Oocyte vitrification: advances, progress and future goals

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Abstract

Background Recent advances in vitrification technology have markedly improved the efficacy of oocyte cryopreservation in terms of oocyte survival and pregnancy, as well as live birth rates. However, there still remains room for improvement in terms of vitrification techniques.

Objective The remaining challenges include the development of a less cytotoxic vitrification solution and of a safe vitrification device in order to have vitrification techniques considered as a standard clinical laboratory procedure.

Methods A systematic electronic literature search strategy has been conducted using PubMed (Medline) databases with the use of the following key words: oocyte, vitrification, cryoprotectant, preservation, pregnancy, and live birth. A list of published papers focused on the improvement of vitrification techniques to have the vitrification protocol standardized have been evaluated in full text for this review. Only key references were cited.

Conclusions Vitrification technology has made significant advancements and holds great promise, but many issues remains to be addressed before it becomes a standardized procedure in clinical laboratories such as the fact that oocyte vitrification may not require a high concentration of cryoprotectant in the vitrification solution when it has a suitable cooling and warming rate. There is also no consistent

evidence that indicates the absence of risk to the vitrified oocytes when they are stored for a prolonged period of time in direct-contact with liquid nitrogen. The long-term development of infants born as a result of this technology equally remains to be evaluated.

Keywords Oocyte · Vitrification · Cryoprotectant · Preservation · Pregnancy · Live birth

Introduction

The cryopreservation of embryos, oocytes, and ovarian tissues are the three main options for female fertility preservation. Each of these options have their specific advantages and disadvantages. For example, embryo cryopreservation is not a feasible option for pre-pubescent girls, for women who object to the use of donor sperm, and for women who do not agree with embryo cryopreservation for various personal, financial, or ethical reasons. Oocyte cryopreservation is equally not a viable option for pre-pubescent girls, which makes ovarian tissue cryopreservation the only remaining alternative for them. As such, there is a growing consensus that oocyte cryopreservation can be considered as an important component of human assisted reproductive technology [1, 2].

A recent guideline from the Practice Committees of the American Society for Reproductive Medicine (ASRM) and the Society for Assisted Reproductive Technology (SART) indicated that mature oocyte vitrification and warming should no longer be considered as an experimental procedure [3] because this technology is recommended in cases of gonadotoxic therapies when there is a lack of alternative options for fertility preservation. However, mature oocyte vitrification and thawing is still being considered as an experimental procedure because, to date, there is not enough evidence presented to be able to recommend it in the

Capsule The efficacy of oocyte vitrification has propelled significant advancements in the field and it holds great promises, but many issues remains to be addressed and studied before it can be considered as a standard procedure in clinical laboratories.

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circumvention of reproductive aging. In addition, the guideline suggested that more widespread clinic-specific data are required on the safety and on the efficacy of oocyte vitrification in the population who require fertility preservation (excluding egg donors) before universal oocyte banking can be recommended.

Initial attempts to freeze oocytes employed the same slow-freezing methods that were considered as the golden standard for embryo cryopreservation. However, the slow-freezing of oocytes were met with a very low survival and pregnancy rates [4–6] and this intervention was thus considered as an experimental procedure [1]. With time, the efficacy of the slow-freezing of oocytes has been improved by increasing the sucrose concentration in the freezing solution [7–10]. Recently, with the introduction and advancement of vitrification techniques, there has been a significant improvement in the efficacy of oocyte cryopreservation [11–20]. Several groups worldwide have reported the high survival and pregnancy rates as well as the high live birth rates as a result of vitrified oocytes [21–28]. However, it appears that the majority of the live births were from egg donor programs [29] rather than from a clinic-specified population who are seeking to use this technology for fertility cryopreservation.

Nevertheless, the success of oocyte vitrification technology made the possible the establishment of egg banks and it has also facilitated the logistics of coordinating egg donors with their recipients. Oocyte vitrification technology allows for a temporary quarantine of donor eggs to test for transmissible diseases in the donors. As a consequence, with the advances of oocyte vitrification technology, it will come to be expected in the near future that many effective egg-banking programs will be established worldwide for female fertility preservation despite the fact that the technology is still struggling to attain its goals.

Candidates for oocyte cryopreservation

An effective oocyte vitrification technology will have a significant impact on the clinical practice of assisted reproduction. In addition to providing a fertility preservation option for young women requiring potentially sterilized (medical and surgical) treatments, cryobanking of oocytes will benefit a large population of women who wish to delay motherhood because of personal and professional reasons.

Avoids legal and ethical restrictions of embryo cryopreservation

Oocyte vitrification benefits infertile couples with moral or religious objections to the cryopreservation of embryos. In fact, the cryopreservation of oocytes has become one of

leading options of female fertility cryopreservation in countries where the law forbids the cryopreservation of embryos.

Facilitates egg donor program

Currently, women with premature ovarian failure (POF) who wish to conceive must rely on donor oocytes. Oocyte donation can be complicated and time consuming as it requires the hormonal synchronization of the donor and the recipient's menstrual cycles. An efficient oocyte vitrification technology eliminates the need of such synchronization and it enables the establishment of egg banks by eliminating the logistics of coordinating egg donors with their recipients [22, 23]. Furthermore, oocyte vitrification technology allows for the temporary quarantine of donor eggs in testing the donors for transmissible diseases [28]. The establishment of egg banks will then likely facilitate and improve the current shortage of eggs donors.

Option for delaying motherhood

Oocyte vitrification may be considered as an option for women who are trying to postpone childbearing while they pursue their professional careers. The impact of ageing on female fertility is well recognized [30], as it is evident that women's best chances of conceiving a healthy child are through a natural reproduction at a relatively early age [31]. The five specific age-related factors that can negatively affect a pregnancy are declining fertility, miscarriage, chromosomal abnormalities, hypertensive complications, and stillbirth [32]. The fertility of women declines throughout the reproductive lifespan and the rates of spontaneous abortions increase significantly after their mid-30's. The risks of conceiving a baby with Down syndrome or with any chromosomal abnormalities are well known to be higher in the oocytes produced by 'older' women [33]. Despite the awareness of these issues, many women continue to delay childbearing in favour of their professional development. The option of oocyte vitrification due to "social" reasons may offer the possibility of a compelling alternate strategy for women that wish to preserve their reproductive potential. Because most of the risks associated with childbearing at an advanced maternal age are due to the age of the eggs, and efficient oocyte vitrification may be considered as an option to potentially avoid risks.

Cryoprotectant for oocyte vitrification

The introduction of oocyte vitrification technology has significantly improved the outcome of oocyte cryopreservation, which lead to pregnancy and live birth rates comparable to those achieved in *in vitro* fertilization (IVF) with the use of fresh oocytes [3, 21]. However, there are many concerns about

the technology used for oocyte vitrification in the presence of a relatively high concentration of cryoprotectant, such as ethylene glycol (EG), dimethyl sulphoxide (DMSO), and propylene glycol (1, 2-propanediol, PROH). The exposure of oocytes to a high concentration of cryoprotectant is known to damage the oocytes via both cytotoxic and osmotic effects [34]. Although there is no standard protocol for oocyte vitrification, it seems that all existing oocyte vitrification procedures use a minimum of 3.0 M concentration of cryoprotectant (with a single cryoprotectant or in a mixture of two cryoprotectants) [11–28].

The presence of a suitable concentration of cryoprotectant in the freezing or vitrification solution usually considerably increases the cell's survival rates. Although the specific role a cryoprotectant actually plays in the freezing or vitrification solution is still unclear, the cryoprotectant takes part in lowering the freezing point and in reducing or preventing the formation of ice-crystals in aqueous solutions. The addition of cryoprotectant into solutions results in a significant increase of their osmolarity. For instance, at 2.0 M concentration of cryoprotectant, the osmolarity of a solution with EG, DMSO, or PROH is approximately more than 2,500 mOsm/kg, respectively [35]. Therefore, the addition and the removal of cryoprotectant from the oocytes create an osmotic imbalance across the oocyte membrane which may result in large volumetric changes (Fig. 1) and cause damages to the oocyte's morphology, cytoskeleton structures, and function.

Studies made with the animal model have reported that oocyte vitrification induces changes in histone H4 acetylation and histone H3 lysine 9 methylation (h3K9) [36]. Both the slow-freezing and vitrification methods differentially modify the gene expression profile of human metaphase-II oocytes [37]. Whereas a report based on a novel which paired randomized controlled trial using DNA fingerprinting technology indicated that the oocyte vitrification method does not increase the risk of embryonic aneuploidy or reduce the implantation potential of blastocysts created after intracytoplasmic sperm injection (ICSI). However, it is to be noted that the group of women that participated in this trial were relatively young and had normal ovarian reserves [38].

During the thawing or warming procedure, the transfer of the oocytes from a solution containing a high concentration of cryoprotectant to an isotonic solution can also lead to a reverse osmotic shock or over-swelling [39] and this may be lethal if the threshold tolerance is exceeded [40]. As a result, it is important to determine oocytes' tolerance to an osmotic stress in order to understand the functional role of a cryoprotectant during oocyte vitrification. If the oocyte is directly exposed to an isotonic solution, a swelling of the oocyte occurs because the inward diffusion of water by osmosis is more rapid than the outward cryoprotectant diffusion from the oocyte [41]. This phenomenon is also known as an osmotic swelling injury or an osmotic shock. An osmotic shock can be prevented by thawing the oocytes in a hypertonic solution containing a non-

Fig. 1 The morphological changes of human oocytes in equilibration solution (ES) and vitrification solution (VS). Normally, ES contained high concentration of cryoprotectants 7.5 % (v/v) ethylene glycol (EG) and 7.5 % (v/v) 1,2-propanediol (PROH) (or dimethyl sulfoxide, DMSO), and VS contained 15 % (v/v) 15 % EG and 15 % PROH (or DMSO) as well as 0.5 M sucrose in the base solution. The oocyte shrank almost 40 %

its volume in ES 1 min after transferred to ES (a–b), and recovered to normal shape within 5 min (c). The oocyte shrank almost 50 % its volume again within 1 min in VS (d), and at this stage, the oocyte was loaded onto the vitrification device for cryopreservation. Development of less cytotoxic vitrification solution with low concentration of cryoprotectant is the key issue in the field. Bar indicates the length of 60 µm

permeating cryoprotectant, such as sugar [42]. Various mono- and disaccharides with sucrose being the most common sugar have been used in thawing or warming solution as an osmotic counterforce in restricting water permeation into the oocyte (Fig. 2) and in preventing a swelling injury [43–46]. Nonetheless, the protective action of sucrose may be more complex and its performance in oocyte vitrification and thawing/warming needs to be investigated furthermore.

The key to the success of the vitrification of oocytes is to strike a balance between the use of a minimal concentration of cryoprotectant without compromising their cryoprotective actions. Interestingly, it has been reported that oocyte vitrification may not require a high concentration of cryoprotectant in the vitrification solution [47, 48]. Current oocyte vitrification protocols need to be refined for a less cytotoxic vitrification solution.

Cooling and warming rates for oocyte vitrification

The cryopreservation protocols for oocytes can be divided into: 1) slow-freezing/rapid thawing (cooling rates of 0.3–2°C/min); and 2) rapid cooling/warming or vitrification (cooling rates are believed to be at least >20,000 °C/min) [49]. Freezing induces precipitation of water into ice, leading to the separation of water from the dissolved substances. The presence of both intracellular ice crystals and a high concentration of solute can be lethal to the oocytes or embryos during the freezing procedure. Therefore, a slow-cooling rate has been proposed in order to maintain the delicate balance between ice crystallization, osmotic, and chilling damages [50].

The cooling rate is of 1°C/min from –5 to –9°C, and ice crystallization is induced by the process known as “seeding”, which results in a heterogeneous ice nucleation that is more stable than a super-cooling or homogenous nucleation. After seeding, the cooling rate is reduced to 0.3 to 0.5°C/min until a lower temperature is reached (usually between –30 and –150°C) and then the cells are stored in liquid nitrogen (LN₂).

Thawing is a rapid procedure with temperature changes that can exceed >360°C/min [51]. This procedure prevents the occurrence of re-crystallization, a process where water enters the oocytes or embryos and transforms into a solid state around previously formed small ice crystals. An alternative to prevent the formation of ice crystals is to transform the oocytes or embryos into a vitreous- or glass-like state by rapid freezing/thawing techniques [52]. Although this approach may improve the viability of the cells, a high concentration of cryoprotectant is required in order to prevent ice-crystal formation [44].

The definition of vitrification is a glasslike solidification of a solution at a low temperature. In another words, vitrification is the ice-free solidification of an aqueous solution. It is the concept that living cells can be successfully frozen if they are cooled so rapidly that ice crystallization does not occur [53]. Strictly speaking, a glass-like state also occurs when slow-freezing procedures are applied at the glass transition temperature (–130 °C). The mechanism of vitrification proposed is the use of a high concentration of cryoprotectant and an extremely rapid cooling/warming rates to pass through the glass transition temperature and avoid intra- and extracellular ice formation [54–58].

Fig. 2 The morphological changes of human oocytes in thawing solutions (TS). The shape of the oocyte did not change in TS-1 containing 1.0 M sucrose (a) in the base solution, but it swelled gradually in TS-2 containing 0.5 M sucrose (b) and TS-3 containing 0.25 M sucrose (c), respectively. Finally, the oocyte was recovered to its normal shape in TS-1 containing no sucrose (d). Whole thawing procedure may take 10–12 min in order to prevent suddenly swelling of the oocyte. This procedure could restrict water permeation into the oocytes and prevent swelling injury. Bar indicates the length of 60 µm

The majority in the field believe that the cooling rate is the most critical factor for oocyte vitrification. In order to accomplish this, a variety of devices have been used to obtain relatively high cooling rates. Initially, replacement devices, such as electron microscopy (EM) grid [11, 59], open pull straw (OPS) [60, 61], nylon mesh [62], cryoloop [63], or plastic sticker [64] have been employed to plunge the devices into LN₂ directly. Subsequently, commercial devices, such as Cryotop [49, 64] and Cryoleaf [13, 19, 20] have been developed and considered as the vitrified samples as they were able to obtain >20,000 °C/min cooling rate through direct contact with LN₂ [49]. However, many concerns about the direct contact with LN₂ have been raised with these vitrified samples [65–68].

Bacteria and fungi contaminations of stored semen and embryos that were in direct contact with LN₂ have been reported [65–68]. Interestingly, there was an idea that the vitrification procedure could be performed with a sterilized LN₂ [69] and that could store the vitrified oocytes with vapour LN₂ [70, 71] to prevent contaminations that occurred by the direct contact of LN₂ during the cryo-storage. But the main issue remains to be whether or not it is absolutely necessary for the vitrified samples to be in direct contact with LN₂ in order to achieve the essential cooling rate for vitrification.

Until recently, it was unknown as to what the optimal cooling and warming rates with a very small amount (<1.0 µl) of solution and samples should be [72]. Recent studies conducted with a mouse model indicated that the lethality of a slow warming process is a consequence of the growth of small intracellular ice crystals by re-crystallization [73, 74]. This means that the cooling rate is of less consequence than the warming rate during vitrification-warming procedures [75]. Interestingly, the authors of such studies have also demonstrated that a very high warming rate, as opposed to a high cooling rate, was the essential element to survival of the oocyte during vitrification-warming procedures [47]. The collected data also indicated that the oocytes can be vitrified successfully with a relatively slow cooling and warming rates (Fig. 3) while having high survival and pregnancy rates [28, 48], suggesting that further understanding of the principle of vitrification is required in the field.

Vitrification of immature or mature oocytes

Although the first human pregnancy and live birth reported from cryopreserved oocytes has been a result of a slow-freezing procedure [76], most of the early records on human oocyte cryopreservation using this method showed dismal survival and pregnancy rates [77–79]. With spindle damage or chromosome mal-alignment as a possible mechanism for the inefficiencies associated with the slow-freezing of mature metaphase-II (M-II) stage oocytes, it has been proposed that

Fig. 3 The cooling curve of the oocyte vitrification without direct contact liquid nitrogen (LN₂). **a** The cooling rate was approximately 442–500 °C/min of the inside of JY Straw when it plunged into LN₂ from 25 to –196 °C required approximately 26–30 s. **b** The warming curve for oocyte thawing. The increase of temperature from –196 to 25 °C required approximately 4–5 s. Therefore, the warming rate was approximately 2,210–2,652 °C/min. It is important to know whether or not the direct contact with LN₂ is absolutely necessary for the vitrified samples in order to achieve the essential cooling rate for vitrification. The information indicates that the optimal cooling and warming rates with a very small amount (<1.0 µl) of solution and samples could be the relatively slow rates, not as to >20,000 °C/min. Reproduced from Wang et al. [48] with permission

the immature oocytes at a germinal vesicle (GV) stage may fair better with the slow-freezing procedure [80]. Theoretically, the risk of polyploidy and aneuploidies can be prevented with the cryopreservation of an immature GV stage oocytes because the chromatids are diffused in the diplotene state of prophase I surrounded by a nuclear membrane of GV that may avoid the spindle de-polymerization [81, 82].

Difficulties still exist with in vitro maturation (IVM) of GV stage oocytes after freezing and thawing procedures. Although the survival rates seem to have improved, poor IVM

and fertilization are major problems associated with an immature oocyte freezing [83–86]. With the development of the vitrification method, there is no significant difference in the survival rate between oocytes vitrified at the immature GV stage and those vitrified at the mature M-II stage [87]. From the few studies that have explored the question, the evidence suggested that oocytes should be vitrified at the mature M-II stage following IVM rather than at the immature GV stage [88–91]. To date, only a few live births resulting from the cryopreservation of oocytes at the immature GV stage [92] or after IVM have been reported [19, 20].

Duration of oocyte storage

Theoretically, the oocytes stored in LN₂ should be relatively safe without any obvious undesirable biological and metabolic changes in the ooplasm. It is generally believed that the biological activities of cells would be stopped completely when they are stored at the temperature of LN₂ (−196°C). So far, most studies examining a prolonged period of storage have been focused on the quality of embryos cryo-stored at −196°C, indicating that the quality of embryos is not influenced by the duration during cryo-storage [93–95]. Other studies have also indicated that a long-term cryo-storage of human embryos did not affect the pregnancy outcome [96, 97]. Surprisingly, an early study has indicated that the pregnancy rate was higher when the embryos were cryo-stored in LN₂ for 1–2 months compared to the embryos that were cryo-stored for more than 2 months [98].

Most of the reports on oocyte cryopreservation so far have been of a relatively short duration in LN₂ before warming. Recently, there has been a report of a live twin birth after IVF of oocytes that were cryopreserved using a slow freezing method in a low sodium medium for almost 12 years [99]. It has been reported that human oocytes can be safely cryopreserved for several years by a slow freezing method with a plastic straw, based from the results of live birth rates of oocytes stored in LN₂ [100]. It seems that the longest storage of human oocytes in LN₂ after vitrification will be of more than 5 years and less than 10 years, but the evidence provided by those reports do not lend support for the practice of oocyte cryopreservation in long-term cryo-banking requiring a long duration of cryo-storage [101].

Regarding cryo-storage systems, especially those related to the vitrification method, there are two storage systems, ie, ‘closed’ and ‘open’ systems to store oocytes or embryos in LN₂ after vitrification. The ‘closed’ system refers to the oocytes that do not come into direct contact with LN₂ during storage, and the ‘open’ system refers to the oocytes that come into direct contact with LN₂ during storage. Many studies have shown that an oocyte vitrification with the ‘open’ system does not have a detrimental effect on the subsequent

fertilization, embryo development, and clinical pregnancy rates [12–22, 102]. However, there is limited information available about the effects on the duration of the oocyte cryo-storage following an ‘open’ system on the subsequent cryo-survival, fertilization, and embryonic development. It has been reported that the vitrified oocytes stored in a vapour-phase LN₂ storage freezer for up to 10 months do not adversely affect the oocyte cryo-survival, fertilization, embryonic development as well as the clinical pregnancy rates compared to a traditional LN₂ tank storage when the ‘open’ vitrification system was applied. This suggests that the vapour-phase LN₂ storage system may eliminate the concern about the risk of direct-contact with LN₂ during storage [21].

Other than the concerns about the risk of contamination from bacteria and fungi during the storage in direct-contact with LN₂, it has been reported, with a mouse model, that the cryo-survival, fertilization rate and embryonic development of the vitrified oocytes are affected significantly, in an adverse manner, by the cryo-storage duration in LN₂ [103]. It is unclear whether or not this is due to the direct-contact of the oocytes to LN₂ during storage. It is commonly believed that there is no thermally driven reactions that occur in cells in aqueous systems at −196 °C. Nevertheless, it has been reported that many important photo-physical events or chemical syntheses occurred to produce some form of hazards in LN₂ [104]. With the result of photo-physical events in LN₂, it has been discovered that direct ionizations from background irradiation or cosmic rays are a source of damage at such low temperatures. This damage occurs as a consequence of the formation of free radicals and the production of breaks in macromolecules. Due to the rapid decomposition of ozone (O₃), ionizing radiation on oxygen dissolved in LN₂ causes the formation of free radicals [105]. Several evidences indicated that oxides of nitrogen are formed in LN₂, which enhances the yield of ozone by a catalytic effect [106, 107]. Ozone production may deleterious damage DNA and cause DNA breaks since an enzymatic repair cannot occur in LN₂ [108].

Many factors could affect the outcome of vitrified human oocytes, such as the age of the women, duration of infertility, and different stimulation protocols. As such, it would be premature to draw a solid conclusion that the cryo-storage duration affects the subsequent cryo-survival, fertilization, and embryonic development. As discussed previously, there are many potential risks attributed with the direct contact of vitrified oocytes into LN₂. Further study is required to confirm whether the cryo-storage duration could affect subsequent oocytes survival, fertilization as well as pregnancy rates after warming.

Obstetric and long-term outcome with vitrified oocytes

Although the number of live births from cryopreserved oocytes has increased rapidly over the past decade, this

technological development must be accompanied by data concerning obstetric and perinatal outcomes as well as a long-term development evaluation of the infants. At the present, the clinical safety issue with vitrified oocytes cannot be completely assessed because of the lack of well-controlled clinical trials. One preliminary data study analyzed the obstetric and perinatal outcomes of 165 pregnancies and 200 infants conceived following oocytes vitrification [18]. The average birth weight and the incidence of congenital anomalies are comparable to that of infertile women undergoing IVF treatment or to spontaneous conceived fertile women, suggesting that conceived pregnancies and infants born from oocytes vitrification are not associated with the increased risk of adverse obstetric and perinatal outcomes. It has to be noted that this preliminary data was not from a well-designed, randomized, and controlled trial.

In addition, a study analyzing a total of 58 reports from 1986 to 2008, which included 936 live born babies (308 from slow-freezing, 616 from vitrification and 12 from both methods), indicated that 1.3 % have been noted to have birth anomalies [109]. It is comparable with the prevalence of major birth defects (MBD) with the spontaneous conception per birth ranged between 0.2 % and 2 % in the individual registries reported to International Clearinghouse for Birth Defects Surveillance and Research (ICBDSR) [110]. It has been estimated worldwide that more than several thousands babies have been born from cryopreserved oocytes. Italy alone has approximately 2,000 babies born from cryopreserved oocytes to date with no reported increases in birth anomalies (personal communication with Dr. Giulia Scaravelli, Istituto Superiore di Sanita, Rome, Italy). However, there are limitations in the safety literature to date due to the lack of data in women of more advanced reproductive age and to the lack of data in oocytes cryopreserved for a long period of storage. More importantly, most of the published data have been performed in clinics with the highest success rates of donor eggs. Thus, these information may not be generalizable to the field of reproductive medicine.

Recently, an analysis of molecular genetics in animal models has suggested that the generation of live offspring from the vitrified oocytes was different compared to naturally conceived offspring [111]. Therefore, apart from the standard of vitrification protocol with minimal concentration of cryoprotectant and proper cryo-storage method in LN₂, the questions still remain to be further addressed on whether or not oocyte vitrification can ensure the adequate safety issue, especially for the long-term development evaluation of the infants.

Conclusions

Vitrification technology has made significant advancements and holds great promise, but many issues need to be addressed

before it becomes the standard clinical laboratory procedure. The first issue is the use of a lower concentration of cryoprotectant without a compromise on the effects of the cryoprotective action. The challenges at the present are to develop less cytotoxic vitrification solutions. The second issue is to find an optimal cooling and warming rate with an appropriate vitrification device. The third question is to confirm whether or not the cryo-storage duration affects the subsequent cryo-survival. Lastly, the fourth matter is to further evaluate infants born from the vitrified oocytes in order to ensure safety of the vitrification technology.

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