FERTILITY AND STERILITY®
VOL. 73, NO. 3, MARCH 2000

Published by Elsevier Science Inc.
Printed on acid-free paper in U.S.A.

1

In vitro blastocyst formation of human oocytes obtained from unstimulated and stimulated cycles after vitrification at various maturational stages

Hyung M. Chung, Ph.D., Seung W. Hong, M.S., Jeong M. Lim, Ph.D., Sook H. Lee, M.D., Won T. Cha, M.T., Jung J. Ko, Ph.D., Se Y. Han, M.D., Dong H. Choi, M.D., and Kwang Y. Cha, M.D.

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

Objective: To evaluate the developmental competence and chromosomal normality of oocytes vitrified at various times after maturation culture.

Design: In vitro model study.

Setting: A university-affiliated hospital.

Patient(s): Unstimulated women who underwent cesarean section or oophorectomy and infertile women who underwent a long protocol of GnRH stimulation.

Intervention(s): Retrieved oocytes were vitrified at 0 or 48 hours after culture in unstimulated cycles and at 0, 8–15, or 24–28 hours after culture in stimulated cycles.

Main Outcome Measure(s): Postthaw morphologic normality, maturation, fertilization, cleavage, blastocyst formation, and chromosome number.

Result(s): In the 53 oocytes that were obtained from unstimulated cycles, no statistically significant differences were found in rates of morphologic normality (range, 56%–63%) or fertilization (range, 31%–37%) according to the time of vitrification. In the 50 oocytes that were obtained from stimulated cycles, more of those that were vitrified at 24–28 hours were morphologically normal than those that were vitrified at 0 or 8–15 hours. Regardless of these differences, high cleavage rates (83%–100%) were obtained that did not differ significantly among the treatment groups. In both cycles, 20%–43% of cleaved oocytes developed to the blastocyst stage by 6 days after IVF. All the karyotyped blastocysts, three from unstimulated cycles and four from stimulated cycles, had a normal number of chromosomes.

Conclusion(s): Vitrified and thawed oocytes from unstimulated or stimulated cycles developed to the blastocyst stage, regardless of when vitrification occurred; the number of chromosomes in the blastocysts was normal. (Fertil Steril® 2000;73:545–51. ©2000 by American Society for Reproductive Medicine.)

Key Words: Human, oocyte, vitrification, in vitro maturation, chromosomal normality

Received April 30, 1999; revised and accepted September 7, 1999.

Presented at the 16th World Congress on Fertility and Sterility, San Francisco, California, October 4–9, 1998.

This work was supported by a grant (No. 1999-2-205-002-5) from the Interdisciplinary Research Program of the Korean Science and Engineering Foundation.

Reprint requests: Hyung M. Chung, Ph.D., 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: artchung@netsgo.com).

0015-0282/00/$20.00
PII S0015-0282(00)00546-4

Techniques for the long-term preservation of human oocytes have been developed and pregnancies have been achieved after the transfer of embryos derived from cryopreserved oocytes (1–5). In these reports, the technique used involved slow freezing and rapid thawing with propylene glycol or dimethylsulfoxide. However, these cryopreservation programs have a limited ability to support oocyte growth and development, and they frequently induce fertilization failure and meiotic spindle and chromosomal abnormalities that adversely affect subsequent embryo development (6, 7). In addition, because of the low freezing capacity of immature (germinal vesicle [GV]) oocytes (8, 9), most studies have focused on the cryopreservation of mature (metaphase II) oocytes, with only a few exceptions (5, 10). This limits the use of cryopreservation programs to patients who have a large number of immature oocytes.

Therefore, we turned our attention to the use of a vitrification protocol instead of the slow freezing method. Vitrification results in less cellular damage than slow freezing. Recent re-

TABLE 1

Patient demographics and morphologic normality, maturation, and fertilization of oocytes vitrified at different times after maturation culture.

Induction program	Total no. of patients	Mean (±SD) age (y)	Mean duration (±SD) of infertility (y)	No. of oocytes allotted	Time of vitrification (hours after culture)	No. of oocytes vitrified	No. (%) of vitrified oocytes		
							Morphologically normal*	Matured*	Developed to the 2PN stage after ICSI†
Unstimulated	66	33.2 ± 5.8	—	30	0	30	19 (63)	12 (40)	7 (37)
				23	48	16‡	9 (56)	—	5 (31)
Stimulated	50	34.1 ± 3.8	5.7 ± 3.5	17	0	17	11 (65)	9 (53)	6 (55)
				16	8–15	14§	9 (64)‖	9 (64)	5 (56)
				17	24–28	12‡	12 (100)‖	—	10 (83)

Note: 2PN = pronuclear stage.

* Percentage of the number of oocytes vitrified. Morphologic normality was evaluated immediately after thawing and maturation of oocytes was examined at 48 hours and 24–28 hours after maturation culture in the oocytes retrieved from unstimulated and stimulated patients, respectively.

† Development of oocytes to the 2PN stage (with male and female pronuclei) was examined at 18 hours after ICSI. Percentage of the number of oocytes that were morphologically normal.

‡ Only oocytes that had a first polar body and expanded cumulus cells were vitrified.

‖ $P<.05$ within the same column of each cycle (P value for model effect was .0001).

§Only oocytes with expanded cumulus cells and no visible GV were vitrified.

Chung. Vitrified oocytes. Fertil Steril 2000.

ports in animals (11–13) demonstrated that vitrification is useful for freezing oocytes at both the GV stage and the metaphase II stage. Blastocysts and live offspring have resulted from vitrified oocytes in some species (14, 15). In a previous study using human oocytes (unpublished data), we found a high rate of morphologic normality when GV-stage oocytes were cryopreserved by a vitrification method in which ethylene glycol was used as a permeable cryoprotectant.

We conducted this study to assess the usefulness of the vitrification method in clinical practice. We obtained oocytes from unstimulated and stimulated cycles and vitrified them at various times after maturation culture. We evaluated the morphologic normality, developmental competence to the blastocyst stage, and chromosomal normality of the resulting blastocysts with an in vitro culture system using the intracytoplasmic sperm injection (ICSI) technique.

MATERIALS AND METHODS

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

Patients

Sixty-six women with normal ovarian function (unstimulated cycles) and 50 infertile women undergoing controlled ovarian hyperstimulation by a long protocol with a GnRH agonist and gonadotropins were enrolled in the study. As shown in Table 1, the mean (±SD) age of the unstimulated women was 33.2 ± 5.8 years and that of the stimulated, infertile women was 34.1 ± 3.8 years. The mean (±SD) duration of infertility was 5.7 ± 3.5 years in the stimulated women.

Media

The basic medium used for the aspiration of oocytes was Ham's F-10 medium supplemented with heparin (30 IU/mL; GIBCO BRL, Grand Island, NY). The basic medium used for in vitro maturation and IVF was a tissue culture medium (TCM-199; GIBCO BRL) with Earle's balanced salt solution, to which 20% (vol/vol) fetal bovine serum (collected from cattle free of bovine spongy encephalitis; GIBCO BRL), 0.05 mg/mL of penicillin G, and 0.075 mg/mL of streptomycin sulfate were added.

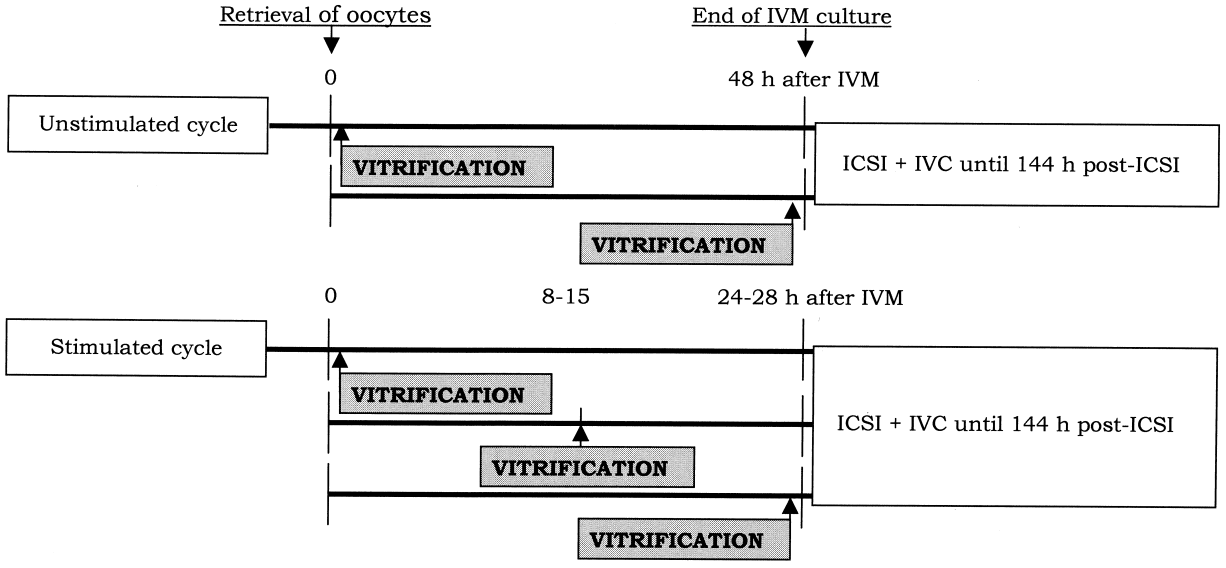
For in vitro maturation of oocytes, 10 IU/mL of pregnant mare serum gonadotropin (Sigma Co., St. Louis, MO) and 10 IU/mL of hCG (Sigma Co.) were added to the TCM-199. This in vitro maturation medium yielded the highest maturation rate compared with the media used in our preliminary experiment (16). For in vitro culture of vitrified oocytes, preimplantation medium (P1 medium; Irvine Scientific Co., Santa Ana, CA) supplemented with 10% (vol/vol) synthetic serum substitute (Irvine Scientific Co.) was used.

Retrieval of Oocytes

Oocytes were retrieved from unstimulated women who were undergoing caesarean section or oophorectomy by direct aspiration of ovarian follicles with a 21-gauge needle attached to a 10-mL syringe filled with aspiration medium (17). In stimulated patients, an ultrasound-guided transvaginal oocyte aspiration technique was used to retrieve oocytes. Oocytes retrieved from all patients were washed four times

FIGURE 1

Experimental design of this study. IVC = in vitro culture; IVM = in vitro maturation.



Chung. Vitrified oocytes. Fertil Steril 2000.

in maturation medium and cultured for designated periods before vitrification.

Vitrification and Thawing of Oocytes

The vitrification method used in this study was based on the method developed by Martino et al. (13). In brief, oocytes were placed in a cryoprotectant consisting of Dulbecco's phosphate-buffered solution (GIBCO BRL) supplemented with 5.5 M of ethylene glycol and 1 M of sucrose for 20 seconds. One or two oocytes were mounted on an electron microscopic copper grid (EM grid; Gilder Co., Westchester, PA) using a fine glass pipette, and surplus cryoprotectant was removed using sterilized filter paper. The EM grids containing the oocytes were immediately plunged into liquid nitrogen at −196°C and stored for 7–31 days.

For thawing, the EM grid was transferred sequentially to Falcon plastic culture dishes (catalog number 1008; Becton and Dickinson, Lincoln Park, NJ) containing Dulbecco's phosphate-buffered solution supplemented with 10% (vol/vol) fetal bovine serum, to which 0.5 M, 0.25 M, 0.125 M, or 0 M of sucrose was added at intervals of 1 minute at 37°C. Thawed oocytes then were washed 4–6 times in maturation medium and reserved for subsequent experimental procedures.

Experimental Design, and In Vitro Maturation, ICSI, and In Vitro Culture of Vitrified Oocytes

The total duration of the maturation culture period was 48 hours for oocytes obtained from unstimulated cycles and 24–28 hours for oocytes obtained from stimulated cycles. For maturation, oocytes were cultured in Falcon organ culture dishes (catalog number 3037; Becton and Dickinson) containing 2 mL of maturation medium at 37°C under 5% CO_2 in humidified air. In unstimulated cycles, retrieved oocytes were vitrified at either 0 hours or 48 hours after maturation culture by random assignment. In stimulated cycles, retrieved oocytes were assigned randomly to three groups that underwent 0 hours, 8–15 hours, or 24–28 hours of maturation before vitrification.

For vitrification at the onset of culture in both cycles, only oocytes that had normal morphology, with an evenly granulated cytoplasm and a distinct GV, were used. An experienced embryologist verified the presence of a GV in the cytoplasm under an inverted microscope. Cumulus cells that enclosed some of the oocytes, obscuring the cytoplasm, were not removed but were partially dispersed with a fine pipette.

At 8–15 hours after maturation culture in stimulated cycles, oocytes that had an evenly granulated cytoplasm but no visible GV were vitrified. For vitrification at the end of maturation culture in both stimulated (24–28 hours) and unstimulated (48 hours) cycles, only oocytes that had normal morphology and an extruded first polar body were used.

Oocytes that were vitrified immediately (0 hours) in unstimulated cycles were cultured further for 48 hours in maturation medium after thawing. In stimulated cycles, oocytes that were vitrified immediately (0 hours) and at 8–15 hours after maturation culture were cultured further for 24–28

TABLE 2

In vitro development of oocytes vitrified and thawed at different times after maturation culture to the blastocyst stage.

Source of oocytes	Time of vitrification (hours after culture)	No. of oocytes cultured*	No. (%) of oocytes developed to the indicated stage	
			Two cell† (48 h after ICSI)	Blastocyst† (192 h after ICSI)
Unstimulated cycle	0	7 (23)	7 (100)	3 (43)
	48	5 (22)	5 (100)	2 (40)
Stimulated cycle	0	6 (35)	5 (83)	2 (33)
	8–15	5 (31)	5 (100)	1 (20)
	24–28	10 (59)	10 (100)	4 (40)

* Oocytes developed to the pronuclear stage were cultured up to 192 hours after ICSI.
† Percentage of the number of oocytes cultured.

Chung. Vitrified oocytes. Fertil Steril 2000.

hours and 13–16 hours, respectively, in maturation medium after thawing. Oocytes that were vitrified and thawed at the end of maturation culture in both cycles underwent ICSI immediately.

The maturity of oocytes that were vitrified at different times after maturation culture was defined by the presence of fully expanded cumulus cells and a first polar body in the perivitelline space at the end of maturation culture. Mature oocytes derived from each experimental group were fertilized in vitro by ICSI using donor's fresh semen that, in general, had >90% motility. The oocytes that had a second polar body and two pronuclei (pronuclear-stage embryos) subsequently were cocultured on a confluent Vero cell monolayer (ATCC, Rockville, MD) in 2 mL of culture medium until 6 days after ICSI. The number of pronuclei in the cytoplasm was counted to verify normal fertilization of vitrified oocytes 16–19 hours after IVF, and cleavage and development to the blastocyst stage of normal pronuclear-stage embryos (with male and female pronuclei) were monitored at 2 days and 6 days after ICSI, respectively. All the experimental procedures are depicted in Figure 1.

Evaluation of Chromosomal Normality

Chromosome preparation of blastocysts derived from vitrified oocytes was undertaken according to the modified air-dry technique of Tarkowski (18). Blastocysts were placed in a microdrop of hypotonic sodium citrate solution (0.9% vol/vol) on a grease-free slide. Blastocysts were fixed with 25% (vol/vol) acetoethanol. The slides then were dried in air for an hour and stained with 5% (vol/vol) Giemsa solution. The chromosomes in the blastocysts were examined under a Cytovision Karyotyper (Cytovision Ultra; Applied Imaging, Sunderland, United Kingdom).

Statistical Analysis

The maturation, fertilization, and preimplantation development of vitrified oocytes were analyzed as binomial data using the generalized linear model procedure (PROC-GLM) with analysis of variance in the SAS program (19). When significance of the main effects was detected in each experimental parameter, the treatment effects were compared by the least-squares method. $P<.05$ was considered statistically significant.

RESULTS

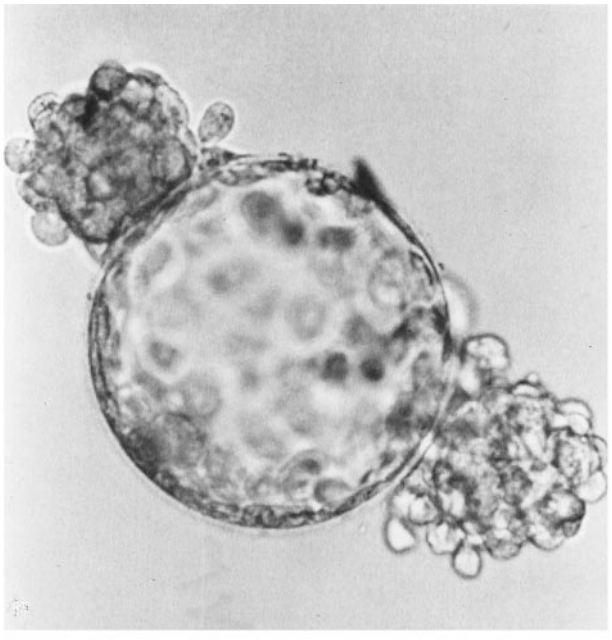
Of a total of 66 unstimulated cycles, oocytes could not be obtained from 20 cycles because no follicle was present in the collected ovarian tissue; 1 or 2 oocytes per patient (total, 53 oocytes) were obtained from the rest of the unstimulated cycles. In the stimulated cycles, 1 oocyte per cycle (total, 50 oocytes) was retrieved from 50 patients. Therefore, a total of 103 oocytes were used for this study.

Of the 53 oocytes that were retrieved from unstimulated cycles, 30 oocytes were vitrified immediately (0 hours after culture) and the remaining 23 oocytes were cultured in maturation medium. Because 16 (70%) of the latter 23 oocytes had expanded cumulus cells and an extruded first polar body at 48 hours after culture, only these oocytes were vitrified. Of the 50 oocytes that were retrieved from stimulated cycles, 17 oocytes were vitrified immediately and the remaining 33 oocytes were cultured further for either 8–15 hours or 24–28 hours. Of the 16 oocytes that were cultured for 8–15 hours, 14 (88%) had normal morphology with no GV and were vitrified. Twelve (71%) of the 17 oocytes that were cultured for 24–28 hours had expanded cumulus cells and a first polar body and were vitrified.

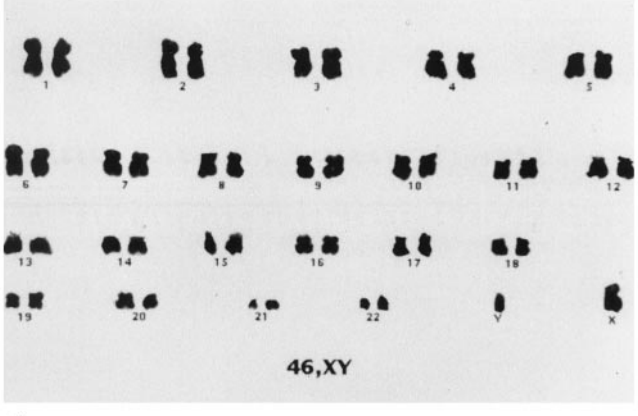
Among the oocytes that were obtained from unstimulated cycles, no statistically significant treatment effect was found in the number of morphologically normal oocytes after vitrification (56%–63%; Table 1). Twelve oocytes (40%) that were vitrified immediately extruded a first polar body after 48 hours of maturation culture. In this group, 37% of the morphologically normal oocytes developed to the normal

FIGURE 2

Morphology and number of chromosomes of a blastocyst developed from a vitrified oocyte. A distinct inner cell mass and expanded blastocele are visible (**A**), and diploid chromosomes consisting of 23 autosomes and sex chromosomes (XY) are present (**B**).



A



B

Chung. Vitrified oocytes. Fertil Steril 2000.

pronuclear stage at 16–19 hours after ICSI, whereas 31% of the oocytes that were vitrified at the end of maturation culture developed to the pronuclear stage. There was no statistically significant difference in the number of pronuclear-stage embryos according to the time of vitrification.

Among the oocytes that were obtained from stimulated cycles, a statistically significant treatment effect ($P<.0001$) was found in the number of morphologically normal oocytes after vitrification and thawing. More oocytes were morphologically normal after vitrification at 24–28 hours (100%) of maturation culture than after vitrification immediately or at 8–15 hours of culture (64%–65%). The maturation rate of the oocytes that were vitrified immediately or at 8–15 hours after maturation culture was 53%–64%, and no statistically significant effect was detected. In addition, there was no statistically significant effect of the treatments on the percentage of vitrified oocytes that developed to the normal pronuclear stage (55%–83%).

As shown in Table 2, 32 (97%) of 33 pronuclear-stage embryos developed from vitrified oocytes cleaved at 48 hours after IVF; this finding was independent of the time of vitrification and the use of hormonal induction. Blastocyst formation occurred in 20%–43% of all vitrified oocytes.

A total of 12 blastocysts derived from oocytes that were vitrified at different times after maturation culture were analyzed for their number of chromosomes. As shown in Table 3, 7 blastocysts were analyzed successfully; 3 were derived from vitrified oocytes obtained from unstimulated cycles and 4 from vitrified oocytes obtained from stimulated cycles. All these blastocysts had a normal number of chromosomes, and the sex ratio was 3:4 (male to female).

DISCUSSION

The results of this study demonstrate that a vitrification method that includes an EM grid and a short equilibration process is useful for the cryopreservation of human oocytes retrieved from both unstimulated and stimulated cycles. This freezing method can be used for immature and maturing oocytes as well as mature oocytes. Fifty-six percent to 100% of the oocytes were morphologically normal when they were vitrified at different maturational stages, and 37%–83% of the morphologically normal oocytes developed to the normal pronuclear stage after ICSI. Twenty percent to 43% of the normal pronuclear embryos developed to the blastocyst stage, and all karyotyped blastocysts had a normal number of chromosomes (Fig. 2).

The vitrification method used in this study is unique in that it includes an EM grid and a relatively short equilibration process compared with other methods. The EM grid enhances heat conduction to the oocyte cytoplasm and the short equilibration procedure helps prevent severe osmotic damage from high concentrations of the vitrification solution. Given that no development was found in vitrified oocytes stored in 0.25-mL plastic straws in our preliminary study (data not shown), use of the EM grid with a short equilibration procedure might improve the postthaw viability of vitrified oocytes. The high rate of morphologic normality found in vitrified oocytes (Table 2) suggests that mechanical damage of oocytes induced by their attachment to the EM grid is negligible with this vitrification method. Another advantage of this method is that it is a rapid process and

TABLE 3

Detailed data of the karyotyping of the blastocysts derived from the vitrified oocytes.

Source of oocyte	Patient	Time of vitrification (hours after culture)	Stage of blastocyst examined	Results of karyotyping	
				No. of chromosomes	Chromosomal status
Unstimulated cycle	A	0	Expanded blastocyst	46, XX	Normal
	B	0	Early blastocyst	Failed to analyze	—
	B	0	Expanded blastocyst	46, XY	Normal
	D	48	Expanded blastocyst	Failed to analyze	—
	E	48	Hatched blastocyst	46, XY	Normal
Stimulated cycle	F	0	Early blastocyst	46, XX	Normal
	G	0	Expanded blastocyst	Failed to analyze	—
	H	8–15	Early blastocyst	Failed to analyze	—
	I	24–28	Expanded blastocyst	46, XX	Normal
	J	24–28	Early blastocyst	46, XX	Normal
	K	24–28	Hatched blastocyst	Failed to analyze	—
	L	24–28	Expanded blastocyst	46, XY	Normal

Chung. Vitrified oocytes. Fertil Steril 2000.

requires less equipment than conventional slow freezing methods.

The duration of oocyte cryopreservation critically influences the developmental competence of oocytes in various species (8–10), and oocytes at the GV stage are more vulnerable to cryoinjury than oocytes at the metaphase I and metaphase II stages. In our preliminary study (17), GV-stage oocytes retrieved from stimulated patients reached GV breakdown metaphase I at 6–18 hours of maturation culture and metaphase II at 21–27 hours of culture. In this study, no critical differences in the postthaw developmental competence of oocytes that were vitrified at different times after maturation culture were found in stimulated cycles. A similar trend in developmental capacity was found in vitrified oocytes obtained from unstimulated cycles, except for their morphologic normality immediately after thawing.

This study represents further progress toward the successful cryopreservation of immature and maturing oocytes with the use of a vitrification method in both stimulated and unstimulated cycles. With this approach, blastocyst formation occurred in all groups of vitrified oocytes. Although only a limited number of oocytes (12–30 in each experimental treatment) were tested in this study, the results suggest that the vitrification protocol described herein may contribute to the development of assisted reproductive technology programs for the use of immature, maturing, and mature oocytes obtained from both unstimulated and stimulated cycles. Thus, this vitrification method increases the options available to women with various causes of infertility.

We performed chromosomal analyses to evaluate the normality of the blastocysts derived from the vitrified oocytes. Although only a limited number of blastocysts developed from vitrified oocytes, all the karyotyped blastocysts had a normal number of chromosomes. These findings suggest that vitrification is a safer method for maintaining normal chromosome numbers and spindle configuration during cryopreservation than is slow freezing. However, the structural normality of the blastocysts that developed from the vitrified oocytes remains unclear because we did not perform further chromosomal analyses because of the limited number of blastocysts we evaluated. Future studies will be designed to confirm the structural normality of oocytes or embryos after vitrification.

To optimize the vitrification program, we compared different thawing protocols after the completion of this study, and our findings recently were published (20). All our experiments are aimed at developing an effective cryopreservation system for human oocytes, which has great potential value in reproductive medicine.

Acknowledgments: The authors thank Rogerio A. Lobo, M.D., Department of Obstetrics and Gynecology, Columbia University Medical Center, for his valuable suggestions during the preparation of this manuscript.

References

1. Chen C. Pregnancy after human oocyte cryopreservation. Lancet 1986; 1:884–6.
2. Van Uem JFHM, Siebzehnrubl ER, Schuh B, Koch R, Trotnov S, Lang N. Birth after cryopreservation of unfertilized oocytes. Lancet 1987;1: 752–3.
3. 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;68:724–6.
4. Polak de Fried, E, Notrica J, Rubinstein M, Marazzi A, Gonzalez MG. Pregnancy after human donor oocyte cryopreservation and thawing in association with intracytoplasmic sperm injection in a patient with ovarian failure. Fertil Steril 1998;69:555–7.

5. Tucker MJ, Wright G, Mortin PC, Massey JB. Birth after cryopreservation of immature oocytes with subsequent in vitro maturation. Fertil Steril 1998;70:578–9.
6. Al-Hasani Diedrich K, van der Ven H, Reinecke A, Hartje M, Krebs D. Cryopreservation of human oocytes. Hum Reprod 1987;2:695–700.
7. Park SE, Lee KA, Son WY, Ko JJ, Lee SH, Cha KY. Chromosome and spindle configurations of human oocytes matured in vitro after cryopreservation at the germinal vesicle stage. Fertil Steril 1997;68:920–6.
8. Lim JM, Fukui Y, Ono H. Developmental competence of bovine oocytes frozen at various maturation stages followed by in vitro maturation and fertilization. Theriogenology 1992;37:351–61.
9. Fuku E, Liu J, Downey BR. In vitro viability and ultrastructural changes in bovine oocytes treated with a vitrification solution. Mol Reprod Dev 1995;40:177–85.
10. Mandelbaum J, Junca AM, Plachot M, Alnot MO, Salat-Baroux J, Alvarez S, et al. Cryopreservation of human embryos and oocytes. Hum Reprod 1988;3:117–9.
11. Shaw PW, Bernard AG, Fuller BJ, Shaw RW. Morphological and functional changes in unfertilised mouse oocytes during a vitrification procedure. Cryoletter 1990;11:427–32.
12. Fuku E, Kojima T, Shioya Y, Marcus GJ, Downey BR. In vitro fertilization and development of frozen-thawed bovine oocytes. Cryobiology 1992;29:485–92.
13. Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol Reprod 1996; 54:1059–69.
14. Sathananthan AH, Ng SC, Trounson AO, Bongso A, Ratnam SS, Ho J, et al. The effects of ultrarapid freezing on meiotic and mitotic spindles of mouse oocytes and embryos. Gamete Res 1988;21:385–401.
15. Hamano S, Koikeda A, Kuwayama M, Nagai T. Full-term development of in vitro matured, vitrified and fertilized bovine oocytes. Theriogenology 1992;38:1085–90.
16. Cha KY, Chian RC. Maturation in vitro of immature human oocytes for clinical use. Hum Reprod Update 1998;4:103–20.
17. Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY, Yoon TK. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. Fertil Steril 1991;55:109–13.
18. Tarkowski AK. An air-drying method for chromosome preparations from mouse eggs. Cytogenetics 1966;5:394–400.
19. Anonymous. SAS user's guide. Statistics. Cary (NC): Statistical Analysis System Institute, 1992.
20. 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.