

Edward E. Wallach, M.D.  
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## Oocyte cryopreservation

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**Objective:** To review historical and contemporary advances in oocyte-cryopreservation techniques and outcomes.  
**Design:** Publications related to oocyte cryopreservation were identified through MEDLINE and other bibliographic databases.

**Conclusion(s):** Oocyte cryopreservation can be used as an adjunct to conventional IVF and as an option for fertile women to electively cryopreserve their gametes. Recent reports indicate pregnancy rates comparable to those for cryopreserved embryos by either slow-freeze or vitrification methods. Larger prospective trials are needed to determine the true efficacy and safety of oocyte cryopreservation. Until a sufficient number of births is reached and adequate outcome data are collected, oocyte cryopreservation should continue to be considered experimental and to be performed under the oversight of an institutional review board. (*Fertil Steril*® 2006;86(Suppl 3): 1037–46. ©2006 by American Society for Reproductive Medicine.)

**Key Words:** Oocyte cryopreservation, slow-freeze, vitrification, fertility preservation, egg freezing

The incorporation of oocyte cryopreservation into the clinical practice of assisted reproduction long has been a goal of many practitioners. Oocyte cryopreservation complements assisted reproduction by extending its application to fertile women. It may be used to avoid long-term embryo cryopreservation, to rescue cycles complicated by ovarian hyperstimulation syndrome or failure to obtain sperm, and to avoid synchronization issues in oocyte-donation cycles. Fertile women may take advantage of this technology to electively delay childbearing or as a strategy for fertility preservation when faced with a new diagnosis of cancer and sterilizing therapies such as chemotherapy and radiation or extirpative surgery. Recent advances in assisted reproduction and embryology, including improved culture media, fertilization with intracytoplasmic sperm injection (ICSI), and optimization of cryoprotectants, have made oocyte cryopreservation a viable reality. This review focuses on the etiology of oocyte cryopreservation, history of oocyte cryopreservation, recent advances, and clinical outcomes.

### PRINCIPLES OF CRYOPRESERVATION

To maintain long-term viability after long-term storage, living cells must be brought into a state of suspended animation in which they remain for indefinite periods of time, and from which they can be brought back to viability at some point in

Received July 29, 2006.

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the future. The temperature that generally is used for storage of mammalian cells,  $-196^{\circ}\text{C}$ , the temperature of liquid nitrogen, appears to be adequate for these purposes, although a precise threshold value for this temperature is not known. At these low temperatures, water exists only in a solid state, and no known biological reactions take place. The only danger to cryopreserved cells is thought to be damage to DNA that is caused by background radiation. It further has been estimated that with the usual terrestrial background radiation levels of 0.1 cGy/y, mammalian cells may survive for hundreds or even thousands of years (1).

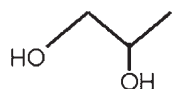
Because human oocytes are viable at  $37^{\circ}\text{C}$  and no biological activity takes place at  $-196^{\circ}\text{C}$ , the time of greatest danger during cryopreservation appears to be during the transitions of temperature: during cooling to  $-196^{\circ}\text{C}$  and during subsequent rewarming to  $37^{\circ}\text{C}$ .

When water is cooled to below its freezing point, it solidifies in a crystalline structure known as ice. Because ice is less dense than liquid water, it necessarily follows that ice crystals occupy a greater volume than does the liquid water from which they were formed. As adjacent volumes of liquid water within a cell solidify, their expansion into ice causes pressure and shearing forces on intracellular organelles, which can suffer considerable damage. Avoidance of ice-crystal formation therefore is one of the principal goals of successful cryopreservation.

As water transitions from liquid to ice, any solutes in the liquid phase are excluded from the solid. This lowers the

**FIGURE 1**

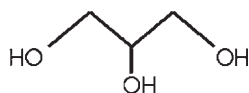
Chemical structure of commonly used permeating cryoprotectants.



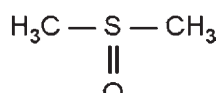
Propylene Glycol  
(PROH)



Ethylene Glycol



Glycerol



DMSO

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freezing point of the remaining unfrozen solution. As the temperature drops and the solid form proliferates, the concentration of electrolytes and other solutes can reach very high levels (2). These concentrations can be quite toxic to intracellular proteins, and thus avoidance of these *solution effects* is a second major goal of successful cryopreservation.

During rewarming, the solid ice melts and releases free water, resulting in decreasing osmolarity of the surrounding solution. When rewarming is slow, there is danger of free water thawing and recrystallizing, thus causing further damage. When rewarming is rapid, sudden drops in extracellular osmotic pressure may lead to rapid shifts of free water across and into the cell, leading to swelling and cell damage (1). This is called *osmotic shock*, and its avoidance is a third major goal of successful cryopreservation.

Therefore, simply immersing oocytes into liquid nitrogen is not an effective strategy for successful cryopreservation. All successful methods must avoid these three issues: ice-crystal formation, solution effects, and osmotic shock. Up to this point in time, all cryopreservation strategies, including all methods of oocyte cryopreservation, have used additional chemicals to avoid cell damage. These chemicals are called cryoprotectants and generally can be divided into two categories, *permeating* and *nonpermeating*.

## PERMEATING CRYOPROTECTANTS

Permeating cryoprotectants are small molecules that readily permeate the membranes of cells. They form hydrogen bonds with water molecules and prevent ice crystallization. At low concentrations in water, they lower the freezing temperature of the resulting mixture. However, at high-enough concentrations, they inhibit the formation of the characteristic ice crystal and lead to the development of a solid, glasslike, so-called vitrified state in which water is solidified, but not expanded. In this manner, the permeating

cryoprotectants satisfy the first goal of successful cryopreservation, the avoidance of ice crystals. The chemical structure of commonly used permeating cryoprotectants is depicted in Figure 1.

Of the permeating cryoprotectants, propylene glycol (1,2 propanediol; PROH) most commonly is used in oocyte cryopreservation. The physical properties of PROH are listed in Table 1. Propanediol mixes readily with water and forms hydrogen bonds with the water molecules. When used in conjunction with slow-freeze protocols, it commonly is added as a cryoprotectant in a concentration of 1.5 M. At this concentration, a hypothetical 1 L of solution would contain 110 mL of PROH and 890 mL of H<sub>2</sub>O. The toxicity of PROH at this concentration is low, but its ability to prevent ice-crystal formation is limited. At this concentration, one molecule of PROH is surrounded by approximately 33 molecules of H<sub>2</sub>O.

At increasing concentrations, PROH exerts an increasing influence on ice-crystal formation. If the concentration is doubled to 3 M, one molecule of PROH is surrounded by approximately 14 molecules of H<sub>2</sub>O, and at a concentration of 6 M, one molecule of PROH is surrounded by approximately 5 molecules of H<sub>2</sub>O. At this concentration, the solution contains approximately 44% PROH by volume; it is difficult for water to crystallize around the intervening molecules of PROH, and a glasslike solid state of water is achieved. However, the toxicity of PROH at this concentration is quite high, and thus the cell can be exposed to this solution either for a very short period of time (as with vitrification techniques) or at very low temperatures, at which the metabolic rate of the cell is very low (as occurs during slow-freezing protocols).

Permeating cryoprotectants play a second important role in cryopreservation, and this is to protect the cell from solution effects. They achieve this goal by remaining in solution and by thus effectively diluting the remaining electrolytes. This effect is described by the *phase rule*, which states that in a two-phase system, such as liquid water and ice at a given pressure, the total solute concentration in the liquid phase is constant for a given temperature (3). Thus, as free water solidifies into ice, the remaining solution will contain progressively higher concentrations of both PROH and electrolytes. Because the total concentration of PROH and electrolytes must be constant, the higher the concentra-

**TABLE 1**

### Physical properties of PROH.

Parameter	Data
Chemical formula	C <sub>3</sub> H <sub>8</sub> O <sub>2</sub>
Melting temperature	-59°C
Molecular weight	76.09 g/M
Density	1.036 g/cm <sup>3</sup>

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tion of PROH, the lower the concentration of electrolytes. Thus, the PROH in solution effectively dilutes the electrolytes, thus limiting their toxicity.

### NONPERMEATING CRYOPROTECTANTS

In contrast to the permeating cryoprotectants, nonpermeating cryoprotectants remain extracellular. They act by drawing free water from within the cell, thus dehydrating the intracellular space. As a result, when they are used in combination with a permeating cryoprotectant, the net concentration of the permeating cryoprotectant is increased in the intracellular space. This further assists the permeating cryoprotectant in preventing ice-crystal formation.

The nonpermeating cryoprotectants play an important role during thawing. During thawing, the water generated by the melting ice rapidly decreases the extracellular osmotic pressure. Osmotic shock may occur if the intracellular cryoprotectant cannot diffuse out quickly enough to prevent excessive influx of free water and the swelling, or even rupture, of the cell. Therefore, freezing and thawing protocols commonly use a high concentration of nonpermeating cryoprotectants during the thawing phase. The most commonly used nonpermeating cryoprotectant is sucrose, but other disaccharides and other nonpermeating agents also may be used.

A novel use for nonpermeating cryoprotectants has been reported for trehalose (4). Rather than being added to the extracellular solution, trehalose is injected directly into the cell. In this manner, it remains intracellular, where it appears to act like a permeating cryoprotectant by preventing ice-crystal formation during cooling.

### SLOW-FREEZE METHOD

The slow-freeze method relies on low initial cryoprotectant concentrations, which are associated with lower toxicity, while the oocyte is still at a temperature at which it is metabolizing. Because cell metabolism is thought to decrease by approximately 50% for every 10°C decrease in temperature, toxicity is limited by having concentrations of cryoprotectants and other solutes increase only after the cell has been cooled to temperatures at which metabolism is quite slow. Cryoprotectants typically are added at room temperature, although in his landmark article, Chen (5) used a prechilled cryoprotectant (DMSO) to minimize its toxic effects. The temperature then is lowered gradually (about 2°C/min) to the seeding temperature at -6°C. During this time, all of the solution remains liquid. The calculated approximate metabolic rate of the oocyte now is <1/16 that of its baseline at 37°C.

At a temperature of -6°C, ice crystals may be induced in the solution by introducing a *seed*, a small crystal of ice that allows other water molecules to undergo crystallization. Seeding commonly is accomplished by touching the outside of the cryopreservation vessel with a very cold instrument,

such as a forceps, which then induces a small ice crystal to form in the area at which the cryovessel is touched.

The resulting formation of ice releases energy known as the *heat of fusion*. Therefore, solutions are commonly held at this temperature for some period of time, such as 10 to 30 minutes, to allow equilibration. The growing ice crystals exclude solutes, thus gradually increasing their concentration in the remaining solution. Therefore, seeding is performed in an area distant from the oocyte, so that the ice crystal grows gradually toward the oocyte.

After equilibration, the temperature gradually is decreased to a final temperature of -32°C. During this time, the ice crystal gradually propagates in the extracellular medium, thus further increasing the concentration of cryoprotectants, particularly in the intracellular space, which further is dehydrated through the use of nonpermeating cryoprotectants. The very slow rate of cooling (0.33° per minute) allows gradual diffusion into the oocyte of additional permeating cryoprotectant while maintaining equilibrium with the extracellular space. At a final temperature of -32°C, PROH still is well above its freezing temperature (Table 1), but its concentration has increased considerably. The metabolic rate of the oocyte now is quite slow, further limiting the toxicity of the increasing concentrations of the cryoprotectants. The freezing vessel now is plunged into liquid nitrogen, and the remaining nonsolidified solution is converted to a solid, vitrified state.

During thawing, a rapid transition of temperature is preferred to prevent recrystallization of water with the potential for ice-crystal damage. Here caution must be taken to avoid osmotic shock from the permeating cryoprotectant, which now is at a very high concentration in the intracellular space. Therefore, additional nonpermeating cryoprotectant is used. As the permeating cryoprotectant gradually diffuses out of the oocyte, the concentration of the nonpermeating cryoprotectant gradually is decreased, until the oocyte is returned to standard culture medium.

### RAPID FREEZE (VITRIFICATION)

During vitrification, permeating cryoprotectants are added at a high concentration while the cell is at room temperature. Because the toxicity of this high concentration of permeating cryoprotectant is substantial, the oocyte cannot be kept at this temperature for long. Instead, a very short time is allowed for equilibration, after which the oocytes are plunged directly into liquid nitrogen. To further protect against ice-crystal formation, an extremely rapid rate of cooling is used. For this reason, novel cryovessels have been used that allow direct contact between liquid nitrogen and the oocyte-containing solution and that have in common a very high surface-to-volume ratio (6-8). This form of ultrarapid freezing must be followed by ultrarapid thawing to prevent ice recrystallization.

## EARLY CLINICAL EXPERIENCE

The first human pregnancy after oocyte cryopreservation was reported by Chen in 1986 (5), using a slow-freeze technique with DMSO. That investigator reported very good survival and fertilization rates of 80% and 83%, respectively, in a sample of 40 oocytes. Over the subsequent decade, investigators struggled to achieve comparable thaw and fertilization rates, and very few live births were reported (9–14). Progress was slow, partly because of the difficulty with fertilization of thawed oocytes during this pre-ICSI era.

## DISRUPTION OF THE MEIOTIC SPINDLE

The microtubular spindle apparatus is a dynamic structure that facilitates segregation of chromosomes, into daughter cells during mitosis and into the first and second polar bodies during meiosis. It must function correctly to achieve accurate chromosome segregation and avoid aneuploidy (15). Although several investigators have raised concerns about possible embryonic aneuploidy after observing disruption of the spindle apparatus during oocyte cooling (9, 16–19), more recent studies suggest that the spindle reforms (15). By using a Polscope system, which permits noninvasive visualization of spindles in living oocytes, Rienzi et al. (20) reported the reappearance of spindles in all surviving oocytes after a slow-freeze method of cryopreservation and concluded that all spindles observed in thawed oocytes resulted from postthaw reconstruction. Further reassuring data were generated by Gook et al. (21), who showed no increase in the number of abnormal or stray chromosomes in previously cryopreserved oocytes. Fluorescence in situ hybridization was used by Cobo et al. (22) to show that the incidence of chromosomal abnormalities in human embryos that were obtained from cryopreserved oocytes was no different than that of control embryos.

Among the >150 live births reported in the world literature after oocyte cryopreservation, there is a single report of a congenital anomaly, a ventricular septal defect (23). In the same report, no intellectual or developmental deficits were detected in 16 children who were born from cryopreserved oocytes after 3 years of follow-up (23). Because the largest demand for oocyte cryopreservation most probably is going to come from women who wish to delay childbearing electively, it is quite likely that several years will be required before sufficient births have occurred to determine the true safety of cryopreserved oocytes. For this reason, and in agreement with the Practice Committee of the American Society for Reproductive Medicine, cryopreserved oocytes should be considered an experimental technique, only to be performed under investigational protocol under the auspices of an institutional review board (24).

## ZONA PELLUCIDA HARDENING

There is evidence that cryopreservation induces zona pellucida hardening through premature cortical-granule release, which then acts as a barrier to fertilization (25–28). It is remarkable that standard insemination techniques were able

to achieve fertilization in the early era of cryopreserved oocytes, although it is clear in retrospect that fertilization was a major rate-limiting factor in the progress of cryopreserved oocytes. After introduction of ICSI by Palermo et al. in 1992 (29), investigators were able to bypass the hardened zona, leading to improved fertilization and live-birth rates (14, 30–32). Larman et al. (33) recently reported that DMSO and ethylene glycol can induce a large transient increase in intracellular calcium concentration in mouse metaphase II oocytes, comparable to the initial increase that is seen at fertilization. Removal of extracellular calcium from the medium failed to affect the response induced by DMSO but significantly reduced the ethylene glycol-induced calcium increase. Those investigators suggested that the source of the DMSO-induced calcium increase was from the intracellular calcium pool, whereas ethylene glycol caused an influx of calcium across the plasma membrane from the external medium. Vitriifying oocytes in calcium-free media reduced zona hardening, increased subsequent fertilization, and did not adversely affect embryonic development to the blastocyst stage. Further studies of calcium physiology with other cryoprotectants ultimately may eliminate zona hardening and the requirement for ICSI.

## RECENT DEVELOPMENTS WITH SLOW-FREEZE METHOD

The largest clinical experience with human oocyte cryopreservation has been published by Porcu et al. (34) and Fabbri et al. (35), in Italy. Their method uses a slow-freeze, rapid-thaw protocol with 1.5 M PROH and 0.2 M sucrose as the permeating and nonpermeating cryoprotectants, respectively. After equilibration and exposure to sucrose, the oocytes are loaded into plastic straws and cooled to  $-8^{\circ}\text{C}$ , manually seeded, and then cooled to  $-30^{\circ}\text{C}$  before transfer to liquid nitrogen. For thawing, straws are removed from liquid nitrogen, held at room temperature, and then submerged in a  $30^{\circ}\text{C}$  waterbath before stepwise dilution of PROH and exposure to 0.2 M sucrose. In their initial series, 338 oocytes from 23 women with tubal-factor infertility were cryopreserved (34). Oocyte survival rate was 59.5% and was found to be independent of the duration of cryopreservation (24 hours vs. 2 to 4 months) or of the presence or absence of the cumulus (62% vs. 52%, respectively). The fertilization rate using ICSI was 64.4%, with a subsequent cleavage rate of 90.8%. A mean ( $\pm$  SD) of  $3.1 \pm 1.3$  embryos were transferred per patient, yielding three pregnancies and one live birth. Subsequently, the same group reported a second series consisting of 1,769 cryopreserved oocytes from 96 patients (35). Of these, 1,502 were thawed, with a 54.1% survival rate, a 57.7% fertilization rate with ICSI, and a cleavage rate of 91.2%. Sixteen pregnancies were achieved, with 9 live births of 11 healthy children (7 singletons and 2 twins). Higher survival rates were obtained when oocytes were cryopreserved in 0.3 mol/L vs. 0.2 mol/L sucrose (60% vs. 82%) and when longer exposure to the cryoprotectant (10.5 to 15 min) was used. Other investigators using comparable slow-freeze methods reported similar

survival and fertilization rates but higher pregnancy rates, ranging from 33% per embryo transfer with autologous oocytes (36), up to 57.1% when cryopreserved donor oocytes were used (37, 38).

## CHOLINE SUBSTITUTION

Substitution of sodium by choline in the cryopreservation media appears to enhance cryopreservation outcome. Choline is a dietary compound that is an essential nutrient for human phospholipid biosynthesis and cell signaling (39). In addition to solution effects, sodium in the culture medium is thought to accumulate intracellularly as a result of impairment of the plasma-membrane Na-K pump during cryopreservation (40–43). Unlike the sodium ion, choline is not thought to cross the cell membrane. Therefore, it is not expected to contribute to intracellular osmolarity or toxicity. Stachecki et al. (40, 41) reported improved survival and developmental rates of mouse oocytes when Na-depleted, choline-substituted media was used for freezing.

Quintans et al. (42) were the first to report live births with the new culture medium. They achieved successful oocyte cryopreservation by using a phosphate-buffered saline-based medium in which sodium chloride was replaced by choline chloride. They observed an oocyte survival rate of 63%, a fertilization rate of 59%, an implantation rate of 25%, a clinical-pregnancy rate per transfer of 50%, and a live-birth rate per transfer of 2/12 (16.7%). Boldt et al. (43) used a similar approach but raised the seeding temperature to  $-6^{\circ}\text{C}$ , used 0.5 mol/L sucrose in the thawing solution, and added assisted hatching of all transferred embryos with acid Tyrode's. They reported a survival rate of 74.4%, a fertilization rate of 59%, an implantation rate of 16.7%, a clinical-pregnancy rate per transfer of 36.4%, and a live-birth rate per transfer of 4/11 (36.4%). Jain et al. (44) used a comparable technique but substituted a modified human tubal fluid medium for phosphate-buffered saline as described by Quinn (45) and reported three ongoing clinical pregnancies (now live births) from four embryo transfers. Recently, Boldt et al. (46) reported their experience with phosphate-buffered saline-based vs. the same modified human tubal fluid-based, sodium-depleted, choline-substituted medium. The overall survival rate was 60.4%, the fertilization rate was 62%, the implantation rate was 13.3%, the clinical-pregnancy rate per transfer was 32.6%, and the live-birth and ongoing-pregnancy rate was 10/14 (71.4%) for the entire study sample. Although higher implantation (15.9% vs. 10.6%) and clinical-pregnancy rates per transfer (37.5% vs. 26.3%) were observed with modified human tubal fluid medium, the differences did not reach statistical significance. It should be noted that the overall pregnancy rates reported in this study may represent the lower limit of success because the number of oocytes thawed in each cycle (mean, 6.8 oocytes) was small.

## TREHALOSE

A recent innovation involves the use of the sugar trehalose as a cryoprotectant (4, 47, 48). A wide variety of organisms in nature, including arctic frogs, salamanders, insects, and fungi, are able to survive extreme temperatures by accumulating large amounts of intracellular sugars (49, 50). Eroglu et al. (51) described a technique for injecting trehalose into discarded human oocytes by using calibrated micropipettes and reported  $>60\%$  survival rates when cells were cooled to  $-60^{\circ}\text{C}$ . The same microinjection technique was tested in mice and showed normal blastocyst development and no difference in implantation sites, viable fetuses, or mean fetal weight when compared with the case of controls (52, 53). The amount of intracellular trehalose progressively decreased as embryos developed (54).

## VITRIFICATION

Kuleshova et al. (55) reported the first birth from vitrified human oocytes in 1999 after vitrification of 17 oocytes by using ethylene glycol (40%) and 0.6 M/L sucrose in open pulled straws. The first large series of human oocyte vitrification was published by Yoon et al. in 2003 (56). They cryopreserved 474 cumulus–oocyte complexes (mature and immature oocytes) by using vitrification with 5.5 M ethylene glycol and 1.0 M sucrose as cryoprotectants. To maximize cooling rates, the oocytes were loaded on an electron-microscope grid. Those investigators reported a survival rate of 68.7%, a fertilization rate of 71.7%, an implantation rate of 6.4%, and a clinical-pregnancy and live-birth rate per transfer of 6/21 (21.4%). Chian et al. (57) used a combination of ethylene glycol, PROH, and sucrose to vitrify 180 oocytes in an open container called a Cryoleaf. They reported a survival rate of 93.9%, a fertilization rate of 74.6%, an implantation rate of 20.4%, and a clinical-pregnancy rate per patient of 7/15 (46.7%). The average number of embryos transferred ( $3.6 \pm 1.3$ ) was relatively high, given the young age ( $31.7 \pm 3.7$  y) of the patients. Kuwayama et al. (58) reported their experience with oocyte cryopreservation by the Cryotop method, using a combination of ethylene glycol and sucrose as cryoprotectants. Of 64 vitrified oocytes, 90.8% survived and 89.6% fertilized, yielding a pregnancy rate of 41.4% per transfer and a live-birth and ongoing-pregnancy rate per transfer of 10/29 (34.5%). The mean number of embryos transferred was 2.2. Lucena et al. (59) also used the Cryotop method for oocyte vitrification and reported an overall pregnancy rate of 13/23 (56.5%) per patient. These high pregnancy rates may be attributed partly to the fact that the majority of transfers used donor oocytes and involved the transfer of a high number of embryos (mean of 4.5).

To achieve the rapid cooling rates seen with vitrification, oocytes are placed in small volumes of media containing cryoprotectants and exposed directly to liquid nitrogen. Several open-carrier systems have been used, including electron-microscope grids, open pulled straws, and cryoloops (6, 7, 8). These carrier systems require direct contact between the

oocyte-containing solution and liquid nitrogen. The potential for disease transmission through contaminated liquid nitrogen recently was proposed (60). As models for human and animal viral pathogens, three bovine viruses, bovine viral diarrhea virus, bovine herpesvirus-1, and bovine immunodeficiency virus were used to experimentally contaminate liquid nitrogen. Thereafter, vitrified bovine embryos in either open or sealed containers were plunged into the contaminated liquid nitrogen. Of the 61 batches of embryos in unsealed containers, 21.3% tested positive for viral association to bovine viral diarrhea virus and bovine herpesvirus-1, whereas none of the embryos in closed containers tested positive for viral association. These same investigators elsewhere reported bacterial and fungal contamination of embryos and semen that were cryopreserved in sealed plastic straws and stored  $\leq 35$  years in liquid nitrogen (61).

Recent data comparing a closed-carrier method for vitrification, the Cryotip, with the open Cryotop method showed similar blastocyst survival (93% vs. 97%), pregnancy rates (51% vs. 59%), and live-birth rates (48% vs. 51%) (62). Isachenko et al. (63) compared the outcomes of vitrification of human pronuclear zygotes inside open pulled straws or open pulled straws inside a closed 0.5-mL straw (aseptic system). The open system achieved cooling at a speed of 20,000°C/min, whereas the closed system yielded a cooling rate of 200°C/min. Embryo development to the expanded blastocyst stage was 15% in the open system vs. 14% in the closed system, suggesting that the closed system is as efficient as conventional vitrification with open-carrier systems.

### OOCYTE-CRYOPRESERVATION OUTCOMES

Oktay et al. (64) recently reviewed published data evaluating the efficiency of cryopreservation. Reports of cryopreservation from January 1997 to 2005 were classified according to the cryopreservation method: slow freeze or vitrification. The slow-freeze data showed a gradual improvement in oocyte-cryopreservation efficiency over time, with live-birth rates increasing from 21.6% per transfer between 1996 and 2004 to 32.4% between 2002 and 2004. Outcomes of oocyte cryopreservation using the slow-freeze method, as reviewed by Oktay et al. (64), are summarized in

**Table 2.** The vitrification data followed a similar trend, with live-birth and ongoing-pregnancy rates increasing from 29.4% before 2005 to 39% after 2005.

The spontaneous-abortion rate after oocyte cryopreservation initially appeared to be quite high. However, a trend toward decreasing abortion rates that parallels the gradual rise in live-birth rates has been observed. Fabbri et al. (35) reported a 43.8% loss rate in 19 pregnancies, Quintans et al. (42) reported a 50% abortion rate in 6 pregnancies, Levi Setti et al. (65) reported a 33% loss rate in 18 clinical pregnancies, and Boldt et al. (46) reported a 28.6% loss rate in 14 pregnancies. In contrast, Borini et al. (66) reported a 20% loss rate in 15 pregnancies, and Chen et al. (36) reported no losses in 7 pregnancies. In the slow-freeze studies included in the Oktay et al. review (64), there were 19 abortions reported among 95 clinical pregnancies for an overall abortion rate of 20%. Although this rate potentially may be subject to publication bias, it is comparable to the 25% loss rate after spontaneous pregnancies (67). The number of births after vitrification still is too low to properly assess the spontaneous-abortion rate, but initial data have suggested very low abortion rates (56, 58, 64).

### COMPARISON WITH EMBRYO CRYOPRESERVATION

Given the limited data and experimental nature of oocyte cryopreservation, embryo cryopreservation remains, at present, the standard recommendation for fertility preservation. However, embryo cryopreservation has many limitations for single women, including discomfort with anonymity of the sperm donor; issues related to paternity and to legal obligations, in the case of directed sperm donation; and disposition of frozen embryos when marriage occurs. There also are potential difficulties in the case of cancer patients who are at increased risk for disability and death. Death of the oocyte provider may pose dilemmas related to disposition of the embryos and potential conflict with family members if a clear directive was not in place before cryopreservation.

Although pregnancy rates after oocyte cryopreservation appear to be lower than those achieved with fresh embryos (64), it may be more appropriate to compare outcomes after

**TABLE 2**

**Results of oocyte cryopreservation using the slow-freeze method.**

Parameter	1996–2004	2002–2004
Mean age (y) $\pm$ SD	33.0 $\pm$ 0.2	31.1 $\pm$ 0.6
Survival, % (n)	55.4 (2,217/4,000)	69.2 (476/688)
Fertilization rate, % (n)	60.7 (1,346/2,217)	64.9 (309/476)
Implantation rate, % (n)	13.1 (122/929)	15.3 (34/222)
Live birth rate per transfer, % (n)	21.6 (76/351)	32.4 (24/74)

Note: Adapted from Oktay et al. (64).

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oocyte cryopreservation with those achieved with cryopreserved embryos. Live-birth rates per transfer of 32.4% with slow freeze and of approximately 39% with vitrification compare favorably with those reported for cryopreserved donor embryos (30.1%), which yield the highest pregnancy rates and are most representative of fertile women (64, 68).

## **OOCYTE-CRYOPRESERVATION APPLICATIONS**

### **Fertility Preservation for Cancer Patients**

Perhaps the clearest indication for oocyte cryopreservation relates to women with newly diagnosed cancers who face sterilizing therapy. In 2001, approximately 8% of the 625,000 women diagnosed with cancer were under the age of 40 years (69, 70). Over the past 25 years, the 5-year relative survival rate in women for all cancers combined improved from 56% to 64% (71), and the desire to have children is high among cancer survivors (72).

Chemotherapy, especially alkylating agents, and ionizing radiotherapy, when used for bone-marrow transplantation, can lead to high rates of ovarian failure (73, 74). Although ovarian suppression using GnRH agonists and antagonists have been proposed as a means to limit ovarian damage from gonadotoxic therapies, the biologic mechanism and effectiveness of these approaches remain controversial (75–77). Chemotherapy and radiation are thought to damage oocytes through induction of apoptotic pathways. Morita et al. (78) showed that injection of sphingosine-1-phosphate, a potent antagonist of the proapoptotic factor ceramide, 2 hours before exposure to radiation protected the immature follicle pool in young adult mice. Before such methods can be used in human beings, there must be certainty that blockade of apoptosis does not counteract the effectiveness of the cancer therapy.

Oocyte cryopreservation offers a method to preserve fertility before gonadotoxic therapies, but the time required to stimulate follicles and perform oocyte retrieval may pose a problem for women and their oncologists, who are eager to commence cancer treatment. One way to expedite oocyte collection is to harvest ovarian cortex laparoscopically. The ovarian cortex of young women contains thousands of primordial follicles that can be harvested independent of the menstrual-cycle phase, thus allowing the patient to proceed with cancer therapy with minimal delay. Although cryopreservation of human ovary has proven difficult because of the ovary's large size, heterogeneous cell population, and susceptibility to ischemic damage, restoration of hormonal function and pregnancies have been achieved after transplantation to both orthotopic and heterotopic sites (79–84). Because there have been reports in both animal models and human beings of residual malignant cells in cryopreserved and transplanted tissues, transplantation of cryopreserved ovarian tissue from patients with systemic or disseminated malignancies should be undertaken with caution until reliable screening methods become available (84, 85).

Another strategy to facilitate rapid collection of oocytes is to harvest and cryopreserve oocytes at the germinal-vesicle stage, when chromosomes are protected within the nuclear membrane and the spindle is not yet formed (80, 86, 87). Although germinal-vesicle oocytes may have a higher survival rate than metaphase II oocytes (88), in vitro maturation techniques still are unreliable (89) and limit the success of this strategy. Although Tucker et al. (90) reported the first birth from in vitro-matured, cryopreserved germinal-vesicle oocytes in 1998, subsequent success has been limited.

Conventional ovarian stimulation with gonadotropins gives rise to high serum estrogen levels, which may stimulate estrogen-dependent malignant cells (91, 92). Breast cancer is the most common cancer in reproductive-age women, affecting >200,000 women per year, of whom 25% are premenopausal (93). To reduce estrogen exposure, Oktay et al. (94) investigated the use of tamoxifen and letrozole for ovarian stimulation. Sixty women with breast cancer were assigned to stimulation consisting of tamoxifen alone (Tam-IVF), tamoxifen and low-dose FSH (TamFSH-IVF), or letrozole with low-dose FSH (letrozole-IVF). Compared with tamoxifen alone, the TamFSH-IVF and letrozole-IVF groups had greater number of follicles, mature eggs, and embryos. Peak estradiol levels were lower in the letrozole-IVF (380 pg/mL) and Tam-IVF (419 pg/mL) groups, compared with in the TamFSH-IVF group (1,182 pg/mL). After a mean follow-up period of 554 days, cancer recurrence rate was similar between the IVF groups and the 31 control patients who elected to not undergo IVF.

### **Delayed Childbearing**

In present-day society, women increasingly are delaying childbearing. As a result, many of them have difficulty conceiving because of the well-known age-related decline in fertility. The advent of oocyte donation showed that reproductive aging is primarily, if not entirely, related to the age of the oocyte (95–97). Because age of the recipient does not appear to play a role in the probability of success of oocyte donation (98–100), oocyte cryopreservation offers fertile young women an opportunity to act as their own potential oocyte donors in the future. Pregnancies at an advanced maternal age are associated with increased complications, but good outcomes can be anticipated in the absence of underlying medical conditions in the mother and with careful obstetrical management (101–103).

Given the present lack of long-term data, counseling of women regarding the advisability of oocyte cryopreservation for future fertility is necessarily complicated. It is a truism that such counseling must be individualized. For example, it has not yet been established how many cryopreserved oocytes are needed to yield a so-called satisfactory pregnancy rate. On the basis of selected recent studies, approximately 20 oocytes are needed to achieve a pregnancy in women under the age of 35 years (Table 3). Because most studies to date only have included women younger than 35 years of

TABLE 3

Success rates of selected recently reported series using slow-freeze and vitrification methods.

Author (y; reference no.)	Method	Survival rate, n (%)	Fertilization rate, n (%)	No. of oocytes per pregnancy
Fabbri (2001 [35])	Slow-freeze	796/1,502 (53)	632/796 (79)	94
Chen (2005 [36])	Slow-freeze	119/159 (75)	80/119 (67)	23
Boldt (2006 [46])	Slow-freeze (sodium depleted)	218/361 (60)	134/218 (61)	26
Yoon (2003 [56])	Vitrification	325/474 <sup>a</sup> (68.6)	142/198 (71.7)	79
Kuwayama (2005 [58])	Vitrification	58/64 (91)	52/58 (90)	5

<sup>a</sup> Cryopreserved and thawed cumulus–oocyte complexes.

Jain. Oocyte cryopreservation. *Fertil Steril* 2006.

age, there are limited data for use in appropriately counseling older women on the likelihood of success with oocyte cryopreservation.

### Ethical Opposition to Embryo Cryopreservation

Oocyte cryopreservation offers an alternative for couples who are opposed to cryopreserving embryos. This strategy also is being used in some countries in which embryo cryopreservation is illegal (104). Most individuals and governmental bodies have less difficulty disposing of unfertilized oocytes or perhaps even with donating excess unfertilized eggs. Disposition of oocytes, as opposed to embryos, also may be easier in cases of separation or divorce. Similarly, IVF practices may have less ethical difficulty disposing of cryopreserved oocytes vs. embryos in the case of abandonment. Ultimately, if pregnancy rates after oocyte cryopreservation continue to be high, more conservative practices, such as fertilizing fewer oocytes and transferring fewer fresh embryos, may lower the rate of multiple pregnancies.

### Oocyte Donation

Oocyte donation is a proven technology that is in widespread use. Current limitations of traditional oocyte donation include donor availability, cost, need to synchronize donor and recipient schedules, travel requirements, and the inability to quarantine oocytes. These shortcomings all can be improved by using cryopreserved donor oocytes. With this technology, donors can undergo ovarian stimulation and oocyte retrieval at a time that is convenient, without regard to the recipient's schedule. Oocytes also can be quarantined by having the donor return for repeat infectious-disease screens at 3- to 6-month intervals. The recipient then can obtain oocytes without waiting for donor availability and can plan embryo transfer at a convenient time, without the risk of a dropped cycle (as can occur with traditional fresh-oocyte donation). Other benefits of this application include the potential for shipping cryopreserved oocytes to allow recipients to stay under the care of their own physicians. Costs may also be less, because oocytes from one donor could be shared by more than one recipient.

### CONCLUSIONS

Oocyte cryopreservation has applications for both infertile and fertile women and is gaining momentum as more centers report good success rates from both slow-freeze and vitrification methods. Prospective trials comparing the two techniques using well-controlled conditions and including women older than 35 years of age are needed to provide better counseling. Until a sufficient number of births is reached and adequate outcome data are collected, oocyte cryopreservation should continue to be considered experimental and should be performed under the oversight of an institutional review board.

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