Vitrification of immature mouse oocyte using stepwise equilibration before or after in vitro maturation

Immature mouse oocytes before or after in vitro maturation (IVM) were vitrified by the use of either conventional one-step or three-step equilibration. Although vitrification after IVM using the stepwise method does not affect the cleavage rate, the early embryonic development was profoundly impaired in both protocols. (Fertil Steril® 2009;92:1153–7. ©2009 by American Society for Reproductive Medicine.)

Key Words: Immature oocyte, in vitro maturation, stepwise equilibration, vitrification

Freezing oocytes is becoming an important and integral part of infertility treatment and fertility preservation. The introduction of new freezing techniques such as oocyte vitrification has significantly increased both oocyte survival and resulting pregnancy rates (1–6). However, to date, the majority of pregnancies reported have been the result of frozen-thawed mature oocytes that were collected after ovarian stimulation.

Unfortunately, patients seeking fertility preservation may not have enough time to undergo ovarian stimulation or may suffer from a medical condition prohibiting hormonal stimulation (i.e., estrogen-receptor positive breast cancer). When there is not enough time for ovarian stimulation and/or when stimulation should be avoided, immature oocytes can be collected from the ovaries without hormonal stimulation.

In most centers, the retrieved immature oocytes are usually allowed to be matured in vitro first and then vitrified at metaphase II stage (7, 8). Combining ovarian tissue cryobanking with retrieval of immature oocytes followed by in vitro maturation (IVM) and vitrification has also been suggested as a novel strategy in patients who want to preserve their fertility (9–11). This strategy can also be applied for infertility treatment and oocyte banking. However, little is known about whether vitrified IVM oocytes can survive after thawing and be fertilized, or whether the transfer of these embryos can result in a viable pregnancy (12).

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An earlier study indicated that IVM human oocytes obtained from unstimulated donors could be successfully vitrified and fertilized, and could also generate chromosomally normal blastocysts (13). Immature oocytes obtained from stimulated cycles had a similar post-thaw survival rate compared with in vivo–matured oocytes, but they had significantly lower fertilization and blastocyst-forming rates (14). The lower developmental competency of vitrified IVM oocytes also was reported in a mouse model (15). Although a 20% live-birth rate per cycle (four among 20 patients) has been reported after vitrification of IVM oocytes (16), the vitrification protocol has had only limited success for IVM oocytes (17). Therefore, more progress is needed to enhance the developmental potential of vitrified IVM oocytes and finally to achieve good clinical results.

On the basis of these findings, we applied an alternative approach using a modified stepwise vitrification protocol; that is, we used a stepwise addition of cryoprotectants to slowly equilibrate IVM mouse oocytes, as described previously elsewhere (18). We also attempted to vitrify germinal vesicle (GV) stage oocytes using either the conventional or the stepwise vitrification method.

Female mice (8 to 10 week old CD1) were injected with 5 IU of pregnant mare serum gonadotropin (PMSG, Folligon; Intervet UK Ltd, Cambridge, UK) and were killed 48 hours later by cervical dislocation. Ovaries were collected in 1 mL of oocyte-washing medium (SAGE-IVF, Inc., Trumbull, CT) and the cumulus-enclosed oocytes (CEOs) were obtained by puncture of the antral follicles. Only CEOs with a uniform covering of cumulus cells were used in this study. The study was approved by the ethics committee of Seoul National University Bundang Hospital.

After washing them three times in oocyte-washing medium, we performed IVM on the CEOs. The CEOs were transferred to microdrops of IVM medium, and then they were incubated for 17 to 19 hours. The IVM medium consisted of a commercial IVM medium (SAGE-IVF, Inc.)

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supplemented with 20% fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO) and follicle-stimulating hormone and luteinizing hormone (75 mIU/mL for each; Serono, Geneva, Switzerland). In all cultures, the groups of up to 10 oocytes were placed in a 50-μL microdrop of medium under mineral oil (Sigma) within 35 $\times$ 10 mm Petri dishes (Falcon; Becton Dickinson, Franklin Lakes, NJ) and were stored at 37°C in humidified 5% CO_2 in air. After IVM, all oocytes were denuded by means of treatment with 75 μg/mL of hyaluronidase (Cook, Brisbane, Australia) or mechanical pipetting, and their maturation was assessed. Polar body extrusion observed under the inverted microscope ($\times$200 magnification) was used as the maturation criterion.

The matured oocytes were then cryopreserved using two vitrification protocols.

Some oocytes were cryopreserved by the conventional ultrarapid vitrification method, as previously described elsewhere (15). Briefly, up to five oocytes were suspended in modified human tubal fluid (m-HTF)–based equilibration medium containing 7.5% ethylene glycol (EG; Sigma Chemical) and 7.5% propanediol (PROH; Sigma Chemical) for 5 minutes; then they were transferred to vitrification medium (15% EG, 15% PROH, and 0.5 mol/L sucrose) for 45 to 60 seconds at room temperature.

In the stepwise protocol, the oocytes were placed at the center of a 30-mm well dish (Genesis, Elmwood, WI) filled with 170 μL of holding medium at room temperature. Then, a 10-μL mixture of 50% EG + 50% PROH was added along the edge of the holding medium with a micropipette (Eppendorf, Hamburg, Germany), avoiding direct exposure of the oocytes during addition of the mixture. After addition, the dish was gently, slowly rotated for mixing and was allowed to settle for 2 minutes, followed by a next-to-final (third) addition by the same manner. The final concentration of the equilibrium solution reached 7.5% EG + 7.5% PROH in the holding medium of the dish. After equilibrium, all oocytes were transferred to the same vitrification medium for 45 to 60 seconds at room temperature.

The oocytes were loaded onto a specially designed device (Cryoleaf; MediCult, Jyllinge, Denmark) and were immediately plunged into liquid nitrogen for storage. For thawing, the Cryoleaf was directly inserted into a 37°C thawing medium (1.0 mol/L sucrose) for 1 minute. Thawed oocytes were transferred to 0.5 mol/L and 0.25 mol/L sucrose solutions for 3 minutes each. Oocyte survival was assessed by morphologic appearance. The surviving oocytes were transferred to fertilization medium (SAGE-IVF, Inc.).

The epididymal spermatozoa were retrieved from the cauda epididymis of 10- to 12-week-old CD1 mice, and the sperm suspensions were preincubated for 1.5 hours in capacitation medium (SAGE-IVF, Inc.). The survived oocytes were then inseminated by sperm at a final dilution of 2 $\times$ 10^6/mL and were incubated at 37°C in humidified 5% CO_2 in air. Inseminated oocytes were washed away from sperm by gentle pipetting 6 hours later, and then they were cultured up to 5 days. Fertilization was assessed by the formation of two cells on day 1 after insemination. Two-cell embryos were transferred to embryo maintenance medium (SAGE-IVF, Inc.), and the development into blastocysts was recorded on day 5 after insemination.

The intact CEOs were cryopreserved using the two previously described vitrification protocols. After thawing, CEO survival was assessed by morphologic appearance, and the surviving CEOs were subjected to IVM. All CEOs were denuded, and only matured oocytes were inseminated (in the same manner). Some CEOs died during IVM, and they were also counted as nonsurviving CEOs. Fertilization was assessed by the formation of two cells on day 1 after insemination, and their development into blastocysts was recorded on day 5.

All data in our study were analyzed using SPSS (Windows version 12.0; SPSS, Inc., Chicago, IL). Student's *t*-test was used to compare parametrical variables between two groups, and the chi-square test was used for the comparison of two proportions. $P<.05$ (two-tailed) was considered statistically significant.

After 17 to 19 hours of IVM culture, 69.8% and 82.1% GV stage oocytes matured to the metaphase II stage (Table 1). These rates were similar to the nonvitrified control. After vitrification and thawing, high survival rates (>90%) were noted in both the conventional and stepwise groups. After insemination, the cleavage rate was statistically significantly lower in the conventional group but were similar in the stepwise group when compared with the nonvitrified control. Despite high survival rates after vitrification of IVM oocytes, either by conventional or stepwise protocol, the blastocyst formation rate was lower than for the nonvitrified control. After vitrification at the GV stage, survival rates were relatively high, and the maturation rates were similar to the nonvitrified controls. However, the cleavage rates were statistically significantly lower than that of the nonvitrified controls. No blastocysts were obtained after vitrification of GV-stage oocytes. Although vitrification after IVM using the stepwise method does not affect the cleavage rate, the early embryonic development was profoundly impaired in both protocols.

We performed a modified protocol for stepwise pre-equilibrium to ultrarapidly vitrify immature mouse oocytes. This ultrarapid vitrification by increasing the steps for pre-equilibration to cryoprotectants was introduced by Kuwayama et al. (19) and later modified by Aono et al. (18, 20). Increasing the steps for pre-equilibration requires increasing the frequency of handling oocytes, which might lead to a loss of cumulus cells of the CEO or oocyte during handling (20). The modified serial dilution method introduced by Aono et al. (18), which is simple and easy to use, could minimize the loss of cumulus cells in the case