A review of best practices of rapid-cooling vitrification for oocytes and embryos: a committee opinion

Practice Committees of the American Society for Reproductive Medicine and Society of Reproductive Biologists and Technologists

The focus of this paper is to review best practices for rapid-cooling cryopreservation of oocytes and embryos. The discussion of best practices includes the types of cryoprotectants and cryo devices typically used. Key performance indicators of rapid-cooling vitrification success are defined. (Fertil Steril® 2021;115:305-10. ©2020 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

DEFINITIONS

Embryo and Oocyte Warming. Commonly but incorrectly referred to as "thawing" or "rewarming," refers to the relatively rapid increase in temperature of cells stored in liquid nitrogen to room temperature or above under defined laboratory conditions (1, 2).

Rapid Cooling: A reduction in temperature, typically at rates of more than −2,500°C/min, before storage in liquid nitrogen at −196°C. Commonly referred to in the literature as "vitrification."

Slow Cooling. A gradual reduction in temperature, typically at rates of −0.1 to −3°C/min, to −30°C or lower before storage in liquid nitrogen at −196°C. Ice crystal formation occurs extracellularly.

Slow Freezing. A misnomer for slow cooling that implies the presence of intracytoplasmic ice crystals, which ideally does not happen.

Vitrification. Formation of an amorphous solid or glass-like state (noncrystalline). Vitrification depends on cooling rate and solution composition and can occur with both slow and rapid cooling.

HISTORY OF GAMETE AND EMBRYO CRYOPRESERVATION

In 1985, a conventional embryo cryopreservation method using 1, 2-propanediol (PROH) as a cryoprotectant and a programmed slow-cooling method was reported (3). Successful pregnancies were achieved from slow-cooled human embryos but rarely achieved from slow-cooled human oocytes. The first report of a successful pregnancy using cryopreserved oocytes with a slow-cooling and rapid-warming method was in 1986 (4). Additional studies of oocyte cryopreservation were reported, but the overall efficiency of these protocols remained low. The technique came under scrutiny when it was suggested that cryopreserved oocytes showed higher levels of chromosomal anomalies compared with fresh oocytes (5–7), thus tempering enthusiasm for the technology. In the 9 years that followed the first reported pregnancy, a total of only five births from cryopreserved-warmed oocytes were reported (8–10). Subsequent research determined that there was no increase in aneuploidy after oocyte cryopreservation (11, 12). In 1998, the first baby was born from a cryopreserved immature oocyte (13).

Improvements of culture media and laboratory techniques led to a resurgence of research toward improving oocyte and blastocyst cryopreservation (14–18). In 1998, a landmark publication described the use of an open pulled straw (OPS) and ethylene glycol and dimethyl sulfoxide (DMSO) that allowed for a minimum volume of 1–2 μL of medium to be used for cryopreservation of bovine ova (19). The combination of cryoprotectants, rapid-cooling rate (greater than −10,000°C/min), and small volume with an "open" device allowed the cells to survive plunging into liquid nitrogen from room temperature. Rapidly cooled–warmed oocyte survival rates of >90% were seen and live births reported (20–22).

PRINCIPLES OF VITRIFICATION

The idea of vitrification, achieving a glass-like state, was first described in 1860, and then again in 1937 (23). It was not until nearly 50 years later, in 1985, that rapid cooling from above-zero temperatures was described as a potential alternative to slow cooling (24). As the temperature of a liquid decreases to below the glass-transition temperature, molecules remain in the disordered pattern of a liquid. However, the physical properties become more similar to those of a rigid solid.

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Molecules become locked in place as though the liquid were frozen in time. The resulting "solid liquid" is called a glass or an amorphous glass-like state.

Vitrification can be described in an equation with four variables: cooling rate, warming rate, viscosity, and sample volume (25). Current rapid-cooling vitrification procedures involve exposure of cells suspended in very small volumes to relatively high concentrations of cryoprotectant(s) for brief periods of time to avoid chemical toxicity, followed by rapid cooling in liquid nitrogen. The high osmolarity of the vitrification solutions rapidly dehydrates the cell, and submersion into liquid nitrogen quickly solidifies the cell so that the remaining intracellular water does not have time to form damaging ice crystals. The cell undergoes a temperature transition from room temperature to −196°C in <2 seconds, resulting in extremely fast rates of cooling (greater than −10,000°C/min) (26). To facilitate rapid heat transfer and reduce chemical toxicity, minimal volumes and small, open cryopreservation devices are used. The warming rate is at least, if not more, important than the cooling rate, as elegantly demonstrated in experiments using mouse oocytes (27). The investigators concluded that the lethality of a slow warming rate is a consequence of allowing time for the development and growth of small intracellular ice crystals by recrystallization.

Even though there are numerous rapid-cooling solutions and methodologies available that work well, these are largely modifications of the DMSO-based protocol described in 1998. During the same period, another vitrification system that did not use DMSO as the main permeable cryoprotectant was also developed (28). The different cryopreservation devices used to store the cells are all microvolume devices that will hold 1–3 μL of medium along with several cells. They all have roughly the same rapid-cooling rates (greater than −10,000 to −50,000°C/min) when plunged into liquid nitrogen from room temperature. A list of key papers describing the evolution of rapid-cooling vitrification are listed in Supplemental Table 1 (available online at www.fertstert.org) (4, 13, 19, 24, 28–40).

OOCYTE RAPID-COOLING VITRIFICATION BEST PRACTICES

Cryoprotectants

The two sets of solutions discussed here, DMSO based and non–DMSO based, are not in opposition; rather, they represent two separate and very different systems to obtain vitrified oocytes and embryos. To date, efficient rapid-cooling vitrification of MII oocytes has been achieved using both DMSO and non-DMSO solutions in a number of mammalian species, including mice, rabbit, bovine, porcine, felid, and humans, in combination with various commonly used devices, many of which are listed in Supplemental Table 2 (available online at www.fertstert.org). When done properly, both systems have very good results. Both represent current and best viable options for vitrification of human oocytes and embryos. There are differences between the systems (volumes, cooling rate, etc.). The major difference is the fact that DMSO is present or absent, because this changes the dynamics of the system; the absence of DMSO allows for the use of slower cooling rates, large volumes, and different carriers. The potential toxicity of cryoprotectants, including DMSO, is widely published on (24, 41).

Outcomes following rapid-cooling vitrification are closely related to the skills of the operators who perform the procedure. Therefore, a well trained team is mandatory to succeed and to obtain consistent results. A strict quality-control program must be applied to the application of rapid-cooling vitrification, which includes controlling learning curves, analysis of the operator's outcomes, vendor lots of solutions used, etc. (42, 43).

Cryopreservation Devices

At least 30 different carrier tools have been described, and at least 15 versions are commercially available (Vajta 2015). Most of them are slightly modified versions of the initially introduced carrier tools, such as the OPS (19), Cryoloop (44, 45), and Cryotop (33). All of these systems are open systems in their original form. Some closed systems are the results of the modifications of these open systems (46). Less commonly used devices include electron microscope grids, drops into liquid nitrogen, and gel loading tips, to name a few.

The Cryotop system, developed in Japan, was mass-marketed and became a best-selling microvolume storage device (36). Owing to its technically challenging nature, the Cryotop system, in conjunction with the ethylene glycol/DMSO/sucrose process, was originally slow to gain widespread acceptance for oocyte rapid-cooling vitrification (47, 48). However, after minor methodologic modifications, two large comparative studies established its place in oocyte cryopreservation history (49, 50). Most laboratories using rapid-cooling vitrification solutions also use open devices for both cooling and storage in liquid nitrogen (39). This is due to the faster rates of cooling.

The possibility of viral contamination of the liquid nitrogen has been suggested after the experimental spiking of liquid nitrogen storage vessels with high viral titers (51). Furthermore, the contamination of vitrification carriers immersed in liquid nitrogen with high microbial and fungal contamination levels has been demonstrated (52). However, there are no published reports of actual cross-contamination of cryopreserved embryos when open storage containers are used (26). Microbial contamination of liquid nitrogen has been reported, but again, there is no evidence that an embryo has been contaminated by direct contact with the liquid nitrogen (53). With these studies in mind, the concerns about contamination during liquid nitrogen storage remain theoretical, even more so when considering the fact that slow-cooled embryos have been stored in cryovials for a number of years, allowing liquid-nitrogen leakage during long-term storage (54). In reproductive biology, including mammalian and human assisted reproduction, no disease transmission caused by liquid nitrogen–mediated cross-contamination, or other cryopreservation-related source, has been reported (46). There are few commercially available vitrification devices that completely meet the requirement of sterility, and reported data are still limited (46). Straightforward procedures can be performed to minimize any potential contamination risk

during vitrification, warming, shipping, and cryo storage using open or semiclosed carriers (55).

There is a reluctance to vitrify using closed devices because of the hypothetical reduction in cooling rates, which may be produced in closed systems owing to thermoisolation and may increase the possibility of ice crystal formation during the cooling process and of recrystallization on warming (56). A systematic review and meta-analysis including seven studies reporting survival, implantation, clinical pregnancy, or live birth rates after closed or open rapid-cooling vitrification of blastocysts was published (57). There were no statistically significant differences in survival rates (risk ratio [RR] 1.00, 95% confidence interval [CI] 0.98–1.02), implantation rates (RR 1.02, 95% CI 0.93–1.11), clinical pregnancy rates (RR 0.99, 95% CI 0.89–1.10), or live birth rates (RR 0.77, 95% CI 0.58–1.03) between closed and open rapid-cooling vitrification. Although there was no statistically significant difference, the trend toward lower live birth rates with closed rapid-cooling vitrification than with open rapid-cooling vitrification is potentially concerning. The closed systems commonly in use are listed in Supplemental Table 2.

KEY PERFORMANCE INDICATORS

With this background as context, practical recommendations to optimize patient outcomes with oocyte rapid-cooling vitrification include the following:

- A structured program for training and proficiency in oocyte cryopreservation should be developed. Similarly to other technically sensitive assisted reproductive technologies, operator metrics to demonstrate acquisition of competence should be recorded and evaluated, including the number of oocytes vitrified and the percentage surviving warming without evidence of damage. Meaningful benchmarks for proficiency must be determined and applied in operator evaluations.
- Because the number of oocytes at retrieval may vary widely among patients and stimulation cycles, a plan for cryopreservation will be prudent to develop in advance. This can include decisions concerning whether to cryopreserve all oocytes or only mature oocytes and how to distribute the oocytes, i.e., how many oocytes will be loaded in or on each cryo device.
- A validated technique with specific cryopreservation and warming solution formulations and cryopreservation devices should be used for oocyte vitrification. Composition of cryopreservation and warming solutions should be those associated with optimal outcomes.
- Handling of the oocytes and timing of the vitrification and warming procedures should be associated with optimal outcomes.
- The technical proficiency of the embryologists involved should be continually monitored through competency assessment and quality management system audits.
- A database should be maintained, allowing tracking and analysis of outcome parameters from the oocyte cryopreservation program, including such variables as:
  a) total number of oocytes retrieved.
  b) number of oocytes cryopreserved and stratified by maturational status.
  c) number of oocytes warmed.
  d) number of oocytes survived and inseminated by intracytoplasmic sperm injection.
  e) number of oocytes fertilized.
  f) number of embryos acquiring a developmental and quality stage consistent with transfer or cryopreservation.
  g) number of embryos transferred.
  h) number of embryos cryopreserved.
  i) implantation rate.
  j) clinical pregnancy rate.
  k) live birth rate.
  l) number of embryos or blastocysts warmed and transferred for vitrified-warmed embryo transfer (FET) cycles.
  m) FET cycle outcome data.
  n) clinically important information on the pregnancy/delivery/neonates.
- The same tenets that comprise a total quality management program in cryopreservation management should apply to cryopreserved oocytes. Best practices for management of cryopreserved tissues, including minimum standards and requirements for critical cryo storage, have been published (58).

EMBRYO AND BLASTOCYST RAPID-COOLING VITRIFICATION

Cleavage-stage embryos and blastocysts on day 5–7 can be rapidly cooled by the same, or slightly modified, protocols used to vitrify oocytes. Reports indicate that artificial shrinkage of the large blastocele of a day-5–7 blastocyst might lessen cryoinjury during both cooling and warming phases (59). Manual puncture of the trophectoderm by needle or laser before rapid-cooling vitrification has been demonstrated to improve survival rates of rapid-cooled blastocysts and results in a higher percentage of high-quality and hatching blastocysts, but not necessarily in improved implantation rates.

A study published in 2016 demonstrated that transfer of rapid-cooled day-3 and day-5 embryos did not adversely affect the neonatal health of offspring compared with transfer of fresh embryos. Furthermore, neonatal outcomes were not different after transfer of rapid-cooled blastocysts compared with rapid-cooled cleavage-stage embryos (60). There are many other studies that support the effectiveness of modern rapid-cooling vitrification techniques for both oocytes and embryos.

FUTURE PERSPECTIVES

Vitrification via rapid cooling is strongly recommended as standard of care for cryopreservation of human oocytes and embryos. Rapid-cooling strategies are being developed for ovarian tissue and for sperm, particularly for patients with oligospermia or for patients with either nonobstructive or obstructive azoospermia for whom testicular sperm must be cryopreserved in very low numbers.

SUMMARY

- Implementation of quality-control measures are necessary to obtain consistent high-quality results with rapid-cooling vitrification of oocytes and embryos.
- Selection of optimized protocols, along with operator training, will result in increases in efficiency, consistency, reliability, and safety.
- Current vitrification systems work well, with survival rates of embryos approaching 100% and pregnancy rates that are similar to if not better than fresh transfer rates.

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Una revisión de las mejores prácticas de vitrificación de enfriamiento rápido para ovocitos y embriones: una opinión del comité.

El objetivo de esta publicación es revisar las mejores prácticas de enfriamiento rápido para la criopreservación de ovocitos y embriones. La discusión incluye tipos de crioprotectores y dispositivos utilizado normalmente. También se definen los indicadores clave de rendimiento del enfriamiento rápido y la vitrificación.