Slow Freezing or Vitrification of Oocytes: Their Effects on Survival and Meiotic Spindles, and the Time Schedule for Clinical Practice

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SUMMARY

Both the slow-freezing method with increased sucrose concentration and new vitrification techniques significantly improve the results of cryopreservation of human oocytes. The recent perfection for vitrification includes the concepts of increase of cooling and warming rates using minimum volume methods, and decrease of toxicity by reducing the concentration of cryoprotectants. In the recent literature, the survival of cryopreserved oocytes ranged from 74% to 90% using the slow-freezing method and from 84% to 99% by vitrification. Overall, the survival rate of oocytes from vitrification (95%, 899/948) appeared higher than that of the slow-freezing method (75%, 1,275/1,683). The microtubules of meiotic spindles are vulnerable to the thermal changes and will depolymerize. After incubation, the microtubules repolymerize. Spindle recovery is faster after vitrification than slow freezing. Even so, after 3 hours of incubation, spindle recuperation is similar between vitrification and slow freezing. Considering both aspects of spindle recovery and oocyte aging, the time schedule for oocyte cryopreservation program makes fertilization in the optimal time. Intracytoplasmic sperm injection is performed for oocytes at 3 hours of post-thaw incubation from the slow-freezing method and 2 hours from vitrification, with restoration of meiotic spindles. The pregnancy potential of cryopreserved oocytes is comparable to that of fresh oocytes or frozen embryos. Cryopreservation of oocytes would importantly contribute to oocyte donation and preservation of fertility for cancer patients. [*Taiwan J Obstet Gynecol* 2009;48(1):15–22]

Key Words: meiotic spindle, oocyte cryopreservation, vitrification

Introduction

The cryopreservation of oocytes is valuable for the treatment of infertility. Oocyte cryopreservation has wider clinical implications than embryo freezing [1]. Women who have no partner or are about to lose their ovarian function because of surgery, chemotherapy or radiotherapy could store their oocytes for future use [2–4]. It also provides an alternative to embryo preservation to avoid ethical issues and legal restrictions [5]. For patients undergoing *in vitro* fertilization, freezing the excess oocytes could avert repeated oocyte retrieval from the patients themselves or be a source for oocyte donation [6]. This is especially important in countries that authorize donation of oocytes, but not embryos, to infertile couples.

Cryopreservation of human oocytes has been significantly improved by both the slow-freezing methods with increased sucrose concentration and new vitrification techniques [7–11]. The slow-freezing method using a programmed cryo-machine is traditionally employed for the cryopreservation of oocytes [12]. These procedures usually take several hours. Vitrification with higher concentrations of cryoprotectants and a fast cooling rate, it transforms cells into an amorphous glassy state without ice crystal formation [13]. Vitrification is time-saving and does not require special equipment. Minimum volume methods with less concentrated vitrification solution have replaced the conventional straw

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method [9–11,14,15]. The superiority regarding survival of oocytes, effect on the meiotic spindles, and pregnancy by the slow-freezing method or vitrification deserves further investigation.

The Slow-freezing Method with 1.5M 1,2-Propanediol (PROH) and 0.3M Sucrose Increases Survival of Oocytes

Using the same freezing solution with 1.5M PROH and 0.1M sucrose of the slow method, mature human oocytes had lower survival rates of 25–34% than do embryos of 75–80% [16,17]. The cytoplasmic membranes of oocytes, which have fewer submembranous actin microfilaments, are more fragile to cryopreservation [18]. The volume-to-surface ratio of oocytes is greater, making the dehydration process more difficult. Porcu et al [19] used the regime of 1.5M PROH with a higher sucrose concentration of 0.2M and obtained a 59% post-thaw survival rate. Fabbri et al [7] showed that increasing the sucrose concentration to 0.3M and exposing oocytes for 15 minutes to cryoprotectants yielded a higher oocyte survival rate of 82%. The regime is thought to dehydrate the oocytes more adequately and reduce intracellular ice formation.

Vitrification Leads to Significant Improvement in the Survival of Oocytes

Another important method to improve the survival of cryopreserved oocytes is vitrification. The recent improvements for vitrification include the concepts of increase of cooling and warming rates using minimum volume methods and decrease of toxicity by reduction of concentration of cryoprotectants [20,21].

Minimum Volume Methods Replace Conventional Straws for Vitrification

A 0.25-mL conventional straw was initially used for vitrification of oocytes. The cooling rate was around 2,500°C/minute and the warming rate was 1,300°C/minute [13]. The limited speed of thermal change of the conventional straw needs more concentrated cryoprotectants to achieve vitrification during cooling and to prevent devitrification during warming. The higher concentrated cryoprotectants are more toxic to oocytes.

Using minimum volume methods, a higher cooling rate can facilitate vitrification with less concentrated cryoprotectants, and a higher warming rate will prevent devitrification. In addition, the high speed of cooling and warming of minimum volume methods can rapidly pass through a damaging temperature zone liable for chilling injury between 15°C and −15°C [22]. The chilling injury harms mainly the cytoplasmic lipid droplets and meiotic spindle of oocytes.

Landa and Tepla [23] performed vitrification by dropping the mouse embryos directly into the liquid nitrogen. But to form a drop needs a relatively large amount of solution (approximately 5 μL). When the drop reaches the liquid nitrogen, it will not sink immediately but float on the surface for several seconds. The drop leads to a strong evaporation at its surface that decreases the cooling rate. Besides, it is difficult to find the embryos for performing thawing.

Further improvements using various carriers to minimize the volume of vitrification solution and to submerge the sample swiftly into the liquid nitrogen were broadly studied. Steponkus et al [24] first utilized electron microscopic copper grids as a carrier for the minimum volume–direct contact approach. They successfully cryopreserved chill-sensitive *Drosophila* embryos by vitrification. Martino et al [22] applied this technique for bovine oocytes, and achieved higher growth potential than using conventional straws. Hong et al [25] and Yoon et al [26] achieved successful pregnancies from vitrified human oocytes using the grid method.

Arav [27] reported the other minimum drop size method that a small droplet of vitrification solution containing oocyte was placed on a thin glass surface. That was then immersed into liquid nitrogen to achieve vitrification. They observed that the minimum droplet (0.5 μL) of less concentrated vitrification solution can prevent ice crystal formation and fracture injury [28].

Vajta et al [29] developed open pulled straws (OPS) to hold bovine oocytes with a small amount of vitrification solution (1 μL). The idea was to reduce the volume of the sample using a pulled straw. The straws were heated and pulled by hand, then cut at the tapering end. As the result, the diameter and the wall thickness of the straw decreased to approximately half of the original. The cooling and warming rates are higher for OPS (16,700°C/minute and 13,900°C/minute, respectively) than the conventional straw. They found that OPS achieved better results than conventional vitrification. Kuleshova et al [30] applied OPS for vitrification of human oocytes and achieved a successful pregnancy.

Lane et al [31] first used cryoloops (Cryoloop; Hampton Research, Laguna Niguel, CA, USA) for vitrifying human blastocysts. The device of a Cryoloop consists of a small nylon loop attached to a holder and equipped with a vial as a container. Oocytes or embryos are suspended on a film of vitrification solution, bridging

the hole of the loop that is then plunged into liquid nitrogen. The size of droplet in the Cryoloop is limited by this special design.

Vanderzwalmen et al [32] developed the hemi-straw system in which the carrier was a cut open straw. Kuwayama and Kato [33] developed the Cryotop (Kitazato Supply Co., Fujinomiya, Japan) method using a fine polypropylene strip for a minimum volume method. With the Cryotop, the achievable cooling rate and warming rate were significantly increased and were 23,000°C/minute and 42,100°C/minute, respectively [34]. Chian et al [10] modified the Cryotop to be the Cryoleaf (MediCult, Jyllinge, Denmark) with a different way to apply the protection sheath. Both Cryotop and Cryoleaf achieved a high success rate in vitrification of human oocytes [9,10].

However, the direct contact system may have the problem of potential contamination from liquid nitrogen. Chen et al [35] modified the loading of OPS to into a closed system, called closed pulled straws (CPS). CPS had the characteristics of OPS as a rapid thermal change method, and of conventional straws as being a non-contact mode. The vitrification medium containing oocytes was isolated by two small segments of air and medium. Through this closed loading system of CPS, the oocytes will not directly contact with liquid nitrogen, which may occur with OPS. Kuwayama et al [34] developed a Cryotip (Kitazato Supply Co., Fujinomiya, Japan) method. Basically, it was a heat-sealed pulled straw technique. The speed of cooling and warming was smaller in Cryotip than Cryotop. But the thermal speed accompanied by the Cryotip was still high enough to obtain adequate vitrification. The use of the closed system may eliminate the possible contamination. The efficacy of CPS or Cryotip deserves further investigation.

Using Less Concentrated Cryoprotectants for Vitrification Reduces Toxicity

Rall and Fahy [13] first successfully vitrified mouse eight-cell embryos using conventional straws with the medium consisting of 20.5% (w/v) dimethyl sulfoxide (DMSO), 15.5% (w/v) acetamide, and 10% (w/v) propylene glycol and 6% (w/v) polyethylene glycol. The treatment of vitrification solution for embryos must be performed at a low temperature of 4°C. Ali and Shelton [36] developed an ethylene glycol (EG)-based vitrification solution consisting of 5.5M EG and 1.0M sucrose for reducing toxicity, which permitted the equilibration steps to be performed at room temperature. Chen et al [14] used this formulated solution for vitrification of human oocytes and attained high survival rates by conventional straws.

EG, with the characteristics of low toxicity and rapid permeation of the cell, is an important component of vitrification solutions. Some authors mixed other permeating agents, such as DMSO or PROH, to reduce the concentration of single cryoprotectant, which may decrease the individual specific toxicity [10,37]. Non-permeable cryoprotectants, such as sucrose, can facilitate dehydration and vitrification, which reduce the required concentration of permeable cryoprotectants [21]. Recently, the less concentrated vitrification solution consisting of 15% (v/v) EG, 15% (v/v) DMSO or PROH, and 0.5M sucrose can be vitrified with the minimum volume method [10,37]. This strategy further reduces the toxicity of vitrification solution.

Treatment of Cryoprotectants with Stepwise Equilibration at Room Temperature

Another important strategy to reduce toxic effects from vitrification solution is the stepwise equilibration of cryoprotectants. A two-step strategy is mainly used [21]. The pretreatment solution contains 20–50% concentrations of cryoprotectants of the vitrification solution. The lower concentration of cryoprotectants in the pretreatment solution is much less toxic than the vitrification solution. Oocytes in the pretreatment solution shrink initially and gradually re-expand to their original volume. This observation indicates the entry of the permeating cryoprotectants into the oocytes. It reduces the time needed for exposure to the subsequent vitrification solution that is more toxic for oocytes. In the vitrification of human oocytes, it has been demonstrated that oocytes pretreated with equilibration solution had a significantly higher survival rate than those without pretreatment [14]. One-step vitrification without pretreatment has the possibility of insufficient permeation of the cryoprotectants, which may result in intracellular ice formation during cooling or warming.

For oocyte vitrification, some investigators perform exposure to cryoprotectants at room temperature [9, 10,14], but other investigators operate the procedures at 35–37°C [26,29]. The higher temperature enhances the passage of the permeating cryoprotectants across the cell membrane, but the toxicity is also increased. Therefore, at 37°C, 2–3 minutes are used for the pretreatment solution, and 20–30 seconds are exposed for the vitrification solution [26,29]. In contrast, at room temperature, 5–15 minutes are used for the pretreatment solution, and 30–60 seconds are exposed for the vitrification solution [9,10,14]. Prolonged exposure to the concentrated cryoprotectants may induce a toxic effect.

The human oocytes treated in vitrification solution for 120 seconds had a poorer fertilization outcome than those vitrified in 60 seconds [14].

Warming at 37°C with Stepwise Dilution

The speed of warming is important to prevent devitrification. Minimum volume methods, such as electron microscopic grid, Cryoloop or Cryotop, can be directly submerged into the dilution medium. The oocytes contact with the dilution medium immediately. The vitrified oocyte is sensitive to osmotic changes after warming. A stepwise dilution with sucrose medium as osmotic buffer is commonly used to prevent excessive swelling as the permeating cryoprotectants leave. Most investigators performed warming at 37°C and dilution at room temperature [11,15]. Dilution at 37°C may lessen spindle damage during the procedures, which deserves further study [38]. Two-to-three step dilutions with sucrose solutions were usually used, and the effects may merit further investigation.

The Cryotop Method for Vitrification of Oocytes

The Cryotop developed by Kuwayama and Kato [33] is now commonly used as a carrier for vitrification of oocytes, and the procedures are described as follows. Oocytes were equilibrated in 7.5% (v/v) EG and 7.5% DMSO in TCM199 (tissue culture medium 199) medium with 20% synthetic serum substitute at room temperature for 15 minutes. They were then placed into vitrification solution of 15% EG and 15% DMSO with 0.5M sucrose. After 1 minute in this solution, oocytes were placed on the Cryotop. The Cryotop has a fine polypropylene strip (0.4 mm wide × 20 mm long × 0.1 mm thick) (Kitazato Supply Co., Fujinomiya, Japan) attached to a plastic holder and equipped with a protective plastic tube [9]. Oocytes are loaded on the strip with minimal solution (0.1 μL), and the solution is further almost completely removed by aspiration. The Cryotop is immersed into filtered liquid nitrogen. Then, the strip is covered with the plastic tube in liquid nitrogen to protect it during storage. For warming, the protective cover is removed from the Cryotop while it is still submerged in liquid nitrogen. The strip is immersed directly into the dilution solution of 1.0M sucrose solution for 1 minute at 37°C. The thawed oocytes were transferred to 0.5M and 0.25M sucrose solutions for 3 minutes at room temperature, and then washed twice with culture medium.

Liquid Nitrogen Slush Increases Cooling Rate but Not Warming Rate

For enhancing the cooling rate and vitrification, one approach is to employ liquid nitrogen slush instead of liquid nitrogen. Nitrogen slush can be produced from liquid nitrogen by using vacuum, in which part of the liquid nitrogen evaporates and the rest of it cools down. The mixture of nitrogen slush and cooled liquid nitrogen is cooled to −205°C. It is commercially available with the construction of the Vit-Master (IMT, Israel) [39]. The concentration of cryoprotectants may be lowered using liquid nitrogen slush, which merits further study. However, the speed of warming may not be changed to prevent devitrification. Besides, most investigators achieve satisfactory results of vitrification of human oocytes using liquid nitrogen only [9–11]. Nonetheless, Cai et al [40] found that the faster cooling rate with Vit-Master had fewer adverse effects on the spindle configuration of vitrified rabbit oocytes than using liquid nitrogen only. The superiority of the Vit-Master compared with liquid nitrogen only still needs further elucidation.

Injury and Recovery of Meiotic Spindles of Frozen–Thawed Oocytes from Slow Freezing or Vitrification

The meiotic spindles of oocytes consist of microtubules that are constructed by polymerization of tubulin dimmers of α- and β-tubulin. Microtubules start from microtubular organizing centers at both poles and anchor chromosomes at the kinetochores. The chromosomes align at the equatorial plane of the meiotic spindles. The tubulin dimmer would polymerize and depolymerize at various stages of a cell cycle. The meiotic spindles are crucial for the events following fertilization in the completion of meiosis, second polar body formation, migration of the pronuclei, and formation of the first mitotic spindle [41].

The spindle is very sensitive to cryoprotectants and low temperature. Oocytes analyzed immediately after thawing displayed severe disorganization or disappearance of spindles using slow or vitrification methods [35,42,43]. Incubation for 1–3 hours at 37°C resulted in recovery of spindles in various degrees, which were dependent on time intervals after thawing and the freezing methods (Table 1).

In vitrified mouse oocytes, Chen et al [43] observed that post-thawing incubation for 1 hour allowed recovery of normal spindle and chromosomes to diverse degrees. The OPS, CPS, and electron microscopic grids

Table 1. Effect of various methods of cryopreservation on the meiotic spindles of oocytes and their recovery

Freezing methods	Oocytes	Examination	Results	Authors
Vitrification	Mouse	Immunostain	OPS preserve better spindle than conventional straws	Chen et al [43]
Vitrification	Mouse	Immunostain	After 2 or 3 hr of incubation, the spindle patterns significantly better than 1 hr	Chen et al [35]
Vitrification	Bovine	Immunostain	Cryotops preserve better spindle than OPS	Morato et al [44]
Slow	Human	Immunostain	60% thawed oocytes with normal spindles after 1 hr of incubation, 81% for controls	Gook et al [18]
Slow	Human	Polscope	Spindle disappeared after thawing, but all reappeared by 3 hr of incubation	Rienzi et al [45]
Slow	Human	Immunostain	0.3M sucrose in the freezing solution preserves spindle better than 0.1M	Coticchio et al [46]
Vitrification *vs.* slow	Human	Polscope	Spindle recovery faster in vitrification than in slow freezing	Ciotti et al [38]
Vitrification *vs.* slow	Human	Immunostain	After 3 hr of incubation, comparable spindle recovery from vitrification and slow freezing	Cobo et al [15]

OPS = open pulled straws.

preserved the spindle morphology and chromosomal pattern better than conventional straws. The rapid thermal change of minimum volume methods may quickly traverse the temperature that is damaging to the spindle, assumed to be 15°C to −15°C [22]. Moreover, oocytes of minimum volume methods are directly warmed in the dilution solution and immediately diluted. This reduces exposure of oocytes to inappropriate temperatures and concentrated cryoprotectants [21]. Chen et al [35] further demonstrated that incubation for 2 or 3 hours resulted in higher percentages of normal spindles than 1 hour. Morato et al [44] found that cryotops achieved better spindle preservation than OPS for vitrification of bovine oocytes.

For human oocytes with slow cryopreservation, Gook et al [18] noticed that 60% of oocytes were composed of normal spindles at 1 hour of incubation after thawing, compared with the control specimens of 81%. Using a computer-assisted polarization microscopy (PolScope, SpindleView; CRi, Woburn, MA, USA), Rienzi et al [45] observed the disappearance of the spindle in all of the oocytes after thawing and washing. The spindle reappeared by 3 hours of incubation. Coticchio et al [46] found that spindles were significantly affected after the slow-freezing method using 0.1M sucrose concentration, while they were unchanged using the 0.3M sucrose protocol. Ciotti et al [38] reported that spindle recovery was faster in vitrification than in slow freezing. Cobo et al [15] found comparable spindle recovery from vitrification and slow freezing after 3 hours of incubation.

Time Schedule for Freezing/Thawing Oocytes and Fertilization: Considering Both Aspects of Spindle Recovery and Oocyte Aging

The changes and recovery of the spindles from freezing and thawing have been linked to the functional effects of oocytes on fertilization and development [35,42]. Eroglu et al [42] observed that slowly cryopreserved mouse oocytes inseminated immediately after thawing exhibited impairment of the spindle rotation, second polar body formation, and an increased rate of digyny. Chen et al [35] found that the percentages of fertilization and blastocyst formation of vitrified mouse oocytes inseminated immediately or at 1 hour of incubation were significantly lower than those of the control specimens, but they were improved when inseminated at 2 or 3 hours of incubation.

The mature oocyte maintains at the metaphase of meiosis II with organized spindle system. After fertilization by entry of a spermatozoon, the intracellular calcium increases and the cytostatic factors decrease [47]. The oocyte completes meiosis II and extrudes the second polar body. The mature oocyte must be fertilized at the adequate time. Otherwise, it will undergo apoptosis. Delayed insemination of mature oocytes results in compromised embryos [48]. Dozortsev et al [49] observed that the optimal time of intracytoplasmic sperm injection (ICSI) for human oocytes was from 37 to 41 hours after administration of human chorionic

gonadotropin (hCG). Those fertilized oocytes achieved the highest implantation rate.

Therefore, choosing the optimum time for fertilization, considering both aspects of spindle recovery and oocyte aging, is important for a successful oocyte cryopreservation program. The oocyte retrieval is usually performed 35 hours post-hCG. Cryopreservation of oocytes is performed at 2 hours after oocyte retrieval. For the slow-freezing protocol, the equilibration time before freezing and the dilution time after thawing take approximately 1 hour. ICSI is performed at 3 hours post-thaw (post-hCG 41 hours) [1,8]. For the vitrification method, the equilibration and dilution is around 10 minutes, which could be negligible. ICSI is usually performed at 2 hours post-thaw (post-hCG 39 hours) [11,15]. Therefore, the timing of insemination for frozen–thawed oocytes is in an optimal interval.

Superiority of the Slow-freezing Method or Vitrification

The slow-freezing method with a higher concentration of sucrose (0.3M) or minimum volume vitrification using Cryotop or Cryoleaf with less concentrated vitrification solution has been mainly used in *in vitro* fertilization centers. We reviewed the results of the two methods reported in the recent literature, which are summarized in Table 2. The survival of cryopreserved oocytes using the slow-freezing method ranged from 74% to 90% [5,6,8,50,51] and ranged from 84% to 99% for the vitrification method [9–11,15,52]. Overall, the survival rate of oocytes from vitrification (95%, 899/948) appeared greater than that of the slow-freezing method (75%, 1,275/1,683). The pregnancy rate ranged from 9% to 75% for the slow-freezing method and 33% to 57% for vitrification. However, the case number was small in most of the series. There are still no prospective randomized control studies to compare the survival, implantation potential and pregnancy rates of these two methods, which may deserve further investigation [53].

Comparable Results of Cryopreserved Oocytes to Fresh Oocytes and Frozen Embryos

In our center, we use the protocol of 1.5M PROH and 0.1M sucrose for cryopreservation of pronuclear or 2–8-cell embryos [54]. The embryos cryopreserved at the pronuclear stage were randomly allocated, unlike those at the cleavage stage which are usually frozen after selection for fresh transfer of those with better morphology. The percentages for survival, pregnancy and implantation for frozen–thawed pronuclear embryos were similar to those of frozen–thawed oocytes [8]. These findings were consistent with the results from Boldt et al [55]. In addition, Yang et al [56] found that the outcome parameters from thawed oocytes and embryos derived from sibling oocytes were also similar. Recently, Cobo et al [15] reported that oocyte vitrification provides a similar pregnancy rate to that obtained using fresh oocytes.

Conclusion

Significant improvement of cryopreservation of oocytes has been achieved by both slow freezing and vitrification. The minimum volume vitrification facilitates increase of cooling and warming rates, reduction of concentration of cryoprotectants, and recovery of meiotic spindle. Vitrification appears to have a higher survival rate than slow freezing. Considering the effects of both oocyte aging and spindle recovery, the proposed time schedule for oocyte cryopreservation program allows for fertilization in the optimal time. Because of comparable survival and pregnancy rates, oocyte freezing provides an alternative to embryo freezing for couples with religious or

Table 2. Survival and clinical pregnancy of cryopreserved oocytes from slow freezing (0.3M sucrose) and vitrification (Cryotop or Cryoleaf)*

Slow freezing			Vitrification†		
Survival	Pregnancy	Authors	Survival	Pregnancy	Authors
119/159 (75)	7/21 (33)	Chen et al [8]	58/64 (90)	12/29 (41)	Kuwayama et al [9]
73/81 (90)	7/15 (47)	Li et al [6]	169/180 (94)	7/15 (47)	Chian et al [10]
687/927 (74)	18/201 (9)	Borini et al [50]	120/143 (84)	13/23 (57)	Lucena et al [11]
68/79 (86)	3/4 (75)	Barritt et al [51]	328/330 (99)	39/120 (33)	Antinori et al [52]
328/437 (75)	16/83 (19)	Parmegiani et al [5]	224/231 (97)	11/23 (48)	Cobo et al [15]

**Data are presented as n (%); †Chian et al [10] used Cryoleaf, and the other authors used Cryotop.*

ethical concerns. Cryopreservation of oocytes would meaningfully contribute to oocyte donation and preservation of fertility in patients about to lose ovarian function.

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