Chapter 6
The Role of In Vitro Maturation in Fertility Preservation

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Introduction

In vitro maturation (IVM) is an advanced embryology laboratory technique whereby immature oocytes, which are removed from ovarian follicles prior to completing their growth in vivo, are cultured from the germinal vesicle (GV) to the metaphase II (MII) stage. Upon reaching maturity in vitro, these eggs can then be fertilized and the resulting embryos can be cultured using conventional in vitro fertilization (IVF) techniques and transferred or cryopreserved.

Except in the most experienced hands, IVM is a labor-intensive, experimental technology requiring meticulous laboratory skills in order to maximize embryonic potential. For that reason, its current place among other assisted reproductive technologies is controversial. Nevertheless, IVM has several unique characteristics that make it an attractive option for fertility preservation. Most notably, IVM allows for extreme flexibility in treatment timing and does not require hormonal stimulation prior to oocyte retrieval from the ovary. This facilitates rapid fertility preservation interventions in the face of impending cancer treatment. It also allows women with estrogen-sensitive cancers to avoid supraphysiologic elevations in serum estradiol levels with ovarian stimulation (Table 6.1).

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Table 6.1 Comparison of IVM to IVF in the setting of fertility preservation

	IVM	IVF
Minimum time to oocyte aspiration	1–2 days	10–14 days
Injectable medications	Single injection	Multiple injections over 10–14 days
Typical number of office visits, ultrasounds, and blood draws	1–2	4–6
Supraphysiologic estradiol levels	No	Yes
Oocyte retrieval at any time in cycle	Yes	No
May be performed in conjunction with other fertility preservation techniques	Yes	No

IVM in vitro maturation, *IVF* in vitro fertilization

The concepts advanced in this chapter represent the current thinking of the faculty in the Division of Reproductive Endocrinology and Infertility at the University of Louisville. Studies generated in our laboratory among our patients form the basis for our treatment protocols utilizing the IVM procedure for fertility preservation.

Historical Background of IVM

The physiologic concept of IVM was first recognized in the 1930s by Dr. Gregory Pincus when, upon removing immature oocytes from rabbit ovarian follicles, he documented their spontaneous resumption of meiosis to the M2 stage [1]. Following the first successful clinical application of IVF in 1978, Cha and colleagues reported the first live birth from IVM a decade later [2]. This seminal case, describing retrieval of immature oocytes from a peripartum ovary, immediately highlighted one of the most attractive clinical features of IVM: assisted reproduction that can be performed completely independent of gonadotropin stimulation or cycle timing.

Initial attempts to create widespread clinical application of IVM were limited by sporadic successes and inefficient pregnancy rates. In 1999, Chian and colleagues introduced the concept of ovarian "priming" to clinical IVM whereby a single injection of human chorionic gonadotropin (hCG) administered 36 h prior to oocyte retrieval appeared to be beneficial to oocyte maturation and fertilization rates [3]. To determine if the efficacy of IVM could be improved with addition of gonadotropins, Fadini and colleagues [4] prospectively randomized 400 women into four equal groups: group A, no stimulation; group B, 10,000 IU hCG intramuscular injection 36–38 h prior to retrieval; group C, follicle-stimulating hormone (FSH) stimulation; or group D, FSH stimulation followed by an hCG injection prior to retrieval (Table 6.2). The four groups represented non-primed, hCG-primed, FSH-primed, and FSH-plus hCG-primed cycles, respectively. The FSH-primed cycles consisted of FSH 150 IU/day for 3 days starting on cycle day 3. Enrolled women were 24–38 years of age, with ovulatory cycles 24–35 days in length and early follicular-phase FSH levels less than 12 IU/L. Follicular growth was monitored by transvaginal ultrasound until a lead follicle was no more than 13 mm in diameter

Table 6.2 Effect of different gonadotropin priming protocols on IVM of oocytes from women with normal ovaries

	Group A	Group B	Group C	Group D	
	No priming	hCG-primed	FSH-primed	FSH-+hCG-primed	Overall
In vivo-matured MII oocytes (%)	0	28 (5.7)	0	109 (20.3)	137 (6.9)
In vitro-matured MII oocytes after 30 h (%)	231/477 (48.4)	284/470 (57.9)	234/461 (50.8)	431/525 (82.1)	1180/1933 (61.0)
Clinical pregnancy rate per embryo transfer (%)	15.3	7.6	17.3	29.9	18.3

IVM in vitro maturation, *hCG* human chorionic gonadotropin, *FSH* follicle-stimulating hormone, *MII* meiosis II
Composite table from Fadini et al. [4]

and the endometrial thickness was greater than 4 mm. Oocyte retrieval was scheduled to occur within 24 h in the non-primed group. In the FSH-primed group, oocyte retrieval was performed after coasting for 36–72 h. In women primed with FSH and hCG, the hCG injection was administered 24–48 h after the last FSH injection and oocyte retrieval was performed 36–38 h later. Only women who received hCG priming had mature MII oocytes at the time of retrieval. The clinical pregnancy rate (CPR) was 29.9% in the women who were enrolled in the FSH- and hCG-primed groups, and this was significantly higher than the CPR of 15.3% in women who received no priming ($P=0.023$). FSH- and hCG-primed cycles demonstrated no significant effects on clinical outcome.

While these efforts to improve the IVM priming protocol continue, techniques in the embryology laboratory have evolved [5]. Yet despite advances in both oocyte stimulation and embryology laboratory procedures, IVM implantation rates do not exceed those of traditional IVF and, for this reason, IVM remains a second-line treatment choice.

Patient Selection

Women who are facing potentially sterilizing cancer therapy and are interested in pursuing fertility preservation are met with a variety of obstacles. At the forefront of the medical decision-making process are two questions: (1) Is exposure to exogenous gonadotropins considered safe? (2) Is it safe to delay cancer treatments in order to undergo traditional ovarian stimulation? Because traditional ovarian stimulation with oocyte or embryo cryopreservation has been associated with higher success rates, it is generally recommended that patients complete traditional stimulation as long as it is deemed safe. However, in patients for whom traditional stimulation is not considered safe, IVM is an alternative.

Although there is a paucity of data on the long-term outcomes of women with estrogen-sensitive cancers who are exposed to controlled ovarian hyperstimulation (COH), there is concern that exposure to supraphysiologic levels of estradiol prior to cancer therapy may stimulate tumor growth [6]. Because IVM does not markedly increase serum estradiol levels, it may be the preferred fertility preservation intervention for patients with estrogen-sensitive cancers. Certainly, a case can be made for IVM in the presence of estrogen receptor (ER)-positive breast cancers, but the same rationale may also be applied to breast cancer patients with unknown ER status, a scenario that may not be that uncommon. Other malignancies with general contraindications to COH, such as early uterine and some ovarian cancers, should also be considered as part of this group.

One of the most valuable and unique characteristics of IVM is its ability to be performed in a very short time period. Whereas conventional IVF requires a minimum of 10–14 days to harvest eggs, oocyte retrieval for IVM can theoretically be completed in a matter of 1–2 days. Rao and colleagues reported an ovum pick-up of 17 immature oocytes on cycle day 12, 2 days after the initial patient encounter for fertility preservation [7]. As consultation for fertility preservation is frequently

initiated under time-sensitive circumstances, this core feature of IVM makes it worthy of consideration in many clinical scenarios.

Proponents of IVM also highlight its advantage in combating a common and rarely life-threatening side effect of exogenous gonadotropin use: ovarian hyperstimulation syndrome (OHSS). Even in the face of novel strategies to reduce the incidence of OHSS, we believe that even mild or moderate hyperstimulation may have a significant impact in the cancer patient population and every effort to safeguard these at-risk patients should be made [8]. Other potential applications of IVM include benign disease in which women are undergoing fertility threatening surgeries, medical diseases that require gonadotoxic therapies, or elective indications in which women are delaying childbearing [9, 10, 11].

Timing of the IVM Procedure

One of the key advantages of IVM over IVF in the setting of fertility preservation is the ability of IVM to be performed at any time point in the menstrual cycle. This feature allows for a rapid intervention in the face of imminent chemotherapy. Oktay et al. highlighted the utility of IVM in their case report describing a breast cancer patient who was unable to complete conventional ovarian stimulation and embryo cryopreservation because of a premature luteinizing hormone (LH) surge [12]. Despite subsequent ovulation of mature follicles, the investigators were able to harvest immature oocytes in the luteal phase, which were then successfully matured and fertilized in vitro.

Critics of luteal-phase aspirations have contended that the postovulatory hormonal milieu tends to minimize oocyte potential. However, a recent retrospective study comparing follicular-phase to luteal-phase aspirations for IVM could not identify any differences in the number of retrieved oocytes, maturation rates, fertilization rates, or the total number of oocytes and embryos that were cryopreserved [13].

Another fertility preservation strategy that leverages the timing flexibility of IVM is the use of sequential oocyte retrievals. In a case series report, Demirtas et al. described two patients who underwent sequential ovarian aspirations with time intervals between procedures as short as 16 days [14]. This approach can maximize oocyte yield within a limited time frame.

Oocyte Harvesting Methods

Transvaginal Oocyte Aspiration

From the operator's vantage, transvaginal oocyte aspiration (TVOA) of immature follicles can be challenging, primarily due to the hypermobility of the ovary and the density of the ovarian tissue. In contrast to the enlarged, relatively fixed, and stable

ovaries in the stimulated patient, unstimulated ovaries are small with a thick capsule and readily move away from the aspiration needle tip upon attempted penetration. Once the needle tip is inside the ovarian capsule, further manipulation is hindered by the dense ovarian stroma and thickness of the unexpanded follicular wall. Frequently, more forceful needle advancements are required, raising the risk for damage to pelvic vasculature and viscera adjacent to the ovary.

As in any surgical procedure, the individual operator's skills develop with time and each settles on his or her preferred techniques and instruments to overcome these difficulties. Based on initial experience with transvaginal aspiration of immature oocytes, several modifications to conventional aspiration techniques have been proposed to facilitate the procedure, including changes in needle gauge, bevel size, aspiration pressure, and the use of a single- versus double-lumen needle [15]. As experience with IVM grows, this list of adjustments has inevitably expanded; unfortunately, this has also led to a lack of a general consensus on the superior approach.

Our group has found it advantageous to employ follicular flushing in the setting of immature oocyte retrieval [16]. This approach is particularly useful in cycles that do not use hCG priming, in which the unexpanded cumulus cells make it very difficult to remove the cumulus-oocyte complex from the follicular wall. We utilize a double-lumen needle (Ref 97109, 1.6×350 mm, tubing 600/600 mm; Swemed V-tip™ by Vitrolife Sweden AB, Kungsbacka, Sweden) and repeat flushing until there is positive identification of an oocyte or the absence of granulosa cells from the aspirates. Our technique also diminishes the number of vaginal wall puncture sites, thus minimizing intraoperative vaginal bleeding.

Whole or Partial Ovarian Tissue Samples

While IVM treatment protocols for the general infertility population almost uniformly involve ultrasound-guided transvaginal aspiration of oocytes from in situ ovaries, we have reported on the isolation of immature oocytes from surgically removed whole and partial ovary specimens [17]. No live births have been reported from this technique. However, in the setting of ovarian pathology of unknown malignancy potential, where preoperative ovarian stimulation is contraindicated or a simple cystectomy is not surgically feasible, IVM offers a valuable "salvage" intervention. Viable ovarian tissue, identified and isolated in the operating room immediately upon surgical removal of the diseased ovary, can be transferred to the embryology laboratory for immature oocyte extraction, maturation, and either cryopreservation or fertilization.

IVM can also be pursued in combination with other fertility-preserving strategies. For patients undergoing oophorectomy for ovarian tissue cryopreservation, we routinely employ the use of extracorporeal immature egg retrieval for IVM just prior to tissue processing. This two-prong therapeutic approach may provide a

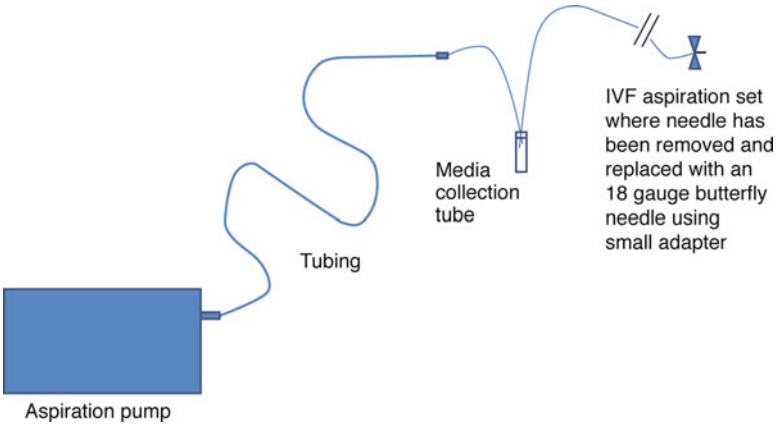

Fig. 6.1 Diagram of the aspiration apparatus for aspiration of immature oocytes includes an aspiration pump, media collection tube, and IVF aspiration set with a butterfly needle

more immediate opportunity for successful conception as some post-thaw tissue strategies, such as in vitro follicular maturation (which attempts to mature entire preantral follicles that have been extracted from frozen-thawed ovarian tissue), are not currently ready for clinical application.

Extraction of immature oocytes from surgically removed whole or partial ovaries can be performed in different ways. Perhaps the simplest technique uses an 18-gauge needle attached to a 5-ml syringe. Before starting the extraction, the syringe is partially filled with modified human tubal fluid (mHTF) media. Using the syringe and needle, all visible follicles are punctured and gently aspirated. With media preloaded into the syringe, any aspirated fluid or tissue is then flushed out into a dish for examination.

We have also used a more complex aspiration setup in which the needle from an IVF needle set is clipped off and, using a small adapter, is spliced onto an 18-gauge Terumo butterfly infusion set needle (Terumo Medical Corporation, Elkton, MD 21921). This modified aspiration needle can then be attached to a standard IVF vacuum pump and operated as usual. A simple diagram of this setup and the use of the aspiration needle are shown in Figs. 6.1 and 6.2, respectively. The butterfly is easy to handle and work with under a dissection scope. The aspiration pump should be set to 60–65 mmHg and can provide consistent, controlled vacuum pressure for removing follicular fluid. A dish of media can be kept close at hand to flush out the needle and line as needed. We have reported a case series using this modified aspiration needle for the extraction of immature oocytes [18].

Regardless of which setup is used, it is easiest to identify any small follicles by first butterflying the ovary in a clean Petri dish and laying it over the top of a light source, such as a dissection scope. The light shining through the tissue helps illuminate the smaller, less obvious follicles.

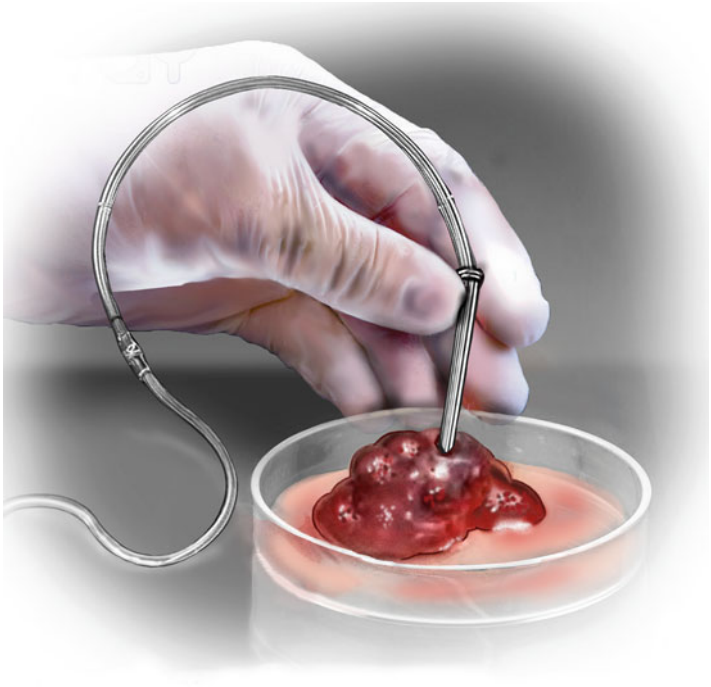

Fig. 6.2 Use of the aspiration needle for the extraction of immature oocytes

Laboratory Techniques

As in IVF, a large part of a successful IVM program is attributed to the meticulousness of the lab technique. The first hurdle is immature oocyte identification. Tightly surrounded by unexpanded cumulus cells, prophase I stage oocytes are not as easy to identify as mature eggs. Even to experienced eyes, efficient identification of immature oocytes takes considerable practice. After initial scanning of aspirates is complete, use of a filtration device may aid in secondary oocyte recovery (BD Falcon, 70-μm Cell Strainer nylon mesh; catalog number 352350, www.bd.com/labware).

Special skill at identifying egg nuclear status with unexpanded cumulus cells is also required. In some cycles, one or more M2 oocytes will be identified at egg retrieval; these oocytes may be fertilized on the same day. It is also important to note eggs at the M1 stage, as the timing of intracytoplasmic sperm injection (ICSI; the preferred fertilization method in IVM) after completion of meiosis may be important [19]. Elucidation of meiotic status can be aided by the use of the "slide technique" whereby tilting of the Petri dish can transiently remove overlying aspirates, flatten the egg, and better reveal its nuclear stage.

Immature oocytes are customarily cultured for up to 48 h while monitoring for the completion of meiosis. This portion of the IVM cycle can be very labor intensive, as maturation can occur at any time. Traditionally, eggs are examined at intervals of 24 and 48 h after retrieval and the time to maturation is known to correlate with oocyte potential [20]. Cycles that yield M2 oocytes at multiple time points require multiple rounds of ICSI, making these cases very protracted for the embryologist.

Optimal IVM culture media has yet to be defined. At the present time, there are three commercially available media sets for IVM (Cook, Medicult, Sage-Cooper). These media have been designed specifically for the culture and nutritional support of the somatic cells and oocyte in immature cumulus-oocyte complexes and contain some elements not found in standard embryo culture media. One of the key differences is the addition of the gonadotropins, FSH, and LH.

Cryopreservation of Embryos and Oocytes from IVM Cycles

Following improvements in fresh cycle IVM pregnancy rates in the late 1990s, Chian et al. reported the first live birth after cryopreservation of zygote-stage embryos derived from in vitro-matured oocytes [21]. This was quickly followed by the first report of a pregnancy resulting from vitrified blastocysts produced from immature eggs [22]. Demonstrating the full potential of IVM embryos, Son et al. reported a live birth following the repeated freezing and thawing of vitrified blastocysts originating from in vitro-matured oocytes [23].

Unfortunately, a relatively common dilemma for the female patient seeking fertility preservation is lack of a male partner which makes the use of donor sperm the only option to pursuing embryo cryopreservation. For pediatric and adolescent cancer populations, where no real consideration of future reproductive options has been previously made, the idea of using donor sperm to preserve fertility may simply be intangible. Additionally, women are six times more likely than men to be abandoned by their partner when surviving a serious medical illness [24]. For all of the above issues, recent fertility strategies have focused on oocyte cryopreservation (described in detail in Chap. 4 of this volume).

As is the case for oocytes originating from stimulated ovaries, vitrification (rapid freezing that results in a glass-like state devoid of ice crystals) appears to be superior to slow freeze methods for cryopreservation of eggs from IVM cycles [25]. Chian et al. reported the first successful pregnancy originating from vitrified oocytes that had been matured in vitro [26]. The same group, however, also noted better egg survival and fertilization rates for in vivo-matured eggs as compared to in vitro-matured oocytes [27].

In the setting of IVM, it is possible to freeze oocytes before or after the completion of meiosis. In theory, freezing at the GV stage may reduce the risk of meiotic disjunction errors and, at least with slow freeze methods, oocyte survival rates are improved. Overall, however, slow freezing of immature eggs is inefficient [28, 29].

Using vitrification methods, post-thaw maturation rates and high-quality embryo development are superior among eggs matured prior to freezing as compared to those frozen at the prophase I stage [25].

Embryo Transfer After Banking

A contributing factor to the suboptimal pregnancy rates seen with fresh IVM cycles (see discussion in next section) may be the lack of synchronization between the ovary and the endometrium. As immature oocytes are retrieved during the mid-follicular phase, the endometrial lining may be underprepared to receive an embryo. Controversy surrounds how best to overcome this dilemma; proposed solutions include the use of supplemental micronized estradiol or limited amounts of exogenous gonadotropins [30, 31]. In the setting of fertility preservation, IVM follows a "freeze all" protocol and no immediate synchronization with the endometrium is needed. As experience with IVM for fertility preservation grows, it will be of interest to determine whether pregnancy rates improve by bypassing the endometrial synchronization factor.

IVM Pregnancy Outcomes

Currently, there are no large-scale published data on outcomes of cryopreserved embryos or eggs derived from IVM, specifically in a fertility preservation population. Given the variability among patients, differences in stimulation protocols, and diversity of embryology laboratories and individual embryologists, reported pregnancy and delivery rates vary widely for IVM procedures. For women with regular menstrual cycles, the recent report by Fadini et al. [4] demonstrates a CPR of 29.9% in women stimulated with FSH and followed by 10,000 IU hCG when the lead follicle was no more than 13 mm (Table 6.2). This prospective randomized trial demonstrated the superiority of FSH- and hCG-primed cycles over non-primed, FSH-only primed, and hCG-only primed cycles. In women with irregular menstrual cycles and polycystic ovaries, the data are more variable [5]. In one of the largest, most recent studies, Benkhalifa et al. [32] reported a CPR of 19.7% per embryo transfer in PCOS patients who underwent hCG priming.

Available data on the health of pregnancies and offspring conceived from IVM are generally reassuring, however. Buckett et al. examined obstetrical outcomes and congenital abnormalities among IVM, IVF, and ICSI pregnancies and could not identify any additional risks to IVM beyond those associated with more conventional assisted reproduction techniques [33]. In a follow-up paper, the same group found a significantly higher incidence of spontaneous early loss among IVM pregnancies [34]. The investigators suggested that this finding may have been secondary to the high prevalence of polycystic ovary syndrome (PCOS) diagnosis among patients undergoing IVM. In support of this theory, Cha et al. showed similar abortion rates,

birth weights, and obstetrical complications for PCOS patients undergoing IVM or IVF [35].

Chian et al. compared obstetrical outcomes between pregnancies conceived from in vitro- and in vivo-matured eggs after vitrification and found higher mean birth weights in the IVM group, again attributed to a higher proportion of PCOS patients [27]. As more data on non-PCOS women undergoing IVM treatment accumulates, improved counseling in the setting of fertility preservation will be possible.

Compared with children from unassisted pregnancies, children conceived by IVM appear to have similar growth and development up to 2 years of life [36, 37].

Future Directions

While changes to ovarian priming protocols, aspiration needles, and laboratory techniques will undoubtedly continue to improve pregnancy rates with IVM, there is most likely a limit to the live birth rate that can be achieved from oocytes removed from the ovary before they have completed their natural maturation process. A related fertility preservation strategy, termed in vitro follicular maturation, attempts to mature entire preantral follicles that have been extracted from frozen-thawed ovarian tissue. While live births have been reported in rodents, this technique remains in the early developmental stages for use in humans [38].

Conclusions

The unique characteristics of IVM place it in a special role in the setting of fertility preservation. Key advantages include limited or no hormonal stimulation, rapidity with which intervention can be completed, and flexibility in cycle timing. Meticulous lab technique and advanced surgical skills are necessary for a successful program. While pregnancy rates remain inferior to those of emergency IVF, IVM should occupy a position in the fertility preservation treatment armamentarium and be made available in a variety of clinical scenarios as both a complementary and, for certain patients, the primary intervention.

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