarian transposition

To avoid or to lower the radiation dose to the ovaries, the ovaries can be transposed out of the direct radiation field. Ovarian transposition should be considered prior to local radiotherapy in cancer patients younger than 40 years in whom gonadotoxic chemotherapy is not indicated. It necessitates a surgical procedure. Laparoscopic transposition is usually preferred[19].

GnRH analogs

Decreasing gonadal function by administering a GnRH analog during chemotherapy cycles may protect the ovaries against the sterilizing effects of chemotherapy; however, although animal studies and some human retrospective studies show some benefit, the mechanism of action is not clear[20]. Furthermore, the efficacy of GnRH analog in reducing ovarian damage is not yet clear.

Apoptosis

Apoptosis, the programmed cell death of the germ cells, may also have a role in chemo- and radiotherapy-induced cell depletion. Some experimental compounds may inhibit the chemotherapy- and radiotherapy-induced oocyte apoptosis. Unfortunately, treatments inhibiting apoptosis are still far away from clinical implementation[21,22].

Ovarian tissue cryopreservation

In cryopreservation of ovarian tissue, hundreds to thousands of immature oocytes may be preserved, and the primordial follicles seem much less susceptible to cryoinjury. However, at least two surgical procedures are needed: one for harvesting the ovarian tissue and at least a second (or in some cases a second and a third) to transplant the cryopreserved ovarian tissue. Furthermore, in cases of transplantation to a heterotopic site (such as the forearm or the abdominal subcutaneous fat), an IVF procedure is needed as well. To date, less then a handful of pregnancies from transplanted ovarian tissue have been reported[23–25].

Although retransplantation of ovarian tissue carries the added benefit of re-instituting ovarian hormonal function as well as the possibility of future fertility, transplantation of the frozen–thawed ovarian cortex tissue strips back to the patient also carries the risk of re-seeding metastatic disease[26]. So far, ovarian graft life seems to be short lived, although the reasons for this are unclear. Possible causes for the poor ovarian function include cryoinjury, a depletion in the number of primordial follicles, and poor vascular perfusion of the transplanted graft.

As an alternative, the primordial follicles ideally could be cultured in vitro to mature oocytes. This would be a very attractive option as many hundreds of primordial follicles would be available, even from very small biopsy specimens. Unfortunately, however, in humans this technology still awaits further research and is far from clinical practice[27,28].

Embryo and oocyte cryopreservation after ovarian stimulation

Mature oocytes can be harvested from the ovaries after controlled ovarian hyperstimulation, allowing generation of embryos and their subsequent cryopreservation. This has the advantage that embryo cryopreservation and subsequent transfer is an established treatment for couples with infertility and is practised around the world.

However, there are two major drawbacks for the conventional IVF with ovarian stimulation and subsequent embryo cryopreservation. The first is the time interval needed for IVF; this ranges from 2 to 6 weeks, beginning at the patient's next menstrual period, which may sometimes be too

long to wait prior to starting chemo- or radiotherapy due to the natural course of the malignant disease. The second is that ovarian stimulation is associated with relatively high estradiol levels that may not be safe in cases of estrogen-sensitive tumors such as breast cancer, or in women with a high likelihood of thromboembolism, as well as for other patients wishing to avoid ovarian stimulation.

To avoid the high estradiol levels associated with ovarian stimulation for cancer patients (particularly breast cancer), letrozole or tamoxifen plus low-dose FSH protocols have been used. These protocols are associated with lower peak estradiol levels, but do not totally avoid ovarian stimulation[29].

After collection of the mature oocytes, the oocytes can be fertilized using the partner's sperm. The resulting embryos will be frozen for future thaw and transfer. Transferring frozen-thawed embryos is an integral part of assisted reproductive technology (ART) programs. Survival rates per thawed embryos are 60–90% and the pregnancy rate per thaw is 20–25%[30,31]. When sperm is not available then oocyte cryopreservation may be considered; this will be discussed in the next section.

IN-VITRO MATURATION FOR FERTILITY PRESERVATION

Avoiding hormonal stimulation

Ovarian stimulation for collection of oocytes can be totally avoided by collecting immature oocytes. As discussed elsewhere in the book, in-vitro maturation (IVM) of oocytes collected via transvaginal ultrasound-guided aspiration has been performed since 1994; almost 1000 babies have been born and the clinical pregnancy rate is currently around 35% per cycle. Immature oocyte collection can be offered for cancer patients with no ovarian stimulation and thus with no concerns regarding aggravating hormone-sensitive disease. The only hormonal treatment needed is a single injection of hCG 36 h prior to the collection to improve the IVM rate[32].

Timing of oocyte retrieval

For women undergoing immature oocyte retrieval for IVM, the collection can be performed during the follicular phase prior to ovulation for normal ovulating patients, and on almost any given day for PCOS patients. Thus, patients scheduled for therapy do not have to wait for the beginning of the next menstrual bleeding and then undergo ovarian stimulation. Patients with PCOS may have the collection at almost any given time 36 h after the hCG injection. Ovulating patients seen first during the follicular phase can have the collection soon after their initial visit. In the cases seen first during the luteal phase, due to lack of sufficient information on collections during the luteal phase, we tend to wait for the subsequent follicular phase. In the normal ovulating patient, a mature oocyte can be collected as well as immature oocytes; the presence of a dominant follicle does not hamper the maturation and developmental potential of the small antral follicles[33]. Therefore, collecting immature oocytes from unstimulated ovaries may save valuable time, time that may have critical importance for patients with malignant disease.

The short time needed may even enable more than one oocyte collection to be performed in the interval available before the gonadotoxic treatment is initiated. Typically this would be in women with breast cancer following surgery and prior to starting chemotherapy. The immature oocytes collected can be cryopreserved as immature germinal vesicle oocytes, or as in-vitro matured mature oocytes, or can be fertilized and cryopreserved as embryos.

Cryopreservation of immature oocytes

Immature oocytes as compared with mature oocytes have a smaller volume and no meiotic

spindle, and the chromosomes are protected within a nuclear membrane. Therefore, it could be presumed that freezing of immature oocytes would yield better survival rates. Nevertheless, cryosurvival and subsequent maturation rates of immature oocytes have been disappointing so far. Pregnancies have been reported, but the overall experience is still poor[34–36].

Cryopreservation of mature oocytes

Conventional cryopreservation

Although the first live birth from a cryopreserved oocyte was reported almost 20 years ago[37], the overall results for mature oocyte cryopreservation have been discouraging. The oocytes are more vulnerable to the freezing process (slow freezing) than are embryos. Intracellular ice formation can cause membrane rupture, abnormal cortical granular reaction, zona hardening, and damage to the meiotic spindle and cytoskeleton. The meiotic spindle damage might result in chromosomal abnormalities. The reported survival rates for mature oocytes (collected from stimulated ovaries) are around 50%[38,39]. There are no data concerning rates of in-vitro matured oocytes surviving conventional oocyte cryopreservation (slow freezing).

Vitrification

Vitrification has been used successfully for the cryopreservation of human oocytes. Cryoprotectants in high concentration are used to induce a glass-like state; the cell is then rapidly frozen, avoiding the formation of intracellular ice. Live births after vitrification of oocytes have been reported, but there are still concerns about the effects of the high concentrations on the vulnerable oocytes[40,41].

Recently we have vitrified mature oocytes collected from stimulated ovaries in two studies. In the first study, 15 patients over-responding to ovarian stimulation underwent oocyte collection and oocyte vitrification, instead of cycle cancellation or converting to IVF. The oocytes were thawed and fertilized, and the subsequent embryos were transferred within the following few months. The oocyte survival, fertilization, and cleavage rates were 85.8%, 73.7%, and 82.5%, respectively. The clinical pregnancy and implantation rates were 46.7% and 22.4%, respectively. In the second study, 17 patients underwent ovarian stimulation for IVF. The collected oocytes were vitrified after collection. Within the following months the oocytes were thawed, fertilized, and transferred. The oocyte survival, fertilization, and cleavage rates were 89%, 72.5%, and 80.4%, respectively. The clinical pregnancy and implantation rates were 41.2% and 16.4%, respectively (McGill Reproductive Center, unpublished data). Thus oocyte vitrification seems to be a potentially promising technique with high oocyte survival rates. This technique should probably be implemented in fertility preservation programs for patients wishing to preserve their fertility and who do not have a partner.

Patients who cannot undergo ovarian stimulation or those who do not want to have ovarian stimulation have the option of immature oocyte collection from unstimulated ovaries. The immature oocytes will be matured in vitro and then vitrified for future use. However promising this option may be, the patients should be strongly advised of the experimental nature of this work.

Embryo cryopreservation after IVM

Patients who have a male partner and are wishing to preserve oocytes fertilized by their partner's sperm should undergo immature oocyte collection from unstimulated ovaries and then the oocytes would be fertilized utilizing ICSI. The resulting embryos would be vitrified or cryopreserved for a future transfer.

As noted above, embryo cryopreservation is an effective and reproducible treatment modality within conventional stimulated IVF. Similarly, many babies have been born

following cryopreservation and subsequent thawing of embryos generated from IVM oocytes[42].

IVM FOR FERTILITY PRESERVATION – THE McGILL EXPERIENCE

We have recently reported the retrieval of immature oocytes from unstimulated ovaries of a cancer patient before gonadotoxic therapy for oocyte vitrification purposes. A 33-year-old stage II breast cancer patient was seen on day 10 of the menstrual cycle, hCG was given, and 19 immature oocytes collected on day 12. Six oocytes matured on day 13 and 11 more matured on day 14. Altogether, 17 mature oocytes were vitrified without any delay in chemotherapy[43].

Since that report, 32 patients with various malignant diseases have undergone 35 immature oocyte collections – 16 patients with breast cancer, 9 patients with Hodgkin's lymphoma, and 7 further patients with other malignancies (non-Hodgkin's lymphoma, anaplastic oligodendroglioma, Ewing's sarcoma, rhabdomyosarcoma, endometrial carcinoma, abdominal desmoid tumor, and desmoplastic ovarian tumor). The indications for immature oocyte collection without ovarian stimulation were at least one of the following:

(1) Hormone-sensitive disease

(2) Patient's request to avoid ovarian stimulation and

(3) Lack of sufficient time for an IVF cycle prior to starting chemo- or radiotherapy.

Of these patients 20 who underwent 23 oocyte collections without ovarian stimulation elected to have their oocytes cryopreserved, rather than generate embryos, because they did not have a partner. One patient had a partner but the couple chose not to use the husband's sperm for fertilization, and to cryopreserve oocytes. From these women, a total of 226 oocytes were collected; 18 were mature on the day of collection, 8 were at the metaphase I stage, and 200 were germinal vesicle oocytes. Of these occytes, 143 were matured in vitro and a total of 160 mature oocytes were vitrified; the average maturation rate was 66.3% ± 30.5%.

The other 12 patients, with a partner, underwent 12 oocyte collections without ovarian stimulation. For these couples, a total of 97 oocytes were collected; 8 were mature on the day of collection and 89 were germinal vesicles (GV) oocytes. Sixty oocytes matured in vitro and the average maturation rate was 64.8% ± 27.8%. Following ICSI, 55 mature oocytes were fertilized, giving an average fertilization rate of 84.4% ± 15.9% and 53 resulting embryos were vitrified. To date, none of the cancer patients has returned for an embryo transfer.

CONCLUSIONS

Fertility preservation is of the utmost importance to patients undergoing gonadotoxic chemo- and/or radiotherapy, and also for patients facing the risk of premature ovarian failure as well as for others wishing to preserve their fertility potential.

The options vary between ovarian protection, ovarian tissue cryopreservation, and oocyte or embryo cryopreservation. Collection of immature oocytes from unstimulated ovaries followed by IVM of the oocytes does not carry the theoretic risk of re-instituting the metastatic malignant disease that transplanting ovarian tissue carries. Collection of immature oocytes from unstimulated ovaries followed by IVM of the oocytes can be performed for patients with a hormone-sensitive disease without risk of aggravating the disease. Collecting the immature oocytes does not require the same time needed for ovarian stimulation and the waiting time for the beginning of the next menstrual cycle. Thus precious time may be saved and therapy is not delayed.

Oocytes of patients without a partner should be vitrified after IVM and oocytes of

patients with a partner should be fertilized after IVM and the resulting embryos then vitrified. However promising IVM of oocytes may seem, especially for patients for whom this may be the only safe procedure for preserving fertility, the patients and their families should be informed of the experimental nature of these treatments and of the lack of information concerning survival, fertilization, and pregnancy potential of oocytes frozen/vitrified after IVM.

REFERENCES

1. Jemal A, Murray T, Samuels A et al. Cancer statistics, 2003. Cancer J Clin 2003; 53: 5–26.
2. Bleyer WA. The impact of childhood cancer on the United States and the world. CA Cancer J Clin 1990; 40: 355–67.
3. Oktay K, Kan MT, Rosenwaks Z. Recent progress in oocyte and ovarian tissue cryopreservation and transplantation. Curr Opin Obstet Gynecol 2001; 13: 263–8.
4. Familiari G, Caggiati A, Nottola SA et al. Ultrastructure of human ovarian primodial follicles after combination chemotherapy for Hodgkin's disease. Hum Reprod 1993; 8: 2080–7.
5. Warne GL, Fairley KF, Hobbs JB et al. Cyclophosphamide induced ovarian failure. N Engl J Med 1973; 289: 1159–62.
6. Meirow D, Lewis H, Nugent D et al. Subclinical depletion of primordial follicular reserve in mice treated with cyclophosphamide: clinical importance and proposed accurate investigative tool. Hum Reprod 1999; 14: 1903–7.
7. Goldhirsh A, Gelber RD, Castiglione M. The magnitude of endocrine effects of adjuvant chemotherapy for premenopausal breast cancer patients. The International Breast Cancer Study Group. Ann Oncol 1990; 1: 183–8.
8. Koyama H, Wada T, Nishizawa Y et al. Cyclophosphamide-induced ovarian failure and its therapeutic significance in patients with breast cancer. Cancer 1977; 39: 1403–9.
9. Bines J, Oleske DM, Gobleigh MA. Ovarian function in premenopausal women treated with adjuvant chemotherapy for breast cancer. J Clin Oncol 1996; 14: 1718–29.
10. Byrne J, Fears TR, Gail MH et al. Early menopause in long-term survivors of cancer during adolescence. Am J Obstet Gynecol 1992; 166: 788–93.
11. Larsen EC, Muller J, Schmeigelow K et al. Reduced ovarian function in long term survivors of radiation and chemotherapy treated childhood cancer. J Clin Endocrinol Metab 2003; 88: 5307–14.
12. Huong DLT, Amoura Z, Duhaut P. Risk of ovarian failure and fertility after intravenous cyclophosphamide. A study in 84 patients. J Rheumatol 2002; 29: 2571–6.
13. Gosden RG, Wade JC, Fraser HM et al. Impact of congenital or experimental hypogonadotrophism on the radiation sensitivity of the mouse ovary. Hum Reprod 1997; 12; 2483–8.
14. Damewood MD, Grochow LB. Prospects for fertility after chemotherapy or radiation for neoplastic disease. Fertil Steril 1986; 45: 443–59.
15. Wallace WH, Thompson AB, Kelsey TW. The radiosensitivity of the human oocyte. Hum Reprod 2003; 18: 117–21.
16. Hreinsson JG, Otala M, Fridstrom M. Follicles are found in the ovaries of adolescent girls with Turner's syndrome. J Clin Endocrinol Metab 2002; 87(8): 3618–23.
17. Bretherick KL, Fluker MR, Robinson WP. FMR1 repeat sizes in the gray zone and high end of the normal range are associated with premature ovarian failure. Hum Genet 2005; 117(4): 376–82.
18. Heffner LJ. Advanced maternal age – how old is too old? N Engl J Med 2004; 351: 1927–9.
19. Tulandi T, Al-Sharani A. Laparoscopic fertility preservation. Obstet Gynecol Clin N Am 2004; 31: 611–18.
20. Blumenfeld Z, Eckmen A. Preservation of fertility and ovarian function and minimization of chemotherapy-induced gonadotoxicity in young women by GnRH-a. J Natl Cancer Inst Monogr 2005; (34): 40–3.
21. Tilly JL. Molecular and genetic basis of normal and toxicant induced apoptosis in female germ cells. Toxicol Lett 1998; 102: 497–501.

22. Morita Y, Perez GI, Paris F et al. Oocyte apoptosis is suppressed by disruption of acid sphingomyelinase gene or by sphingosine 1 phosphate therapy. Nat Med 2000; 6: 1109–14.

23. Oktay K, Buyuk F, Veeck L et al. Embryo development after heterotropic transplantation of cryopreserved ovarian tissue. Lancet 2004; 363: 837–40.

24. Donnez J, Dolmans MM, Demylle D et al. Livebirth after orthotopic tranplantation of cryopreserved ovarian tissue. Lancet 2004; 364: 1405–10.

25. Meirow D, Levron J, Eldar-Geva T et al. Pregnancy after transplantation of cryopreserved ovarian tissue in a patient with ovarian failure after chemotherapy. N Engl J Med 2005; 21: 353: 318–21.

26. Shaw JM, Bowles J, Koopman P et al. Fresh and cryopreserved ovarian tissue samples from donors with lymphoma transmit the cancer to graft recipients. Hum Reprod 1996; 11: 1668–73.

27. O'Brien MJ, Pendola JK, Eppig JJ. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. Biol Reprod 2003; 68: 1682–6.

28. Picton HM, Danfour MA, Harris SE et al. Growth and maturation of oocytes in vitro. Reprod Suppl 2003; 61: 445–62.

29. Oktay K, Buyuk E, Libertella N et al. Fertility preservation in breast cancer patients: a prospective controlled comparison of ovarian stimulation with tamoxifen and letrozole for embryo cryopreservation. J Clin Oncol 2005; 23: 4347–53.

30. Wang JX, Yapp YY, Mathews CD. Frozen-thawed embryo transfer: influence of clinical factors on implantation rate and risk of multiple conception. Hum Reprod 2001; 16: 2316–19.

31. Assisted Reproductive Technology in the United States: 2000 results generated from the American Society for Reproductive Medicine/Society for Assisted Reproductive Technology Registry. Fertil Steril 2004; 81: 1207–20.

32. Chian RC, Gulekli B, Buckett WM et al. Priming with human chorionic gonadotropin before retrieval of immature oocytes in women with infertility due to the polycystic ovary syndrome. N Engl J Med 1999; 341: 1624–6.

33. Chian RC, Buckett WM, Abdul Jalil C et al. Natural-cycle in vitro fertilization combined with in vitro maturation of immature oocytes is a potential approach in infertility treatment. Fertil Steril 2004; 82: 1675–8.

34. Toth TL, Lanzendorf SE, Sandow BA et al. Cryopreservation of human prophase I oocytes collected from unstimulated follicles. Fertil Steril 1994; 61: 1077–82.

35. Wu J, Zhang L, Wang X. In vitro maturation, fertilization and embryo development after ultrarapid freezing of immature human oocytes. Hum Reprod 2001; 121: 389–93.

36. Tucker MJ, Morton PC, Wright G et al. Clinical application of human egg cryopreservation. Hum Reprod 1998; 13: 3156–9.

37. Chen C. Pregnancy after human oocyte cryopreservation. Lancet 1986; 327: 884–6.

38. Fabbri R, Porcu E, Marsella T et al. Human oocyte cryopreservation: new prospectives regarding oocyte survival. Hum Reprod 2001; 16: 411–16.

39. Sonmezer M, Okatay K. Fertility preservation in female patients. Hum Reprod Update 2004; 10: 251–6.

40. Arav A, Yavin S, Zeron Y et al. New trends in gamete cryopreservation. Mol Cell Endocrinol 2002; 187: 77–81.

41. Yoon TK, Kim TJ, Park SE et al. Live births after vitrification of oocytes in a stimulated in vitro fertilization-embryo transfer program Fertil Steril 2003; 79: 1323–6.

42. Chian RC, Gulekli B, Buckett WM et al. Pregnancy and delivery after cryopreservation of zygotes produced by in vitro matured oocytes retrieved from a woman with polycystic ovarian syndrome. Hum Reprod 2001; 16: 1700–2.

43. Rao GD, Chian RC, Son W et al. Fertility preservation in patients undergoing cancer treatment. Lancet 2004; 363: 1829.