Optimizing human oocyte cryopreservation for fertility preservation patients: should we mature then freeze or freeze then mature?

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Objective: To evaluate the maturation and post-thaw survival rates of immature oocytes to determine whether in vitro maturation (IVM) should be attempted prior to or after cryopreservation.
Design: Nonrandomized observational study.
Setting: Private academic and clinical reproductive center.
Patient(s): Patients (n = 71) who donated immature unusable oocytes after vaginal oocyte retrieval (VOR) after undergoing controlled ovarian hyperstimulation using a standard GnRH antagonist protocol.
Intervention(s): Germinal vesicle (GV), metaphase I (MI), and metaphase II (MII) oocytes (n = 175) were obtained from consenting IVF patients for fresh IVM, post-thaw IVM, or control group. In the fresh IVM group, GV- and MI- stage oocytes (n = 69) were cultured for 24 hours, matured in vitro (IVM-MII), cryopreserved, thawed, and evaluated for survival. In the post-thaw IVM group, GV- and MI- stage oocytes (n = 27) were frozen on day 0, thawed, evaluated for survival, and cultured for 24-hour IVM. MII donor oocytes (n = 79) were cryopreserved and thawed as a control.
Main Outcome Measure(s): Survival postfreeze and oocyte development to the MII stage was analyzed using a χ^2 analysis.
Result(s): Fresh IVM had a significantly higher maturation rate than post-thaw IVM.
Conclusion(s): Oocyte cryopreservation is important for patients at risk of ovarian cancer, elective fertility preservation, and, potentially, for ovum donation. The superior maturation rate of GV and MI oocytes in the fresh versus post-thaw groups provides strong evidence for maturing oocytes to the MII stage before cryopreservation. (Fertil Steril® 2013;99:1356–62. ©2013 by American Society for Reproductive Medicine.)
Key Words: Elective oocyte cryopreservation, in vitro maturation, germinal vesicle, metaphase I, survival

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The goal of oocyte cryopreservation for fertility preservation is generally to freeze mature oocytes for later thawing and fertilization with IVF. Unfortunately, some centers have observed a number of oocytes retrieved from stimulated cycles at the time of vaginal oocyte retrieval (VOR) to be nonoptimal for cryopreservation owing to a delay in maturation (1–3). Rather than discard these immature oocytes, they are often frozen with the hope that upon thawing they could be coaxed along the maturation pathway. Investigations into the potential for these oocytes to be matured in vitro (IVM) have led to conflicting conclusions. We investigated

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whether to induce the maturation of germinal vesicle (GV) or metaphase I (MI) oocytes to mature metaphase II oocytes (MII) before cryopreservation or whether the embryologist should wait until thawing to induce the maturation process. An optimally timed freeze-thaw protocol would ensure the maximal utility of reproductive tissue and prospectively could influence post-thaw survival and maturation rates of immature oocytes, possibly enhance fertilization rates, and increase the probability of successful pregnancy when IVF is attempted with these thawed oocytes. Our study investigates the application of IVM and seeks to define an optimal protocol to maximize the survival, maturation, and overall viability of immature oocytes retrieved during oocyte cryopreservation cycles.

Oocyte cryopreservation can support a patient's physical, moral, and ethical choices (1). Patients who are diagnosed with cancer or other medical conditions may choose to freeze their embryos but could benefit from the potential benefits from oocyte cryopreservation (2–5). Preservation of the maximal quantity of viable reproductive tissue should ultimately optimize the probability of future reproductive success.

Successful cryopreservation and thawing of oocytes has provided challenges in reproductive medicine, and its successful implementation has lagged behind sperm and embryo cryopreservation for many years (6).

In addition, optimal acquiescence of oocyte cryopreservation with IVM has challenged many physicians and researchers in its methodology. The application of IVM has not been a typical treatment route for infertility patients, as previous ovulation induction cycles with oocyte retrieval of mature MII oocytes are far more effective (2). Although cryoinjury, such as cell degradation, fragmentation, and the sensitivity of microtubule spindles (7), is a major concern when freezing oocytes that have used IVM, proper technical methodology could enhance the opportunity for developmentally delayed oocytes to achieve reproductive success. A recent case reported by Dowling-Lacey et al. demonstrated the feasibility of recovering large numbers of immature oocytes from unstimulated polycystic ovaries, followed by maturation in vitro, exposure to a slow freeze-thaw protocol, and fertilization with intracytoplasmic sperm injection. The investigators achieved embryonic competence, implantation, and healthy term births (8). In many labs, after retrieval of immature oocytes, the standard operating procedure involves maturation in vitro to the MII stage, followed by freezing and then thawing when they are ready to be used (9, 10). Several studies have compared the slow freezing of immature oocytes before and after IVM (11–16), but results were found to be conflicting and analysis was limited by experimental design. A recently published study (17) addressed such limitations and found optimization in survival of GV oocytes when IVM was used before a slow freeze cryopreservation/thaw procedure.

Our study's analysis mirrored the most recently published experimental design but in addition instituted the use of MI oocytes in the sample set. Our study investigates whether to attempt IVM before or after oocyte cryopreservation to compare our results to recent findings. We present the outcome of an Institutional Review Board–approved study of 96 donated immature oocytes. We evaluated two alternate cryopreservation and IVM procedures. The fresh IVM group used IVM before freeze-thaw; and the post-thaw IVM group used freeze-thaw and then IVM. The post-thaw survival and maturation rates of immature oocytes in both groups were evaluated to determine whether development with IVM should be attempted before or after human oocyte cryopreservation. Our findings attempt to provide a more defined technique in the joint application of IVM and cryopreservation of oocytes and to confirm previous findings related this topic.

MATERIALS AND METHODS

Institutional Review Board approval was obtained for this nonrandomized observational study. Patients (n = 71) who donated their oocytes (mean age, 39.3 ± 6.6) underwent controlled ovarian hyperstimulation using a standard GnRH antagonist protocol. When at least two of the leading follicles reached 18 mm in diameter, final oocyte maturation was achieved by the administration of 250 g of recombinant hCG. Vaginal oocyte retrieval was performed 36 hours after administration, with the use of transvaginal ultrasound guidance and under anesthesia on an outpatient basis.

Oocyte Retrieval, Hyaluronidase, and Oocyte Segregation

Once oocytes were identified and isolated from the follicular fluid produced during VOR, they were placed into culture for approximately 3 hours, before being denuded through brief exposure to hyaluronidase. Oocytes were evaluated on this first day (day 0) for the presence of a first polar body in the periviteline space and were classified as immature (GV, MI) or mature (MII). GV and MI oocytes were placed into two separate testing groups, and MII oocytes were used for the patient's IVF cycle. GV and MI oocytes were allocated to either the fresh IVM or post-thaw IVM experimental groups. The fresh IVM group (GV-38 [56%] and MI-31 [44%]) first followed an IVM protocol for GV and MI oocytes to the MII (IVM-MII) stage and then exposed the IVM oocytes to a freeze-thaw protocol, while the post-thaw IVM group (GV-12 [44%] and MI-15 [56%]) first followed a freeze-thaw protocol and then attempted an IVM protocol of thawed GV and MI oocytes to the MII stage of development (GV-MII and MI-MII).

Maturation–IVM

The same maturation procedure was used for both the fresh IVM and the post-thaw IVM groups. Oocytes were cultured for 24 hours in 95% Quinn's Advantage Cleavage Medium supplemented with 5% human serum albumin, 100 mIU/mL hCG, and 75 mIU/mL FSH. Samples were incubated for 24 hours at 37°C in a 5.5% CO_2 atmosphere before maturation was analyzed.

Oocyte Cryopreservation

Cryopreservation of both the matured fresh IVM and the immature post-thaw IVM oocytes was performed using a modified slow freeze protocol with oocyte freeze medium

(Medi-Cult). Oocytes were briefly washed in a human tubal fluid medium and then equilibrated in a solution containing 1.5 M 1,2-propanediol (PrOH) for 10 minutes. The oocytes were placed in a 1.5-M concentration of PrOH and 0.3 M sucrose for 15 minutes. A maximum of two oocytes were placed on straws and slowly cooled at a rate of −2°C/minute to a temperature slightly below the melting point of the solution (−7°C), at which point the solution was "seeded" to avoid supercooling (33). Oocytes were then cooled to −30°C at a rate of −0.3°C/minute and finally plunged into liquid nitrogen at −197°C.

Oocyte Thawing

Both fresh IVM-MII and immature post-thaw IVM oocytes were thawed using the same protocol. Thawing was performed using oocyte thaw medium (Medi-Cult) by rapidly warming the oocytes for 30 seconds in air and then for 40 seconds in a water bath at 30°C. Sequential washes exposed oocytes to decreased levels of PrOH from 1.0 M for 5 minutes, to 0.5 M for 5 minutes, and finally to a concentration of 0.0 M PrOH for 30 minutes before the cells were returned to normal oocyte culture medium and 37°C.

Cryopreservation/Thaw Survival

All oocytes were evaluated for survival post-thaw, with the post-thaw IVM group being cultured for 24 hours and evaluated for possible maturation.

Statistical Analysis

A χ^2 analysis was performed.

RESULTS

From February to April 2009, immature oocytes were donated consecutively by consenting patients (n = 71) during each of their GnRH antagonist cycles. A total of 69 immature oocytes (GV-38 [1.9 ± 0.9 per patient] and MI-31 [1.3 ± 0.7 per patient]) from consenting patients (n = 32) were included in the fresh IVM group and were initially matured in vitro (fresh-IVM). If they matured to MII (fresh IVM-MII) within 24 hours, they were frozen and then subsequently thawed for survival analysis. Another 27 donated immature oocyte (GV-12 [4 ± 1.4 per patient] and MI-15 [5 ± 2.8 MI per patient]) from consenting patients (n = 4) were included in the post-thaw IVM group and were initially frozen as immature GV and MI oocytes. The surviving oocytes (GV [10/12] and MI [13/15]) were then placed in IVM culture for 24 hours to attempt maturation to the MII stage (GV-MII or MI-MII). A control group of mature oocytes from clinical patients, who consented to research, consisting of 79 mature MII oocytes not requiring IVM were also evaluated using the same freeze-thaw protocol.

The post-thaw survival percentages of GV, MI, fresh IVM-MII, and control MII oocytes did not differ significantly (83.3% [n = 9], 86.7% [n = 12], 83% [n = 57], and 86% [n = 68], respectively). Overall, combined survival and maturation was significantly higher ($P<.05$) in the fresh IVM group at 63.8 % (44/69) compared with the post-thaw IVM group at 33.3% (9/27; Fig. 1). Although the MI oocytes matured to MII at a higher rate than the GV oocytes, in both the fresh and post-thaw IVM groups, a 50% decrease in maturation potential was shown for all oocytes post-thaw (Fig. 2).

DISCUSSION

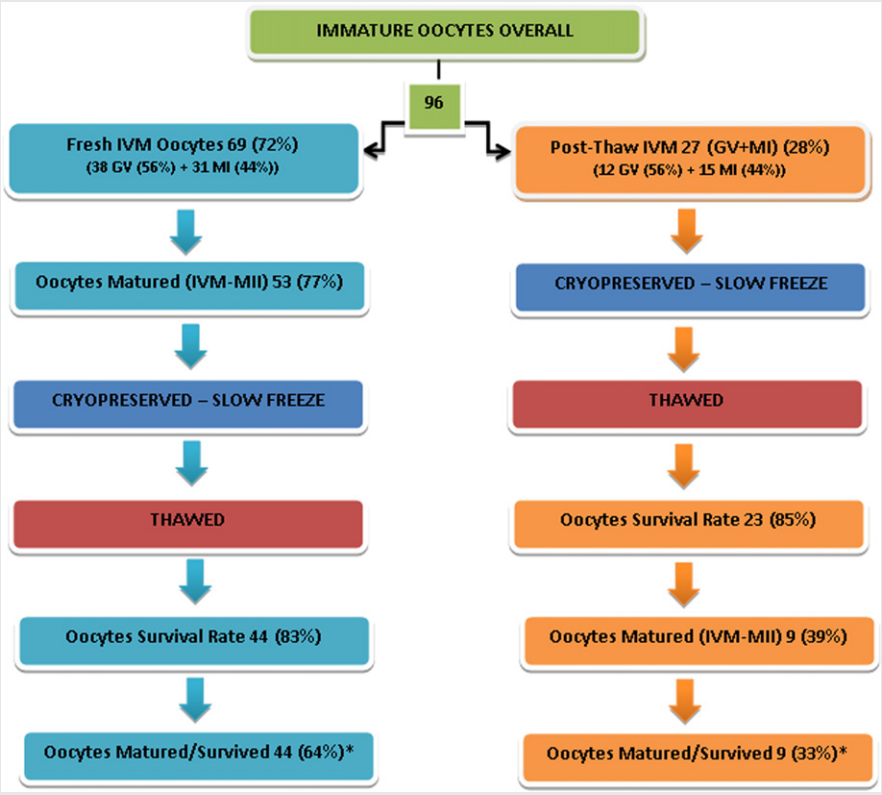
Although IVM has garnered conflicting views in practice within reproductive medicine, it has been shown to facilitate salvage of the reproductive viability of immature oocytes (1). Conservation of retrieved immature oocytes after VOR has become a major concern for patients as they strive to maximize the reproductive viability of all oocytes obtained during treatment. Optimal methodology conformity of oocyte IVM and cryopreservation procedures has challenged many in reproductive medicine.

Our study's findings support a protocol that initially matures oocytes to the MII stage in vitro and then exposes them to a freeze-thaw procedure. The survival rates did not differ significantly among the post-thaw survival of GV, MI, fresh IVM-MII, and control MII groups (83.3% [n = 9], 86.7% [n = 12], 83% [n = 57], and 86% [n = 68], respectively). The overall combined survival and maturation rates between the groups (fresh IVM group at 64% [n = 22], post-thaw at 33% [n = 8]) and the maturation rates of the different maturities (GV to MII fresh-50% [n = 19] vs. post-thaw-25% [n = 3]; and MI to MII Fresh-81% [n = 25] vs. post-thaw-40% [n = 6]) were all superior in maturation percentage potential by nearly double (Fig. 2). A significant increase ($P<.05$) was observed in the overall maturation rate of the groups. We observe that our results may be potentially considered biased by underlying factors owing to the uneven ratio of immature oocytes between the testing groups, although we propose our experiment's results to be warranted due to its relation to similar studies. Our data support instituting a fresh IVM protocol for immature GV and MI oocytes, where IVM is initially performed and thereafter mature oocytes are exposed to a freeze-thaw protocol.

The joint practice of IVM and cryopreservation has raised logistical questions in reproductive medicine, and establishing a mutually beneficial protocol where both techniques nurture an optimized environment for survival, development, and potential reproductive viability is essential for successful treatment. Oocyte cryopreservation has increasingly been more efficiently applied in the field of assisted reproductive technology (18–24) and now allows flexibility for the patient and assisted reproduction programs during treatment cycles (5). Continued developments in the IVM, cryopreservation, and thaw techniques have also improved and increased patient treatment options.

A number of studies suggested freezing IVM oocytes at the MII stage to avoid development limitations (8, 9, 15, 17, 25–28), although conflicting results were ascribed to variability in experimental design. Studies conducted by Cao et al., using vitrification, and another more recent comprehensive publication by Wang et al., using slow freeze, evaluated the best time to use IVM and cryopreservation in relation to survival rates (15, 17). In the

FIGURE 1



Experimental design flow diagram. *$P<.05$.

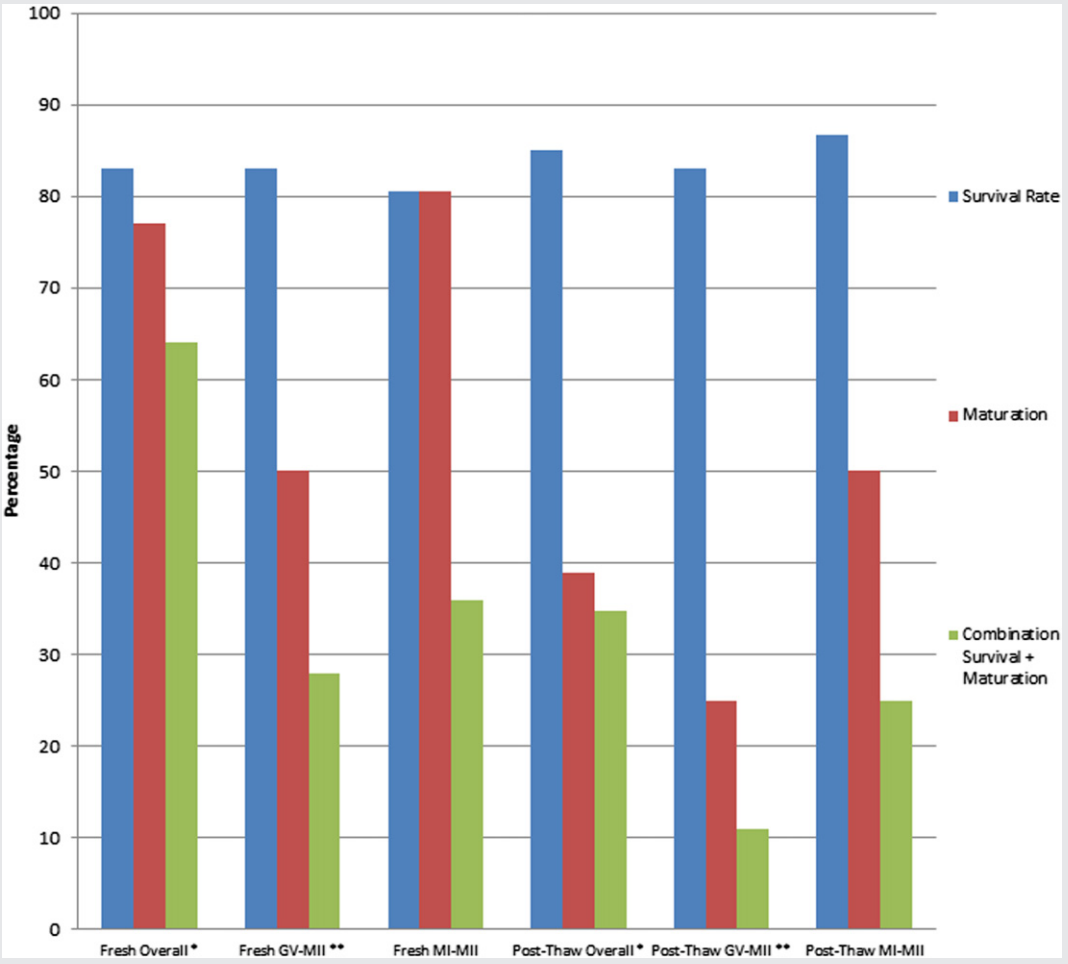
Lee. Late maturing oocyte cryopreservation. Fertil Steril 2013.

same vein as our study, the survival rate was analogous to the work conducted by both groups. Notably, the percentages of survival of Cao et al.'s group freeze samples (GV and fresh IVM, 85.4% and 86.1%, respectively) and those of Wang's group (GV, fresh IVM MII, and control MII, 69.7%, 70.5%, and 70%, respectively) (15, 17) were similar to our percentages of survival for freeze samples (GV, fresh IVM MII, and control MII; 83.3%, 83%, and 86%, respectively). The difference between our study and the studies of Cao et al. and Wang et al. was the use of an MI sample set; survival with this freeze group was (86.7%). Maturation to the MII stage, termed polar body extrusion, in the comparative studies was found to be lower in the post-thaw GV group versus in the GV-MII fresh group (Cao et al.: 50.8% vs. 85.4%; Wang et al.: 51.3% vs. 75.7%) (15, 17). Although our post-thaw GV and GV to MII fresh had lower maturation rates (25% vs. 50%), potentially attributable to a smaller sample size, we support the same conclusions that Cao's and Wang's groups came to in using IVM and cryopreservation of GV oocytes. In addition to our study and not previously reported, post-thaw MI and MI to MII fresh maturation (40% vs. 81%) showed a similar trend in using an IVM protocol first. Our study serves as an additional study that provides corroboration for defining the best time to contingently expose immature oocytes to IVM in combination with cryopreservation.

We speculate that the different oocyte maturation rates among the post-thaw IVM Group compared with the fresh IVM group may be related to the cryopreservation process. Exposure to a number of mechanical, thermal, and chemical disturbances during cryopreservation (29) at any stage of development leaves oocytes potentially susceptible to developmental dysfunction (30). Such deterrents could subsequently curtail cellular function, incite aneuploidy, decrease reproductive viability, and cause cellular death (31). Studies in bovine models have shown that oocytes retrieved in later phases of follicular development have more abundant levels of messenger RNA (mRNA) transcripts compared with oocytes from less developed follicles, correlating well with future embryonic developmental quality (32, 33). With this knowledge, we suspect a genetic discord, possibly cessation in mRNA signaling and downstream protein production, which may be introduced by cryopreservation of oocytes.

Cryobiologists have attempted to evaluate the effect of cryoprotectants and the cooling process on meiotic cell division. A previous study by Rienzi et al. studied analyzed MII oocytes using a slow freeze method and viewed the reappearance of spindles in surviving oocytes, concluding that all spindles observed in thawed oocytes resulted from post-thaw reconstruction (34). Wang et al. recently expanded upon this notion with an investigation of chromatin and spindle formation chromatin via the use of confocal microscopy

FIGURE 2



Immature oocyte thaw survival, maturation, and combination of survival and maturation of the fresh IVM and post-thaw IVM. All groups treated with a standard IVF gonadotropin-releasing hormone (GnRH) antagonist protocol. Combintation Survival + Maturation is based off a hypothetical percent with a sample size (n = 100). Fresh overall (n = 69): Survival (n = 57); Maturation (n = 44); Combitnation Survival + Maturation (n = 22). Fresh GV-MII (n = 38): Survival Rate (n = 32); Maturation (n = 19); Combination Survival + Maturation (n = 11). Fresh MI-MII (31): Survival Rate (n = 25); Maturation (n = 25); Combination Survival + Maturation (n = 11). Post-Thaw Overall (n = 27): Survival (n = 23); Maturation (n = 9); Combintation Survival + Maturation (n = 8). Post-Thaw GV-MII (n = 12): Survival Rate (n = 9); Maturation (n = 3); Combination Survival + Matuartion (n = l). Post-Thaw MI-MII (15): Survival (n = 12); Maturation (n = 6); Combination Survival + Maturation (n = 3). *,**$P<.05$ for maturation only; no significance observed in survival or combination percentage among all groups.

Lee. Late maturing oocyte cryopreservation. Fertil Steril 2013.

with three-dimensional imaging (17). Interestingly, the percentage of oocytes with organized spindles was significantly higher ($P<.05$) in nonfrozen oocytes compared with either GV- or MII-stage oocytes that were frozen and thawed; and when the two frozen groups were compared, no statistical difference was observed (17). This new knowledge could explain for the near 50% decrease in maturation rate in our study's oocytes after a freeze-thaw protocol. Surprisingly, Wang et al.'s analysis of length, width, and total microtubule volume of bipolar spindles of testing and control groups showed no significant difference (17), leading to the conclusion that further knowledge of specific genetic and protein markers is necessary and could clarify the events in maturation during the GV-MII or MI-MII period of oocyte development.

Wang et al. addressed the effect of IVM on meiotic cell division in their analysis of bipolar spindles in MII oocytes. They demonstrated no detectable irregularities and showed cryosurvival to be significantly lower in the testing samples (freeze group) than in control samples (control group; post-thaw GV, 0.9%; GV-MII fresh, 5.6%; and control MII, 39.5%; $P<.0001$). Other studies have found that oocytes undergoing IVM in unstimulated cycles or stimulated cycles have resulted in healthy live births (35–39). A study by Lim et al. demonstrated that IVM alone is an efficient treatment and yields acceptable pregnancy rates but considers the isolation of patient-specific conclusions per case as a necessary measure to foster the best treatment options for patients (40).

Further investigation will ultimately demonstrate how cryopreservation of GV and MI oocytes negatively affects the specific sequence of maturation events (notably the synchronicity of nuclear and cytoplasmic maturation) after thawing and IVM (17). We are currently furthering this analysis in considering the MI chromatin and microtubules arrangements to better conclude whether further timing of meiotic changeover is essential for peerless survival and maturation. Targeted freezing techniques (slow freeze or vitrification) and precise timing of cryopreservation procedures dictated by the oocyte's meiotic stage could ensure optimization and increase the reproductive probability of such oocytes. Cao et al.'s group used a vitrification protocol in their experiment and showed similar results to our study's results (15). Another study conducted by Combelles et al. compared GV oocytes for survival and maturation in either a slow freeze or vitrified group. No difference in survival was observed between the two groups (slow freeze [70.4%] vs. vitrified [67.3%]), nor was a difference shown in maturation (slow freeze [60.0%] vs. vitrified [58.8%]) (16). Further investigation by confocal analysis revealed a higher proportion of mature oocytes with a normal bipolar spindle, as compared with slow freezing, influencing this group to conclude that vitrification maybe a slightly superior protocol over slow freezing (16). Presently, our study is currently investigating whether pre- and postmaturation vitrification in our same experimental design corroborates these previous studys' results.

Some researchers have suggested the need for a study that investigates the survival, maturation, and reproductive potential of oocytes that have either been denuded or have maintained intact granulosa and cumulus cells before cryopreservation (16, 19). A few studies investigating the potential stress to membrane permeability of cryoprotective agents on denuded human and nonhuman primate oocytes and their findings suggest a need to study intact cumulus oocytes during cryopreservation (41, 42). Limited progress has been observed in this research field recommendation owing to the exclusion of cumulus-intact oocytes usage during cryopreservation procedures, an impediment that obstructs the integral experimental design for such a study (16). The potential findings of such a study could enhance optimization in oocyte maturation when implementing cryopreservation, but without a viable experimental design, we can only speculate as to whether the inclusion or exclusion of cumulus cells would support the optimal release of inhibition and achieve optimal maturation after cryopreservation. Once modifications are made to create a cryopreservation protocol to include cumulus-intact oocytes, then a study comparing it with a standard cryopreservation protocol can be conducted to select the best approach to oocyte maturation.

Our study demonstrates that immature oocytes should be matured in vitro before cryopreservation. We have incorporated our findings into standard laboratory procedure and now routinely apply fresh IVM to immature oocytes before gamete cryopreservation. Although IVM has improved, it remains inchoate, requiring continued assessment of oocyte cryopreservation techniques. Additional investigation and longitudinal follow-up of subsequent embryo genomics and neonatal outcome are necessary to educate us on how to achieve optimal reproductive potential in all patients' treatment cycles.

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