In vitro maturation of human immature oocytes for fertility preservation

Ri-Cheng Chian, Ph.D.,[a,b] Peter S. Uzelac, M.D.,[c] and Geeta Nargund, M.D.[d]

[a] State Key Laboratory for Reproductive Medicine, Nanjing Medical University, Nanjing, People's Republic of China; [b] Department of Obstetrics and Gynecology, McGill University, Montreal, Quebec, Canada; [c] Marin Fertility Center, San Francisco, California; and [d] Create Health Clinic, London, United Kingdom

Cryopreservation of embryos, oocytes, or ovarian tissues is the main option for female fertility preservation. Oocyte cryopreservation has emerged as especially important: the dramatic increase in the number of infants born from vitrified oocytes indicates that it is becoming one of the most important intervention options. However, oocyte cryopreservation with standard controlled ovarian hyperstimulation may not be feasible for some cancer patients as there are serious concerns about the effect of ovarian stimulation with hormones on the risk of cancer recurrence. Also, urgent gonadotoxic cancer treatment may not allow sufficient time for a patient to undergo hormonal ovarian stimulation. Thus, immature oocyte retrieval from ovaries without ovarian stimulation followed by in vitro maturation and vitrification is a promising fertility preservation option for women who cannot undergo ovarian stimulation or cannot delay their gonadotoxic cancer treatment. Immature oocytes can be collected from the ovaries during both the follicular and luteal phases, which maximizes the possibility for fertility preservation. The combination of ovarian tissue cryopreservation with immature oocyte collection from the tissue followed by oocyte vitrification via in vitro maturation represents another promising approach of fertility preservation in young women with cancer. (Fertil Steril® 2013;99: 1173–81. ©2013 by American Society for Reproductive Medicine.)

Key Words: Cancer, fertility preservation, IVM, oocytes, vitrification

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/chianrc-in-vitro-maturation-oocytes-fertility-preservation/

Use your smartphone to scan this QR code and connect to the discussion forum for this article now.*

* Download a free QR code scanner by searching for "QR scanner" in your smartphone's app store or app marketplace.

Over the last few decades, the incidence of cancer in females has increased by up to 20% even as mortality rates have declined due to progress in cancer treatments (1). With aggressive chemotherapy and/or radiotherapy coupled with bone marrow transplantation, more than 90% of teenage girls and young women affected by some malignancies will survive (2). However, chemotherapy with alkylating agents and radiation therapy with a field that includes the pelvis have an adverse effect on ovarian reserve, which may lead to premature ovarian failure (POF) and infertility (3). The number of cancer survivors is increasing every year, leaving a growing number of women of reproductive age faced with the risk of POF and infertility. One of the major concerns is whether these women will be able to have healthy children after their cancer treatment. Therefore, fertility preservation is an important issue that should be addressed with women who are at risk of POF after gonadotoxic chemotherapy or radiotherapy treatment for cancer, autoimmune, and hematologic disorders or potentially sterilizing surgical procedures. A suggested strategy for female fertility preservation for cancer patients has been proposed (12) (Fig. 1).

The clinical options for female fertility preservation can be divided into two broad categories: surgical interventions and cryopreservation cells/tissues. The surgical procedures include ovarian transposition in patients who require pelvic irradiation (4), and radical trachelectomy (5) for selected low-grade and early stage cancer patients, such as those with cervical, endometrial, or epithelial ovarian cancer (6, 7). Female patients have three main cryopreservation options for fertility preservation: embryos, oocytes, and/or ovarian tissues. The American Society of Clinical Oncology (ASCO) (8) and American Society for Reproductive Medicine (ASRM) (9) have published guidelines for female fertility preservation before gonadotoxic oncologic treatment, both indicating that embryo cryopreservation is the only viable strategy at present.

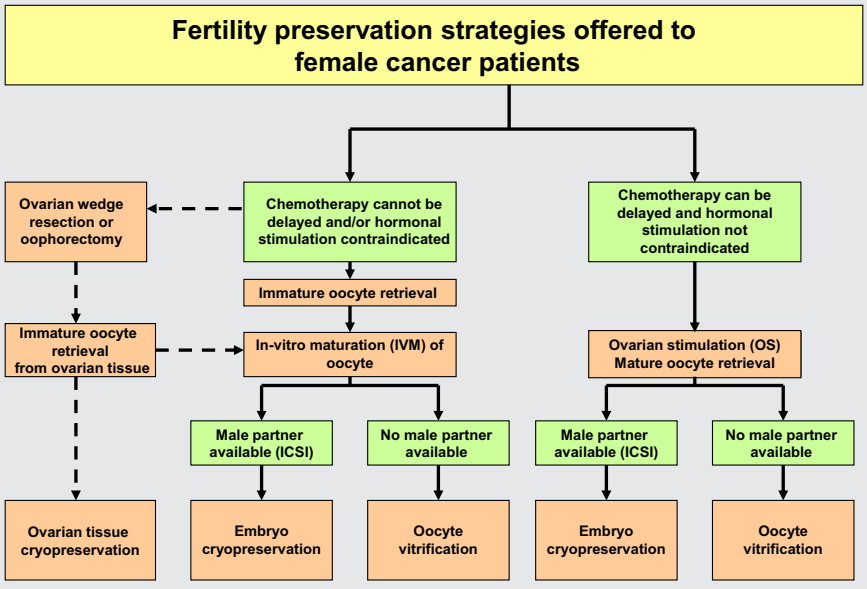
Embryo production involves ovarian stimulation with gonadotropin followed by retrieval of mature oocytes and in vitro fertilization (IVF) using sperm from a male partner or donor,

Received December 7, 2012; revised and accepted January 25, 2013; published online February 20, 2013.
R.-C.C. has nothing to disclose. P.S.U. has nothing to disclose. G.N. has nothing to disclose.
Reprint requests: Ri-Cheng Chian, Ph.D., Royal Victoria Hospital, Women's Favilion F3-36, 687 Pine Avenue West, Montreal, Canada H3A 1A1 (E-mail: ri-cheng.chian@mcgill.ca).

Fertility and Sterility® Vol. 99, No. 5, April 2013 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2013.01.141

FIGURE 1



The proposed fertility preservation strategies offered to female cancer patients. Reproduced from Chian et al. (12) with permission.

Chian. IVM oocytes for fertility preservation. Fertil Steril 2013.

which may need several weeks for preparation. The window of opportunity for fertility preservation is small. Ovarian stimulation with follicle-stimulating hormone (FSH) may not be suitable for some patients who cannot delay their cancer treatment. Therefore, embryo cryopreservation may not be a feasible option for many cancer patients. Also, embryo cryopreservation is not a feasible option for women who lack a male partner or those who object to the use of donor sperm. Furthermore, embryo cryopreservation is excluded for prepubertal girls who are at risk of POF, for whom cryopreservation of ovarian tissues is the only method of fertility preservation. Although there have been several successful live births after ovarian tissue cryopreservation and orthotopic transplantation (10, 11), the procedures still are considered experimental (9).

Oocyte cryopreservation may be considered an important option for female fertility preservation. Normally, oocyte cryopreservation involves ovarian stimulation, mature oocyte retrieval, and cryopreservation. However, oocyte cryopreservation with the standard ovarian stimulation procedure may not be feasible for some cancer patients. Immature oocyte retrieval from the ovaries without prior ovarian stimulation followed by in vitro maturation (IVM) and cryopreservation is a promising fertility preservation option for women who cannot undergo hormonal ovarian stimulation or who cannot delay their gonadotoxic cancer treatment.

PROBLEMS WITH HORMONAL OVARIAN STIMULATION IN SOME CANCER PATIENTS

Breast cancer remains the most common cancer in women, representing approximately 30% of all female cancers (13). A risk of breast cancer is associated with persistently elevated blood estrogen levels. Ovarian stimulation using FSH causes a state of high estrogen concentrations in serum (14), so the safety of ovarian stimulation with FSH in breast cancer patients is a primary concern, especially in women who have estrogen-positive breast cancer.

Although the special stimulation protocol with the combination of letrozole and low-dose FSH has been applied to ovarian stimulation in breast cancer patients (15, 16), the effect of a temporary increase in the level of estrogen in serum is an increased risk of breast cancer recurrence. Recent findings also suggest that there is an indirect mitogenic effect of estrogen on hormone receptor-negative breast cancer (17). In addition, other oncologic or nononcologic diseases such as systemic lupus erythematosus, desmoids tumors, or severe endometriosis are also considered to be estrogen sensitive (18).

The influence of the level of estrogen elevation on some cancer patients remains unclear and controversial. It has been suggested that, if possible, avoiding the elevation of estrogen concentrations to supraphysiologic levels is a good option for fertility preservation in breast cancer patients, regardless of their estrogen receptor status (19). As a result, immature oocyte retrieval followed by IVM and cryopreservation has been proposed as a safer alternative (20). The advantages of this method for fertility preservation are that the patients are not exposed to elevated estrogen levels and that the treatment can be completed in a shorter period of time compared with the standard protocols of ovarian stimulation for oocyte or embryo cryopreservation.

DEVELOPMENT OF IVM AS INFERTILITY TREATMENT

The first live birth from human IVF was produced from natural cycle IVF without ovarian stimulation (21). Later, natural cycle IVF was slowly replaced by ovarian stimulation IVF because it was believed that the number of oocytes retrieved

relates to the number of embryos available to transfer, thus directly affecting the chance of a successful pregnancy (22–24). In the beginning, relatively inexpensive medication such as clomiphene citrate was used to stimulate the ovaries to produce multiple follicles. However, current ovarian stimulation protocols use expensive gonadotropin-releasing hormone (GnRH) agonists or antagonists in combination with FSH for ovarian stimulation. Some women are very sensitive to stimulation with exogenous gonadotropins and are at increased risk of developing ovarian hyperstimulation syndrome (OHSS). Also, there is anxiety that the long-term side effects of repeated ovarian stimulation may increase the risk of ovarian, endometrial, and breast cancers (25).

It is interesting that the initial reports of pregnancies from in vitro matured oocytes were from stimulated cycles where the immature oocytes retrieved were followed by IVM and IVF (26, 27), and the morphologically immature and mature oocytes had been retrieved from the stimulated cycle. Robert Edwards, the pioneer of IVF, believed that recovery of immature oocytes followed by IVM could be a potentially useful treatment for women with infertility (28). In comparison with ovarian stimulation IVF, the major advantages of IVM treatment can be considered to be avoidance of the risk of OHSS, reduced cost, and simplified treatment (29).

IVM of Immature Oocytes from Cesarean Delivery

Human IVF technology was developed with in vitro matured oocytes derived from surgical wedge resection or oophorectomy materials. In the beginning, although embryos were produced successfully from the in vitro matured oocytes (30), it was impossible to perform embryo transfer with the produced embryos due the nature and source of the immature oocytes from oophorectomy. A successful live birth from IVM was reported in 1991 using immature oocytes that had been collected for an oocyte donation program during a cesarean delivery (31). An important question is why oocytes derived from a patient during a cesarean delivery should still have maturational and developmental potential sufficient to produce a healthy live birth, as the endocrinologic environment of the ovaries during pregnancy may not be the same as that found during a normal menstrual cycle. Subsequently, it was shown that immature oocytes could be retrieved from the ovaries regardless of follicular or luteal phase (32), and that the number of immature oocytes collected was related mainly to the age of the women, pathology, day of the menstrual cycle, and cyclic versus noncyclic ovaries. It seems that the number of immature oocytes collected from the ovary decreases as women's age increases, but there is no statistically significant difference in oocyte maturation and cleavage rates among donors in the different age groups (32).

Immature oocytes from the luteal phase have a significantly higher maturation rate compared with those collected from the follicular phase (33). Although a pregnancy resulting from the fertilization of immature oocytes derived at the time of a cesarean delivery was confirmed by another report (34), the oocyte maturation rate seemed relatively low when these immature oocytes were cultured in vitro (35). Nevertheless, it has been reported that immature oocytes collected from the small follicles at cesarean delivery followed by IVM and IVF can develop to blastocyst stage when the embryos are cocultured with human ampullary epithelial cells (36). Immature oocytes derived from cesarean delivery followed by IVM may be an available source for a donor oocyte program. More research is required to clarify the efficiency and safety issues surrounding the use of immature oocytes derived from cesarean delivery.

IVM of Immature Oocytes from Women with PCO or PCOS

A large number of antral follicles in the ovaries are seen in infertile women with polycystic ovaries (PCO) or polycystic ovary syndrome (PCOS). These patients are sensitive to ovarian stimulation with gonadotropins and have an increased risk of OHSS compared with women who have normal ovaries. Since the first report of a pregnancy in a woman with anovulatory PCOS after undergoing IVM and IVF (37), several groups have made efforts to develop this treatment for infertile women with PCO or PCOS. The technique involves modified IVM treatment, with priming with FSH or human chorionic gonadotropin (hCG) before immature oocyte retrieval; the current clinical pregnancy and implantation rates are approximately 35% and 15%, respectively (38). The primary candidates for IVM treatment were women with PCO or PCOS, regardless of whether they were in ovulatory or anovulatory cycles (37), and the pregnancy rates after IVF or IVM treatment correlated with the number of oocytes retrieved.

Priming with FSH before immature oocyte retrieval. As an alternative approach, a truncated course of ovarian stimulation with FSH before immature oocyte retrieval has been used, indicating that FSH pretreatment promotes efficient recovery of immature oocytes and maturation in vitro for infertile women with PCOS (39). It has been reported that priming with recombinant FSH before the harvesting of immature oocytes from patients with PCOS improves the maturational potential of the oocytes, with significantly higher implantation and pregnancy rates compared with women who did not have FSH priming (40). However, another report indicated that FSH priming with 75 IU/day for 6 days in combination with hCG priming 36 hours before immature oocyte retrieval had no additional benefit in women with PCOS (41). Although these results are conflicting on the benefits of using primed follicles for IVM in women with PCO or PCOS associated with irregular menstrual cycles, theoretically the use of FSH priming of follicles at the beginning of the luteal or follicular phases is likely to enhance more follicular development. A report has shown that improved pregnancy and implantation rates (46.7% and 45.2%, respectively) were obtained in women with PCOS for single-embryo transfer after priming with FSH before immature oocyte retrieval, IVM, and intracytoplasmic sperm injection (ICSI) (42).

Priming with hCG before immature oocyte retrieval. It has been demonstrated that the time course of oocyte maturation in vitro is hastened and the rate of oocyte maturation is increased by priming with 10,000 IU hCG 36 hours before

retrieval of immature oocytes from women with PCO or PCOS (43, 44). Therefore, it is possible that pregnancy rates will potentially be improved by priming with hCG (45). This notion was confirmed further by other studies (41, 46–48), where hCG priming not only promoted the initiation of some oocytes in their maturation process to metaphase-I stage from follicles ($\geq$ 10 mm in diameter) in vivo but also enhanced some oocytes in germinal vesicle (GV) stage from small follicles to acquire maturational and developmental competence in vivo. This was because the rate of oocyte maturation starting from the same GV stage was significantly different among cumulus-oocyte complexes (COCs) with different morphology (49). Priming with hCG before immature oocyte retrieval for IVM treatment in infertile women with PCO or PCOS has become a routine procedure in some clinics, and has achieved promising results in terms of oocyte maturation, fertilization, and pregnancy rates as well as live births (38, 50).

Immature Oocytes from Overresponders or Delayed Responders

Currently, most infertility treatments employ ovulation stimulation protocols to increase the number of oocytes available for fertilization, but a significant number of these stimulated cycles must be canceled because of the risk of hyperstimulation or a delayed response of gonadotropin stimulation. Immature oocyte retrieval followed by IVM and IVF may provide an effective management strategy for such patients, with no need to cancel the treatment cycle.

It has been reported that a 47.1% clinical pregnancy rate was achieved after immature oocyte retrieval and IVM in a group of patients who were a high risk of developing OHSS (51). For the prevention of OHSS during treatment, the gonadotropin stimulation was halted, and hCG was administered when the leading follicle had reached a mean diameter of 12–14 mm; oocyte collection was performed 36 hours later. Approximately 12% of the oocytes collected had already become mature at time of oocyte collection. Although the administration of hCG is invariably associated with exacerbation of the OHSS condition, no OHSS occurred when hCG was administered once the leading follicle reached to 12–14 mm in diameter (52). Therefore, patients who have a potential of developing OHSS can be provided with this option, with or without hCG administration, followed by oocyte retrieval and IVM rather than canceling the treatment cycle.

In addition, some patients during an ovarian stimulation treatment cycle appear to respond to the stimulation but have a low estrogen level or few or slow-growing follicles. Normally, this group of women have their cycles canceled or are given prolonged stimulation and a higher dose of gonadotropins. It has been reported that pregnancies can be established in these women by immature oocyte retrieval after hCG administration followed by IVM (53). An approximately 40% pregnancy rate has been obtained after immature oocyte retrieval and IVM without hCG administration before immature oocyte collection (54), suggesting that IVM may be a viable alternative to cancellation of the stimulation cycle in poor responders. It should be noted that with hCG administration there will be some mature oocytes collected. When these mature oocytes are pooled together with the immature oocytes collected, the potential for successful IVM/IVF treatment is maximized without cancelation of the ovarian stimulation treatment cycle.

IVM of Immature Oocytes from Women with Regular Menstrual Cycles

IVM treatment has also been applied to infertile women with normal ovaries and regular menstrual cycles. To obtain a greater number of immature oocytes, gonadotropins (FSH or hCG or FSH combined with hCG) have been used to provide mild ovarian stimulation. These different approaches have produced inconsistent results. In such cases, mature oocyte collection should be the first choice if FSH stimulation was employed; then consideration should be given to immature oocyte retrieval followed by IVM and IVF.

Priming with FSH alone or FSH combined with hCG before immature oocyte retrieval. Priming with a fixed dose of FSH (150 IU/day) for 3 days from day 3 of the menstrual cycle for IVM treatment did not increase the number of oocytes obtained per aspiration and does not improve oocyte maturation, cleavage rates, or embryonic development in women with normal cycling ovaries (55). The same investigators reported that the rates of oocyte maturation, fertilization, cleavage, or implantation were not different between "coasting" for 2 days or 3 days before immature oocyte retrieval when women with normal menstrual cycles were given 150 IU FSH/day for 3 days started from day 3 (56). However, it has been shown that using low-dose FSH priming starting from the luteal phase improves the efficiency of immature oocyte recovery and maturation as well as the rates of fertilization with IVM treatment in women with regular menstrual cycles (57).

Recently a study was designed to determine whether the efficiency of IVM in women with normal ovaries can be improved by gonadotropin administration (58). Four hundred women were randomly allocated to the following four groups: [1] nonprimed cycles, [2] hCG-primed cycles, [3] FSH-primed cycles, or [4] FSH- plus hCG-primed cycles. In groups where hCG was used, the overall maturation rate was higher, and the percentage of total available mature oocytes was higher. The conclusion was that a more favorable result can be obtained with a combination of FSH plus hCG priming; FSH priming or hCG priming alone had no significant effect on the clinical outcome.

Priming with hCG before mature and immature oocyte retrieval. In women with normal ovaries and a regular menstrual cycle, there is no advantage to priming with hCG alone for the sole purpose of immature oocyte retrieval. In these women, consideration should be given to natural cycle IVF and administration of hCG when the leading or dominant follicles reach a certain size (preferably 12–14 mm in diameter). In such cases, one or two mature oocytes together with several immature oocytes can be retrieved at the time of oocyte retrieval 36 hours after hCG priming (59). A protocol has been developed for infertile women with normal ovaries

and regular menstrual cycles: the combination of natural cycle IVF and IVM (60, 61).

Natural Cycle IVF-IVM

There has been increased interest in natural cycle IVF, because it is more comfortable for the woman, with fewer side effects and less physical burden. A study using life table analysis to calculate the cumulative success rates after successive cycles of treatment with natural cycle IVF indicated that the cumulative probability of pregnancy was 46% with an associated live-birth rate of 32% after four treatment cycles (62). Thus, the question must be asked as to which is the best option for the infertile patients: to undergo two or three cycles of natural IVF treatment or one ovarian stimulated cycle, covering the same time span with similar pregnancy and live-birth rates but a higher risk of complications?

In a natural cycle, normally only a single follicle develops to the preovulatory stage and ovulates its mature oocyte for potential fertilization. However, many small follicles also grow in the ovaries during the same follicular phase of the menstrual cycle. It is interesting that atresia does not occur in the nondominant follicles, even after dominant follicle selection; immature oocytes retrieved at this stage have been successfully matured in vitro and fertilized, and they have resulted in several pregnancies and healthy live births (63, 64). Animal model studies also support the finding that oocyte quality and early embryonic developmental competence of immature oocytes after IVM are not affected by the presence of the dominant follicle in the ovaries (65, 66).

However, prevention of ovulation from the dominant follicle due to a natural luteinizing hormone (LH) surge is important when the patients are treated with natural cycle IVF-IVM. It has now been established that 10,000 IU hCG can be administered 36 hours before oocyte retrieval when the size of the leading follicle has reached 12–14 mm in diameter (60, 61). Most oocytes collected from the leading follicles were mature at metaphase 2 (MII) stage. It is now possible to combine natural cycle IVF with IVM as an alternative to natural cycle IVF, and clinical pregnancy rates of 35% have been achieved for a selected group of women with various causes of infertility without recourse to ovarian stimulation (60). It is interesting that it has been reported that natural cycle IVF-IVM together with IVM-alone treatments can achieve acceptable pregnancy and implantation rates in more than 50% of infertile women (61), suggesting that natural cycle IVF-IVF is an efficient infertility treatment for all indications, especially for women under 35 years of age.

Although the strategy of natural IVF-IVM has been successfully applied to women with normal ovaries and regular menstrual cycles, it is frequently asked whether there are differences in pregnancy and implantation rates between the presence and absence of mature oocytes collected at time of the retrieval during natural cycle IVF-IVM treatment. A recent study reported that the clinical pregnancy rates are not different regardless of whether mature oocytes were collected at the time of oocyte retrieval, but the live-birth rate was statistically significantly higher ($P<.05$) when mature oocytes were collected at the time of oocyte retrieval (67), indicating that natural cycle IVF-IVM may be a more suitable treatment for younger women who have regular menstrual cycles.

SAFETY ISSUE WITH IVM OOCYTES

The number of live births from IVM oocytes has been increasing over the past two decades. There are many concerns about IVM infants as to obstetric and perinatal outcomes as well as long-term development. At the present, the issue of clinical safety of using IVM oocytes cannot be completely assessed because of the lack of well-controlled clinical trials. Several studies have reported that the mean birth weight and the incidence of congenital anomalies seem to be comparable with spontaneous conceptions or conceptions of infertile women undergoing IVF treatment (68–70). More recently, it was reported that 196 infants conceived after IVM of immature oocytes were not associated with an increased risk of adverse obstetric or perinatal outcomes compared with children conceived by in vivo matured oocytes or children conceived by conventional ovarian stimulated ICSI cycles (71).

As IVM treatment gains more widespread use, large and better designed studies are needed to investigate the short- and long-term health of IVM infants (72). It has been estimated that more than 5,000 IVM infants have been born worldwide. We are trying to collect data on the obstetric and perinatal outcomes of pregnancies and live births after IVM treatment. So far, approximately 1,500 infants born from IVM treatment have been collected in the database. Preliminary analysis indicates that birth weight and incidence of congenital anomalies appear to be comparable to births to infertile women who were treated with standard ovarian stimulation and ICSI cycles (Chian et al., unpublished data).

VITRIFICATION OF IN VITRO MATURED OOCYTES

Recent advances in vitrification techniques have markedly improved the efficacy of oocyte cryopreservation in terms of oocyte survival and pregnancy rates as well as live-birth rates that are now comparable to those achieved with fresh oocytes for IVF. The number of live births from vitrified oocytes has increased rapidly over the past decade. It has been estimated that worldwide several thousand babies have been born from cryopreserved oocytes. In Italy alone, approximately 2,000 babies have been born from cryopreserved oocytes, half of them from vitrified oocytes (personal communication with Dr. Giulia Scaravelli, Istituto Superiore di Sanita, Rome, Italy). To date, most live births were from in vivo matured oocytes produced from standard ovarian stimulation cycles, and only a few live births were from cryopreserved IVM oocytes (12, 73, 74). However, as yet no pregnancies have been reported from cycles for fertility preservation of cancer patients from in vitro matured oocytes after cryopreservation.

A major question is whether immature oocytes should be cryopreserved before or after IVM. With the special structure of immature oocytes with their GV compared to mature MII-stage oocytes, it has been proposed that cryopreservation at the immature GV stage may reduce damage to the oocytes from the freezing procedure (75). Theoretically, the use of

immature GV-stage oocytes circumvents the risk of polyploidy and aneuploidy because the chromatins are diffuse in the diplotene state of prophase I and are surrounded by a nuclear membrane, which may avoid spindle depolymerization (76). However, difficulties still exist with IVM of GV-stage oocytes after freezing-thawing. Although the survival rates seem to be improving, poor IVM and fertilization rates are still major problems associated with immature oocyte freezing (77–81). With the development of vitrification techniques, it was found that there is no difference in the survival rate between oocytes vitrified at the immature GV stage and those vitrified at the mature MII stage (82). But the potential of oocyte maturation was reduced significantly by vitrification of immature oocytes at the GV stage, suggesting that oocytes should be vitrified at the mature MII stage after IVM rather than at the immature GV stage (83–85).

Collection of Mature and Immature Oocytes at Follicular Phase for Fertility Preservation

Oocyte vitrification is an important alternative for women with cancer who require fertility preservation, and immature oocyte retrieval followed by IVM is especially important. A published case report describes a single 33-year-old woman with stage II breast cancer who came to our IVF center on day 10 of her menstrual cycle to investigate her options with respect to fertility preservation before undergoing chemotherapy the next week (86). The patient had no ovarian stimulation, and on day 12 of her menstrual cycle she underwent oocyte retrieval after administration 10,000 IU hCG given 36 hours previously. Although no mature oocytes were retrieved from the leading follicle, a total of 19 immature oocytes were collected; of these, 6 matured in vitro the next day, and 11 more on the day after. In total, 17 in vitro matured oocytes were cryopreserved for this patient. The next week, she underwent chemotherapy with no delays.

It is important to note that sometimes mature oocytes can be collected from the ovaries if the oocyte retrieval is performed in the follicular phase after hCG priming, even though there has been no ovarian stimulation with FSH (87). Particularly in women with breast cancer, fertility preservation has included retrieval of immature oocytes followed by IVM and then either vitrification of the oocytes or the embryos after IVF (88). These investigators have reported that for oocyte vitrification the average number of oocytes retrieved was 11.4 $\pm$ 8.8, the IVM rate was 64.2%, and an average of 7.9 $\pm$ 6.6 mature oocytes were vitrified per patient treated. The median duration from the first evaluation to the oocyte retrieval was 8 days. In addition, for embryo vitrification, the average number of oocytes retrieved was 9.7 $\pm$ 6.4, and the maturation rate was 53.2%; an average of 5.8 $\pm$ 2.7 mature oocytes was available for fertilization per patient. The fertilization rate was 77.8%, resulting in 4.5 $\pm$ 2.7 embryos vitrified per patient. The median duration from the first evaluation to oocytes collection was 13 days. Importantly, there were on average 1.6 $\pm$ 1.2 in vivo matured oocytes, and 9.0 $\pm$ 0.8 immature oocytes were retrieved from each patient. Therefore, this method provides a useful option for fertility preservation in breast cancer patients without ovarian stimulation and with no delay in cancer treatment.

It is interesting that it has also been proposed that IVM can be used as a complementary strategy to improve the mature oocyte yield in breast cancer patients who are undergoing ovarian stimulation with a modified letrozole-FSH protocol for fertility preservation (89). This indicates that immature oocytes retrieved during oocyte and embryo cryopreservation cycles should not be discarded, which should improve the future potential of fertility.

Collection of Immature Oocyte at Luteal Phase for Fertility Preservation

Another advantage of immature oocyte retrieval followed by IVM and vitrification is the possibility of retrieving oocytes regardless of the phase of the menstrual cycle (87), indicating that it is possible to perform immature oocyte retrieval sequentially in either the follicular or luteal phases of the menstrual cycle in breast cancer patients without affecting the quantity or maturation rate of the oocytes. Although no pregnancies or live births have been reported from immature oocytes retrieved during the luteal phase, they are a distinct possibility; there is evidence, mentioned previously, that pregnancies and live births have occurred after the removal of immature oocytes from pregnant women during cesarean delivery (31). Thus, in cancer patients under severe time constraints, immature oocyte retrieval in the luteal phase followed by IVM and vitrification can be considered before chemotherapy to maximize the possibility of fertility preservation, as has been suggested by various investigators (90, 91). It is interesting that there were no statistically significant differences found in the number of retrieved oocytes, maturation rate, fertilization rate, or total number of cryopreserved oocytes and embryos when immature oocyte retrieval followed by IVM of oocytes for fertility preservation was performed during the follicular phase compared with the luteal phase of the cycle (92).

COMBINATION OF OVARIAN TISSUE AND IVM OOCYTE VITRIFICATION

Ovarian tissue cryopreservation has been the primary method of fertility preservation for prepubertal girls who are at risk of POF (93). Ovarian tissue cryopreservation followed by later transplantation may be offered as a method of fertility preservation for pubertal cancer patients who cannot receive ovarian stimulation due to time constraints or contraindications. Cryopreservation of ovarian tissues would only preserve primordial and primary follicles. It has been reported that immature oocyte retrieval from the visible antral follicles after ovarian wedge resection or oophorectomy and subsequent IVM and vitrification of those oocytes represents an additional strategy for fertility preservation, indicating that this method of fertility preservation can be offered as an adjunct to ovarian tissue cryobanking (94). In addition, it has been also reported that the combination of ovarian tissue cryopreservation with immature oocytes collection from the tissue followed by IVM-vitrification of oocytes represents a promising

approach of fertility preservation for young women with mosaic Turner syndrome (95, 96).

CONCLUSIONS

Retrieval of immature oocytes followed by IVM of oocytes or embryo vitrification provides the optimal option for female fertility preservation, especially for patients who cannot be stimulated with FSH or who have time constraints related to their cancer treatment. Immature oocytes can be collected from the ovaries in both the follicular and luteal phases, which maximizes the possibility of fertility preservation for cancer patients. The combination of ovarian tissue cryopreservation with immature oocyte retrieval from the ovarian tissue followed by IVM and vitrification of oocytes represents another novel strategy of fertility preservation for young women before gonadotoxic treatment.

REFERENCES

1. Heron M, Hoyert D, Murphy S, Xu J. National vital statistics reports. Natl Health Stat Report 2009;57:14.
2. American Cancer Society. Cancer facts and figures 2009. Available at: http://www.cancer.org/research/cancerfactsfigures/cancer-facts-figures-2009.
3. Poniatowski BC, Grimm P, Cohen G. Chemotherapy-induced menopause: a literature review. Cancer Invest 2001;19:641–8.
4. Tulandi T, Al-Took S. Laparoscopic ovarian suspension before irradiation. Fertil Steril 1998;70:381–3.
5. Del Priore G, Ungar L, Smith JR. Complications after fertility-preserving radical trachelectomy. Fertil Steril 2006;85:227.
6. Liou WS, Yap OW, Chan JK, Westphal LM. Innovations in fertility preservation for patients with gynecologic cancers. Fertil Steril 2005;84:1561–73.
7. Plante M. Fertility preservation in the management of gynecologic cancers. Curr Opin Oncol 2000;12:497–507.
8. 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.
9. Ethics Committee of the American Society of Reproductive Medicine. Fertility preservation and reproduction in cancer patients. Fertil Steril 2005;83:1622–8.
10. Donnez J, Silber S, Andersen CY, Demeestere I, Piver P, Meirow D, et al. Children born after autotransplantation of cryopreserved ovarian tissue. a review of 13 live births. Ann Med 2011;43:437–50.
11. Anderson RA, Wallace WHB. Fertility preservation in girls and young women. Clin Endocrinol 2011;75:409–19.
12. Chian RC, Gilbert L, Huang JYJ, Son WY, Holzer H, Cui SJ, et al. Obstetrical outcomes following vitrification of oocytes matured in-vivo or in-vitro. Fertil Steril 2009;91:2391–8.
13. Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, et al, American Cancer Society. Cancer statistics 2004. Ca Cancer J Clin 2004;54:8–29.
14. Oktay K, Buyuk E, Libertella N, Akar M, Rosenwaks Z. 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.
15. Oktay K, Hourvitz A, Sahin G, Oktem O, Safro B, Cil A, et al. Letrozole reduces estrogen and gonadotropin exposure in women with breast cancer undergoing ovarian stimulation before chemotherapy. J Clin Endocrinol Metab 2006;91:3885–90.
16. Madrigrano A, Westphal L, Wapnir I. Egg retrieval with cryopreservation does not delay breast cancer treatment. Am J Surg 2007;194:477–81.
17. Gupta PB, Kuperwasser C. Contributions of estrogen to ER-negative breast tumor growth. J Steroid Biochem Mol Biol 2006;102:71–8.
18. Morice P. Borderline tumours of the ovary and fertility. Eur J Cancer 2006;42:149–58.
19. Gupta PB, Proia D, Cingoz O, Weremowicz J, Naber SP, Weinberg RA, et al. Systemic stromal effects of estrogen promote the growth of estrogen receptor-negative cancers. Cancer Res 2007;67:2062–71.
20. Huang JYJ, Buckett WM, Gilbert L, Tan SL, Chian RC. Retrival of immature oocytes followed by in vitro maturation and vitrification: a case report on a new strategy of fertility preservation in women with borderline ovarian malignancy. Gynecol Oncol 2007;105:542–4.
21. Steptoe PC, Edwards RG. Birth after re-implantation of a human embryo. Lancet 1978;312:366.
22. Lopata A, Brown JB, Leeton JF, Talbot JM, Wood C. In vitro fertilization of preovulatory oocytes and embryo transfer in infertile patients treated with clomiphene and human chorionic gonadotropin. Fertil Steril 1978;30:27–35.
23. Johnston I, Lopata A, Speirs A, Hoult I, Kellow G, du Plessis Y. In vitro fertilization: the challenge of the eighties. Fertil Steril 1981;36:699–706.
24. Jones HW Jr, Jones GS, Andrews MC, Acosta A, Bundren C, Garcia J, et al. The program for in vitro fertilization at Norfolk. Fertil Steril 1982;38:14–21.
25. Brinton LA, Moghissi KS, Scoccia B, Westhoff CL, Lamb EJ. Ovulation induction and cancer risk. Fertil Steril 2005;83:261–71.
26. Veeck LL, Wortham JW Jr, Witmyer J, Sandow BA, Acosta AA, Garcia JE, et al. Maturation and fertilization of morphologically immature human oocytes in a program of in vitro fertilization. Fertil Steril 1983;39:594–602.
27. Prins GS, Wagner C, Weidel L, Gianfortoni J, Marut EL, Scommegna A. Gonadotropins augment maturation and fertilization of human immature oocytes cultured in vitro. Fertil Steril 1987;47:1035–7.
28. Edwards RG. Are minimal stimulation IVF and IVM set to replace routine IVF? Reprod Biomed Online 2007;14:267–70.
29. Edwards RG. 2007 IVF, IVM, natural cycle IVF, minimal stimulation IVF—time for a rethink. Reprod Biomed Online 2007;15:106–19.
30. Edwards RG, Bavister BD, Steptoe PC. Early stages of fertilization in vitro of human oocytes matured in vitro. Nature 1969;221:632–5.
31. Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY, Yoon TK. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their cultured in vitro and their transfer in a donor oocyte program. Fertil Steril 1991;55:109–13.
32. Cha KY, Do BR, Chi HJ, Yoon TK, Choi DH, Koo JJ, Ko JJ. Viability of human follicular oocytes collected from unstimulated ovaries and matured and fertilized in vitro. Reprod Fertil Dev 1992;4:695–701.
33. Cha KY. In vitro fertilization using immature follicular oocytes harvested from ovarian tissue. In: Behrman SJ, Patton GW, Holtz G, editors. Progress in infertility. Boston: Little, Brown; 1994:99–112.
34. Hwang JL, Lin YH, Tsai YL. Pregnancy after immature oocyte donation and intracytoplasmic sperm injection. Fertil Steril 1997;68:1139–40.
35. Hwang JL, Lin YH, Tsai YL. In vitro maturation and fertilization of immature oocytes: a comparative study of fertilization techniques. J Assist Reprod Genet 2000;17:39–43.
36. Hwu YM, Lee RKK, Chen CP, Su JT, Chen YW, Lin SP. Development of hatching blastocysts from immature human oocytes following in-vitro maturation and fertilization using a co-culture system. Hum Reprod 1998;13:1916–21.
37. Trounson A, Wood C, Kausche A. In vitro maturation and fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. Fertil Steril 1994;62:353–62.
38. Chian RC, Lim JH, Tan SL. State of the art in in-vitro oocyte maturation. Curr Opin Obstet Gynecol 2004;16:211–9.
39. Wynn P, Picton HM, Krapez J, Rutherford AJ, Balen AH, Gosden RG. Pretreatment with follicle stimulating hormone promotes the number of human oocytes reaching metaphase II by in vitro maturation. Hum Reprod 1998;13:3132–8.
40. Mikkelsen AL, Lindenberg S. Benefit of FSH priming of women with PCOS to the in vitro maturation procedure and the outcome: a randomized prospective study. Reproduction 2001;122:587–92.
41. Lin YH, Hwang JH, Huang LW, Mu SC, Seow KM, Chung J, et al. Combination of FSH priming and hCG priming for in-vitro maturation of human oocytes. Hum Reprod 2003;18:1632–6.
42. Junk SM, Yeap D. Improved implantation and ongoing pregnancy rates after single-embryo transfer with an optimized protocol for in vitro oocyte maturation in women with polycystic ovaries and polycystic ovary syndrome. Fertil Steril 2012;98:888–92.

43. Chian RC, Buckett WM, Too LL, Tan SL. Pregnancies resulting from in vitro matured oocytes retrieved from patients with polycystic ovary syndrome after priming with human chorionic gonadotropin. Fertil Steril 1999;72:639–42.
44. Chian RC, Gulekli B, Buckett WM, Tan SL. 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.
45. Chian RC, Buckett WM, Tulandi T, Tan SL. Prospective randomized study of human chorionic gonadotrophin priming before immature oocyte retrieval from unstimulated women with polycystic ovarian syndrome. Hum Reprod 2000;15:165–70.
46. Hwang JL, Lin YH, Tsai YL, Hsieh BC, Huang LW, Huang SC, et al. Oocyte donation using immature oocytes from a normal ovulatory woman. Acta Obstet Gynecol Scand 2002;81:274–5.
47. Nagle F, Sator MO, Juza J, Huber JC. Successful pregnancy resulting from in-vitro matured oocytes retrieved at laparoscopic surgery in a patient with polycystic ovary syndrome. Hum Reprod 2002;17:373–4.
48. Son WY, Yoon SH, Lee SW, Ko Y, Yoon HG, Lim JH. Blastocyst development and pregnancies after IVF of mature oocytes retrieved from unstimulated patients with PCOS after in-vivo hCG priming. Hum Reprod 2002;17:134–6.
49. Yang SH, Son WY, Yoon SH, Ko Y, Lim JH. Correlation between in vitro maturation and expression of LH receptor in cumulus cells of the oocytes collected from PCOS patients in hCG-primed IVM cycles. Hum Reprod 2005; 20:2097–103.
50. Huang JYJ, Chian RC, Tan SL. Ovarian hyperstimulation syndrome prevention strategies: in vitro maturation. Semin Reprod Med 2010;28:519–31.
51. Lim KS, Son WY, Yoon SH, Lim JH. IVM/F-ET in stimulated cycles for the prevention of OHSS. Fertil Steril 2002;76(Suppl):S11-O-25.
52. Lim JH, Park SY, Yoon SH, Yang SH, Chian RC. Combination of natural cycle IVF with IVM as infertility treatment. In: Tan SL, Chian RC, Buckett WM, editors. In-vitro maturation of human oocytes, basic science to clinical application. London: Informa Healthcare Press; 2007:353–60.
53. Check ML, Brittingham D, Check JH, Choe JK. Pregnancy following transfer of cryopreserved-thawed embryos that had been a result of fertilization of all in vitro matured metaphase or germinal stage oocytes. Case report. Clin Exp Obst Gyn 2001;28:69–70.
54. Liu J, Lu G, Qian Y, Mao Y, Ding W. Pregnancies and births achieved from in vitro matured oocytes retrieved from poor responders undergoing stimulation in in vitro fertilization cycles. Fertil Steril 2003;80:447–9.
55. Mikkelsen AL, Smith SD, Lindenberg S. In-vitro maturation of human oocytes from regularly menstruating women may be successful without follicle stimulating hormone priming. Hum Reprod 1999;14:1847–51.
56. Mikkelsen AL, Host E, Blaabjerg J, Lindenberg S. Time interval between FSH priming and aspiration of immature human oocytes for in-vitro maturation: a prospective randomized study. Reprod Biomed Online 2003;16:416–20.
57. Suikkari AM, Tulppala M, Tuuri T, Hovatta O, Barnes FL. Luteal phase start of low-dose FSH of follicles results in an efficient recovery, maturation and fertilization of immature human oocytes. Hum Reprod 2000;15:747–51.
58. Fadini R, Del Canto MB, Mignini Renzini M, Brambillasca F, Comi R, Fumagalli D, et al. Effect of different gonadotrophin priming on IVM of oocytes from women with normal ovaries: a prospective randomized study. Reprod Biomed Online 2009;19:343–51.
59. Chian RC, Buckett WM, Abdul Jalil AK, Son WY, Sylvestre C, Rao D, 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.
60. Lim JH, Yang SH, Chian RC. New alternative to infertility treatment for women without ovarian stimulation. Reprod Biomed Online 2007;14: 547–9.
61. Lim JH, Yang SH, Xu Y, Yoon SH, Chian RC. Selection of patients for natural cycle in vitro fertilization combined with in vitro maturation of immature oocytes. Fertil Steril 2009;91:1050–5.
62. Nargund G, Waterstone J, Bland J, Philips Z, Parsons J, Campbell S. Cumulative conception and live birth rates in natural (unstimulated) IVF cycles. Hum Reprod 2001;16:259–62.
63. Paulson RJ, Sauer MV, Francis MM, Macaso T, Lobo RA. Factors affecting pregnancy success of human in-vitro fertilization in unstimulated cycles. Hum Reprod 1994;9:1571–5.
64. Thornton MH, Francis MM, Paulson RJ. Immature oocyte retrieval: lessons from unstimulated IVF cycles. Fertil Steril 1998;70:647–50.
65. Smith LC, Olivera-Angel M, Groome NP, Bhatia B, Price CA. Oocyte quality in small antral follicles in the presence or absence of a large dominant follicle in cattle. J Reprod Fertil 1996;106:193–9.
66. Chian RC, Chung JT, Downey BR, Tan SL. Maturational and developmental competence of immature oocytes retrieved from bovine ovaries at different phases of folliculogenesis. Reprod Biomed Online 2002;4:129–34.
67. Yang SH, Patrizio P, Yoon SH, Lim JH, Chian RC. Comparison of pregnancy outcomes in natural cycle IVF/M treatment with or without mature oocytes retrieved at time of egg collection. Systems Biol Reprod Med 2012;58: 154–9.
68. Buckett WM, Chian RC, Holzer H, Dean N, Usher R, Tan SL. Obstetric outcomes and congenital abnormalities after in vitro maturation, in vitro fertilization, and intracytoplasmic sperm injection. Obstet Gynecol 2007;110: 885–91.
69. Shu-Chi M, Jiann-Loung H, Yu-Hung L, Tseng-Chen S, Ming-I L, Tsu-Fuh Y. Growth and development of children conceived by in-vitro maturation of human oocytes. Early Hum Dev 2006;82:677–82.
70. Söderström-Anttila V, Salokorpi T, Pihlaja M, Serenius-Sirve S, Suikkari AM. Obstetric and perinatal outcome and preliminary results of development of children born after in vitro maturation of oocytes. Hum Reprod 2006;21: 1508–13.
71. Fadini R, Mignini Renzini M, Guarnieri T, Dal Canto M, De Ponti E, Sutcliffe A, et al. Comparison of the obstetric and perinatal outcomes of children conceived from in vitro or in vivo matured oocytes in in vitro maturation treatments with births from conventional ICSI cycles. Hum Reprod 2012;27: 3601–8.
72. Basatemur E, Suteliffe A. Health of IVM children. J Assist Reprod Genet 2011;28:489–93.
73. Tucker MJ, Wright G, Morton PC, Massey JB. Birth after cryopreservation of immature oocytes with subsequent in vitro maturation. Fertil Steril 1998;70: 578–9.
74. Chian RC, Gilbert L, Huang JYJ, Demirtas E, Holzer H, Benjamin A, et al. Live birth after vitrification of in vitro matured human oocytes. Fertil Steril 2009; 91:372–6.
75. Toth TL, Baka SG, Veeck LL, Jones HW Jr, Muasher S, Lanzendorf SE. Fertilization and in vitro development of cryopreserved human prophase I oocytes. Fertil Steril 1994;61:891–4.
76. Toth TL, Lanzendorf SE, Sandow BA, Veeck LL, Hassen WA, Hansen K, et al. Cryopreservation of human prophase I oocytes collected from unstimulated follicles. Fertil Steril 1994;61:1077–82.
77. Son WY, Park SE, Lee KA, Lee WS, Ko JJ, Yoon TK, et al. Effects of 1,2-propanediol and freezing on the in vitro developmental capacity of human immature oocytes. Fertil Steril 1996;66:996–9.
78. Cooper A, Paynter SJ, Fuller BJ, Shaw RW. Differential effects of cryopreservation on nuclear or cytoplasmic maturation in vitro in immature mouse oocytes from stimulated ovaries. Hum Reprod 1998;13:971–8.
79. Isachenko EF, Nayudu PL. Vitrification of mouse germinal vesicle oocytes: effect of treatment temperature and egg yolk on chromosomal normality and cumulus integrity. Hum Reprod 1999;14:400–9.
80. Mandelbaum J, Belaïsch-Allart J, Junca AM, Antoine JM, Plachot M, Alvarez S, et al. Cryopreservation in human assisted reproduction is now routine for embryos but remains a research procedure for oocytes. Hum Reprod 1998;13(Suppl 3):161–77.
81. Mandelbaum J, Anastasiou O, Lévy R, Guérin JF, de Larouzière V, Antoine JM. Effects of cryopreservation on the meiotic spindle of human oocytes. Eur J Obstet Gynecol Reprod Biol 2004;113(Suppl 1):S17–23.
82. Cao YX, Xing Q, Zhang ZG, Wei ZL, Zhou P, Cong L. Cryopreservation of immature and in-vitro matured human oocytes by Vitrification. Reprod Biomed Online 2009;19:369–73.
83. Cao YX, Chian RC. Fertility preservation with immature and in vitro matured oocytes. Semin Reprod Med 2009;27:456–64.
84. Borini A, Bianchi V. Cryopreservation of mature and immature oocytes. Clin Obstet Gynecol 2010;53:763–74.
85. Fasano G, Demeestere I, Englert Y. In-vitro maturation of human oocytes: before or after vitrification? J Assist Reprod Genet 2012;29:507–12.

86. Rao GD, Chian RC, Son WS, Gilbert L, Tan SL. Fertility preservation in women undergoing cancer treatment. Lancet 2004;363:1829–30.
87. Huang JYJ, Chian RC, Gilbert L, Fleiszer D, Holzer H, Dermitas E, et al. Retrieval of immature oocytes from unstimulated ovaries followed by in vitro maturation and vitrification: a novel strategy of fertility preservation for breast cancer patients. Am J Surg 2010;200:177–83.
88. Shalom-Paz E, Almog B, Shehata F, Huang J, Holzer H, Chian RC, et al. Fertility preservation for breast-cancer patients using IVM followed by oocyte or embryo vitrification. Reprod Biomed Online 2010;21:566–71.
89. Oktay K, Buyuk E, Rodriguez-Wallberg KA, Sahin G. In vitro maturation improves oocytes or embryo cryopreservation outcome in breast cancer patients undergoing ovarian stimulation for fertility preservation. Reprod Biomed Online 2010;20:634–8.
90. Demirtas E, Elizur SE, Holzer H, Gidoni Y, Son WY, Chian RC, et al. Immature oocytes retrieval in the luteal phase to preserve fertility in cancer patients. Reprod Biomed Online 2008;17:520–3.
91. Oktay K, Demirtas E, Son WY, Lostritto K, Chian RC, Tan SL. In vitro maturation of germinal vesicle oocytes recovered after premature luteinizing hormone surge: description of a novel approach to fertility preservation. Fertil Steril 2008;89:228.e19–22.
92. Maman E, Meirow D, Brengauz M, Raanani H, Dor J, Hourvitz A. Luteal phase oocytes retrieval and in vitro maturation is an optional procedure for urgent fertility preservation. Fertil Steril 2011;95:64–7.
93. Moffa F, Biacchiardi CP, Fagioli F, Biasin E, Revelli A, Massobrio M, et al. Ovarian tissue cryostorage and grafting: an option to preserve fertility in pediatric patients with malignancies. Pediatr Hematol Oncol 2007;24:29–44.
94. Huang JY, Tulandi T, Holzer H, Tan SL, Chian RC. Combining ovarian tissue cryobanking with retrieval of immature oocytes followed by in vitro maturation and vitrification: an additional strategy of fertility preservation. Fertil Steril 2008;89:567–72.
95. Huang JY, Tulandi T, Holzer H, Lau NM, Macdonald S, Tan SL, et al. Cryopreservation of ovarian tissue and in vitro matured oocytes in a female with mosaic Turner syndrome: Case Report. Hum Reprod 2008;23:336–9.
96. Lau NM, Huang JYJ, MacDonald S, Elizur SE, Gidoni Y, Holzer H, et al. Feasibility of fertility preservation in young females with Turner syndrome. Reprod BioMed Online 2009;18:290–5.