REVIEW

In-vitro maturation of germinal vesicle and metaphase I eggs prior to cryopreservation optimizes reproductive potential in patients undergoing fertility preservation

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Purpose of review
To evaluate current and previous findings related to a timely implementation of in-vitro maturation (IVM) of germinal vesicle, metaphase I and metaphase II oocytes with an optimal cryopreservation to determine whether IVM should be attempted prior to (fresh IVM) or IVM after cryopreservation (postthaw IVM). Mitochondrion, chromatin and spindle formation in both groups were interpreted from referenced studies to establish best management of all oocytes.

Recent findings
The postthaw survival of germinal vesicle, metaphase I, fresh IVM-metaphase II and control metaphase II 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 were significantly higher ($P<0.05$) in the fresh IVM group at 63.8% (44 of 69) compared with the postthaw IVM group at 33.3% (nine of 27).

Summary
Conservation of retrieved immature oocytes after vaginal oocyte retrieval has become a major concern for patients, as they strive to maximize the reproductive viability of all oocytes obtained during treatment. Oocyte cryopreservation is important for patients at risk of ovarian cancer, elective fertility preservation and potentially for ovum donation. The superior maturation rate of germinal vesicle and metaphase I oocytes in the fresh IVM vs. postthaw groups provides strong impetus to mature oocytes to the metaphase II stage prior to cryopreservation.

Keywords
elective oocyte cryopreservation, germinal vesicle, in-vitro maturation, metaphase I and survival

INTRODUCTION

In-vitro maturation (IVM) involves extended culture of immature oocytes to allow resumption of meiotic division following transvaginal oocyte retrieval. During IVM, oocytes that failed to mature *in vivo* (either arrested at the germinal vesicle stage of prophase I, or those which have resumed meiosis but remain within the meiosis I stage) are cultured *in vitro* in an attempt to extend maturation to the metaphase II stage. In early attempts at oocyte cryopreservation, retrieved germinal vesicle and metaphase I oocytes were frozen along with the mature metaphase II oocytes and demonstrated suboptimal postthaw survival and maturation. Recent advances in oocyte maturation and freezing protocols have provided an opportunity to investigate the optimal conditions for the cryopreservation of immature oocytes in order to increase their developmental potential. In particular, we sought to address the question of whether there was a difference in oocytes quality if they were in-vitro matured before or after cryopreservation. Here, we review the current knowledge of both IVM and cryopreservation and describe an optimized joint approach.

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KEY POINTS

- Germinal vesicle and metaphase I oocytes should be matured *in vitro* prior to cryopreservation in order to optimize the reproductive potential of all retrieved oocytes.
- Derivations in mitochondrion, chromatin, spindle formation and COC discussed by referenced studies, suggest the necessity for further evaluation of events in the maturation during the germinal vesicle-metaphase II or metaphase I-metaphase II period of oocyte development.
- Additional investigation and longitudinal follow-up of membrane permeability stressors, subsequent embryo genomics and neonatal outcome are necessary to educate us on how to achieve optimal reproductive potential in all patients' treatment cycles.

IN-VITRO MATURATION: PATIENT APPLICATION

Several types of patients benefit from the use of IVM. Female cancer patients preparing to undergo gonadotoxic chemotherapy or pelvic radiation therapy, as well as those for whom ovarian stimulation is contraindicated because of hormone-sensitive tumors, may have their fertility preserved through the retrieval, maturation and cryopreservation of immature oocytes [1]. A number of clinical studies have also examined the application of IVM on immature oocytes retrieved from polycystic ovarian syndrome patients prone to ovarian hyperstimulation, and fertility patients who do not respond well to routine doses of exogenous hormones [2]. Oocytes retrieved vaginally from stimulated IVF cycles during the follicular or luteal phases may be suboptimal for immediate use because of a delay in their maturation [3–5]. Rather than discard these patients' germinal vesicle and metaphase I oocytes, IVM may maximize the yield of retrieved oocytes for immediate IVF treatment or for storage and later use via cryopreservation.

CRYOPRESERVATION: SLOW-FREEZE AND VITRIFICATION APPLICATION

The successful implementation of oocyte cryopreservation lagged behind sperm and embryo cryopreservation for many years, despite the fact that the first birth from a cryopreserved oocyte reported in 1986 [6]. The relatively large cellular volume and high water content of oocytes leads to a to a number of potential mechanical, thermal, osmotic and chemical disturbances of intracellular structures during cryopreservation, which render oocytes particularly susceptible to cell degradation, fragmentation and the disruption of the cytoskeleton [7–10]. These stressors may hinder cellular function; incite aneuploidy and apoptosis, decreasing reproductive viability [11]. Cryoprotectant agents (CPAs), such as sucrose, dimethylsufoxide, 1,2-propanediol and ethylene glycol, are used to dehydrate oocytes prior to freezing to prevent intracellular ice crystal formation, thereby minimizing cellular damage. Successful oocyte cryopreservation was first achieved via the slow-freezing technique, which employs a low concentration of CPAs in an effort to limit chemical toxicity while slowly dehydrates the oocyte without inducing osmotic shock [12–14]. Vitrification is a newer method of oocyte cryopreservation that has recently gained wide acceptance, demonstrating exceptionally high oocyte survival rates and has led to numerous successful live births [15,16]. Vitrification involves an ultrarapid cooling of the oocyte in high concentrations of CPAs, and thereby the prevention of intracellular ice crystals by the formation of a vitreous (or glass-like) ooplasm. An increasing number of recent studies suggest an increased benefit of vitrification for both immature and mature oocytes with respect to viability and developmental outcomes [16–19]. Combelles *et al.* [17] investigated the survival, maturation and cytoskeletal and chromosome organization of sibling immature oocytes that were slow-frozen, vitrified or not cryopreserved. All groups shared similar rates of survival (67–70%) and polar body extrusion (59–79%). Vitrification has been associated with a higher proportion of mature oocytes with a normal bipolar spindle, as compared with slow-freezing [17]. Nevertheless, the overall yield of oocytes with bipolar spindles is lower when compared with that of oocytes that were never frozen, thereby indicating the need for further optimization of vitrification protocols for immature oocytes.

IN-VITRO MATURATION AND CRYOPRESERVATION: TIMING OF APPLICATION TO FOSTER OPTIMAL MATURATION

The joint application of IVM and cryopreservation remains a novel treatment option for infertility patients. A case report describing the first successful human birth resulting from the slow-freezing of a germinal vesicle oocyte demonstrated the feasibility of immature oocyte freezing followed by IVM [20]. Since then, numerous studies have observed lower oocyte maturation rates in oocytes that underwent IVM after cryopreservation when compared with the fresh oocytes that are matured *in vitro*, an effect that is likely related to the cryopreservation process

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[21▪▪,22▪▪,23▪,24]. Germinal vesicle oocytes were initially hypothesized to be less vulnerable to cryoinjury compared with metaphase II oocytes, due to their intact nucleus and lack of temperature and chemical-sensitive meiotic spindle [25]. Cryopreservation of immature germinal vesicle stage oocytes was thought to minimize the risk of aneuploidy during cryopreservation due to decondensed chromosomes in the diplotene state of prophase I, which may prevent the missegregation of genetic material due to meiotic spindle depolymerization of metaphase-aligned chromosomes or chromatids [26]. In contrast to what was initially theorized, current evidence demonstrates germinal vesicle oocytes to be especially vulnerable to cryoinjury [27▪▪,28]. Human germinal vesicle oocytes have been shown to lack acetylated microtubules, which have been shown to confer increased stability [17]. Cryopreservation of germinal vesicle oocytes may interfere with major processes involving membrane-bound organelles, including redistribution of cortical granules, smooth endoplasmic reticulum and mitochondria. Although the meiotic spindle may be able to recover after freeze-thaw, damages to these cellular components in a germinal vesicle oocyte may be irreversibly detrimental.

PREVIOUS RESEARCH: IN-VITRO MATURATION THEN CRYOPRESERVATION OR CRYOPRESERVATION THEN IN-VITRO MATURATION?

Several recent studies [21▪▪,27▪▪,29–34] have performed side-by-side comparisons of freezing either at the germinal vesicle or post-IVM metaphase II stage, in an attempt to eliminate any confounding effect of differing laboratory and manipulator conditions. All studies used denuded (cumulus-free) immature oocytes retrieved from stimulated cycles, which had failed to mature *in vivo*. Baka *et al.* [29] employed conventional epifluorescence to demonstrate improved spindle and chromosome integrity with slow-freezing at the germinal vesicle stage when compared with the metaphase II stage. Boiso *et al.* [30] also concluded that it is best to slow-freeze oocytes at the germinal vesicle rather than the metaphase II stage, based on superior maturation rates, yet reported similar survival rates and comparable levels of spindle and chromosome abnormalities. Goud *et al.* [33] showed lower survival, but similar maturation and cleavage-stage development, in immature oocytes compared with oocytes that underwent slow-freeze after IVM. Versieren *et al.* [34] reported decreased maturation when slow-freezing at the germinal vesicle stage, with no differences in parthenogenetic activation and development for immature oocytes slow-frozen either before or after IVM. Cao *et al.* [32] allocated 472 immature oocytes to a group that was vitrified at the germinal vesicle stage and another group that was first underwent IVM, followed by vitrification. A third group, which was not vitrified, underwent fresh IVM, serving as controls. There was no significant difference between the survival rates of the oocytes vitrified at germinal vesicle stage and those vitrified at metaphase II stage (85.4 vs. 86.1%). However, oocyte maturation rates were significantly reduced when oocytes were vitrified at immature germinal vesicle stage followed by IVM (50.8%) compared with the control group (70.4%). Following insemination by intracytoplasmic sperm injection, there was no difference in the fertilization (62.1 vs. 58.8%), cleavage (69.5 vs. 67.5%) and blastocyst development (0.0 vs. 0.0%) rates between these two groups. However, these results were significantly lower than those achieved in the control group. It is difficult to draw a valid conclusion from these conflicting findings, as studies differed in their use of slow-freezing [29–31,33] or vitrification [32]. Furthermore, in all of these studies comparisons were made using oocytes from different patients, which may contribute to significant confounding by patient-specific variables [29–33].

Further studies sought to address this and optimize their experimental design with the use of sibling oocytes from the same patient allocated randomly to either test group [21▪▪,27▪▪,31]. Fasano *et al.* [31] randomly allocated 100 oocytes to vitrification at metaphase II 24–48 h after IVM and compared them with 84 immature oocytes that were immediately vitrified at the germinal vesicle or metaphase I stage followed by postthaw IVM. The groups exhibited similar survival rates (86.9 vs. 84.5%). However, the oocytes that were matured prior to vitrification exhibited significantly higher maturation rates (46 vs. 23.8%) and number of metaphase II oocytes inseminated per oocyte collected (40 vs. 23.8%). Of note, the investigators included both sibling germinal vesicle and metaphase I oocytes, which failed to mature *in vivo*; and it is unclear if germinal vesicle oocytes from a single patient were randomly allocated to either treatment group. Wang *et al.* [27▪▪] also used sibling germinal vesicle stage oocytes, subjecting them to cryopreservation by slow-freezing either prior to or after IVM followed by analysis of chromatin, microtubules and microfilaments by three-dimensional imaging. Cryopreserved oocytes were compared with oocytes matured *in vitro* but never frozen. Survival was similar between oocytes frozen before or after IVM (69.7 vs. 70.5%). The maturation rate after IVM was lower in oocytes frozen at the germinal vesicle stage vs. those matured then frozen (51.3 vs. 75.7%) or

not frozen (75.4%). Slow-frozen germinal vesicle oocytes also demonstrated an elevated incidence of spontaneous activation – failing to maintain normal metaphase II arrest or progress normally through metaphase I. Spindle and chromosome configurations were disrupted to similar extents in both immature and mature frozen-thawed oocytes with similar length, width and volume of bipolar metaphase II spindles in all groups. Oocytes frozen at the germinal vesicle stage had a reduced volume of microtubules in nonbipolar spindle structure, which may reflect disturbances in cytoplasmic maturation after freeze-thawing immature oocytes [27▪▪].

RECENT FINDINGS

Our recent analysis further expanded upon the question of the optimal stage at which to freeze oocytes undergoing IVM, with the addition that post-IVM metaphase II oocytes were also compared with a cohort of metaphase II donor oocytes that were cryopreserved and thawed as a control. We evaluated two alternate cryopreservation and IVM procedures using immature sibling oocytes, which failed to mature during ovarian stimulation [21▪▪]. The fresh IVM Group utilized IVM prior to undergoing slow-freeze; and the postthaw IVM Group underwent slow-freeze and then IVM. The postthaw survival and maturation rates of immature oocytes in both groups were evaluated to determine whether IVM should be attempted prior to or after slow-freezing. The postthaw survival percentages of germinal vesicle, metaphase I, metaphase II oocytes frozen after IVM and control metaphase II oocytes did not differ significantly (83.3, 86.7, 83 and 86%, respectively). Overall, combined survival and maturation (germinal vesicle to metaphase II fresh-50% vs. postthaw-25%; and metaphase I to metaphase II fresh-81% vs. postthaw-40%) were significantly higher in the metaphase II oocytes that were matured *in vitro* prior to cryopreservation [21▪▪].

DISCUSSION: CRYOPRESERVATION TECHNIQUE, CHROMATIN STABILITY AND CUMULUS-OOCYTE COMPLEX FUTURE DIRECTION(S)

Overall, the comparative studies to date suggest that the reproductive potential of immature eggs is improved by first maturing then cryopreserving at the metaphase II stage rather than cryopreserving then maturing them postthaw. In general, the timing of cryopreservation with respect to IVM appears to have the most dramatic impact on maturation rates and potential. On the basis of our findings [21▪▪] and the current literature, others and we now routinely apply IVM on fresh immature oocytes from stimulated cycles to maximize maturation and survival prior to cryopreservation. Despite the growing body of literature focused on defining an optimal protocol, implantation rates of oocytes that are immature when retrieved remain low. There are several studies published on the subject (ref [1–8]), each having both strengths and methodological weaknesses. Three of the studies focused on an assessment of spindle and chromosome organization rather than testing the developmental competence *in vitro* [27▪▪,29,30], and demonstrated varying results. This may be attributed to differences in assessment methodology and criteria. Further studies are needed to better establish the spindle organization of immature oocytes cryopreserved at either the germinal vesicle or post-IVM stage. Given that Wang *et al.*'s [27▪▪] analysis of length, width and total microtubule volume of bipolar spindles of testing and control groups showed no significant difference, future research should consider the role of other elements, which potentially mediate oocyte maturation, such as spindle proteins, genetic ploidy, DNA fragmentation, oocyte metabolism and specific genetic and protein markers.

Although immature oocytes were shown to survive better with IVM than with cryopreservation, more work is required to advance the methodology of cryopreservation for oocytes at any stage of maturation. Studies comparing fresh vs. postthaw IVM have used both slow-freezing [21▪▪,27▪▪,29,30,33,34] and vitrification protocols [31,32]. Some of the slow-freezing studies used a choline-based rather than sodium-based protocol [27▪▪,33]. Given the differing outcomes studied, it is difficult to speculate upon the potential influence of using the conventional vs. sodium-substituted protocol. Goud *et al.* [33] compared both protocols within a single study that did not use sibling oocytes and demonstrated improved maturation and cleavage rates when germinal vesicle oocytes underwent slow-freezing using a choline-based protocol. Research efforts may benefit from tailor cryopreservation according to meiotic stage of an oocyte, thus understanding the optimal protocol and cryoprotectant for the lipid composition and membrane properties for both germinal vesicle and metaphase II oocytes [10,35,36].

The presence of cumulus cells, which play a role in mediating optimal oocyte maturation, may influence the success of cryopreservation of immature oocytes. However, cryopreservation of the intact cumulus-oocyte complex (COC) has been shown to be limited by an inability to preserve the delicate and highly dynamic interactions between oocyte and

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cumulus cells, which may reflect ineffectiveness of cryoprotectants to sufficiently penetrate the COC. Ultrastructural evaluation of vitrified bovine and mouse COCs demonstrated disruption of the intercellular communications between the oocyte and cumulus cells [37,38]. Luciano *et al.* [39] demonstrated the feasibility of cryopreserving cumulus-free bovine immature oocytes followed by coculture with intact COCs during IVM, with encouraging results. If cryopreservation of cumulus-free oocytes remains the best option available, IVM methodology could potentially improve by reestablishing cumulus–oocyte interactions during coculture after both cell types are cryopreserved separately [40]. The development of currently used IVM media was tailored to maturing intact COCs, which were not the predominate source of oocytes examined in joint IVM and cryopreservation [41▪▪]. This may explain why IVM remains an inefficient process, without significant improvements in recent years.

CONCLUSION

On the basis of the most recent literature, we recommend observing the maturation of germinal vesicle or metaphase I oocytes to metaphase II oocytes before cryopreservation rather than waiting until thawing to induce the maturation process. Improvements to the current maturation methodology, the use of noninvasive markers to select high-quality oocytes for IVM and further evaluation of cryopreservation technology may maximize the probability of developmentally delayed oocytes achieving reproductive success. Future research within the field of fertility preservation and reproductive medicine will undoubtedly include focus on the improved cryopreservation techniques as well as enhanced IVM strategies to optimize the reproductive potential of immature oocytes.

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Conflicts of interest

There are no conflicts of interest.

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Papers of particular interest, published within the annual period of review, have been highlighted as:

- ▪ of special interest
- ▪▪ of outstanding interest

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