Vitrification of oocytes produces high pregnancy rates when carried out in fertile women

Thomas J. Kim, M.D.,[a,b,c] Larry R. Laufer, M.D.,[b] and Seung Wook Hong, M.Sc.[c,d]

[a] Reproductive Medicine Associates of New Jersey, Morristown, New Jersey; [b] Naval Medical Center, San Diego, California; [c] CHA Fertility Center, Los Angeles, California; and [d] Chabiotech Co. Ltd., Pochon CHA University, Seoul, South Korea

Objective: To determine the efficiency of our vitrification technique when applied in young fertile women.
Design: Clinical research and application.
Setting: In vitro fertilization center.
Patient(s): Twenty-one women were recruited from the navy community with 19 patients finishing the study.
Intervention(s): Vitrified oocytes with use of the electron microscopic grid method were warmed 6 months after vitrification. Surviving metaphase II oocytes were microinjected for fertilization, and clinical results were evaluated.
Main Outcome Measure(s): Survival, fertilization, and cleavage rate. Pregnancy and implantation rate.
Result(s): Three hundred ninety-five oocytes were warmed, of which 320 oocytes (81.0%) survived. Two hundred eighty-five metaphase II oocytes were microinjected for fertilization; 206 of them (72.3%) fertilized, and 53 embryos were transferred to 19 patients (in 20 warming cycles). Twenty-four of 53 transferred embryos (45.3%) implanted as confirmed by ultrasound examination. Of the 20 transfers, 16 resulted in clinical pregnancy (80%), 3 miscarried (15%), and 13 (65%) went on to produce 20 live births, respectively. This is much higher in comparison with our previous data using supernumerary oocytes where the rates of implantation and pregnancy were 6% and 21%. Live-birth rates per warmed oocyte and per injected oocyte were 5.1% and 7.2%, respectively.
Conclusion(s): High pregnancy and implantation rates were observed after 6 months of cryopreservation by vitrification when oocytes from fertile woman were used. Proper screening of candidates for oocyte cryopreservation is of crucial importance to assure a favorable pregnancy outcome. (Fertil Steril® 2010;93:467–74. ©2010 by American Society for Reproductive Medicine.)

Key Words: Human oocyte cryopreservation, vitrification, electron microscope grid, ethylene glycol, clinical outcome

Successful cryopreservation of human oocytes has been one of the most elusive tasks for reproductive scientists for many years. Since the first successful pregnancy from frozen human oocytes was reported 20 years ago (1), many researchers around the world have exerted great effort attempting to make this part of the infertility treatment armamentarium. For the most part, efforts have been focused on the conventional slow-freezing method (2–13). More recently, vitrification methods have been adopted and studied by many researchers (14–25). However, despite various permutations of the freezing protocol with different types and concentrations of cryoprotectant, a pregnancy rate (PR) of only 10% to 25% has been achieved. The total number of live births from oocyte cryopreservation on record is just over 200 infants worldwide. Yoon et al. (17) published our experience with vitrification using electron microscope (EM) grids for placement of human oocytes collected from infertile patients, which demonstrated a 21.4% PR with a 6.4% implantation rate, resulting in seven live births. One point worth emphasizing from this report is that the oocytes used for the study were surplus oocytes from patients undergoing IVF-ET, which were stored for future use. We postulated that if better-quality oocytes were used, instead of surplus oocytes after IVF, the resultant PR might have been higher.

The main purpose of this study was to learn more about the efficiency of vitrification cryopreservation by using better-quality oocytes from a proved fertile population. Because pregnancy outcome is influenced by oocyte quality as well as the technologic efficiency of cryopreservation, we wished to assess our technology when applied to women with proved fertility who presumably have good oocyte quality. Because this study was not intended to compare our vitrification technique with other oocyte freezing methods or with fresh cycle IVF results, there was no designated control group for the study.

In this prospective Institutional Review Board–approved study, we report our experiences with vitrification of human oocytes from a selected group of fertile women. Although there is no guarantee that proved fertile women always produce good-quality oocytes, there is less chance of oocyte quality being a significant confounding variable when trying to evaluate specifically the technologic aspect of the vitrification process. We recruited young women who had undergone tubal ligations after their last pregnancy, then desired to have more children. The only difference in their treatment

compared with conventional IVF-ET was that between oocyte retrieval and ET, the retrieved oocytes underwent 6 months of oocyte cryopreservation (quarantine) with use of our vitrification method.

MATERIALS AND METHODS
Study Population
After Institutional Review Board (Western Institutional Review Board study No. 1064078) approval was obtained for the study, patients were recruited among those referred for reversal of sterilization at the Naval Medical Center San Diego. Some patients had failed tubal anastomosis procedures, and some did not have adequate length of fallopian tube segments remaining for reparative surgery. All potential study subjects were offered the alternatives of participating in the study, undergoing tubal anastomosis with minimal waiting time (where applicable), or undergoing conventional IVF-ET through the Naval Medical Center San Diego assisted reproductive technologies program. All potential volunteers were presented with the goal of the study, protocol of ovulation induction, egg retrieval, oocyte cryopreservation and thawing, and ET. They were informed that this was an experimental procedure and they could drop out of the study at any time during the process. All medications, ultrasound monitoring, and surgical and laboratory procedures were provided at no cost to the volunteers. No other financial or material compensation was offered. Informed consent was obtained from all study participants before starting ovulation induction.

Inclusion criteria for the study were [1] age 35 years or younger at the time of recruitment, [2] prior tubal ligation after the last child, [3] body mass index <30, and [4] basal antral follicle counts of ≥10. A total of 21 women were recruited from the navy community with 19 patients finishing the study. One patient underwent 2 warming cycles; thus, a total of 20 warming cycles were assessed. Two patients failed to follow up by the close of the study and therefore were not included in the data. They did not return to undergo ET because the service members were on active duty overseas. All women were evaluated with basal blood hormonal screening, infectious diseases screening, and baseline sonogram, as well as office hysteroscopy.

Ovarian Stimulation and Oocyte Retrieval
Controlled ovarian hyperstimulation was performed with use of either midluteal phase GnRH agonist (Lupron; TAP Pharmaceuticals, North Chicago, IL) down-regulation or GnRH antagonist (Ganirelix acetate; Organon, Roseland, NJ) suppression along with a combination of recombinant FSH (225–300 IU) (Follitropin beta; Organon) and daily low dose (20 IU) hCG (Pregnyl; Organon), followed by surge dose (10,000 IU) of hCG when at least two follicles had a mean diameter of 18 mm. Transvaginal oocyte retrieval was performed 36 hours after the hCG administration. All oocytes were counted and vitrified for cryopreservation within 3 to 4 hours of retrieval.

For transfer of embryos derived from cryopreserved oocytes, the endometrial lining was prepared for approximately 2 weeks by using E_2 transdermal patches (two patches per week) (Vivelle, 0.1 mg; Novartis Pharmaceutical, East Hanover, NJ), and the medication dosage was adjusted depending on the progression of the endometrial lining thickness. Once the desired endometrial thickness was achieved, oocytes were warmed for intracytoplasmic sperm injection (ICSI). Progesterone (100 mg/d) was started on the day the oocytes were warmed and continued until the time of β-hCG assay; vaginal P was used for 6 more weeks for those who conceived.

Preparation of the Vitrification and Warming Solutions
The solutions for equilibration, vitrification, and warming were prepared with use of a base solution made with human tubal fluid with HEPES (SAGE IVF, Pasadena, CA) or Dulbecco's phosphate-buffered saline solution (GIBCO Invitrogen, Carlsbad, CA) plus 20% human serum albumin (HSA; SAGE IVF). The equilibration solution contained 1.5 mol/L ethylene glycol (EG; Sigma-Aldrich, St. Louis, MO) in base solution. The vitrification solution contained 5.5 mol/L EG and 1.0 mol/L sucrose (Sigma-Aldrich) in base solution. The warming solutions were made of 1.0, 0.5, 0.25, 0.125, and 0 mol/L sucrose with base solution.

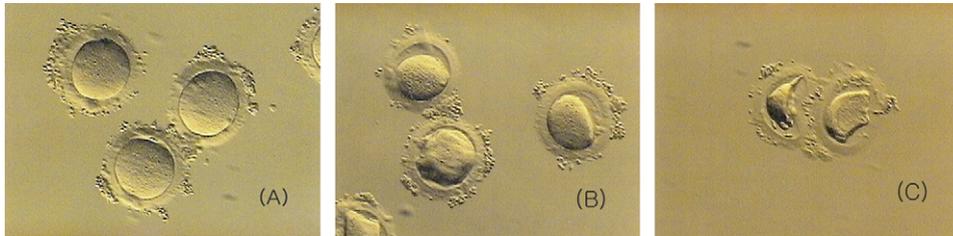### Vitrification of the Oocytes
Vitrification was initiated routinely within 3 to 4 hours after oocyte retrieval. Retrieved oocytes were incubated in four-well multidishes containing 0.5 mL G-Fert medium (Vitrolife, Englewood, CO) supplemented with 5 mg/mL HSA (Vitrolife) overlaid with light paraffin oil (Ovoil; Vitrolife) at 37°C in a humidified atmosphere of 6.5% CO_2, 5% O_2, 88.5% N_2 until they were vitrified. The oocyte-cumulus complexes were exposed briefly to hyaluronidase (50–80 IU/mL, type IV-S; Sigma-Aldrich) for partial removal of cumulus cells 1 hour before vitrification. Equilibration and vitrification solutions were prewarmed on the stage maintained at 37°C. The oocytes were placed into 1.0 mL of prewarmed equilibration solution for 2.5 minutes. The oocytes then were placed into the vitrification solution for 20 seconds (Fig. 1). Three or four oocytes were mounted on an EM grid (Ted Pella Inc., Redding, CA) with use of a fine pipette, and excess cryoprotectant solution was removed with underlying paper. The grid holding oocytes was plunged immediately into the container filled with liquid nitrogen, and the grid was put into a grid holder with use of fine forceps. Then the holder was capped before immersion in a storage tank.

Warming of the Oocytes
The EM grid holding the oocytes was taken from the grid holder with fine forceps under liquid nitrogen, then immediately transferred sequentially into Falcon plastic culture dishes (two-well; Becton Dickinson, Franklin Lakes, NJ) containing 1.0 mL of warming solutions of 1.0, 0.5, 0.25, 0.125, and 0 mol/L of sucrose at intervals of 2.5 minutes at 37°C. The oocyte-cumulus

Oocytes during exposure to cryoprotectant solution. (**A**) After partial removal of cumulus cell mass. (**B**) After exposure to equilibration solution (1.5 mol/L EG). (**C**) After exposure to vitrification solution (5.5 mol/L EG + 1.0 mol/L sucrose).

Kim. Vitrification of human oocytes. Fertil Steril 2010.

complexes were detached from the grid by pipetting after the last warming step. The oocytes were washed several times in base solution, then placed into preequilibrated 0.5 mL G-Fert medium supplemented with 5 mg/mL HSA overlaid with Ovoil at 37°C in a humidified atmosphere of 6.5% CO_2, 5% O_2, 88.5% N_2 until they were inseminated by ICSI.

Sperm Preparation and Insemination (ICSI)

Sperm preparation was carried out with use of Sperm Grad solution (90% density gradient medium; Vitrolife) depending on the initial semen parameters. The resulting pellet was washed twice in human tubal fluid with HEPES (SAGE IVF) and G-Sperm (Vitrolife) supplemented with 5 mg/mL HSA. The pellet was layered with G-Fert medium supplemented with 5 mg/mL HSA for swim-up. Insemination was performed routinely by ICSI. The oocyte-cumulus complexes were denuded with use of 50 to 80 IU/mL hyaluronidase 2 to 3 hours after warming. At this time viability and maturity of oocytes were assessed.

To perform ICSI, oocytes were placed in a 25-μL droplet of human tubal fluid with HEPES medium supplemented with 5 mg/mL HSA. The partner's spermatozoa were placed in a 5- to 10-μL droplet of 8% to 10% polyvinylpyrrolidone (G-ICSI; Vitrolife). All droplets were overlaid with oil for tissue culture (SAGE IVF). Intracytoplasmic sperm injection was performed on a Nikon inverted microscope (Nikon Instruments, Melville, NY) with Narishige micromanipulators (Narishige International, East Meadow, NY). The injected oocytes were rinsed and placed in 0.5 mL of G-Fert medium supplemented with 5 mg/mL HSA until fertilization was assessed.

Embryo Culture

Embryo culture was carried out in preequilibrated 50-μL droplets of sequential media (G1.2 or G1.3 and G2.2 or G2.3) under Ovoil at 37°C in a humidified atmosphere of 6.5% CO_2, 5% O_2, 88.5% N_2. Sixteen to 20 hours after ICSI oocytes were checked for the presence of two pronuclei. After the zygotes were rinsed, three or four embryos were transferred to G1.2 or G1.3 medium supplemented with 5

mg/mL HSA for an additional 48 hours. On day 2 (42–44 hours after ICSI) after warming, embryo quality was assessed by cell numbers and fragmentation. The embryos were cultured together according to their quality. On day 3 (66–68 hours after ICSI) after warming, the embryos were assessed and transferred to G2.2 or G2.3 medium supplemented with 5 mg/mL HSA (Fig. 2). On day 5 and 6, blastocysts were classified into three different stages under an inverted microscope (magnification ×200) according to the degree of expansion: early blastocyst, the blastocoele being less than half the volume of the embryo; blastocyst, the blastocoele being greater than half the volume of the embryo; and expanded blastocyst, the blastocoele volume being greater than that of the early embryo along with thinning of the zona pellucida. In most cases, two most expanded blastocysts with big and tight inner cell mass were selected from the given cohort for ET, and surplus blastocysts were refrozen with use of vitrification.

Embryo Transfers and Assessment of Pregnancy

Embryo transfer was performed from day 2 through day 6 after warming depending on the numbers of fertilized embryos and their quality. Assisted hatching was performed with use of acid Tyrode's solution (Irvine Scientific, Santa Ana, CA) at least 1 hour before ET. All ETs were performed with use of an Efficere catheter (Cooper Surgical, Trumbull, CT) under ultrasound guidance. Most patients received either two or three embryos except for two patients who received four embryos on day 3. Biochemical pregnancy was assessed by serum β-hCG level 14 days after warming the oocytes. Implantation and clinical pregnancy were confirmed by the presence of gestational sac(s) and fetal cardiac activity approximately 4 to 5 weeks after ETs.

RESULTS

The mean age (±SD) of the women was 31.7 ± 3.0 years. A total of 20 warming cycles from 19 patients were performed, and there were no cancelled ETs in this study. One patient (No. 3 and No. 7) who produced 40 oocytes at retrieval underwent two warming cycles from the same batch of oocytes collected. The first cycle ended in miscarriage. She conceived

FIGURE 2

Oocytes after warming and embryos developing after ICSI after warming. (**A**) Oocytes after warming. (**B**) Oocytes removed from cumulus and granulosa cells. (**C**) Zygote (two pronuclei) after ICSI. (**D**) Two- to four-cell stage embryos 2 days after ICSI. (**E**) Four- to eight-cell stage embryos 3 days after ICSI. (**F**) Morular and blastocyst stage embryos 5 days after ICSI.
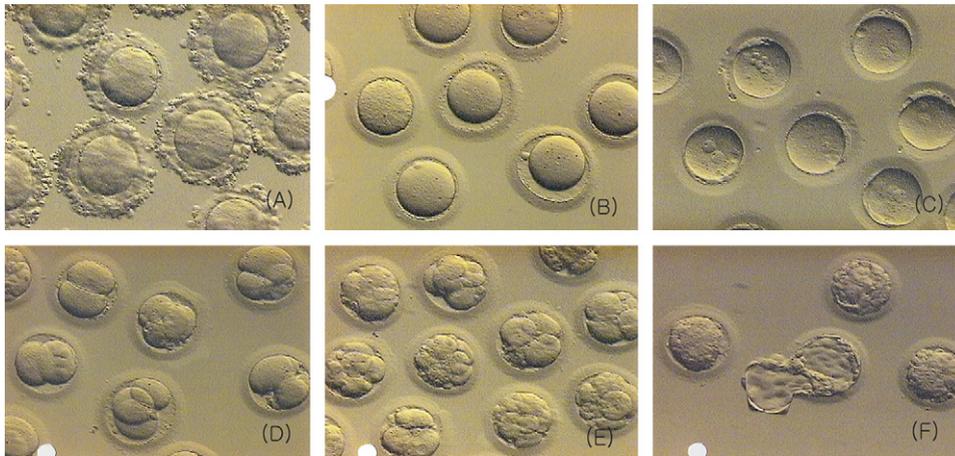
Kim. Vitrification of human oocytes. Fertil Steril 2010.

on the second cycle with triplets and carried the pregnancy successfully to delivery.

A total of 483 oocytes were vitrified, of which 395 oocytes were warmed and 320 (81.0%) of the oocytes survived. Of these, 35 oocytes were either in metaphase I (MI) or germinal vesicle stage (GV) and therefore were not considered further. Thus 285 oocytes were microinjected for insemination, and 206 (72.3%) of the oocytes were fertilized, of which 185 (89.8%) embryos developed to the cleavage stage. Fifty-three embryos were transferred to 19 patients (in 20 warming cycles). The mean number (±SD) of embryos transferred per warming cycle was 2.7 ± 0.7. Of the 53 transferred embryos, 24 (45.3%) were confirmed as having successful implantation by ultrasound examination approximately 4 weeks after ET. Twenty-two surplus blastocysts were refrozen on day 5 or 6 after warming with use of vitrification. Thus if we subtract refrozen blastocysts from warmed oocytes and injected oocytes, the implantation rates per oocyte warmed and warmed oocyte injected were 6.4% (24/373) and 9.1% (24/263), respectively. Of the 20 transfers, 16 resulted in clinical pregnancy; the PR was 80.0% per warming cycle and 84.2% per patient. The clinical summary and detailed accounts of the progression of the vitrified oocytes are depicted in Tables 1 and 2.

Thirteen patients delivered 20 infants. Of the 13 deliveries, there were nine singletons, one set of twins, and three sets of triplets. Live-birth rates per oocyte warmed and oocyte injected were 5.1% (19/373) and 7.2% (19/263), respectively. Of the 20 infants, 11 were male and 9 were female. The mean birth weight (±SD) of the infants was 2,672 ± 1,035 g, and there were no reported complications or birth defects at the time of delivery. All delivered infants had normal physical profiles up to the present time. Three patients (18.8%) had

miscarriages around 6 to 8 weeks gestation. Two patients had a single sac, and one patient had twin sacs (Table 3). There were no therapeutic abortions after amniocentesis or selective reductions during this study.

DISCUSSION

There have been several variations in freezing techniques described. Most laboratories have experiences with the slow-freezing technique. It is known as equilibrium freezing because the fluid exchange between the extracellular and intracellular spaces results in stable freezing without serious osmotic and deformation effects to cells (26). Although it is an accepted safe procedure because of utilization of lower concentrations of cryoprotectants, cells are still susceptible to injury from ice crystal formation. Porcu et al. (2–4) described the freezing of metaphase II (MII) oocytes by the slow-freezing method. Other groups using the slow-freezing protocol reported 43% to 90% survival rates (6, 8, 9, 12, 27) with PRs of 19% to 33%.

Over the last few years, vitrification has emerged as a viable alternative to the slow-freezing method (14–16, 28). It is a nonequilibrium method that prevents ice crystal formation. It requires a rapid cooling rate along with much higher concentrations of cryoprotectants compared with slow freezing (22). Because of high concentrations of cryoprotectants used and by known biologic and physicochemical effects of cryoprotectants, chemical toxicity of these agents is an acknowledged key limiting factor (28). Therefore, balancing the maximal cooling rate and minimizing the cryoprotective concentration is of crucial importance (29). Over the last 5 years, encouraging survival rates have been reported for the vitrification of oocytes (17, 30), cleavage-stage embryos

and zygotes (31, 32), and blastocyst stage embryos (33–37). By 2005, it was suggested, after large comparative studies, that vitrification was a more efficient way of cryopreserving human embryos than the slow-freezing method (31, 38, 39). In general, survival rates after vitrification of human embryos of >85% have been reported (34, 38, 40, 41).

One of the practical advantages of the vitrification method is its simplicity in that one embryologist can perform the entire procedure in minutes without the use of costly equipment (19). However, there are some concerns with the standardization of the method, operator-dependent variation with a steep learning curve, and optimal selection of cryoprotectants or cryocarriers. Even in our program, it took a considerable amount of practice and time for any embryologist to perform the procedure with timely precision according to the protocol. Depending on the level of proficiency, the experimental results varied widely among different embryologists (unpublished data).

Researchers working with the vitrification method have developed their own unique protocols by making alterations in concentration of cryoprotectants, cooling rate, or cryocarriers and have attempted to prove its superiority. However, there is still inadequate data concerning the efficiency and the standardization of the method because of variation in use and application at different stages (22, 42). For example, although we believe our protocol with 5.5 mol/L EG with 1.0 mol/L sucrose for 20 seconds equilibration time produces the best outcome, other protocols, such as 15% EG and 15% dimethyl sulfoxide with 0.5 mol/L sucrose for <60 seconds of equilibration, have also produced excellent oocyte survival (99%), fertilization (93%), and cleavage (96%) rates (19, 25, 31). It appears that the equilibration stage can last up to a minute in these highly concentrated cryoprotectants without causing damage to human oocytes.

Different types of cryocarriers have been used with the goal of maximizing the cooling rate by reducing volume of the vitrification solution. Cryocarriers described in the literature include the open pulled straw (43), microdrops (44), the hemistraw (29), the Flexipet denuding pipette (45), and Cryotop (46). In our laboratory, a copper EM grid was used for its high thermal conductivity. We had good success with EM copper grids (14, 17, 28), but for this study a gold grid was used as a carrier for vitrified oocytes because the copper grid was more difficult to handle as a result of its light weight, which caused too much movement when placed in the grid holder under liquid nitrogen. Gold offers advantages similar to copper; it has high thermal conductivity, and its heavier atomic weight makes the gold grid more stable and hence less prone to movement under LN_2. Moreover, survival rates were not different from that of copper (data not shown).

Our results indicate that initial oocyte quality before cryopreservation is probably the single most significant factor in determining successful pregnancy after warming. Trying to cryopreserve compromised, lower-quality oocytes is expected to produce lower PRs. When we compared our current data with results of our older study (17) where leftover oocytes from IVF cycles were used with the same vitrification protocol, there was a striking difference in PRs (84% vs. 21%) and implantation rates (45% vs. 6%) although the fertilization and cleavage rates were similar between the two studies.

The most remarkable aspect of our result is the implantation rate of 45%. Because we were not sure of the number

TABLE 2

Progression of vitrified oocytes.

Warming cycles (patients)	Age (y)	No. vitrified oocytes	No. warmed oocytes	No. survived oocytes	No. ICSI	No. two pronuclei	No. transferred embryos
1	36	19	19	7	7	3	3
2	33	15	15	12	11	7	4
3[a]	28	40	16	13	11	11	4
4	27	66	32	30	30	18	3
5	31	22	22	14	13	10	2
6	30	31	31	26	24	13	3
7[a]	28	0	24	18	15	10	3
8	29	42	32	30	28	19	2
9	34	29	29	21	15	14	2
10	26	17	17	13	12	10	3
11	30	20	20	19	19	15	2
12	31	8	8	6	5	4	2
13	34	28	24	22	18	15	3
14	35	12	12	6	5	5	3
15	33	16	16	13	9	8	3
16	32	18	18	18	17	10	2
17	31	21	21	16	14	10	2
18	31	11	11	10	8	5	3
19	34	25	25	24	22	17	2
20	37	3	3	2	2	2	2

[a] Patient underwent two warming cycles out of same batch of oocytes retrieved.

Kim. Vitrification of human oocytes. Fertil Steril 2010.

of frozen oocytes required to produce successful pregnancy, large numbers (19.8) of oocytes were used for each warming cycle. It turned out that only 72% of the warmed oocytes were in MII; therefore, 14.3 oocytes were microinjected with sperm (ICSI) per warming. Our past experience was that warmed-oocyte quality of immature (GV) to intermediate (MI) oocytes was greatly inferior to that of MII oocytes (unpublished data). In four patients, there were more than enough good-quality blastocysts left after ET procedures. These blastocysts were revitrified at the patients' request.

From our current study data, though a small sample size, we tried to estimate the number of oocytes needed to produce a healthy infant after a long-term (at least 6 months) cryopreservation in the fertile population. We factored in the refrozen blastocysts for calculation. On the basis of the number of births of 19 along with the estimated probable birth of 8 (22 × 36%) from refrozen blastocysts, and total number of warmed oocytes of 395, it took about 15 (395/27) oocytes regardless of their maturity or 11 (15 × 72%) mature oocytes to produce one live birth in a healthy fertile population <36 years of age. If we subtract refrozen blastocysts from warmed oocytes and injected oocytes, the live-birth rates per oocyte warmed and oocyte injected were 5.1% (19/373) and 7.2% (19/263), respectively. This calculation may be an overestimation given our liberal use of the number of oocytes for this study, but it compares very favorably with the results of fresh IVF-ET cycles and much better than what was previously reported on oocyte cryopreservations.

Recently the efficiency of cryopreserved oocytes by slow freezing and vitrification has been reviewed (47, 48). Live-birth rates per oocyte thawed were 1.9% and 2.0% for slow freezing and vitrification, respectively. Live-birth rates per injected oocyte and ET for slow freezing were 3.5% and 21.6%, respectively. During the same period the live-birth rate per oocyte in fresh cycles of IVF-ET was 6.6%. Thus the live-birth rate per vitrified warmed oocyte of the present study is about the same as that with fresh IVF-ET and twofold to threefold higher than that in the world literature on oocyte freezing PRs.

There were three sets of triplets after three ETs on day 4, 5, or 6 after warming. This indicates that once the embryos develop past the cleavage stage, their developmental potential is almost equal to that of fresh embryos. Therefore, the same criteria used for fresh blastocyst transfers may be applied in determining the number of blastocysts to transfer to reduce multiple pregnancies.

There is no doubt that successful and safe cryopreservation of human oocytes definitely will improve and change the way we practice reproductive medicine. Its safety and efficiency will need to withstand the test of time. We have witnessed steady improvements in efficiency of IVF over the last 25

TABLE 3

Pregnancy outcome of vitrified oocytes.

Patient No.	Age (y)	Day-ET	No. transferred embryos	Pregnancy	Boy (g)	Girl (g)
1	36	2	3	No		
2	33	3	4	Singleton		3,430
3[a]	28	3	4	Miscarriage		
4	27	5	3	Triplets	1,644, 4,616	1,446
5	31	5	2	Singleton	4,961	
6	30	5	3	Singleton	3,855	
7[a]	28	6	3	Triplets	1,559	1,106, 1,928
8	29	6	2	Singleton		3,855
9	34	5	2	Singleton	3,572	
10	26	6	3	Singleton	3,912	
11	30	5	2	Miscarriage		
12	31	3	2	Singleton	2,835	
13	34	6	3	No		
14	35	4	3	Triplets		1,956, 1,814, 2,608
15	33	5	3	Miscarriage		
16	32	6	2	Singleton	2,807	
17	31	4	2	No		
18	31	3	3	Singleton		3,062
19	34	5	2	Twins	2,835, 2,636	
20	37	3	2	No		

[a] Patient underwent two warming cycles out of same batch of oocytes retrieved.

Kim. Vitrification of human oocytes. Fertil Steril 2010.

years after going through many trials and errors along the way. It appears that oocyte cryopreservation is going through a similar path of development. Although statistically small in numbers, many groups around the world have reported much improved efficiency (10%–25% PRs) compared with rates that used to be 1% to 2% merely 10 years ago.

If the current success rate can be maintained, application of oocyte vitrification may go beyond the cases with medical problems. With the modern trend of women delaying childbearing for social and professional reasons, oocyte cryopreservation technology may be able to provide these women with an option of family planning beyond their biologic time clock. Also, with increasing concerns and regulations for transmitting infectious diseases in third-party reproduction, it may be inevitable to quarantine donated oocytes to ensure their safety, as it is a routine practice for donated sperm. Sperm banks around the country have established policies to quarantine sperm samples for a minimum of 6 months to ensure safety from infectious diseases while the donor is rescreened in 6 months.

Some European countries allow only limited numbers of oocytes to be fertilized, therefore, patients are forced to cryopreserve supernumerary oocytes for future use. As more experience and knowledge are gained, it is logical to think that it is only a matter of time before oocyte cryopreservation can be offered in clinical practice to well-qualified patients.

In conclusion, this study shows that oocytes vitrified with use of the EG and EM gold grid system retain excellent developmental competence after warming, resulting in a high PR without any known complications or birth defects. Maintenance of high developmental competence of warmed oocytes could allow us to reduce the number of blastocysts transferred to avoid multiple pregnancies. Oocyte cryopreservation, when applied to properly screened patients, will be a useful technology in reproductive medical practice. More studies are necessary to further develop a better selection method for oocyte quality discrimination and better in vitro maturation methods for immature and intermediate mature oocytes to improve their survival.

REFERENCES

1. Chen C. Pregnancy after human oocyte cryopreservation. Lancet 1986;1:884–6.
2. Porcu E, Fabbri R, Seracchioli R, Ciotti PM, Magrini O, Flamigni C. Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. Fertil Steril 1997;13:724–6.
3. Porcu E. Freezing of oocytes. Curr Opin Obstet Gynecol 1999;11:297–300.

4. Porcu E, Fabbri R, Damiano G, Giunchi S, Fratto R, Ciotti PM, et al. Clinical experience and applications of oocyte cryopreservation. Mol Cell Endocrinol 2000;27:33–7.

5. Tucker MJ, Wright G, Morton PC, Massey JB. Birth after cryopreservation of immature oocytes with subsequent in vitro maturation. Fertil Steril 1998;70:578–9.

6. Fabbri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C. Human oocyte cryopreservation: new perspectives regarding oocyte survival. Hum Reprod 2001;16:411–6.

7. Quintans CJ, Donaldson MJ, Bertolino MV, Pasqualini RS. Birth of two babies using oocytes that were cryopreserved in a choline-based freezing medium. Hum Reprod 2002;17:3149–52.

8. Fosas N, Marina F, Torres PJ, Jove I, Martin P, Perez N, et al. The births of five Spanish babies from cryopreserved donated oocytes. Hum Reprod 2003;18:1417–21.

9. Boldt J, Cline D, McLaughlin D. Human oocyte cryopreservation as an adjunct to IVF–embryo transfer cycles. Hum Reprod 2003;18:1250–5.

10. Chen SU, Lien YR, Chen HF, Chang LJ, Tsai YY, Yang YS. Observational clinical follow-up of oocyte cryopreservation using a slow-freezing method with 1,2-propanediol plus sucrose followed by ICSI. Hum Reprod 2005;20:1975–80.

11. Levi Setti PE, Albani E, Novara PV, Cesana A, Morreale G. Cryopreservation of supernumerary oocytes in IVF/ICSI cycles. Hum Reprod 2006;21:370–5.

12. Borini A, Sciajno R, Bianchi V, Sereni E, Flamigni C, Coticchio G. Clinical outcome of oocyte cryopreservation after slow cooling with a protocol utilizing a high sucrose concentration. Hum Reprod 2006;21:512–7.

13. Bianchi V, Coticchio G, Distratis V, Di Giusto N, Flamigni C, Borini A. Differential sucrose concentration during dehydration (0.2 mol/l) and dehydration (0.3 mol/l) increases the implantation rate of frozen human oocytes. Reprod Biomed Online 2007;14:64–71.

14. Hong SW, Chung HM, Lim JM, Ko JJ, Yoon TK, Yee B, et al. Improved human oocyte development after vitrification: a comparison of thawing methods. Fertil Steril 1999;72:142–6.

15. Kuleshova L, Gianaroli L, Magli C, Ferraretti A, Trounson A. Birth following vitrification of a small number of human oocytes: case report. Hum Reprod 1999;14:3077–9.

16. Chung HM, Hong SW, Lim JM, Lee SH, Cha WT, Ko JJ, et al. In vitro blastocyst formation of human oocytes obtained from unstimulated and stimulated cycles after vitrification at various maturational stages. Fertil Steril 2000;73:545–51.

17. Yoon TK, Kim TJ, Park SE, Hong SW, Ko JJ, Chung HM, et al. Live births after vitrification of oocytes in a stimulated in vitro fertilization–embryo transfer program. Fertil Steril 2003;79:1323–6.

18. Yoon TK, Lee DR, Cha SK, Chung HM, Lee WS, Cha KY. Survival rate of human oocytes and pregnancy outcome after vitrification using slush nitrogen in assisted reproductive technologies. Fertil Steril 2007;88:952–6.

19. Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. Reprod Biomed Online 2005;11:300–8.

20. Kyono K, Fuchinoue K, Yagi A, Nakajo Y, Yamashita A, Kumagai S. Successful pregnancy and delivery after transfer of a single blastocyst derived from a vitrified mature human oocyte. Fertil Steril 2005;84:1017.

21. Lucena E, Bernal DP, Lucena C, Rojas A, Moran A, Lucena A. Successful ongoing pregnancies after vitrification of oocytes. Fertil Steril 2006;85:108–11.

22. Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. Reprod Biomed Online 2006;12:779–96.

23. Camus A, Clairaz P, Ersham A, Van Kappel AL, Savic G, Staub C. [The comparison of the process of five different vitrification devices.]. Gynecol Obstet Fertil 2006;34:737–45.

24. Selman H, Angelini A, Barnocchi N, Brusco GF, Pacchiarotti A, Aragona C. Ongoing pregnancies after vitrification of human oocytes using a combined solution of ethylene glycol and dimethyl sulfoxide. Fertil Steril 2006;86:997–1000.

25. Antinori M, Licata E, Dani G, Cerusico F, Versaci C, Antinori S. Cryotop vitrification of human oocytes results in high survival rate and healthy deliveries. Reprod Biomed Online 2007;14:72–9.

26. Mazur P. Equilibrium, quasi-equilibrium, and nonequilibrium freezing of mammalian embryos. Cell Biophys 1990;17:53–92.

27. Boldt J, Tidswell N, Sayers A, Kilani R, Cline D. Human oocyte cryopreservation: 5-year experience with a sodium-depleted slow freezing method. Reprod Biomed Online 2006;13:96–100.

28. Yoon TK, Chung HM, Lim JM, Han SY, Ko JJ, Cha KY. Pregnancy and delivery of healthy infants developed from vitrified oocytes in a stimulated in vitro fertilization–embryo transfer program. Fertil Steril 2000;74:180–1.

29. Liebermann J, Tucker MJ. Effect of carrier system on the yield of human oocytes and embryos as assessed by survival and developmental potential after vitrification. Reproduction 2002;124:483–9.

30. Kulesshova LL, Lopata A. Vitrification can be more favorable than slow cooling. Fertil Steril 2002;78:449–54.

31. Kuwayama M, Vajta G, Ieda S, Kato O. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. Reprod Biomed Online 2005;11:608–14.

32. Rama Raju GA, Haranath GB, Krishna KM, Prakash GJ, Madan K. Vitrification of human 8-cell embryos, a modified protocol for better pregnancy rates. Reprod Biomed Online 2005;11:434–7.

33. Choi DH, Chung HM, Lim JM, Ko JJ, Yoon TK, Cha KY. Pregnancy and delivery of healthy infants developed from vitrified blastocysts in an IVF-ET program. Fertil Steril 2000;74:838–9.

34. Mukaida T, Nakamura S, Tomiyama T, Wada S, Kasai M, Takahashi K. Successful birth after transfer of vitrified human blastocysts with use of a cryoloop containerless technique. Fertil Steril 2001;76:618–20.

35. Reed ML, Lane M, Gardner DK, Jensen NL, Thompson J. Vitrification of human blastocysts using the cryoloop method: successful clinical application and birth of offspring. J Assist Reprod Genet 2002;19:304–6.

36. Takahashi K, Mukaida T, Goto T, Oka C. Perinatal outcome of blastocyst transfer with vitrification using cryoloop: a 4-year follow-up study. Fertil Steril 2005;84:88–92.

37. Hong SW, Kim TJ, Lee SH, Choi DH, Cha KY. Cryopreserved blastocysts using vitrification protocol give excellent pregnancy and implantation rates after thawing. Fertil Steril 2005;84:S178–9.

38. Huang CC, Lee TH, Chen SU, Chen HH, Cheng TC, Liu CH, et al. Successful pregnancy following blastocyst cryopreservation using supercooling ultra-rapid vitrification. Hum Reprod 2005;20:122–8.

39. Stehlik E, Stehlik J, Katayama KP, Kuwayama M, Jambor V, Brohammer R, et al. Vitrification demonstrates significant improvement versus slow freezing of human blastocysts. Reprod Biomed Online 2005;11:53–7.

40. Mukaida T, Nakamura S, Tomiyama T, Wada S, Oka C, Kasai M, et al. Vitrification of human blastocysts using cryoloops: clinical outcome of 223 cycles. Hum Reprod 2003;18:384–91.

41. Vanderzwalmen P, Bertin G, Debauche CH, Standaert V, Bollen N, Roosendaal EV, et al. Vitrification of human blastocysts with the hemistraw carrier: application of assisted hatching after thawing. Hum Reprod 2003;18:1504–11.

42. Kattera S, Chen C. Cryopreservation of embryos by vitrification: current development. Int Surg 2006;91:S55–62.

43. Chen SU, Lien YR, Chen HF, Chao KH, Ho HN, Yang YS. Open pulled straws for vitrification of mature mouse oocytes preserve patterns of meiotic spindles and chromosomes better than conventional straws. Hum Reprod 2000;15:2598–603.

44. Papis K, Shimizu M, Izaike Y. Factors affecting the survivability of bovine oocytes vitrified in droplets. Theriogenology 2000;54:651–8.

45. Liebermann J, Tucker MJ, Graham JR, Han T, Davis A, Levy MJ. Blastocyst development after vitrification of multipronuclear zygotes using the Flexipet denuding pipette. Reprod Biomed Online 2002;4:146–50.

46. Katayama K, Stehlik J, Kuwayama M, Kato O, Stehlik E. High survival rate of vitrified human oocytes results in clinical pregnancy. Fertil Steril 2003;80:223–4.

47. Oktay K, Cil AP, Bang H. Efficiency of oocyte cryopreservation: a meta-analysis. Fertil Steril 2006;86:70–80.

48. Borini A, Bianchi V, Bonu MA, Sciajno R, Sereni E, Cattoli M, et al. Evidence-based clinical outcome of oocyte slow cooling. Reprod Biomed Online 2007;15:175–81.