

Live births after vitrification of oocytes in a stimulated in vitro fertilization–embryo transfer program

Tae Ki Yoon, M.D.,^a Thomas J. Kim, M.D.,^b Sung Eun Park, M.S.,^b
Seung Wook Hong, M.S.,^b Jung Jae Ko, Ph.D.,^a Hyung Min Chung, Ph.D.,^a and
Kwang Yul Cha, M.D.^a

Pochon CHA University and Infertility Medical Center of CHA General Hospital, Seoul, Korea

Objective: To assess the usefulness of the vitrification method in clinical practice.

Design: Clinical study of vitrification of human oocytes.

Setting: A university-affiliated hospital.

Patient(s): Thirty-four patients who agreed to undergo additional IVF-ET after the failed fresh cycle using the previously vitrified/thawed oocytes.

Intervention(s): Surplus oocytes from the IVF-ET patients were vitrified for the next cycle.

Main Outcome Measure(s): Morphologic normality of post-thawed oocytes, fertilization, cleavage, pregnancy, and implantation rate.

Result(s): Overall morphological survival and fertilization rates of vitrified/liquefied oocytes were 68.6% (325/474) and 71.7% (142/198), respectively. Pregnancy rate and implantation rate per embryo transfer were 21.4% (6/28) and 6.4% (8/125), respectively. All pregnancies resulted in the delivery of healthy babies (1 twin and 5 singletons/6 pregnancies).

Conclusion(s): The feasibility of the vitrification method for human oocytes was confirmed by our clinical results. Subsequent studies on vitrification and thawing procedures should be undertaken for further optimizing the vitrification method. (*Fertil Steril*® 2003;79:1323–6. ©2003 by American Society for Reproductive Medicine.)

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Reprint requests: Tae Ki
Yoon, M.D., College of
Medicine, Pochon CHA
University and Infertility
Medical Center of CHA
General Hospital, 606-5
Yeoksam 1-Dong,
Kangnam-Gu, Seoul, 135-
081, Korea (FAX: 82-2-501-
8704; E-mail:
tkyoon@cha.ac.kr).

^a College of Medicine,
Pochon CHA University
and Infertility Medical
Center of CHA General
Hospital.

^b CHA Fertility Center, Los
Angeles, California.

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Cryopreservation of human oocytes has been one of the most elusive tasks for scientists in the field of assisted reproductive technology. Ever since the first successful cryopreservation of human embryos in the early 1980s, embryo freezing has become a routine procedure in many centers offering assisted reproductive techniques and has resulted in the births of many children. However, embryo freezing may also be associated with legal and ethical issues. Some of the issues involving embryo freezing may be avoided by cryopreserving the oocytes instead. Currently, several centers offer oocyte cryopreservation services to patients who may lose gonadal function because of chemotherapy, radiation treatment for various cancers, or extirpative therapy for pelvic pathology and pain.

Numerous studies have been reported in search of developing an ideal oocyte cryo-

preservation method after the first success of Chen (1), who reported the first pregnancy from frozen human mature oocytes. Since then, several centers around the world have reported successful pregnancies, though limited, that were established after frozen oocyte–derived embryo transfer programs (2–10). Most of these pregnancies were achieved by a slow-freezing method, and the viability of the oocytes after thawing has been highly variable, ranging from 2.2% to 65%. Therefore, there is a pressing need to develop a more efficient way to cryopreserve the oocytes.

Recent reports in animals (11–13) demonstrated that vitrification was used for oocyte freezing at both the germinal vesicle stage and the metaphase II stage. The vitrification used in mammalian models has shown promising results.

We have developed a vitrification method for the cryopreservation of human oocytes. In our previous study, we tried this vitrification method using ethylene glycol and an electron microscope grid for the cryopreservation of human oocytes and demonstrated that this method could yield higher postthaw survival and subsequent embryo development than slow-freezing methods (14, 15). In this paper, we report the interim results of our ongoing study that employed this technology for IVF-ET in patients who have failed fresh-IVF cycles.

MATERIALS AND METHODS

The institutional review board of CHA General Hospital, College of Medicine, Pochon CHA University, Seoul, Korea approved all of the experimental procedures used in this study. All of the study participants gave their informed consent.

Patients

From October 1997 to February 2002, 136 patients agreed to participate in an oocyte cryopreservation protocol using the vitrification method for the extra oocytes while they were undergoing IVF cycles. After failing the fresh-IVF cycles, 34 patients returned for ET using vitrified-thawed oocytes. The mean (\pm SD) age and duration of infertility for the patients were 32.4 ± 5.8 years and 5.6 ± 4.8 years, respectively. Reasons for the IVF-ET were as follows: tubal factors ($n = 10$), male factors ($n = 18$), unexplained causes ($n = 2$), ovum donation ($n = 2$), anovulation ($n = 1$), and endometriosis ($n = 1$).

After proper pituitary regulation and desensitization using GnRH agonist (Buserelin; Hoechst AG, Seoul, Korea) during the luteal phase, controlled ovarian hyperstimulation was initiated with FSH-hMG. Ovulation was triggered with 10,000 IU of hCG (Profasi; Serono, Seoul, Korea) when controlled ovarian hyperstimulation had resulted in the development of at least two follicles >18 mm in diameter. Transvaginal oocyte retrieval was done 34–36 hours after the hCG administration. Using our standard protocol, we prepared some of the retrieved cumulus oocyte complexes for the fresh IVF-ET. The rest of them were used for vitrification. For transfer of embryos derived from cryopreserved oocytes, we performed thawing and intracytoplasmic sperm injection (ICSI) on the day of ovulation in the natural cycle or 14 days after hormone replacement with E_2 valerate (Progynova; Schering, Berlin, Germany). We transferred embryos to the uterus 3 days after the ICSI procedure. Pregnancy was identified by the serum β -hCG level, checked 14 days after ET. On diagnosing the pregnancy, we performed a maternal blood dual test and amniocentesis to exclude fetal anomalies.

Vitrification and Thawing of Oocytes

The vitrification method used in this study was based on the method developed by Martino et al. (11). Cumulus-

oocyte complexes were pre-equilibrated for 2.5 minutes in 2 mL of Dulbecco's phosphate-buffered saline (Gibco BRL, Grand Island, NY) supplemented with 1.5 M of ethylene glycol (E-9129; Sigma, St. Louis, MO) and 10% (vol/vol) fetal bovine serum (Gibco BRL) at 37°C. Oocytes were then placed for the final equilibration in the same volume of Dulbecco's phosphate-buffered saline supplemented with 5.5 M of ethylene glycol, 1.0 M of sucrose, and 10% fetal bovine serum for 20 seconds. In the meantime, two to five oocytes were mounted on an electron microscope grid (Gilder, Westchester, PA) using a fine pipette, and excess cryoprotectant solution was removed with the underlying sterilized filter paper. The grids containing oocytes were immediately plunged into liquid nitrogen, and a cryovial cap and goblet were used for placement of the grid. Oocytes were then stored for 1–43 months before thawing; the mean interval between the fresh and vitrified cycles was 7.4 months. For thawing, the grids were sequentially transferred to culture dishes containing 2 mL of Dulbecco's phosphate-buffered saline supplemented with 1.0, 0.5, 0.25, 0.125, or 0 M of sucrose and 10% (vol/vol) fetal bovine serum at intervals of 2.5 minutes at 37°C.

Intracytoplasmic Sperm Injection, Embryo Culture, and Embryo Transfer

After being washed four to six times, cumulus cells removed from liquefied oocytes with 50 IU/mL of hyaluronidase and then transferred into the preimplantation-1 (P-1) medium (Irvine Scientific, Irvine, CA) with 10% synthetic serum substitute (Irvine Scientific). Oocytes were defined as morphologically survived if they possessed an intact zona pellucida, plasma membrane, and a refractive cytoplasm. Intracytoplasmic sperm injection was performed 3 hours after the culture of oocytes. The number of pronuclei in the cytoplasm was counted to verify normal fertilization of vitrified oocytes 16–19 hours after ICSI. The embryos from 2PN zygotes were cultured in the P-1 medium with 10% synthetic serum substitute and transferred on day 2 or 3 after ICSI.

Statistical Analysis

Data are expressed as mean \pm SD. The χ^2 test was used for statistical comparison. $P < .01$ was considered statistically significant.

RESULTS

Survival, fertilization, cleavage, pregnancy, and implantation rates of vitrified oocytes are summarized in Table 1. Thirty-four patients agreed to undergo transfer of embryos derived from cryopreserved oocytes after failing the fresh-cycle IVF-ET. Of the 868 oocyte-cumulus complexes (25.6 ± 11.9) retrieved from 34 stimulated patients, 394 oocytes were used for fresh IVF-ET cycles, and the remaining 474 oocyte-cumulus complexes were cryopreserved using the vitrification method. A morphological survival rate of the

TABLE 1

Comparison of fertilization, cleavage, pregnancy, and implantation rates of vitrified human oocytes.

Variable	Vitrified (n)
No. of cycles	34
No. of COCs provided oocytes ^a	474
No. of vitrified-thawed oocytes	474
No. of survived oocytes (%)	325 (68.6)
No. of microinjected oocytes	198
No. of fertilized oocytes (%)	142 (71.7)
No. of cleaving 2PN embryos ^b (%)	135 (95.0)
No. of patients undergoing ET	28
No. of embryos transferred (mean \pm SD)	125 (4.5 \pm 1.9)
Pregnancy rate per transfer (%)	6/28 (21.4)
Implantation rate (%)	8/125 (6.4)
Live birth	7

Note: 2PN = male and female pronuclei.

^a Only morphologically normal oocytes were provided for IVF-ET cycles.

^b Surplus cleaved embryos were cryopreserved.

Yoon. Live births after oocyte vitrification. Fertil Steril 2003.

oocytes was 68.6% (325/474). Of those, 61% (198/325) were metaphase II oocytes with morphologically normal cytoplasm suitable for ICSI procedure.

Fertilization rate of these cryopreserved oocytes was 71.7% (142/198), and the cleavage rate on day 2 was 95% (135/142). The average number of embryos transferred was 4.5 (125/28). The mature oocyte-to-baby rate was 4% (8/198).

Detailed pregnancy outcomes from vitrified oocytes are summarized in Table 2. At the time of embryo transfer, six patients did not go through with the ET procedure because of fertilization failure or poor embryo quality. A total of 125 cleaving embryos from vitrified oocytes were transferred to the remaining 28 patients, and 6 patients achieved clinical pregnancies. The pregnancy and implantation rates of vitrified IVF-ET were 21.4% (6/28) and 6.4% (8/125), respec-

TABLE 2

Detailed pregnancy outcome developed from vitrified oocytes in a stimulated cycle.

Patient	Duration of frozen oocytes (mo)	No. of oocytes					No. implanted/transferred	No. delivered (sex)
		Vitrified	Survived (%)	Microinjected	Fertilized (%)	Cleaved		
A	9	14	14 (100)	7	6 (85.7)	6	1/6	1 (M)
B	2	13	11 (84.6)	11	7 (63.6)	7	1/4	1 (M)
C	16	18	15 (83.3)	8	5 (62.5)	5	1/5	1 (F)
D	16	15	13 (86.7)	11	8 (72.7)	7	1/7	1 (F)
E	43	10	6 (60.0)	6	4 (66.7)	4	2 ^a /4	1 (F)
F	32	15	14 (93.3)	7	4 (57.1)	4	2/4	2 (M)

^a One had selective abortion at 12 weeks of gestation.

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TABLE 3

Patients' characteristics and ART cycle profiles.

Variable	Data
No. of patients	34
Mean (\pm SD) age, in y	32.4 \pm 5.8
Mean (\pm SD) infertility duration, in y	5.6 \pm 4.8
Infertility factor (n)	
Tubal	10
Male	18
Unexplained	2
OD	2
Anovulation	1
Endometriosis	1

Yoon. Live births after oocyte vitrification. Fertil Steril 2003.

tively. All pregnancies resulted in the delivery of healthy babies. Maternal blood dual test results for fetal anomaly in the first trimester were normal in all patients. Four patients consented to undergo amniocentesis, and the fetuses were verified to have normal karyotypes. The first baby was a healthy male infant delivered in August 1999. We identified twinning in two pregnancies and performed a selective reduction procedure at 12 weeks of gestation in one case at the patient's request. So far, seven healthy babies have been born of six pregnancies. Detailed characteristics of patients who were involved in this study are summarized in Table 3.

DISCUSSION

In our previous study, we reported the birth of infants derived from vitrified-thawed oocytes (10). In this article, our results further support the notion that oocyte vitrification can be applied to assisted reproductive technology successfully.

The main obstacle with conventional oocyte cryopreservation method (slow freezing and rapid thawing) is low rates

of oocyte survival and fertilization, leading to a poor pregnancy rate due to cryoinjuries. These include damage to the meiotic spindles, microfilaments, zona pellucida, and cortical granules (16).

The vitrification method used in this study is unique in that it includes an electron microscope grid and a relatively short equilibration process compared with other methods. The electron microscope grid enhances heat conduction to the oocyte cytoplasm, and the short equilibration procedure helps prevent severe osmotic damage from high concentration of the vitrification solution. The high rate of survival and cleavage found in vitrified oocytes (Table 1) suggests that mechanical damage of oocytes induced by their attachment to the EM grid is negligible. This method can reduce cryoinjury of oocytes during freezing and optimizes the efficacy of oocyte cryopreservation. Another advantage of this method is that it is a rapid process and requires less equipment than does the conventional slow-freezing method.

However, the disadvantage of vitrification using the electron microscope grid is that oocytes with the vitrification solution come directly in contact with liquid nitrogen during cooling and storage. A recent study by Bielanski et al. (17) reported the possible transmission of viral pathogens to bovine embryos vitrified and stored in contaminated LN₂. To protect the risk of LN₂ contamination, we are currently conducting experiments to develop effective vitrification method by using straws.

Improving the survival rate and the fertilization rate remains a major challenge in cryopreservation technology. Recently, ICSI has been proposed as a solution to cryodamage involving the zona pellucida and cortical granules. Porcu et al. (7) reported the introduction of the ICSI led to an increase in the fertilization rate. In this study, we also performed ICSI to achieve fertilization for the vitrified-thawed oocytes. Fertilization rate (71.7%) in vitrified-thawed oocytes was not significantly decreased when compared with those in fresh IVF-ET cycles (data not shown). Severe cytotoxicity of high concentration of cryoprotectant may adversely affect oocyte development after vitrification. In our previous report, vitrified oocytes had lower spindle and chromosomal abnormalities than did fresh oocytes, which resulted in reduced survival after thawing (18).

This study has confirmed the feasibility of the human oocyte vitrification method. Cryopreservation of oocytes by vitrification, rather than by freezing, can significantly improve the efficacy of various infertility programs, such as oocyte banking and ovum donation. Currently, we are offering oocyte banking to several leukemic patients as well as other types of cancer patients who are scheduled to undergo chemotherapy. If the current success (pregnancy) rate can be maintained or even improved with oocyte vitrification method, application of this technology can go beyond the populations with medical problems. Because more women are delaying childbearing for social and professional reasons,

some of these women would lose their reproductive capabilities with no clear alternative plans. Oocyte banking technology may be able to provide these women with an option, though limited, to preserve and prolong their reproductive potentials. With recent public interest in the area of women's aging and fertility, development of this type of technology will be needed even more. Additionally, oocyte cryopreservation may have the potential to overcome many of the legal and ethical problems associated with the embryo cryopreservation and the disposal of surplus embryos during IVF-ET. More studies on vitrification and thawing procedures are needed to develop more efficient and optimal vitrification methods. Several experimental vitrification protocols using different mixtures and concentration of cryoprotectant compounds are under way.

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