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Review Article

Potential Use of Immature Oocyte to Improve Fertility Preservation Outcome: A Narrative Review

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Abstract

Fertility preservation through gamete vitrification has become one of the critical strategies to secure a childbearing potential in patients who are diagnosed with cancer or risks of infertility. Preserving the gametes would prevent the deleterious effects of cancer drugs or radiotherapy exposure on the quality of the gametes. Furthermore, *in vitro* fertilisation of vitrified mature human oocytes has lately demonstrated promising results that are reflected in the increased survival rate of thawed oocytes and the resultant clinical pregnancy rate. However, limitations in the cryopreservation of mature oocytes of cancer patients persist. Ovarian stimulation protocols which comprise administering gonadotrophin-releasing hormones could aggravate cancer or delay essential cancer therapy. Considering such circumstances, vitrification of immature oocytes would become a rational option. While the vitrification procedure of mature oocytes has been established, the vitrification of immature oocytes remains controversial due to a low post-thaw *in vitro* maturation and fertilisation rate. Apparent cryoinjuries to the immature oocytes post thawing or warming have been observed in both human and animal model oocytes. An alternative strategy was therefore proposed to improve the effectiveness of utilising immature oocytes for fertility preservation by conducting the *in vitro* oocyte maturation process first before vitrification. This method has prevailed, especially in oncofertility patients. Although the success rate of the clinical outcomes remains low, this approach, in conjugation with proper counselling, might provide oncofertility patients with an opportunity to preserve their reproductive potential.

Keywords: *Cancer-related infertility, fertility preservation, immature oocytes, in vitro fertilisation, vitrification*

Introduction

The first successful births of twin human babies from fertilisation of frozen-thawed pre-ovulatory mature oocyte were reported in Australia in 1986.[1] The strategy of utilising vitrified mature oocytes in an *in vitro* fertilisation (IVF) programme, however, has endured a slow acceptance due to the low survival rate of the oocytes post thawing. Experimental research on oocyte cryopreservation indicates the difficulties of vitrifying a large single oocyte cell.[2] The urgency to improve the success rate of oocyte cryopreservation has risen in several countries such as Italy, Austria, Germany and Switzerland due to legislation that restricts embryo cryopreservation.[3] A significant improvement in cancer prognosis after treatments in young adolescent patients has also validated the demand for an oocyte cryopreservation programme as means to preserve the reproductive potential of girls or women who are about

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to undergo cancer therapies.[4,5] Oocyte freezing as an alternative method to treat infertility was first approved in the UK by the Human Fertilisation and Embryology Authority in 2000[6] followed by the American Society for Reproductive Medicine in 2013.[7]

Vitrification is preferably performed when the oocyte is at a mature stage, namely metaphase II (MII). To promote *in vivo* maturation of oocytes, ovarian stimulation begins at day 2 or 3 of a menstrual cycle during the follicular phase. Exogenous gonadotropin hormone is administered once daily to support follicular growth until a minimum of two or three follicles has reached 18 mm. Oocyte maturation trigger utilising gonadotropin-releasing hormone agonist or human chorionic gonadotropin is then injected and the ovum pick-up procedure is commenced 36 h later.[8] Nonetheless, the gonadotropin stimulation approach is unsuitable for certain patients with underlying conditions such as cancer. Women with breast cancer, for instance, are particularly sensitive to the elevation of serum oestradiol (oestrogen-sensitive tumour) and are therefore advised to avoid undergoing ovarian stimulation using the gonadotropin hormone.[9] In addition to the risks of cancer aggravation, the time required for ovarian stimulation procedure could delay crucial cancer treatments. Investigating the potential use of immature oocytes as an alternative option for fertility preservation therefore becomes admissible. Contrary to the collection of *in vivo* matured oocytes, immature oocytes can be retrieved conveniently at any stage of the ovarian cycle.[10] More importantly, the necessity of the ovarian stimulation protocol could be bypassed. A case report published in 2012 demonstrated the convenient method of retrieving immature oocytes from antral follicles during a conservative surgery for ovarian cancer, indicating the feasibility and development prospects of such strategy.[11]

Several investigations were carried out to establish an optimal fertility preservation strategy using immature oocytes that could assure the chance of bearing a child in a specific group of cancer patients.[12-14] In the earlier decade of oocyte cryopreservation history, a low survival rate of cryopreserved mature oocyte post thawing has led to a postulation of a higher cryoinjury in resistance in germinal vesicle (GV) immature oocyte compared to the MII mature oocyte. At that point, GV was believed to be the ideal stage for vitrification rather than MII because of its lack of a microtubular spindle system. Moreover, chromosomes in GV are enclosed by a nuclear membrane, which was considered to reduce the risk of chromosome injuries and prevent the polyploidy or aneuploidy occurrence possibly induced by the extreme cooling condition during vitrification.[15] However, increasing evidence has suggested that the GV-stage oocytes are as vulnerable to cryoinjuries as the mature-stage oocytes.[16-19] Some studies have subsequently recommended performing an *in vitro* maturation (IVM) of the immature oocytes before vitrification,[20-24] while others have opposed this idea.[25-27] This literature provides a comprehensive review on the safety and current progress of vitrifying immature human oocytes for possible use of fertility preservation.

Methods

The literature review was conducted using several search engines including Google Scholar and PubMed. Boolean search strategy (AND, OR, NOT) has been applied to identify the relevant articles using terms such as oocyte, egg, vitrification, freezing, slow freezing, cryopreservation, immature and fertility preservation. Keyword phrases included in the search were 'immature egg freezing', 'immature oocyte vitrification' and 'immature oocyte freezing' [Figure 1].

Discussion

The clinical indications for women who could benefit from fertility cryopreservation using immature oocytes

Several factors including the cancer type and grade, urgency of the cancer treatment and marital status should be taken into consideration when opting for the fertility preservation programme. The clinical algorithm for female cancer patients aiming to retain their reproductive potential has been well defined.[4,9,28] As previously described, gonadotropin ovarian stimulation may be contraindicated in certain cancer patients with hormonal-sensitive tumours such as desmoplastic tumours and breast cancer (especially in oestrogen receptor-positive type and breast adenocarcinoma).[13,29] Administering exogenous gonadotropin during ovarian stimulation could induce an increased oestradiol level up to 15 times more than the natural cycle.[30] Thus, oncologists may advise the patients to undergo oocyte retrieval without ovarian stimulation.[12,13] Likewise, women who cannot delay their cancer treatments for a 2- to 6-week stimulation protocol could benefit from the retrieval and cryopreservation of immature oocytes.[9]

Clinical evidence on the benefits of preserving fertility using immature oocytes was proven in several studies.[10,12-14,29] A 2010 study demonstrated a novel approach of collecting immature oocytes without ovarian stimulation in 38 oncofertility women diagnosed with breast cancer. The oocyte retrieval was performed under sedation 36 h following a 10,000 IU HCG injection.

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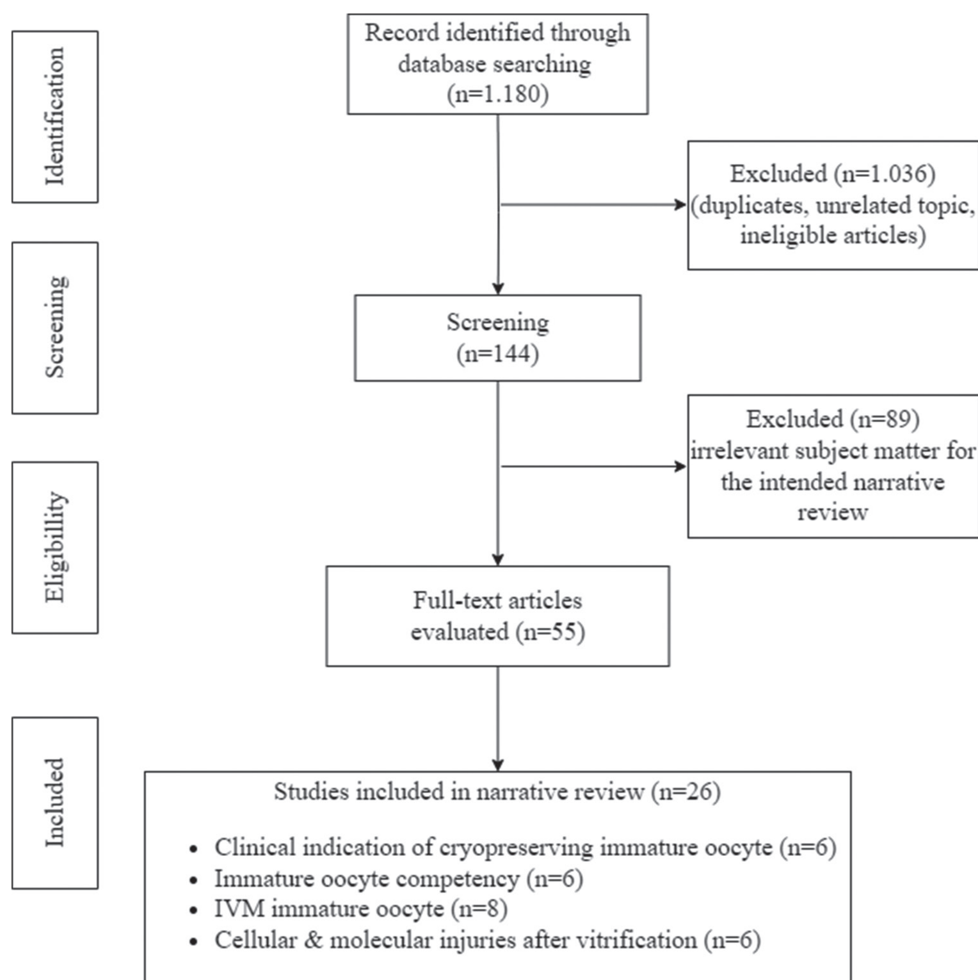

Figure 1: Search strategies for narrative review

GV-stage oocytes were subsequently matured *in vitro* through a 24-h culture and the resultant mature oocytes were used for further treatment. The yield of the *in vitro* mature oocytes ranged between 1 and 22 and the median vitrified embryos was 4 with a range of 1–13. Of the 38 women, 18 opted for the IVM process followed by the oocyte vitrification while the remaining agreed to fertilise their mature oocytes and vitrify the resultant embryos.[13]

An increased number of *in vitro* matured oocytes for vitrification was reported in two oncofertility women through a combination of immature oocyte aspiration at both the follicular and the luteal phase.[29] Moreover, evident benefits of retrieving immature oocytes at the luteal phase were demonstrated in a woman aged 21 who was incapable of suspending her cancer treatment and was advised against receiving the gonadotropin therapy.[29] Supporting the previous results, a prospective study on 248 breast cancer women has proven a similar IVM rate of immature oocytes aspirated at either the follicular or the luteal phase. The increasing yield of mature oocytes post IVM has certainly generated an interest in the practice of such strategy as an urgent approach for fertility preservation.[10]

Another plausible method of fertility preservation is through immature oocytes aspiration from excised ovarian tissue combining with an ovarian tissue cryo-banking. This strategy was applied in four women who were diagnosed with Hodgkin lymphoma, breast cancer and rectal cancer.[12] Immature oocytes were collected from the excised ovarian tissue and were subjected to the IVM culture. The mean oocyte maturation rate post IVM was 79%. All of the oncofertility patients managed to acquire at least one mature oocyte for vitrification. Furthermore, a large retrospective cohort study in 2015 comprising 255 cancer patients has validated the safety and advantages of harvesting the ovarian tissue for immature oocyte collection as means to attain an increased total number of *in vitro* matured oocytes and fertilisation rate.[14]

As the insemination of the *in vitro* matured oocytes was decided based on the marital status, patient preference or

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age, most studies on fertility preservation using immature oocytes have heretofore reported the yield of the *in vitro* matured oocytes as the main outcome.[10,12-14,29] Only few studies managed to describe the downstream IVF outcomes including the total number of embryos derived from the *in vitro* matured oocytes.[13,14] Hourvitz *et al*. reported a mean number of vitrified embryos between 1.67 ± 0.56 and 3.39 ± 0.73 depending on the immature oocyte collection procedure.[14] Although the results were encouraging, clear benefits of utilising immature oocytes to obtain embryos for further treatment are difficult to define due to the small sample sizes of the available reports. Therefore, detailed information regarding the current success rate of fertility preservation should be informed to the patients who wish to undergo the program.

Immature oocyte collection in IVF could also benefit women with polycystic ovary syndrome who are at risk of an ovarian hyperstimulation syndrome subsequent to ovarian stimulation.[31] The fertility preservation programme would also cater to the increasingly modern trend of postponing childbearing due to social or non-medical reasons. A study in the UK provided several background and clinical characteristics of women who underwent fertility cryopreservation. The mean age of the 27 women involved in the study was 36.7 years. They were highly educated, and half of the participants were professionally employed.[32]

Competency of *in vitro* matured germinal vesicle or post germinal vesicle breakdown metaphase I oocytes: A lesson from a stimulated fresh *in vitro* fertilisation cycles

Clinical use of immature oocytes obtained during a stimulated fresh IVF cycle is disputable even without the vitrification processing. *In vitro* matured GV and MI oocytes lack the competency to improve the clinical pregnancy.[33-35] Although a similar fertilisation rate was observed between the *in vivo* and *in vitro* matured oocytes, Shu *et al*. concluded that the clinical pregnancy and live birth rate of transferring embryos derived from the *in vitro* matured oocytes were unsatisfactory.[33] A 2010 study has also shown the inconspicuous effectiveness of using immature oocytes derived from the stimulated IVF cycles. Two hundred and sixty-three immature oocytes subjected to IVM were compared with their sibling *in vivo* matured oocytes (n = 234). Although both groups acquired comparable fertilisation rates, the developmental quality of the day 2 cleavage stage in the immature oocyte group was lower in regard to the blastomeric number and symmetry. Moreover, none of the 17 transferred embryos derived from the *in vitro* matured oocytes were successfully implanted.[34] Another study also observed a low clinical efficacy of the *in vitro* matured oocytes which did not culminate to a single clinical pregnancy in the five cases of embryo transfer.[35]

Nonetheless, a promising utilisation of *in vitro* matured non-GV or germinal vesicle breakdown (GVBD) has recently been reported by Olid *et al*.[36] IVM of GVBD oocytes in G-2™ PLUS media (Vitrolife, Sweden) resulted in a 10% clinical pregnancy rate and a 5.6% live birth rate. Concordantly, an evidence on the clinical benefit of *in vitro* matured MI-stage oocyte was recorded through one successful live birth of a healthy normal baby.[37]

An IVF using *in vitro* matured oocytes is undeniably less favourable than the *in vivo* matured oocytes. However, performing IVM of non-GV immature oocytes is another option, particularly in cycles which yield a low number of mature oocytes. The possible reasons underlying the low developmental competence of *in vitro* matured non-GV stages were implied by Ferrer-Vaquer *et al*.[38] This study highlighted the several differences in the cytoplasmic maturation between *in vivo* and *in vitro* matured oocytes. Alterations in the mitochondrial membrane potential, cluster number of endoplasmic reticulum and actin cytoskeleton were particularly observed in *in vitro* matured oocytes.

Should *in vitro* maturation be performed before or after vitrification?: A lesson from the use of immature oocytes in *in vitro* fertilisation to retain reproductive potential

Following the successful birth of a human female baby derived from a frozen-thawed *in vitro* matured GV immature oocyte in 1998 Georgia – America, cryopreservation of immature oocytes has been proposed as an alternative strategy to preserve the gametes.[39] Over the past two decades, numerous studies have revealed the challenges of immature oocyte vitrification. Several limitations of the approach are well discussed in an existing review as follows: (i) even though the survival rates of the frozen-thawed mature and immature oocyte are similar, the capacity of the surviving immature oocytes to undergo maturation *in vitro* is considerably low; (ii) fertilisation capacity of *in vitro* matured oocytes is also lower compared to the *in vivo* matured oocytes; and (iii) vitrification has been shown to evoke injuries of the chromosomes and cytoplasmic organelles even in the GV stage, disclaiming the theories that were previously suggested. The irreversible injuries of the organelles could be accountable for the lack of maturation and fertilisation capacity of the vitrified immature oocytes.[16,19] However, several contradictory

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studies have proved that the outcomes of vitrifying oocytes at immature stage were not inferior to that of mature oocytes.[25-27]

As presented in Table 1, most studies recommended performing IVM before vitrification to improve the clinical utilisation of the immature oocytes. Nonetheless, three studies suggested otherwise, proving that vitrifying oocytes before IVM or after were equally efficient. An in-depth evaluation is therefore still required to identify the factors which led to the different outcomes and to confidently determine whether vitrification of immature oocytes should be done before or after IVM. The current methodology for IVM is not sufficient enough to induce oocytes with a viability that is on par with the *in vivo* matured oocytes. Advancements in the IVM process might become an essential key to enhance the quality of *in vitro* matured oocytes and a stepping stone towards improvement in the fertility preservation programme.[18]

A standard procedure for IVM is to culture the immature oocytes in media supplemented with human serum albumin and highly purified gonadotrophins for up to 30 h until a polar body extrusion is observed.[40] Of note, the collection of immature oocytes from small antral follicles may slightly be more time – and technically – demanding than the collection of mature oocytes. A cell strainer with minute pores might be utilised to search for immature oocytes with minimal cumulus complex.[40] No particular commercialised brand of media has been deemed more effective in promoting IVM.

Advancements for IVM of oocytes could only be achieved with a clear insight into the *in vivo* maturation process. The mechanism involves a series of complex signalling pathways that are regulated by specific factors expressed through different cells found within the cumulus-oocyte complex. These cells are connected by gap junctions and connexins that need to be maintained *in vitro* to preserve the intra- and intercellular communications. In the pursuit of mimicking an *in vitro* condition which complements the *in vivo* micro-environments, several pre-maturation enhancements of IVM have been attempted. Vanhoutte *et al*.[41] designed a three-dimensional (3D) culture system utilising an extracellular matrix composed of collagen. Retrieved immature oocytes with intact cumulus complex were cultured on polymerised collagen supplied with IVM media and phosphodiesterase-3 inhibitor (meiotic arrester). Subsequently, the oocytes were recovered from the gel, washed and denuded for fertilisation through conventional IVF. The 3D culture managed to maintain the communication framework between cells found in the cumulus-oocyte complex that in turn brought on more meiotically competent oocytes and improved embryo development compared to the conventional IVM method.

In addition to the pre-maturation culture, several studies have shown an enhanced efficacy of IVM by fine-tuning the media with paracrine oocyte maturation-promoting factors such as FSH and amphiregulin[42] and oocyte-secreted factors such as bone morphogenetic protein 15 and growth differentiation factor 9.[43] Nonetheless, while the promising outcomes of these experiments could bridge the gap in the effectiveness between an IVM-IVF cycle and a conventional IVF cycle, large prospective studies are obligatory before these findings could be translated to clinical practice. Additionally, methods to reduce the cryoinjuries during vitrification should also be pursued to accomplish an altogether viable strategy for fertility preservation.

The cellular and molecular causes of low fertility potential in immature oocytes after vitrification

Several studies have revealed the consequences of immature oocyte vitrification in humans[44,45] and animal models[46-49] on a cellular and molecular level. In a 1988 study conducted by Van Blerkom *et al*., mouse GV vitrification was shown to cause premature chromosome condensation, extrusion of chromatin fragmentation in the cytoplasm, microtubule defects, impaired relocation of mitochondria and protein synthesis alteration.[46] An experimental study that examined the post-vitrification cycle cell status and chromosomal integrity of GV-stage rat oocytes also pointed out similar findings. Following a warming procedure, the GV-stage oocytes immediately entered the M-phase cell cycle but failed to maintain chromosomal integrity due to multiple chromatin fusion. The study also highlighted a remarkably low number of F-actin in both the cytoplasm and the cortical area and an irregular retraction of trans-zona pellucida post vitrification.[47] Several defects of the cytoskeleton, aberrant density of the mitochondrial matrix and microtubule were also observed in other animal studies.[48,49]

Utilising transmission electron microscope, frozen-thawed GV oocytes were shown to prematurely release peripheral cortical granules onto the subzonal space during IVM. Additionally, the number of cortical granules was reduced remarkably causing an increased density of the zona pellucida ultrastructure and subsequent zona pellucida hardening. Aggregates of small mitochondria-smooth endoplasmic reticulum (M-SER) were found to be randomly distributed within the cytoplasm.[44] Furthermore, detrimental effects of cryoprotectant exposure on the chromosomes and microtubules of immature human

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Table 1: Summary table of studies evaluated the various outcomes of vitrified immature oocyte before or after *in vitro* maturation

Study design	Year	Main objective	Sample size	Main outcome	Conclusion	References
In vitro experimental study	2009	To compare the efficacy of vitrification before and after IVM of immature oocytes	Immature human oocytes (*n*=472) Group 1 consisted of 219 GV that was vitrified before IVM Group 2 comprised 253 GV that was vitrified after IVM (*n*=79, while the remaining (*n*=99) were setting for control group)	A comparable survival rate post vitrification was observed between Group 1 and 2 (85.4% *vs*. 86.1%, respectively) However, the number of matured oocytes was significantly higher in Group 2 than in Group 1 A similar outcomes including fertilisation rate, cleavage and blastocyst rate were observed	IVM of GV-stage oocytes before vitrification yields better outcomes in terms of maturation rate	Cao *et al*.[20]
In vitro experimental study	2012	Comparing the effectiveness of IVM of immature oocytes before and after vitrification	184 immature (GV or MI) human oocytes were randomly allocated to Group 1 (vitrified after IVM, *n*=100) and Group 2 (vitrified before IVM, *n*=84)	While the survival rates were similar between Group 1 and 2 (86.9% and 84.5%), the maturation rate was significantly higher in Group 1 than Group 2 (46% *vs*. 23.8%) Fertilisation rate and embryo development potency between the Group 1 and 2 were comparable. However, no transferable blastocysts were obtained in both groups	A high number of *in vitro* matured oocytes survived the vitrification procedure because IVM was conducted before vitrification	Fasano *et al*.[21]
In vitro experimental study	2012	To investigate the best stage for vitrification of cumulus-free immature oocytes	Immature oocytes were obtained from 120 IVF patients GV-stage oocytes were allocated to freezing prior to IVM (*n*=109) and after IVM (*n*=107)	A comparable survival rate between vitrified-GV before and after IVM was observed (69.7% *vs*. 70.5%, respectively) However, the low maturation rate (51.3% vs. 75.7%) and high spontaneous cleavage were observed in GV oocytes of vitrified before IVM	Vitrification of *in vitro* matured GV oocytes acquired more preferable outcomes than GV oocytes vitrified before IVM	Wang *et al*[23]
Cross-sectional study	2013	Evaluating the maturation rate and viability of immature oocytes post warming	Infertile human oocytes A total of 53 fresh immature oocytes were subjected to fresh IVM as a control group (fIVM) and 50 immature oocytes were vitrified prior to IVM (vIVM)	Maturation rate of fIVM was superior over vIVM (88.7% *vs*. 56%, respectively, $P<0.001$)	Performing IVM prior to vitrification attained the maturation capacity of immature oocytes and the viability to further support embryonic development	Yazdanpanah *et al*.[24]
Experimental study	2016	Observing cryoinjuries on nuclear integrity such as spindle, DNA, as well as embryonic aneuploidy and ZPD after warming	Infertile human oocytes Thirty-one *in vivo* mature oocytes were used as control (Group A, with or without vitrification) Two hundred-twenty-six immature oocytes were allocated to Group B (IVM group before vitrification, n=113) and Group C (immature group, n=110)	Aneuploidy rate was comparable amongst groups ($P<0.05$). However, comet cells were significantly lower in Group A than Group B and C ($P<0.05$) Group C showed a lower occurrence and retardance value of spindle compared to Group A and B cleavage rate was also lower than that of Group A and B ($P<0.05$)	According to post warming molecular analysis, the most suitable stages for oocyte vitrification are at the *in vivo* mature and *in vitro* matured stages	Song *et al*.[22]

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Table 1: Contd...

Study design	Year	Main objective	Sample size	Main outcome	Conclusion	References
In vitro experimental study	2011	Evaluating the effect of GV vitrification on the methylation profile followed by IVM	184 GV oocytes subjected to vitrification followed by IVM and 120 GV oocytes were matured *in vitro* as a control group	*In vitro* matured oocytes derived from vitrified-GV showed comparable methylation profiles as fresh *in vitro* matured GV oocytes	Oocyte vitrification at the GV stage did not impair the methylation profile	Al-Khtib *et al.*[25]
In vitro experimental study	2016	Assessing the effects of GV vitrification on nuclear configuration, DNA methylation as well as cytoplasmic maturation and maturation rate post warming	Infertile human oocytes A total of 284 oocytes were allocated into three groups *In vivo* mature oocyte group (MII as the control group, n=56) IVM group (vitro-MII, n=106) Immature vitrified group (cryo-MII, n=122)	The cryo-MII group was shown to have the highest abnormal spindle and chromosome configuration amongst the groups (P<0.05) However, global DNA methylation, mitochondria and cortical granules distribution (reflecting cytoplasmic maturation), as well as maturation rate post warming were similar amongst the three groups	Although GV vitrification could disrupt the configuration of spindle and chromosome, GV stage can preserve its viability and undergo IVM after warming	Liu *et al.*[27]
In vitro experimental study	2016	Investigation on whether oocyte vitrification delivers better results before or after IVM in regards to the survival, maturation rate and embryonic development	221 unused human GV from ICSI-IVF cycles were grouped Group 1 as the control group (GV oocytes were maturated up to the MII stage without vitrification) Group 2 (GV-stage oocytes were vitrified and subsequently matured *in vitro* until reaching the MII stage) Group 3 (GV oocytes underwent IVM followed by a vitrification-thawing procedure)	The post warming survival rates between Group 2 and group 3 were similar (P=0.810) Group 2 showed the highest number of *in vitro* matured oocytes (83.7%) compared to Group 1 (63.4) and Group 3 (56.6%) Blastocyst formation rates were low in the overall three groups (P=0.432)	Vitrifying GV-stage oocytes resulted in a higher survival rate and maturation rate post warming	Molina *et al.*[26]

IVM=*In vitro* maturation, GV=Germinal vesicle, MI=Metaphase I, fIVM=Fresh IVM, vIVM=Vitrified IVM, ZPD: Zona pellucida density, ICSI=Intra-cytoplasmic sperm injection

oocytes were also apparent. An increased incidence of abnormal chromosomes and microtubules was exhibited in the *in vitro* matured GV oocytes.[45]

Conclusion

Fertility preservation utilising immature oocytes could increase the availability of mature oocytes and consequently embryos for cryopreservation. Ineffectiveness of immature oocyte vitrification for fertility preservation persists on account of the vitrification injuries observed in frozen-thawed oocytes of both the animal models and humans. Alternatively, performing IVM before vitrification would increase the survival and fertilisation rate of the immature oocytes. Developing methods that could significantly improve IVM and reduce cryoinjuries during vitrification is imperative to deliver the clinical benefits of immature oocyte cryopreservation as means to preserve fertility.

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Conflicts of interest

There are no conflicts of interest.

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