Clinical-pregnancy outcome after vitrification of blastocysts produced from in vitro maturation cycles

This study was conducted to report the clinical-pregnancy outcome after vitrification of the blastocysts produced from in vitro maturation cycles. The survival rate after thawing was 92.0% (92/100). The clinical-pregnancy and implantation rates were 43.8% and 23.6%, respectively. These results suggest that the blastocyst-stage embryos produced from in vitro maturation cycles can be safely cryopreserved through vitrification. (Fertil Steril® 2007;88:1449–51. ©2007 by American Society for Reproductive Medicine.)

Vitrification would be a very attractive alternative to the conventional slow-cooling protocol, with its advantages of the lack of ice crystal formation and ease of operation. In controlled ovarian hyperstimulation cycles, vitrification of blastocysts by using the cryoloop (1), electron-microscope (EM) grids (2), open hemi-straw (3), open polypropylene strip (4, 5), or closed plastic straw with a thin part (4) has already resulted in many pregnancies. The survival rate of expanded blastocysts after vitrification is increased significantly when the blastocoele is artificially shrunk with a glass microneedle (6), 29-G needles (2), pipetting (7), or a laser pulse (8), which is thought to reduce ice crystal formation. Recently, we have developed a vitrification procedure for expanded blastocysts and have achieved a reasonable pregnancy rate in our conventional IVF program (9). This study was performed to report the clinical outcome after vitrification and thawing of the blastocysts produced from in vitro maturation (IVM) cycles by using the established method, and we compared the result with cryothawing of conventional intracytoplasmic sperm injection cycles.

Approval for the study was obtained from the institutional review board of the Maria Infertility Hospital. Patients with polycystic ovary syndrome (PCOS) or with PCO-like ovaries were recruited. The oocytes were collected between cycle days 9 and 16, based on the patient's cycle length and endometrium thickness of >6 mm. The patients were given 10,000 IU of human chorionic gonadotropin (IVF-C; LG chemical, Seoul, Korea) 36 hours before oocyte retrieval. Oocyte recovery was performed according to the protocol described elsewhere (10–12). After collection, immature oocytes were cultured in maturation medium, consisting of Yoon Shanhyun medium with 30% human follicular fluid (FF) supplemented with 1 IU/mL FSH, 10 IU/mL human chorionic gonadotropin, and 10 ng/mL recombinant human epidermal growth factor (Daewoong Pharmaceutical Co., Seoul, Korea) (11). Intracytoplasmic sperm injection was used to fertilize the mature oocytes. Fertilization was assessed 17–19 hours after insemination for the appearance of two distinct pronuclei and two polar bodies. The zygotes were co-cultured with cumulus cells in 10 μL of Yoon Shanhyun medium supplemented with 10% human FF (13). The cumulus cells for co-culture were retrieved from stimulated oocytes at the time of collection and prepared as described elsewhere (13). After embryo transfer, surplus embryos were cultured, and only the embryos that developed to the expanded blastocyst stage (diameter of >160 mm and good quality) until day 6 after intracytoplasmic sperm injection were cryopreserved with vitrification.

All 233 blastocysts from 66 patients were vitrified on an EM grid (IGC 400; Pelco International, CA) after artificial shrinkage (2) between January 2002 and December 2005.

The freezing solution for vitrification, EFS40, was prepared according to a method described elsewhere (2) and consisted of 40% (vol/vol) ethylene glycol (EG; Sigma Chemical Co., St. Louis, MO), 18% (wt/vol) Ficoll (Ficoll 70, average molecular weight, 70,000 Da; Pharmacia Biotech, Uppsala, Sweden), 0.3 mol/L sucrose, and 20% human FF in modified Dulbecco's phosphate-buffered saline. A pretreatment solution (EG20), modified Dulbecco's phosphate-buffered saline containing 20% EG and 20% human FF, was prepared. The blastocysts were placed in EG20 for 1.5 minutes before exposure to the vitrification solution at room temperature. The blastocysts were then incubated in EFS40 at room temperature for 10 seconds and then loaded onto the EM grid. The excess of cryoprotectant was removed by using sterilized filter paper, and then blastocysts were directly plunged into LN_2 within 30 seconds and were stored in an LN_2 tank.

For thawing of vitrified blastocysts, a two-step cryoprotectant dilution method was used in which the EM grids stored in LN_2 were directly transferred into a 100-μL drop of 0.5 mol/L sucrose solution (prepared in Dulbecco's phosphate-buffered saline containing 20% human FF) at room temperature as soon as possible and then were transferred into 100-μL drops containing 20% human FF in Dulbecco's phosphate-buffered saline for 5 minutes at room temperature (9). The embryos were washed three times in

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culture medium and co-cultured with cumulus cells in 10 μL of Yoon Shanhyun medium containing 10% human FF. The postthawing survival of blastocysts was observed approximately 18 to 20 hours after warming under a microscope. Embryo transfer was scheduled on day 5 after ovulation in the spontaneous cycles of ovulatory patients, or on day 19 in artificial cycles that were prepared with exogenous estrogen and progesterone for three anovulatory patients. One to three surviving blastocyst-stage embryos were transferred into the patient's uterus.

A total of 233 blastocyst-stage embryos produced from 66 IVM cycles were vitrified in this period. Of these, 27 patients (32 cycles) who had blastocysts transferred after thawing were studied. The blastocysts had been stored for 3–12 months in LN_2. The age and duration of infertility of the 27 patients (mean ± SD) were 32.6 ± 3.7 years and 3.1 ± 2.7 years, respectively. Table 1 shows the clinical results of the human blastocyst vitrification. Of the vitrified 125 blastocysts, a total of 100 expanded blastocysts were warmed from 32 cycles. Ninety-two blastocysts (92.0%) were reexpanded after warming. Of the 92 blastocysts that survived, 68 were hatching or had hatched from blastocyst stage (73.9%) at the time of transfer. A total of 89 blastocysts were transferred in 32 cycles, and 14 clinical pregnancies (CP; 43.8%, 14/32) and a 23.6% implantation rate (IR; 21/89) were established, which included 2 miscarriages and 2 ongoing and 10 term deliveries (including 6 twin pregnancies). Birth weights of the infants were within the range of 1,800–3,540 g, and all delivered infants had a normal physical profile up to the present.

During the same period, 2,020 expanded blastocysts with good quality were produced from 579 conventional intracytoplasmic sperm injection cycles, and the blastocysts were vitrified. Of them, 264 cycles had transferred blastocysts after thawing. A total of 775 blastocyst-stage embryos were thawed, 707 blastocyst-stage embryos survived (91.2%), and 624 were hatching or had hatched (88.2%) at transfer. Six hundred ninety-seven survived embryos underwent embryo transfer in 264 cycles. The IR was 25.8% (180/697), and the CP rate was 45.4% (120/264). The survival, implantation, and pregnancy rates were comparable to those of IVM blastocyst thawing cycles ($P>.05$).

There is only one report for blastocyst transfer and pregnancy derived from IVM (14) in addition to ours (10–12). Barnes et al. (14) were the first to report successful development to the blastocyst stage in sequential culture medium designed specifically to optimize blastocyst development. A pregnancy resulted from the transfer on day 6 of a single blastocyst after assisted hatching. However, only one of six embryos produced in this case report was competent to develop to the blastocyst stage. Since then, there have not been any reports about the successful clinical application after transfer of blastocysts in IVM programs by a sequential culture system similar to that used in conventional IVF. It has been known that co-culture systems significantly enhance the percentage of embryos developing to the blastocyst stage in IVF cycles (15, 16). Hwu et al. (17) and Cobo et al. (18) have reported that co-culture can be added to IVM programs to enhance blastocyst-stage embryo development. In addition, we have also reported successful pregnancies from blastocyst transfer by using a co-culture method in patients undergoing IVM cycles (10–12). These reports served as evidence that up to now, the co-culture method is the best option for obtaining blastocysts in an IVM program.

TABLE 1

Clinical results for patients receiving blastocysts thawed after vitrification of blastocysts obtained from IVM cycles.

Variable	Value
No. of treatment cycles (no. of patients)	32 (27)
No. of expanded blastocysts thawed	100
No. of expanded blastocysts survived (%)	92 (92.0)
No. of expanded blastocysts hatching or hatched at embryo transfer (%)	68 (73.9)
No. of blastocysts transferred (mean)	89 (2.8)
No. of implantations (%)	21 (23.6)
No. of clinical pregnancies (%)	14 (43.8)

Lee. Vitrification of blastocyst derived from human immature oocytes. Fertil Steril 2007.

Suikkari et al. (19) found that the cryosurvival of the in vitro–matured zygotes and cleaved embryos was very poor, compared with that of embryos generated from in vivo–matured oocytes by using the slow-cooling method. In their study, 8 of 24 cleaved embryos and 14 of 25 zygotes survived after thawing, suggesting that cryopreservation of in vitro–matured embryos may not be an optimal procedure. Alternatively to the slow-cooling method, we have established a vitrification system on EM grids combined with artificial shrinkage in an IVF program and have reported the clinical usefulness of the cryopreservation of human blastocysts produced from IVF or IVM programs (20–22). Recently, we have modified a simple thawing protocol in our cryopreservation program and have reported reasonable CP rates in a conventional IVF program (9). Accordingly, we applied the established vitrification and simplified thawing protocol into the blastocysts produced from IVM cycles in this study. We had a 43.8% CP rate and 23.6% IR, as shown in these results. This clinical outcome was comparable to that of conventional intracytoplasmic sperm injection cycles (CP rate, 45.4%; IR, 25.8%) that were performed in the same period but was relatively lower than that for fresh blastocyst

transfer in our IVM program, as reported elsewhere (CP rate, 51.1%; IR, 26.4%) (12). The reason for the lower clinical outcome in frozen cycles may be attributed to cryopreservation of the blastocysts after selection of the best embryos for fresh transfer.

In conclusion, the blastocyst-stage embryos produced from IVM oocytes retrieved from women with PCOS can be safely cryopreserved by vitrification on an EM grid, and successful pregnancy can be achieved after embryo transfer.

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REFERENCES

1. 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.
2. Son WY, Yoon SH, Yoon HJ, Lee SM, Lim JH. Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele. Hum Reprod 2003;18: 137–9.
3. Vanderzwalmen P, Bertin G, Debauche CH, Standaert V, Bollen N, van Roosendaal E, et al. Vitrification of human blastocysts with the Hemi-Straw carrier: application of assisted hatching after thawing. Hum Reprod 2003;18:1504–11.
4. 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.
5. Liebermann J, Tucker J. Comparison of vitrification versus conventional cryopreservation of day 5 and day 6 blastocysts during clinical application. Fertil Steril 2006;86:21–6.
6. Vanderzwalmen P, Bertin G, Debauche CH, Standaert V, van Roosendaal E, Vandervorst M, et al. Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification. Hum Reprod 2002;17:744–51.
7. Hiraoka K, Hiraoka K, Kinutani M, Kinutani K. Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. Hum Reprod 2004;19:2884–8.
8. 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.
9. Lee SY, Kim HJ, Park SJ, Yoon HJ, Yoon SH, Lee KH, et al. Optimization of a dilution method for human expanded blastocysts vitrified using EM Grids after artificial shrinkage. J Assist Reprod Genet 2006;9:1–5.
10. Son WY, Park SJ, Hyun CS, Lee WD, Yoon SH, Lim JH. Successful birth after transfer of blastocysts derived from oocytes of unstimulated women with regular menstrual cycle after IVM approach. J Assist Reprod Genet 2002;19:541–3.
11. Son WY, Yoon SH, Lee SW, Ko Y, Yoon HG, Lim JH. Blastocyst development and pregnancies after IVF of mature oocytes retrieved from unstimulated patients with PCOS after in-vivo HCG priming. Hum Reprod 2002;17:134–6.
12. Son WY, Lee SY, Lim JH. Fertilization, cleavage and blastocyst development according to the maturation timing of oocytes in in vitro maturation cycles. Hum Reprod 2005;20:3204–7.
13. Yoon HG, Yoon SH, Son WY, Kim JG, Im KS, Lim JH. Alternative embryo transfer of day 3 or day 5 for reducing the risk of multiple gestation. J Assist Reprod Genet 2001;18:262–7.
14. Barnes FL, Crombie A, Gardner DK, Kausche A, Lacham-Kaplan O, Suikkari AM, et al. Blastocyst development and birth after in-vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. Hum Reprod 1995;10:3243–7.
15. Menezo YJR, Guerin JF, Czyba JC. Improvement of human early embryo development in-vitro by co-culture on monolayers of Vero cells. Biol Reprod 1990;42:301–7.
16. Bongso A, Ng SC, Fong CY, Ratnam S. Cocultures: a new lead in embryo quality improvement for assisted reproduction. Fertil Steril 1991;56:179–91.
17. Hwu YM, Lee RK, Chen CP, Su JT, Chen YW, Lin SP. Development of hatching blastocysts from immature human oocytes following in-vitro maturation and fertilization using a co-culture system. Hum Reprod 1998;13:1916–21.
18. Cobo AC, Requena A, Neuspiller F, Aragonés M, Mercader A, Navarro J, et al. Maturation in vitro of human oocytes from unstimulated cycles: selection of the optimal day for ovum retrieval based on follicular size. Hum Reprod 1999;14:1864–8.
19. Suikkari AM, Tulppala M, Tuuri T, Hovatta O, Barnes F. Luteal phase start of low-dose FSH priming of follicles results in an efficient recovery, maturation and fertilization of immature human oocytes. Hum Reprod 2000;15:747–51.
20. Cho HJ, Son WY, Yoon SH, Lee SW, Lim JH. An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. Hum Reprod 2002;17:2419–22.
21. Son WY, Yoon SH, Park SJ, Yoon HJ, Lee WD, Lim JH. Ongoing twin pregnancy after vitrification of blastocysts produced by in-vitro matured oocytes retrieved from a woman with polycystic ovary syndrome. Hum Reprod 2002;17:2963–6.
22. Son WY, Lee SY, Chang MJ, Yoon SH, Chian RC, Lim JH. Pregnancy resulting from transfer of repeat vitrified blastocysts produced by in-vitro matured oocytes in patient with polycystic ovary syndrome. Reprod Biomed Online 2005;10:398–401.