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Improved human oocyte development after vitrification: a comparison of thawing methods

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Objective: To evaluate the developmental competence of vitrified human oocytes thawed using two different methods to establish an effective cryopreservation protocol.

Design: In vitro model study.

Setting: University-affiliated hospital.

Patient(s): Patients who underwent a long protocol of ovarian stimulation with GnRH and gonadotropins.

Intervention(s): Vitrified oocytes from the patients were thawed using either a four-step method with 2.5-minute intervals or a four-step method with 5-minute intervals.

Main Outcome Measure(s): Morphologic normality, maturation, fertilization, and development of the oocytes to the blastocyst stage.

Result(s): The two thawing methods did not significantly affect the morphologic normality (84%–100%), maturation (75%–100%), fertilization (38%–71%), polyspermy (more than three pronuclei; 0%–20%), or parthenogenetic activation (only female pronucleus; 0%–8%) of the vitrified oocytes. However, more of the vitrified oocytes developed to the two-cell (71%–100% versus 50%–67%), four-cell (71%–93% versus 0%–50%), eight-cell (46%–71% versus 0%), and blastocyst (23%–36% versus 0%) stages after thawing using the four-step method with 2.5-minute intervals than using the four-step method with 5-minute intervals.

Conclusion(s): Vitrified human oocytes developed to the blastocyst stage with IVF. A four-step thawing method with 2.5-minute intervals was more effective in supporting preimplantation embryo development than a four-step thawing method with 5-minute intervals. (Fertil Steril® 1999;72:142–6. ©1999 by American Society for Reproductive Medicine.)

Key Words: Human, oocyte, vitrification, thawing protocol, in vitro development

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Technology for the long-term preservation of mammalian gametes and zygotes has improved greatly over the past 20 years (1, 2) and currently is used for supporting various assisted reproductive techniques in human reproductive medicine. Recent progress in cryobiology and its related sciences have made it possible to preserve human embryos effectively (3, 4), and several cryopreservation methods for human oocytes also have been developed. Successful freezing of human oocytes has several advantages (5–7), such as allowing the establishment of oocyte banks. However, the postthaw survival and subsequent development of frozen oocytes has been extremely poor in published studies (8–10), and further research to enhance the viability of cryopreserved oocytes is required.

Recent research suggests that vitrification can be useful in the cryopreservation of human oocytes (11–13) because it prevents cellular injury from intracellular ice crystal formation during freezing. In a previous experiment (14), preimplantation development was demonstrated in vitrified human oocytes after in vitro maturation (IVM), IVF, and in vitro culture (IVC). A high concentration of cryoprotectants was used in the original vitrification method, however, and modification was necessary to decrease the associated osmotic shock and cryoprotectant toxicity.

In this study, we changed the thawing protocol of the vitrification method (14) to reduce osmotic stress on oocytes during removal of the cryoprotectant from the oocyte cytoplasm.

We evaluated the morphologic status of the oocytes immediately after vitrification and thawing, their maturation after culture for IVM, their fertilization and pronuclei (PN) formation after IVF, and their subsequent development to the blastocyst stage. The objective of the study was to evaluate the developmental competence of human oocytes that had been vitrified and thawed using two protocols to establish an effective oocyte cryopreservation method.

MATERIALS AND METHODS

The institutional review board of CHA General Hospital, Pochon CHA University, approved the experimental procedures used in this study.

Patients

A total of 42 patients undergoing a long protocol of controlled ovarian hyperstimulation (COH) with GnRH and gonadotropins consented to participate in this study. The mean (±SD) age of the patients was 32.7 ± 3.9 years and the mean (±SD) duration of infertility was 6.4 ± 3.5 years. The oocytes used in this study were retrieved from the patients by transvaginal oocyte aspiration and numbered >20. The collected oocytes were randomly allotted into each experimental treatment condition.

Media

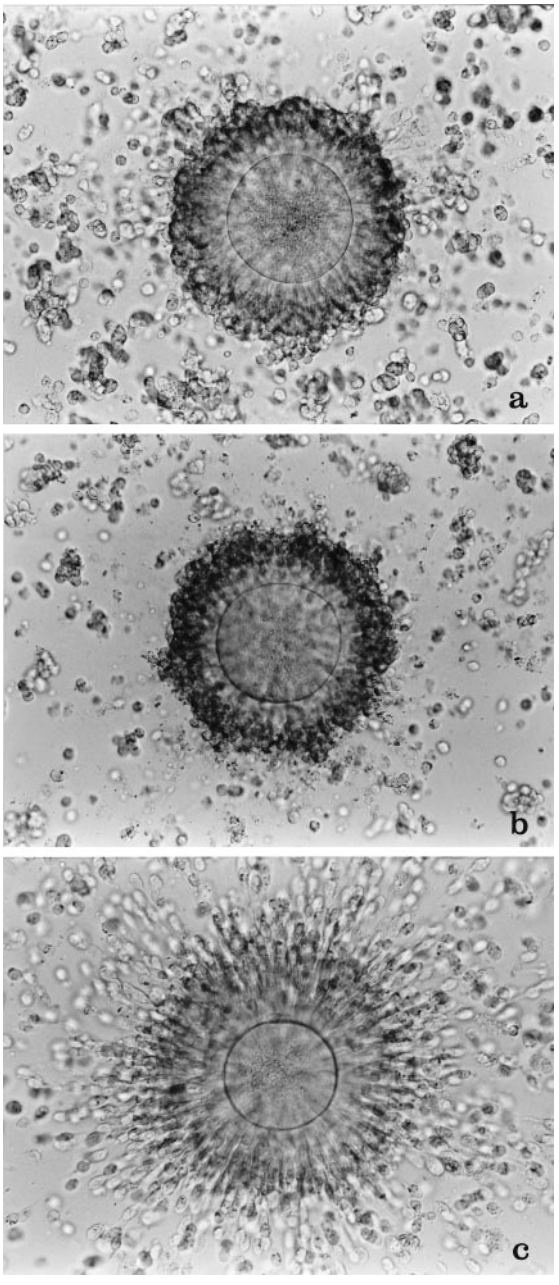
The basic medium used for the aspiration of oocytes was Ham's F-10 medium supplemented with heparin (30 IU/mL; GIBCO BRL, Grand Island, NY). The medium used for IVM was tissue culture medium-199 (GIBCO BRL) with Earle's balanced salt solution supplemented with 20% (vol/vol) fetal bovine serum collected from cattle that were free of bovine spongy encephalitis (GIBCO BRL), 10 IU/mL of pregnant mare serum gonadotropin (Sigma Chemical Co., St. Louis, MO), 10 IU/mL of hCG (Sigma Chemical Co.), 0.05 mg/mL of penicillin G, and 0.075 mg/mL of streptomycin sulfate. For IVF and IVC of vitrified oocytes, Preimplantation 1 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 obtained from the patients undergoing COH with the use of our routine protocol. Only oocytes that had evenly granulated cytoplasm and were surrounded by cumulus cells were used for this study. Retrieved oocytes then were classified into three categories: oocytes with compacted cumulus cells and visible germinal vesicles (GVs) in the cytoplasm, oocytes with compacted cumulus cells but no visible GVs in the cytoplasm, and oocytes with expanded cumulus cells and with or without a first polar body. In the preliminary studies (data not shown), retrieved oocytes in categories 1–3 were at the GV, GV breakdown to metaphase I, and metaphase II stages, respectively. The morphology of these types of oocytes is depicted in Figure 1.

FIGURE 1

Morphology of oocytes retrieved from stimulated infertile patients. (**A**), Compacted cumulus cells and visible germinal vesicles. (**B**), Compacted cumulus cells. (**C**), Expanded cumulus cells. Original magnification, ×200.



Vitrification and Thawing of Oocytes

Vitrification and subsequent thawing was conducted in all three oocyte categories. The oocytes to be vitrified were placed in Dulbecco's phosphate-buffered saline (GIBCO BRL) supplemented with 1.5 M of ethylene glycol (Sigma Chemical Co.) at room temperature (20°–25°C) for 5 minutes. The oocytes then were placed in Dulbecco's phosphate-

buffered saline supplemented with 5.5 M of ethylene glycol and 1 M of sucrose for 20 seconds. In the meantime, 1–3 oocytes were mounted on electron microscopic copper grids (EM grids; Gilder Co., West Chester, PA) using a fine glass pipette. The excess cryoprotectant solution was removed with the underlying sterilized filter paper and the EM grids containing the oocytes immediately were plunged into liquid nitrogen at −196°C and stored for 7–31 days.

For thawing of the vitrified oocytes, the EM grids were divided randomly into two groups. In group A (long protocol), the EM grids were transferred sequentially to Falcon culture dishes (Becton Dickinson, Lincoln Park, NJ) containing 10% (vol/vol) fetal bovine serum in Dulbecco's phosphate-buffered saline supplemented with 1 M, 0.5 M, 0.25 M, and 0.125 M of sucrose at intervals of 5 minutes at 37°C. In group B (short protocol), the EM grids were transferred sequentially to culture dishes containing the same dilution agent at 2.5-minute intervals. After thawing, the oocytes were washed 4–6 times in maturation medium for further experimental procedures.

In Vitro Maturation, IVF, and IVC of Vitrified Oocytes

The vitrified and thawed oocytes from the two experimental protocols were matured in Falcon organ culture dishes (Becton Dickinson) containing 2 mL of maturation medium at 37°C in 5% CO_2 in humidified atmospheric air. A different culture time was used for each category of oocytes. The oocytes with compacted cumulus cells and GVs were cultured in maturation medium for 20–24 hours. The oocytes with compacted cumulus cells and no visible GVs and those with expanded cumulus cells were cultured for 10–16 hours and 4–6 hours, respectively.

At the end of IVM culture, maturation of oocytes, defined as fully expanded cumulus cells and the presence of a first polar body in the perivitelline space, was evaluated. In vitro fertilization of mature oocytes was performed using fresh semen with >90% viability. One hundred thousand spermatozoa per milliliter was added to the medium containing the mature oocytes and incubated for another 16–19 hours. The oocytes that had a second polar body and 2 PN (pronuclear-stage embryos) were cocultured on a confluent Vero cell monolayer purchased from American Type Culture Collection (Rockville, MD) in 2 mL of culture medium until 5 days after fertilization.

The number of PN in the cytoplasm that confirmed normal fertilization (the presence of male and female PN in the ooplasm), polyspermy (>3PN) and parthenogenetic activation (only a female pronucleus) of oocytes, and pronuclear-stage embryos developing into the two-cell, four-cell, eight-cell, and blastocyst stages were monitored at 16–19, 48, 72, 96, and 120 hours after IVF, respectively.

Statistical Analysis

Each category of oocytes with normal morphology after thawing that matured, were fertilized, and substantially cleaved to the 2-cell, 8-cell, 16-cell, morula, and blastocyst stages were assigned a score of 1 (developed). Oocytes that did not develop to the appropriate stages were assigned a score of 0 (undeveloped). The scores at each stage of development were subjected to analysis of variance using the general linear model in the SAS program (15). When significance of the main effects was detected in each experimental parameter, the treatment effects were compared by the least-squares method.

RESULTS

A total of 106 oocytes retrieved from patients undergoing COH were vitrified, and all the vitrified oocytes were recovered after thawing from both protocols. There were no significant effects of vitrification on oocyte morphology ($P>.23$), maturation ($P>.18$), fertilization ($P>.17$), polyspermy ($P>.24$), or parthenogenetic activation ($P>.84$). As shown in Table 1, the percentages of oocytes that had normal morphology immediately after thawing, were mature, and were normally fertilized, were 84%–100%, 75%–100%, and 38%–71%, respectively. Only a small percentage of oocytes was parthenogenetically activated after IVF (0%–8%), and 0%–20% of fertilized oocytes were abnormally fertilized.

A significant treatment effect related to the two different thawing protocols was detected in the development of 2PN-stage embryos to the two-cell ($P<.03$), four-cell ($P<.0001$), eight-cell ($P<.0001$), and blastocyst ($P<.04$) stages. More embryos derived from vitrified oocytes developed to the two-cell (71%–100% versus 50%–67%, $P<.003$), four-cell (71%–93% versus 0%–50%, $P<.0001$), eight-cell (46%–71% versus 0%, $P<.0001$), and blastocyst (29%–36% versus 0%, $P<.006$) stages after thawing using the four-step method with 2.5-minute intervals than after thawing using the four-step method with 5-minute intervals (Table 2). A substantial number of oocytes with expanded cumulus cells at the time of retrieval developed to the four-cell, eight-cell, and blastocyst stages with the short protocol and to the four-cell stage with the long protocol after vitrification and thawing.

DISCUSSION

The results of this study demonstrate that human oocytes retrieved from infertile patients using COH can develop to the blastocyst stage after vitrification and thawing, IVM, IVF, and IVC. The thawing method used is crucial for successful development to the blastocyst stage. The four-step thawing method with 2.5-minute intervals enhanced the preimplantation development of the vitrified oocytes more efficiently than the four-step method with 5-minute intervals. Twenty-three percent to 36% of the pronuclear-stage em-

TABLE 1

Postthaw morphology, maturation, and pronucleus formation of human oocytes retrieved from stimulated infertile patients after vitrification with the use of two thawing protocols.

Thawing protocol	Type of oocytes	No. of oocytes vitrified and thawed	No. (%)* of oocytes with normal morphology	No. (%)* of oocytes matured after IVM culture	No. of inseminated oocytes		
					Fertilized (%)*	3 PN (%)†	1 PN (%)*
Short protocol	Compacted CC plus visible GV	25	21 (84)	19 (76)	13 (52)	0 (0)	0 (0)
	Compacted CC	12	12 (100)	9 (75)	8 (67)	1 (13)	1 (8)
	Expanded CC	21	18 (86)	18 (86)	15 (71)	1 (7)	0 (0)
Long protocol	Compacted CC plus visible GV	16	15 (94)	14 (88)	7 (44)	0 (0)	0 (0)
	Compacted CC	16	16 (100)	14 (88)	6 (38)	0 (0)	0 (0)
	Expanded CC	16	16 (100)	16 (100)	10 (63)	2 (20)	0 (0)

Note: CC = cumulus cells; GV = germinal vesicles; IVM = in vitro maturation; PN = pronuclei.
* Percentage of oocytes vitrified.
† Percentage of oocytes matured.

bryos derived from vitrified oocytes developed to the blastocyst stage after IVC.

Mandelbaum et al. (16) first reported on the cryopreservation of immature human oocytes retrieved from stimulated patients. It has been observed that the maturation (8, 9) and fertilization rates of immature oocytes after freezing and thawing are similar in oocytes retrieved from both unstimulated and stimulated patients. However, the survival and subsequent development of these oocytes is extremely poor compared with that of human embryos that are frozen after fertilization. The poor results are due to structural and functional abnormalities of the oocytes induced by freezing and thawing (17). In our previous studies (18, 19), the incidence of chromosomal and spindle abnormalities was higher in human oocytes that were cryopreserved by a slow freezing method compared with fresh oocytes.

Oocyte cryopreservation capacity might be improved by modifying the freezing method used. In our previous report (20), an increase in oocyte viability was observed with the use of the vitrification method. The results of the present study (Table 2) further suggest that modification of the thawing protocol can enhance postvitrification viability and subsequent development. Reducing the total elapsed time of the process to half the original interval can be beneficial because it decreases the severe osmotic shock that occurs during thawing. Further, the shorter protocol also allows the vitrified oocytes to return to suitable culture conditions more quickly. For whatever reason, the four-step thawing method with 2.5-minute intervals sufficiently removes the 5.5 M of ethylene glycol from the oocyte cytoplasm.

An alternative method of enhancing oocyte viability after vitrification is to vitrify oocytes at the specific stage of maturation at which they are resistant to freezing damage. There have been several reports in other mammalian species that oocytes at the GV stage had lower cryopreservation capacity than oocytes at the metaphase I through metaphase II stages (21, 22). Successful deliveries reported in the previous studies (23, 25) were achieved with oocytes cryopre-

TABLE 2

Postthaw development of human oocytes retrieved from stimulated infertile patients after vitrification with the use of two thawing protocols.

Thawing protocol	Type of oocytes	No. of 2PN embryos cultured	No. (%) of 2PN embryos that developed to the indicated stage			
			Two-cell (48 h after IVF)	Four-cell (72 h after IVF)	Eight-cell (96 h after IVF)	Blastocyst (120 h after IVF)
Short protocol	Compacted CC plus visible GV	13	13 (100)^{a}	10 (77)^{ad}	6 (46)^{a}	3 (23)^{ab}
	Compacted CC	7	5 (71)^{ab}	5 (71)^{acd}	4 (57)^{a}	2 (29)^{ab}
	Expanded CC	14	13 (93)^{a}	13 (93)^{d}	10 (71)^{a}	5 (36)^{a}
Long protocol	Compacted CC plus visible GV	7	4 (57)^{b}	0 (0)^{b}	0 (0)^{b}	0 (0)^{b}
	Compacted CC	6	4 (67)^{ab}	2 (33)^{bc}	0 (0)^{b}	0 (0)^{b}
	Expanded CC	8	4 (50)^{b}	4 (50)^{ac}	0 (0)^{b}	0 (0)^{b}

Note: CC = cumulus cells, GV = germinal vesicles, PN = pronuclei. Different superscript letters in each parameter are significantly different ($P<.05$).

served at the GV breakdown to telophase I stages and the metaphase II stage.

In humans, pregnancy and delivery of normal infants recently were achieved by freezing oocytes at more mature stages (5–7). The results presented in Table 2 reflect these previously reported findings. However, the use of a vitrification program could lessen the difference in cryopreservation capacity observed between GV and metaphase II stage oocytes; no clear differences in developmental competence were found in oocytes that were vitrified and thawed at various maturational stages. Our present data suggest that, with suitable cryopreservation and subsequent IVM, human oocytes of different maturational stages can be cryopreserved successfully with no sustained loss of subsequent developmental competence.

The results of this study confirmed the formation of blastocysts from oocytes vitrified at various maturational stages before fertilization. To evaluate the subsequent developmental competence of vitrified oocytes, studies on implantation and pregnancy after the transfer of embryos derived from oocytes that have been vitrified and thawed with the use of a selective protocol should be conducted. Such a project is under way and, thus far, at least one pregnancy has been achieved in a patient who received embryos derived from vitrified oocytes. This pregnancy is at 14 weeks of gestation and both the β-hCG level and fetal development appear to be normal.

A major advantage of cryopreserving human oocytes is the ability to establish oocyte banks. Oocyte banks will help restore fertility in women with various catastrophic diseases such as malignant cancer. They can further support the development of assisted reproductive technologies by combining oocyte freezing with routine IVM, IVF, and IVC programs. The establishment of oocyte banks can alleviate certain ethical concerns regarding embryo freezing. The disposal of excess oocytes can be avoided, and the surplus cryopreserved oocytes will remain viable until their future use. Thus, oocyte cryopreservation with vitrification uses human gametes in a more efficient and appropriate manner.

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