Female Fertility: Is it Safe to “Freeze?”

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Abstract

Objective: To evaluate the safety and risk of cryopreservation in female fertility preservation.

Data sources: The data analyzed in this review were the English articles from 1980 to 2013 from journal databases, primarily PubMed and Google scholar. The criteria used in the literature search show as following: (1) human; embryo; cryopreservation/freezing/vitrification, (2) human; oocyte/immature oocyte; cryopreservation/freezing/vitrification, (3) human; ovarian tissue transplantation; cryopreservation/freezing/vitrification, (4) human; aneuploidy/DNA damage/epigenetic; cryopreservation/freezing/vitrification, and (5) human; fertility preservation; maternal age.

Study selection: The risk ratios based on survival rate, maturation rate, fertilization rate, cleavage rate, implantation rate, pregnancy rate, and clinical risk rate were acquired from relevant meta-analysis studies. These studies included randomized controlled trials or studies with one of the primary outcome measures covering cryopreservation of human mature oocytes, embryos, and ovarian tissues within the last 7 years (from 2006 to 2013, since the pregnancy rates of oocyte vitrification were significantly increased due to the improved techniques). The data involving immature oocyte cryopreservation obtained from individual studies was also reviewed by the authors.

Results: Vitrifications of mature oocytes and embryos obtained better clinical outcomes and did not increase the risks of DNA damage, spindle configuration, embryonic aneuploidy, and genomic imprinting as compared with fresh and slow-freezing procedures, respectively.

Conclusions: Both embryo and oocyte vitrifications are safe applications in female fertility preservation.

Key words: Embryo Cryopreservation; Oocyte Cryopreservation; Ovarian Tissue Cryopreservation

INTRODUCTION

In recent decades, more and more women have infertility problems due to declining oocyte quality, commonly related to the maternal age,[3] invasive cancer treatment,[2] premature ovarian failure (POF)[3] and polycystic ovary syndrome (PCOS).[1] For example, in USA, 805,500 women were diagnosed as new cases of invasive cancer in 2013, and about 10% of them were <45 years old.[4] The fertility of survivors who underwent cancer treatments such as chemotherapy,[5] radiotherapy[6] and bone marrow transplantation[7] was impaired by the treatments. Thus, it is imperative to reserve these cancer survivors’ fertility before cancer treatments by cryopreserving their oocytes, oocytes or embryos.

Furthermore, some nononcological diseases such as POF and PCOS may also lead to the ovarian dysfunction.[3] Moreover, it is increasingly common for women in many countries to delay childbearing until the age of 35 years or older.[8] Therefore, fertility preservation in women is not only a medical problem, but also a current social issue to be resolved.

Cryopreservation is a crucial option for people seeking fertility preservation, which refers to freezing cells and tissues to sub-zero temperatures in order to stop all biologic activity and preserve them for future use.[9] Thus, a woman could bear children even after chemotherapy or menopause, as well as store eggs extracted for an in vitro fertilization (IVF) cycle.[10] Ever since the first baby born from a frozen embryo in 1983,[11] and the first pregnancy achieved from a frozen oocyte in 1986,[12] interests in cryopreservation techniques are growing tremendously. Over the past 30 years, techniques for cryopreservations of human embryos, oocytes, and ovarian tissues have been developed, with two main techniques commonly being used: the traditional, slow-freezing method, and vitrification, a novel technique combining ultra-rapid cooling time with high cryoprotectant concentration, engendering glass-like formation to avoid damaging ice crystals.[13]

Numerous individual studies investigating the different

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reproductive outcomes of cryopreservation on human embryos, immature or mature oocytes, or ovarian tissues have been reported. Currently, embryo and mature oocyte cryopreservation following IVF are the only methods endorsed by the American Society of Reproductive Medicine (ASRM) and recommended by the American Society of Clinical Oncology (ASCO) for fertility preservation for patients with cancer. However, the overall safety and risks for cryopreservation techniques in female fertility preservation are still uncertain. The aim of this article was to review the current knowledge on the possible effects of freezing methods on human embryos, oocytes, and ovarian tissues, and provide recommendations for clinical applications.

**Clinical Outcome Assessments**

**Better clinical outcomes: frozen embryos versus fresh ones**

Human embryo freezing is the initial method to preserve the fertility for women at reproductive age. In addition, it is the first freezing method of fertility preservation for women endorsed by ASRM. This technique has been investigated by a respectable number of research teams, and hereby we summarized the latest systematic studies to discuss the clinical effects of cryopreservation on human embryos.

In Roque et al.’s systematic meta-analysis, three clinical trials accounting for 633 cycles in women aged 27–33 years old showed that frozen embryo transfer resulted in a statistically significant increase in the ongoing pregnancy rate [the relative risk (RR) and 95% confidence intervals (CI) was 1.32 (1.10-1.59)] and clinical pregnancy rate [RR (95% CI) was 1.31 (1.10-1.56)] compared with the fresh transfer group. Interestingly, the fresh group showed a higher miscarriage rate [RR (95% CI) was 0.83 (0.43-1.60)], but no statistical difference was found when compared with the frozen group. Moreover, Maheshwari et al. systematically quantified the obstetric and perinatal risks for singleton pregnancies after frozen embryo transfer and compared it with those after fresh embryo transfer. Eleven studies included in this meta-analysis indicated better perinatal outcomes in singleton pregnancies after the transfer of frozen-thawed embryos when compared to those after fresh IVF embryos. This was, because the RRs (95% CI) of: antepartum hemorrhage [0.67 (0.55-0.81)], very preterm birth (delivery at < 32 weeks) [0.73 (0.50-1.08)], preterm delivery (delivery at < 37 weeks) [0.84 (0.78-0.90)], small for gestational age [0.45 (0.30-0.66)], low birth weight (birth weight < 2500 g) [0.69 (0.62-0.76)], and perinatal mortality [0.68 (0.48-0.96)] was significantly lower in women who received frozen embryos than those transferred with fresh embryos. The RR differences of very low birth weight (birth weight < 1500 g) [0.72 (0.50-1.04)], congenital anomalies [1.05 (0.81-1.35)], and transfer rate to neonatal intensive care unit [1.0 (0.92-1.08)] between frozen and fresh groups were not available. However, pregnancies from frozen-thawed embryos had an increased risk of cesarean section [1.1 (1.05-1.15)] compared with those after fresh embryos.

The reason for improved clinical outcomes with frozen embryos is not clear. It might be associated with having a well-balanced embryo-endometrium interaction in frozen cycle and lacking controlled ovarian hyperstimulation, which may adversely affect endometrial receptivity during fresh IVF cycle. In addition, when hormone replacement cycles were applied in frozen embryo transfers, estrogen and progesterone were given in physiological doses to mimic natural cycles while supraphysiological doses of gonadotropins were given in fresh cycles. Another feasible explanation is that freezing and thawing may screen the good quality embryos to survive, resulting in better fetal growth.

**Equivalent outcomes: frozen mature oocytes versus fresh ones**

Mature oocyte cryopreservation is a freezing method, which was just approved by ASRM in 2012. Before 2005, the survival rates and pregnancy rates of freezing oocyte were very low because of limited freezing techniques and oocyte fragility. Recently, oocyte vitrification, a novel, and well-developed freezing method, improved the clinical outcomes. Hence, we reviewed the current relevant studies to evaluate the efficacy and safety of mature oocyte cryopreservation.

Five randomized controlled trials were systematically analyzed to assess the efficacy of metaphase II (MII) oocyte vitrification in clinical pregnancy outcomes from 2005 to 2009. These studies concluded that there were no differences between the vitrified MII oocytes and the fresh oocytes in terms of ongoing pregnancy [RR (95% CI) was 1.03 (0.73-1.45)], top-quality embryo [RR (95% CI) was 0.91 (0.83-1.01)], embryo cleavage [RR (95% CI) was 1.0 (0.90-1.11)], fertilization [RR (95% CI) was 1.02 (0.91-1.13)], and implantation [RR (95% CI) was 0.96 (0.75-1.24)]. Furthermore, the incidence of congenital anomalies was not increased in live born babies from oocyte cryopreservation, when compared with natural conception. Thus, on the basis of the evidence provided by Cobo and Diaz’s meta-analysis, oocyte vitrification seemed to be an efficient method of fertility preservation, appearing to have similar clinical outcomes as fresh mature oocytes.

**Reduced oocyte viability: frozen immature oocytes versus fresh ones**

Theoretically, immature oocytes, such as oocytes at the germinal vesicle (GV), GV breakdown, and MI stages, should be more tolerant of cooling, since they do not have a spindle apparatus and are absent during the ovarian hyperstimulation. Thus, immature oocyte cryopreservation seemed to be a safer option for women to reserve the fertility. Unfortunately, attempts to optimize protocols were hindered by the scarcity of clinical materials and the restriction of in vitro maturation (IVM). To date, although normal infants were successfully obtained from embryos...
derived from immature oocytes.\(^ {42-43}\) only a handful of global centers are using immature oocyte cryopreservation as an alternative option for women with cancer.\(^ {44-47}\)

In 1997, Park et al.\(^ {48}\) reported that human oocytes matured in vitro after cryopreservation at the GV stage showed an increased incidence of chromosomal and spindle abnormalities, as well as lower maturation rates. Sixteen years later, both maturation rate and oocyte viability rate are still lower in frozen-thawed immature oocytes than fresh immature oocytes.\(^ {49-53}\) With respect to this problem, it was presumably related to the reduced cortical granules at the cortex of cryopreserved GV oocytes, as well as the appearance of vacuoles and small mitochondria-smooth endoplasmic reticulum complexes in the ooplasm.\(^ {54}\) However, the survival rate of frozen-thawed immature oocytes was better than that of mature MII oocytes.\(^ {54}\)

Considering the above situation, it appeared that cryopreservation of immature oocytes after IVM to MII stage is a superior strategy over freezing immature oocytes directly.\(^ {51,52,55}\) Although the survival rates seemed to have been improved, many problems still need to be solved, including the fertilization and pregnancy outcome of immature oocyte freezing. Therefore, immature oocyte cryopreservation is still an investigated technique used in fertility preservation and requires further exploration before its clinical application.

**Better clinical outcomes: vitrification versus slow-freezing**

There are two cryopreservation methods: slow-freezing and vitrification. Slow-freezing has the advantages of using low concentrations of cryoprotectants, but induces the risk of intracellular ice formation. On the other hand, vitrification is a rapid method and requires high concentrations of cryoprotectants, which is associated with chemical toxicity and osmotic shock yet reducing chilling sensitivity and crystallization damage to gametes.\(^ {56}\) To compare these two methods, we reviewed newly published literatures and found two meta-analysis reviews concerned with human embryos, one meta-analysis review referring to human mature oocytes, and several studies of human immature oocytes.

**Embryo cryopreservation**

In meta-analysis reported by Loutradi et al.\(^ {57}\) the survival rates of the two methods were compared in four eligible studies, including three randomized controlled trials from 1980 to 2006. Furthermore, in systematic review reported by AbdelHafez et al.\(^ {58}\) the primary and secondary clinical outcomes of the two methods were examined in six randomized controlled trials from 1980 to 2008 (among those, two original papers were unavailable, one study was overlapping in both meta-analyses).\(^ {59-64}\) The information summarized by Loutradi et al.\(^ {57}\) indicated that the survival rates of both cleavage stage embryos [RR (95% CI) was 15.57 (3.68-65.82)] and blastocysts [RR (95% CI) was 2.2 (1.50-3.16)] after vitrification were significantly higher when compared with slow-freezing.\(^ {57,63,65-67}\) In addition, AbdelHafez et al.\(^ {58}\) consistently showed that the survival rate [RR (95% CI) was 4.87 (3.01-7.88)], clinical pregnancy rate [RR (95% CI) was 1.55 (1.03-2.32)], ongoing pregnancy rate [RR (95% CI) was 1.82 (1.04-3.20)], and implantation rate [RR (95% CI) was 1.49 (1.03-2.15)] of vitrified embryo transfers were statistically higher than those from slow-freezing embryos.\(^ {59-64}\)

There were no differences in multiple pregnancy rate [RR (95% CI) was 2.11 (0.99-4.52)], a miscarriage rate [RR (95% CI) was 0.57 (0.16-2.03)], and live-birth rate [RR (95% CI) was 0.87 (0.36-2.12)] between vitrification and slow-freezing.\(^ {59-64}\) Vitrification is superior to slow-freezing in human embryo cryopreservation.

**Mature oocyte cryopreservation**

Cobo and Diaz’s\(^ {32}\) meta-analysis analyzed five randomized controlled trials between 2005 and 2009 to compare the clinical efficacy of mature (MII phase) oocyte vitrification with slow-freezing. The survival rate [RR (95% CI) was 2.46 (1.82-3.32)], fertilization rate [RR (95% CI) was 1.5 (1.07-2.11)], top-quality embryo rate [RR (95% CI) was 3.32 (1.37-8.02)], embryo cleavage rate [RR (95% CI) was 2.25 (1.32-3.85)], and pregnancy rate [RR (95% CI) was 3.18 (1.06-9.52)] were remarkably higher in vitrified mature oocyte group when compared with those in slow-freezing group.\(^ {32-37}\) The strength of the evidence provided by these randomized trials proved vitrification to be an efficient method to cryopreserve mature oocytes.

**Immature oocyte cryopreservation**

In 2007, Sarajari et al.\(^ {68}\) compared the survival rate and the resumption rate of meiosis of immature oocytes (GV, MI phases) using vitrification and slow-freezing methods. The data from two groups showed that the survival rate was much higher in the vitrification group when compared with the slow-freezing group, but the maturation rate after IVM was no different between the two groups. Similar to Sarajari et al.’s results, the vitrification protocol of immature oocytes yielded better primary outcomes in survival rate, maturation rate, and cleavage rate as compared with the slow-freezing procedure.\(^ {69}\) Additionally, Combelles et al.\(^ {70}\) investigated the cytoskeletal and chromosome organization of immature oocytes that were either vitrified or slow-frozen, and the results indicated that the survival and maturation rates were comparable between the two groups, with similar rates of spindle morphometrics and chromosome organization; however, fewer oocytes underwent spontaneous activation or appeared to have spindle abnormalities, and more oocytes showed a bipolar spindle after IVM following vitrification when compared with slow-freezing.

**Assessments of DNA integrity, spindle configuration, aneuploidy and genomic imprinting after cryopreservation**

In spite of the excellent clinical outcome of cryopreservation, there are still many more questions about cryo-injury, such as DNA damage, spindle deformation, chromosome abnormalities, and aberrant genomic imprinting when cells are exposed to mechanical, thermal, and chemical stresses during cryopreservation.\(^ {71}\) Multiple centers in the world have analyzed the possible damages to subcellular structures of mature oocytes using slow-freezing and vitrification.
protocols. In general, it has been demonstrated that mature oocytes are less tolerant to cooling than embryos, most probably due to the spindle sensitivity to cryoprotectants and low temperature.\(^{[72]}\)

Studies showed that the meiotic spindle was a dynamically sensitive structure, which disappeared after freezing and reforms after thawing during cryopreservation.\(^{[73‑76]}\) This phenomenon was regulated by precise polymerization and depolymerization of tubulin, and shown to be in a very delicate equilibrium.\(^{[77‑78]}\) The chromosomes were aligned tightly on the spindle even when the spindle was disassembled.\(^{[79]}\) About 3–5 hours after thawing, the spindle reformed completely.\(^{[73,74]}\) Interestingly, no DNA fragmentation was observed by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling assay in both freezing and fresh oocytes.\(^{[80,81]}\) Concomitantly, a randomized controlled trial demonstrated that oocyte vitrification did not increase the risk of embryonic aneuploidy, verified by DNA fingerprinting.\(^{[82]}\)

Given the complications of epigenetic stability during oocyte and early embryo development, only one study investigated the cryogenic effects on the imprinted methylation of a human oocyte. As shown in Al-Khib et al. study,\(^{[83]}\) vitrified GV oocytes followed by IVM to M II acquired full imprint at Kv differentially methylated region 1 (a maternal imprinting center) and generally retained the unmethylated state of H19DMR (a paternal imprinting center) with the same efficiency as fresh GV oocytes in human, which suggested that freezing does not alter the genomic imprinting regardless of IVM. Unquestionably, the genomic imprinting of cryopreserved oocytes was affected by a complex relationship among the impaired fertility, assisted reproductive technologies and cryopreservation.\(^{[84,85]}\) Further studies on imprinting establishment in human oocyte cryopreservation are necessary to reach definitive conclusions. In addition, some studies have compared vitrification of oocytes with slow-freezing by examining morphological appearance, meiotic spindle, DNA integrity, and oocyte configuration. The results proved that vitrification was more effective than slow-freezing, offering higher survival rate, faster cellular volume recovery, faster spindle recovery, and similar DNA fragmentation.\(^{[77,81,86]}\)

**Decreased ovarian function and increased recurrent ovarian failure: cryopreserved ovarian tissue transplantation versus fresh ovarian tissue transplantation**

Cryopreservation of ovarian tissue may be the only available option for prepubertal girls with cancer to reserve the fertility.\(^{[87]}\) One theoretic option, *in vitro* growth of isolated immature follicles before or after ovarian tissue freezing, may avoid the transmission of malignant cells, but it is still at a laboratorial stage.\(^{[88]}\) Alternatively, ovarian tissue cryopreservation with subsequent transplantation has been categorized as an experimental option for fertility preservation by ASRM.\(^{[89]}\) Despite few clinically available applications, ovarian tissue banking has been offered to patients as a clinical service by many global centers.\(^{[90]}\) As of now, only xenografting of ovarian tissue and cryopreservation with subsequent ovarian transplantation have been successfully applied in humans.\(^{[91]}\) Due to the small number of patients, the difficulties with surgical techniques, and the duration of follow-up, it is hard to execute large studies on the clinical outcomes after ovarian tissue transplantation (OTT). So far, only one meta-analysis was reported on the reproductive function after cryopreserved and fresh ovarian tissue grafts.\(^{[92]}\) In Bedaiwy et al.’s systematical review,\(^{[93]}\) 25 individual studies, including 46 unique cases, were analyzed by the time of re-establishment of ovarian function, in which the short-term (<12 months) and long-term (>12 months) ovarian function as well as the pregnancy rate after freezing or fresh OTT were evaluated.\(^{[91‑117]}\) The precise data indicated that cryopreserved ovarian grafts had a decreased trend in the recovery of ovarian function \([RR (95% CI) = 0.41 (0.15‑1.09)]\) and an increased trend of recurrent ovarian failure \([RR (95% CI) = 2.13 (0.89‑5.56)]\) compared with fresh ovarian grafts.\(^{[91‑117]}\) The decreased reproductive potentiality of frozen OTT may be related with the ischemic injury to follicles, caused by the freeze-thaw procedure in cryopreserved ovarian grafts.\(^{[91‑117]}\) As for the long-term efficacy, there was insufficient evidence to tell the difference between fresh and cryopreserved OTT. According to the reported cases, the pregnancy rate of patients from frozen grafted ovaries was similar to those from fresh grafted ovaries.\(^{[91‑117]}\) Consequently, the efficacy of OTT using cryopreserved tissues is not yet equivalent to that of fresh grafts. The ovarian cryopreservation protocols are required to be improved and optimized in the future, supported by strong experimental evidence.

**Clinical Application Recommendation**

1. Embryo vitrification is a well-established method for adult women who have a male partner or wish to use donor sperm for medical and social reasons. However, it is a cost-based procedure, requiring ovarian stimulation, oocyte collection, and the use of assisted reproduction techniques that may take 2–5 weeks, which is not feasible for some patients with hormone-sensitive tumors such as breast cancer or requiring urgent cancer treatment.\(^{[89]}\)

2. Mature oocyte vitrification, an established method for fertility preservation recently endorsed by ASRM and ASCO, is an option for older postpubertal female children and adult women who are single or who have religious or ethical objection to embryo freezing for medical and social reasons.\(^{[118]}\) However, it also requires a cycle of ovarian stimulation before the beginning of chemotherapy or radiation therapy, which could delay the cancer treatment and increase the risks of stimulating hormone-sensitive cancers.\(^{[89]}\)

3. Immature oocyte vitrification is an experimental option for patients who need urgent cancer treatments or...
have hormone-sensitive cancers, providing the benefit of avoiding both ovarian stimulation and expensive medications.\textsuperscript{[79]} Unfortunately, this method is limited because of poor clinical results.

4. Ovarian tissue cryopreservation is considered as an investigational approach. Only subsequent transplantation or IVM offers the advantages of preserving thousands of primordial follicles at one time and potentially restoring temporary endocrine function to cancer survivors and patients with POF.\textsuperscript{[92]} This procedure of cryopreservation may be the only practical option for prepubertal girls with cancer.\textsuperscript{[87]} Additionally, this option currently emerges to serve other specific groups of patients, such as women with hormone-sensitive cancers or women who require an immediate cancer treatment.\textsuperscript{[90]} The disadvantage of this technique is multiple challenges involved, including: oocyte atresia from both ischemia at the time of biopsy and the freeze-thaw process;\textsuperscript{[103]} altered hormonal profiles compared to normal ovaries;\textsuperscript{[109]} surgical trauma of transplantation, short duration of ovarian function (the mean time reported was 4 to 5 years\textsuperscript{[87,90]}) and potential reintroduction of cancer cells.

**Novel Options for Healthy Women**

Fertility cryopreservation is commonly applying for patients who had already developed cancer or aging women at risk of declining ovarian function, considered as an elective method to prevent deterioration of ovarian function. However, more and more women are recently choosing delayed child-bearing family pattern in the United State and some European countries,\textsuperscript{[8]} who prefer to reserve their oocytes or embryos for nonmedical purposes, which is referred as “social freezing”.\textsuperscript{[110]} Although social freezing is legitimate in these countries, it is still a debated issue for many medical, ethical, and social reasons, such as decreasing pregnancy and increasing obstetric complications, questionable long-term effects of cryopreservation, controversial offspring quality from cryopreserved gametes, and the changing of family patterns. Further studies on the new problems after social freezing are urgently needed.

**Conclusions**

Studies provided reassuring data on safety and efficacy of oocyte and embryo cryopreservations, and both embryo and oocyte vitrifications are excellent options for female patients to reserve reproductive fertility. Nonetheless, the risks of oocyte and embryo vitrifications are still retained, particularly from the unclear potential cytotoxic effects of ice crystals and the cryoprotectants, as well as whether oocytes and embryos are ready for long-term storage. With more cryopreservation applications, more problems will be raised, including its unresolved inherent medical risks and emerging social risks concerning the health and outcomes of freezing children and the changing of family patterns. In the future, large-scale studies should be executed to optimize the freeze-thaw protocol in order to reduce the clinical risks and social side-effects.

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