The Effect of In Vitro Maturation (IVM) Protocol Changes on Measures of Oocyte/Embryo Competence

Bruce I. Rose 1,2,* and Kevin Nguyen 2

1 Department of Obstetrics and Gynecology, University of Central Florida, Orlando, FL 32816, USA
2 Brown Fertility, 8149 Point Meadows Way, Jacksonville, FL 32256, USA
* Correspondence: brucerose50@gmail.com; Tel.: +1-610-653-7979

Abstract: Purpose: In vitro maturation (IVM) continues its evolution as new ideas are introduced with the objective of making the IVM procedure easier and more effective. This study combines ideas believed likely to improve the IVM outcome or make the IVM oocyte identification process easier. Methods: A cohort of 45 women underwent an IVM cycle in which letrozole was used with the theoretical objective of improving the competence of small antral follicles, the oocyte aspiration technique was modified to minimize the time between oocyte aspiration and oocyte identification, and blastocysts were transferred during a subsequent cycle with controlled endometrial development. Results: Measures of oocyte competence used for these prospectively followed cycles were as follows: the maturation rate was 90.5%, the fertilization rate was 92.4%, the cleavage rate was 94.6%, the usable blastulation rate per zygote was 50.2%, and the implantation rate was 34.2%. Per transfer, the biochemical pregnancy rate was 63.2%, the clinical pregnancy rate was 55.3% and the ongoing/delivered pregnancy rate at the end of the first trimester was 47.4%. The miscarriage rate for clinical pregnancies in the first trimester was 14.3% and the ongoing twinning rate was 11.1%.

Keywords: in vitro maturation; IVM; oocyte competence; cytoplasmic competence; letrozole; Steiner-Tan needle; IVF

1. Introduction

The application of in vitro maturation (IVM) of human oocytes to in vitro fertilization and embryo transfer (IVF) has been successful in producing offspring for more than 30 years. Yet, compared to IVF, IVM is not commonly utilized [1–3]. Even for the subset of patients for which IVM has been most successful, women with high antral follicle counts, IVM is only used in rare patients. Initially, there was much enthusiasm about a procedure that did not use high doses of gonadotropins, eliminated the risk of uncontrolled ovarian hyperstimulation, and was otherwise gentler for patients than conventional IVF [4–6]. Some programs which have tried to incorporate IVM, have expressed concern that compared to conventional IVF, IVM was more time-consuming, oocyte identification at retrieval was technically different, and the reported clinical pregnancy rates seemed to vary widely between programs, and were often low compared to IVF [1,5,7].

An adjunct preceding an IVM retrieval, called “priming”, is aimed at trying to optimize the competence of the oocytes so that they may become embryos which develop, implant, and become babies. Most commonly, these adjunctive treatments included an injection of human chorionic gonadotropin (hCG) preceding retrieval, treatment with 450 IU of follicle-stimulating hormone (FSH) split over three days, or this same FSH treatment together with an injection of hCG [8,9]. An additional method of priming, which is not commonly used, is treatment with letrozole during the time period that antral follicles are expected to be small [10]. The use of letrozole at the beginning of an IVM cycle is reasonable since normal follicle development from the preantral stage to small antral stage has been shown to be dependent on androgen availability [11,12]. The objectives were to increase androgen...
availability to small antral follicles (<6–7 mm) and augment FSH availability to larger antral follicles (>8–10 mm).

The traditional approach to IVM retrieval involves aspirating several follicles and because of the small volume of those follicles, removing the needle periodically to flush out oocytes contained within the needle collection set [6]. This approach to oocyte aspiration potentially exposes the oocytes to opportunities for prolonged cooling while in the needle or the tubing. The amount of cooling would vary with the protocol for aspiration, such as, how frequently the needle was removed for flushing, the type of needle used, and the speed of the surgeon. It is possible to rapidly flush the oocyte out of the needle and into a collection tube and, theoretically, decrease the potential for oocyte cooling in the needle or collecting tubing. With good needle placement, most antral follicles can be used as a receptacle for flush media. With repeated filling and emptying, enough fluid can be moved through the needle to ensure that fluid in the needle and collecting system tubing, potentially containing oocytes, was moved into the collection tube, which can then be passed to the laboratory [13].

The IVM literature reports a wide range of miscarriage rates after pregnancy using IVM with about half of early programs, which published a miscarriage rate, reporting a clinical pregnancy loss rate of greater than 25% [5,7,14]. This may have been due to the widely differing uterine environments into which fresh embryos were transferred [14–16]. The literature forming the basis for endometrial preparation prior to donor embryo transfer in IVF, and subsequently, frozen embryo transfer (FET), is at odds with the uterine endocrine environments commonly created during fresh transfer IVM cycles which may have low estrogen levels and/or evidence of poor endometrial response [14–16]. IVM transfer could be made more uniform by cryopreserving embryos and transferring them back to the patient during a routine FET cycle. Such an approach has recently been used in a program with low pregnancy loss results [16], but is not uniformly used across all IVM programs [6,7].

The objective of this paper was to evaluate oocyte and embryo competence measures after employing a defined protocol that utilizes all three of the forestated modifications of more common approaches to IVM, to determine if these measures were consistent with expectations after conventional IVF. Again, these three modifications were, priming with letrozole during the small antral follicle stage, using a retrieval technique focused on minimizing the time from oocyte pickup to oocyte identification, and avoiding fresh embryo transfer by utilizing vitrified blastocysts in a routine FET cycle.

2. Materials and Methods

Patients were either self-referred or selected because of prior poor response to letrozole/clomiphene citrate and/or FSH. Patients were also required to be under age 39, have an antral follicle count greater than 25, and an anti-Müllerian hormone (AMH) level greater than 3.5 ng/mL. These criteria were chosen to aid staff in referring patients to the study and to limit the confounding impact of the increase in aneuploidy with advanced age. Participants also were required to have ovaries easily assessable by transvaginal ultrasound and have a body mass index (BMI: mass/(height)^2) under 40 (original protocol 35: changed to better recruit from a polycystic ovarian syndrome (PCO) population). All patients considering IVM were counseled that IVM should be viewed as an experimental procedure, consistent with the American Society of Reproductive Medicine (ASRM) recommendations at the time [7]. They were also informed that the procedure was to be done under an experimental protocol monitored by Baptist Health Institutional Review Board (IRB Number 18-49). This protocol was also enrolled in www.clinicaltrials.com, trial number NCT4149496, (accessed on 1 March 2023). The proposal submitted to the IRB included the use of the Steiner-Tan pseudo double lumen needle, which had not been Food and Drug Administration (FDA) approved for use in the United States. A consultation with the FDA confirmed that the needle could be used under an IRB protocol with appropriate labeling and as long as the use had no commercial component (under 21 CFR 812 (m)).
Patients were treated with letrozole 2.5 mg (generic) for five to seven days at the time follicle were expected to be maximally responsive to androgens [12]. FSH (Gonal-F, Serono, Merck, Darmstadt, Germany) 25 to 75 IU was added for three to five days overlapping the use of letrozole by three days with the objective of providing FSH to the larger antral follicles. Thirty-eight hours prior to the planned retrieval, hCG (Ovidrel 250 mcg, Serono, Merck, Darmstadt, Germany) was given. Operating room and embryologist availability limited IVM retrievals to a specific three days during each cycle month. The variation in medication administration was used to try to optimize patients to fit into the limited available retrieval opportunities. Most patients received 2.5 mg of letrozole for five days and 50 IU of FSH for four days starting on the third day of letrozole. Cycles were canceled if no antral follicles with diameters greater than 5 mm were present or if the patient had follicles greater than 14 mm in diameter prior to available retrieval days. In a program with greater flexibility than ours, letrozole (2.5 mg) would be started on or before cycle day 3 and low dose FSH (25–50 IU) would be added when antral follicles were sufficiently large. Letrozole would be stopped after a 2-day overlap of FSH. FSH would be stopped and hCG given with the plan to move to retrieval with a sufficient cohort of 8–12 mm follicles (Table 1).

The protocol used.

<table>
<thead>
<tr>
<th>Cycle Day</th>
<th>1 to 3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8 to 10</th>
<th>10 to 12</th>
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<tbody>
<tr>
<td>Letrozole (2.5 mg)</td>
<td>*</td>
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<td>FSH (25–50 IU)</td>
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<td>Clinical decision for hCG</td>
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<tr>
<td>Clinical decision for oocyte retrieval</td>
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</table>

* First day of letrozole is based on the appearance of the patient’s ovaries. * Drug given or action taken.

Oocyte retrievals utilized the Steiner-Tan pseudo double lumen 17 gauge needle (IVFETFLEX, Ganz, Austria). This was a 17-gauge needle with a plastic outer sheath which carried the flush media so that it entered the needle about 6 cm from the distal end and so that flush media moved both into the distal and proximal parts of the needle. The technique used was to aspirate an antral follicle and, if needle placement was adequate, repeatedly rapidly flush the follicle with a total of four to ten ml of phosphate-buffered saline (PBS) while continuing to aspirate. The objective was to clear the needle and tubing of each potentially retrieved oocyte as quickly as possible.

Oocytes obtained after IVM were identified as rapidly as possible to minimize their exposure to a nonoptimal environment. Identification of oocytes was done by decanting collection tubes filled with follicular fluid and PBS onto a Petri dish for examination under a dissecting microscope, as done for routine IVF. Approximately four hours after collection, cumulus cells were minimally disrupted to determine the maturity of each oocyte. Immature cumulus oophorus complexes were incubated in a Nunn/Falcon 4-well dishes of IVM media (Sage, Cooper Surgical Medical Devices, Ballerup, Denmark) with 75 mIU FSH/mL and 20% complement deactivated serum, obtained from the patient, under oil in a 5% CO₂/O₂ environment at 36.5 °C. To maximize assessment of the ability of oocytes to mature in vitro, all oocytes were observed for 60 h post retrieval for maturity with checks in approximately 8 to 12 h intervals. Mature oocytes were then incubated for two hours in continuous single culture media (Irvine Scientific/FujiFilm, Santa Ana, CA, USA) with 5% synthetic serum (Cooper Surgical ART-3011) before being fertilized using intracytoplasmic sperm injection (ICSI). Zygotes were incubated in continuous single culture media with 5% synthetic serum and Irvine culture media (90164) under the same environmental conditions for a maximum of six days. Blastocysts were vitrified (Irvine freeze kit 90133-SO or 90188) after grading and stored until needed for transfer. The patients subsequently underwent routine frozen embryo transfer (FET) after blastocyst warming (Irvine thaw kit 90137-SO or 90183). The practice protocol for all FET cycles in this IVF...
program was to transfer two blastocysts if they were available and if it was acceptable to the patient. More than 90% of our patients choose to transfer two embryos.

3. Results

Forty-five IVM retrievals were performed with data recorded prospectively. Three IVM retrieval cycles were excluded from this study, because they violated the protocol. Two of these were excluded because they were IVM “rescue” cycles and used an IVF gonadotropin protocol rather than the above IVM protocol (both patients delivered singletons from these IVM rescue cycles). The last excluded cycle was performed on a 40 year-old woman, who had required IVM in the past to achieve pregnancy. Her cycle resulted in a biochemical pregnancy. Four cycles did not have a transfer. In one cycle, the husband could not produce sperm. In three cycles, no blastocysts were produced. Data from these four cycles were included in the 42 IVM cycles reported on below (except for calculations involving blastocysts and pregnancy rates).

The average age of these patients was 30 ± 4.3, the average BMI was 29 ± 7.2, and the average AMH was 10.2 ± 7.3. The average number of oocytes retrieved was 9.24 ± 4.01 with 90.5% of them becoming mature during the IVM incubation. Of the mature oocytes, 92.4% fertilized after ICSI. Of the zygotes, 94.6% cleaved and 50.2% became transferrable blastocysts. The average number of blastocysts produced per patient was 4.18 ± 2.19.

An average of 0.71 oocytes per cycle were mature on the day of retrieval. Eleven patients had at least one oocyte mature on the day of retrieval.

For the first FET cycle after the IVM cycle, the biochemical pregnancy rate per transfer was 65.8% and the clinical pregnancy (with cardiac motion) rate per transfer was 57.9% (clinical pregnancies based on having a sac on ultrasound: 60.5%). The twelve-week ongoing or delivered pregnancy rate per transfer was 47.4%. The twin live birth rate was 11.1%. The implantation rate for this series was 34.2%. No blastocysts were produced in 7.9% of these IVM retrievals. There were no cases of ovarian hyperstimulation syndrome (OHSS). Three viable clinical pregnancies were lost during the first trimester. After this first FET cycle, 29 patients had 86 cryopreserved blastocysts remaining for future use.

4. Discussion

Prima facie, the IVM protocol utilized provided acceptable clinical and ongoing pregnancy rates that were comparable to those seen in routine IVF [17]. The results were also comparable to recent high IVM results reported in recent publications [18–20]. Based on the 58% clinical pregnancy rate in this series, a randomized trial, with an 80% power to detect a 10% difference in clinical pregnancy rates with a probability less than 5% that it wrongly detects a difference when one is not present and a 20% chance that it fails to detect a difference that truly exists, would require a total of 780 subjects. Such a study would be difficult to perform in a country where advanced reproductive technology was both expensive and often self-pay.

IVM derived oocytes are suspected of lacking cytoplasmic competence compared to IVF [3,6,7,9,20]. Although competence has a complex biochemical basis [9,21–23], its endpoint is most easily seen in an oocyte’s ability to mature, fertilize, cleave, become a blastocyst, become a clinical pregnancy, and result in a live birth. The notion that the IVM process leads to a lack of oocyte competence was most strongly supported by earlier treatment series reflecting a lower pregnancy rate, a lower fertilization rate, and a lower implantation rate in patient cycles using IVM compared to patient cycles using IVF [1,2,5,7,14]. An ASRM guidance committee opinion about IVM suggested that the implantation rate was the most significant reflection of the inferiority of IVM to IVF [7]. This publication noted implantation rates of 5.5% to 21.6% in the IVM medical literature, whereas; in this study, the implantation rate was 34.2%. This paper’s findings suggested that a subset of oocytes, the oocytes that become the best blastocysts, was not compromised by the IVM process. Such oocytes were also adequately numerous to result in a pregnancy rate comparable to IVF.
Since this was not a controlled study, to better understand the results, an appropriate comparison group from our patient population was sought that had undergone IVF treatment using an approach as close to the above IVM protocol as possible. Our program cryopreserved all embryos in patients who were believed to be at high risk for OHSS based on follicle development, or who experienced OHSS symptoms prior to a day-5 transfer. Preceding the program’s adoption of routine use of an agonist trigger for high-risk patients, this was a relatively common occurrence. Such patients were also likely to have high antral follicle counts and frequently had polycystic ovarian syndrome. We identified 60 such patients who had all their embryos frozen because of OHSS concerns and who underwent IVF in the same monthly cycle groups as the IVM patients in this study. All patients in this comparison group utilized ICSI, shared the same environmental conditions, were exposed to the same IVF products (media, oil, protein) after ICSI, used the same FET protocol, and used the same approach to embryo transfer (double embryo transfer) as the IVM group. In spite of the similarities between this group and the IVM group, since the IVF group was selected based on different criteria, using it as a comparison group was a significant limitation of this study. Table 2 is a presentation of both the demographics and the outcomes of this group of IVF patients compared to those in this IVM series. Note the difference in the AMH levels of these two groups (5.7 compared to 10.2 in the IVM group). Although a woman’s AMH level correlates with her primordial follicle density [24], it is not always associated with her follicles’ sensitivity to FSH.

<table>
<thead>
<tr>
<th>Table 2. Appraisal of the IVM series with a comparable IVF group.</th>
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<tr>
<td><strong>IVM Protocol</strong> (n = 38 † or 44 ‡) (SD)</td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>BMI</td>
</tr>
<tr>
<td>AMH</td>
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<tr>
<td>Number oocytes</td>
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<tr>
<td>% Mature</td>
</tr>
<tr>
<td>% Fertilized (of mature)</td>
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<tr>
<td>% Cleaved</td>
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<tr>
<td>Number blastocysts</td>
</tr>
<tr>
<td>% Blastocysts (of fertilized)</td>
</tr>
<tr>
<td>Implantation rate</td>
</tr>
<tr>
<td>% Biochemical pregnancies (of transfers)</td>
</tr>
<tr>
<td>% Clinical pregnancies (heart beat)</td>
</tr>
<tr>
<td>% Ongoing or delivered pregnancies</td>
</tr>
</tbody>
</table>

† Number of transfers; ‡ Number of retrievals; ‡ Fisher’s exact test or two-tailed t-test with significance if p < 0.05; ‡ Includes oocytes that were mature on the day of retrieval; NS = not significant; SD = standard deviation.

To reiterate, this group of IVF patients was selected post hoc and therefore, was not a control group for this study. The rate of blastulation and the number of oocytes and blastocysts produced were significantly higher in the IVF group compared to the IVM group. Other clinical measures of oocyte competence were not a priori different.

The three major differences, between the IVM protocol used here from the more common approaches that have been used for IVM in the past, may be contributors to the increased competence compared to much of the IVM literature. The first major difference was the use of letrozole directed at small antral follicles to potentially enhance oocyte competence. Growth of preantral follicles and small antral follicles is driven by androgens. Androgens, during early antral follicle development, promote granulosa cell mitosis, in-
crease FSH receptors, increase FSH sensitivity, and decrease follicle atresia [11,12]. The use of letrozole directly addressed the issue of potential decreased competence of IVM-derived oocytes. It is only in the late antral follicle phase (9 to 10 mm), that follicles become FSH-dependent for their growth. Theoretically, the impact of androgens on granulosa cell mitosis also aided in oocyte retrieval by creating more larger antral follicles available for easier IVM aspiration [25].

The second difference was in the retrieval technique, which utilized the Steiner-Tan needle to minimize the time that the oocytes were out of their ovarian follicles and delivered to the laboratory. This needle differed from traditional IVF needles because its functional dead space was about 0.06 mL; whereas, a traditional needle (with tubing) had dead space of about 1.5 mL [26]. It differed from a traditional double-lumen IVF flushing needle, which used separate channels for flushing and aspirating, and therefore, had a dead space similar to a single-channel needle (unless copious flushing was done). A commonly used retrieval technique for IVM had been to aspirate several antral follicles into a needle (single or double lumen) before removing the needle from the patient and then rinsing the needle to provide an aspirate for the laboratory to evaluate [27]. In our hands, using that technique, oocytes might remain in the needle and tubing for up to five minutes. Aspirates could be evaluated only after using a cell culture screen to filter out clots and debris. The oocyte identification process was more time-consuming and tedious than routine oocyte identification after IVF. Crane et al., found that a retrieval to incubation interval of more than four minutes in IVF was associated with diminished fertilization [28]. With traditional IVM retrieval techniques (without copious flushing), it would be unusual to not expose some oocytes to residence in the room temperature portion of the collecting system for at least 5 min. This aspect of IVM differed from IVF, where oocytes were usually immediately aspirated into a collection tube and quickly identified by the embryologist. The fluid dilution which occurs with the use of the Steiner-Tan needle and intentional vigorous flushing also made oocyte identification with IVM more similar to oocyte identification with IVF and thus easier [29]. Note that the benefits attributed to the use of the Steiner-Tan needle may also be obtained using a standard double-lumen needle. The surgeon needs to copiously flush each follicle and remain aware of the possible location of the retrieved oocyte at all times.

The third difference in this protocol from most protocols used for IVM was the avoidance of fresh transfers. Immature oocyte retrieval occurred before a patient’s follicles were able to produce much endogenous estradiol, which potentially led to varied environments for endometrial development prior to fresh IVM transfer [15,16]. Some published IVM cycle series have reported a miscarriage rate as high as 50% [5]. Early studies evaluating methods to artificially develop the endometrial linings of patients that were optimally receptive to embryo implantation focused on the duration of adequate estrogen exposure [14,30]. In this series, the potential problems of low estrogen and short duration of estrogen were avoided by using FET. Possibly as a result, the miscarriage loss rate in the first trimester (14.3%) was similar to routine IVF.

The combined use of FSH and hCG during an IVM cycle enabled some oocytes to become metaphase II (MII) on the day of retrieval [8], which potentially confused the interpretation of the results. In the present study, the average number of oocytes, which were MII on the day of retrieval, was 0.71 per IVM cycle. There were 11 patients who had at least one MII oocyte on the day of retrieval. The blastulation rate resulting from the oocytes that were MII on the day of retrieval was 72.4% (compared to 42% for the IVM subgroup excluding these cycles). The subgroup consisting of these eleven women with at least one oocyte having early maturation had an implantation rate of 35.2% and a clinical pregnancy rate of 57.1% (compared to 34.2% and 57.9% for the full IVM group). Thus, there is little difference in the clinical outcome of patients who did or did not have a mature oocyte on the day of IVM retrieval when the entire cohort of oocytes is considered. Those patients, who produced a mature oocyte at IVM retrieval, may have benefited from their likelihood of having more excess blastocysts for use in subsequent FET cycles.
Likely the most important reason for an IVF program to have the capacity to provide IVM is to be able to treat young women diagnosed with cancer, who require a treatment for their cancer that may be toxic to their ovaries [31,32]. Unlike IVF, a plan to undertake IVM can be initiated as soon as a patient presents with this problem. IVM can be undertaken effectively at any point in a woman’s menstrual cycle [33]. To incorporate IVM into an established IVF program, a clinician needs to understand general principles that correlate to producing competent oocytes that can be used for future reproduction. This study suggests three principles that facilitate the production of competent oocytes. Namely, priming based on theoretically sound principles, an aspiration process that attempts to treat oocytes as they are treated during conventional IVF, and the future use of cryopreserved blastocysts once the cancer has effectively been treated. The use of letrozole to limit estrogen production due to exogenous FSH, has been used in women with breast cancer [34].

This objective of using adjuncts to improve oocyte competence prior to IVM based on data from basic research will be an ongoing undertaking. One promising example is the use of inositol isomers either by supplementation before a cycle, during a cycle, or added to the culture media [35–37].

Another example, which has been popularized by an international group, adds a pre-maturation laboratory step to IVM. In this approach, retrieved oocytes underwent a 24 h “pre-maturation” incubation with C-type natriuretic peptide to delay meiosis followed by maturation enhanced by incubation with amphiregulin. This protocol was found to increase oocyte competence, as reflected in a higher maturation rate, an increased percentage of good quality embryos, and a higher clinical pregnancy rate, compared to oocytes obtained using more traditional approaches to IVM [18].

5. Conclusions

The modifications of IVM technology include letrozole use for early antral follicle development, rapid clearing of the collection system to minimize the time between the oocyte leaving the ovary and its identification by the embryologist, and the use of FET to control and standardize embryo transfer for IVM derived blastocysts results in acceptable indicators of oocyte competence difficult to distinguish from IVF derived oocytes. More blastocysts were created from women with a high antral follicle count using IVF than using IVM, suggesting the full cohort of IVM-derived oocytes was less competent than the cohort of IVF-derived oocytes. However, the best IVM-derived blastocysts appeared to function as well as the best IVF-derived blastocysts. Hopefully, the ideas used in this IVM series will be combined with other new approaches to advance the evolution of IVM-technique.

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References


28. Crane, M.M.; Divine, G.W.; Blackhurst, D.W. Conclusions about the effects on fertilization of time from aspiration to incubation and blood in the aspirate depend on the use of appropriate statistical techniques. *Fertil. Steril.* 2014, 81, 1548–1553. [CrossRef]


32. Hart, R.J. Optimizing the opportunity for female fertility preservation in a limited time-frame for patients with cancer using in vitro maturation and ovarian tissue cryopreservation. *Fertil. Steril.* 2019, 111, 258–259. [CrossRef]

33. Creux, H.; Monnier, P.; Son, W.-Y.; Tulandi, T.; Bucket, W. Immature oocyte retrieval and in vitro oocyte maturation at different phases of the menstrual cycle in women with cancer who require urgent gonadotoxic treatment. *Fertil. Steril.* 2017, 107, 198–204. [CrossRef]


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