

Perinatal outcome of blastocyst transfer with vitrification using cryoloop: a 4-year follow-up study

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Objective: To evaluate perinatal outcome of ultrarapid vitrified blastocyst transfer.

Design: Retrospective study.

Setting: Private IVF clinics.

Patient(s): One hundred eight women, who delivered 147 babies.

Intervention(s): Supernumerary blastocysts were vitrified using cryoloop method and transferred after warming.

Main Outcome Measure(s): Survival rate of blastocyst after vitrification, implantation and pregnancy rates, neonatal outcome and congenital birth defects.

Result(s): A total of 1,129 vitrified blastocysts from 435 cycles were warmed and 967 survived (85.7%). In 413 cycles of transfer, the pregnancy, implantation, and abortion rates were 44.1%, 29.0%, and 22.0%, respectively. Of 108 deliveries, 34 (32.9%) were multiple pregnancies and 20 were preterm (18.5%). Out of 147 children born, 50.3% were male and congenital birth defects were observed in 1.4%. These results were similar with those of fresh blastocyst transfer program.

Conclusion(s): The vitrification of blastocyst using cryoloop is a simple, easy, and quick method. This technique yields the same high pregnancy and implantation rates as fresh blastocyst transfer. Congenital defect rate in this study was similar to fresh blastocyst transfer, proving the method to be safe. (Fertil Steril® 2005;84:88–92. ©2005 by American Society for Reproductive Medicine.)

Key Words: Vitrification, blastocyst, neonatal outcome, congenital birth defect

Blastocyst transfer has been proven to be effective for increasing implantation and pregnancy rates (1, 2). High implantation rates can allow us to reduce the number of transferred embryos, thereby reducing the risk of high-order multiple pregnancy (3).

Blastocyst transfer has also been shown to be useful for improving the pregnancy rate in patients with multiple failures of IVF (2).

Human blastocysts have been frozen by the slow-cooling method, but the results had not been consistent (4–6) until recently (7). Because only a small number of blastocysts are available for cryopreservation, a simple, quick, and reliable procedure is required for clinical use to cryopreserve blastocysts to optimize outcome of blastocyst transfer. We reported the first successful birth after transfer of vitrified human blastocysts with use of a cryoloop containerless technique in 2001 (8) and subsequently high implantation and pregnancy rates after 223 cycles (9).

Vitrification would be a preferred method of cryopreservation to the slow-cooling method because of the lack of ice crystal formation and convenience. The cryoloop enables ultrarapid cooling and warming with reduced volume of

vitrification solution. However, a major drawback of vitrification is use of relatively high concentration of cryoprotectants; which may affect the embryo and subsequent growth in the uterus. Therefore, we analyzed reproductive and neonatal outcomes and investigated congenital defects in infants born after vitrification of blastocyst.

MATERIALS AND METHODS

Patients

HART Clinic is a private outpatient IVF clinic. Our policy of IVF/intracytoplasmic sperm injection (ICSI) is to perform conventional day 2 or 3 ET on patients who receive the first IVF/ICSI treatment, and their supernumerary embryos are cultivated by day 5 or 6 when good quality blastocysts are vitrified. Blastocyst transfer program is carried out in patients who failed to conceive after conventional transfer, not only for increasing pregnancy rates but also for diagnosis of quality of their embryos.

Between April 2000 and June 2003, we performed 435 cycles of vitrified-warmed blastocyst transfer programs in our clinics. Of those, 138 were IVF and 297 were ICSI.

During the same period, we performed 662 fresh blastocyst transfer programs. Of those, 184 were IVF and 478 were ICSI.

IVF/ICSI Protocol

All patients used long and short protocol using GnRH analogue and gonadotropin for the ovarian hyperstimulation.

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Oocytes were retrieved 36 hours after hCG administration and the standard IVF/ICSI procedure was described previously (8, 9). Oocytes were inseminated using either conventional IVF or ICSI and incubated in human tubal fluids (hTF) medium containing 5 mg human serum albumin (HSA) or G1.2 medium (Vitrolife, Gothenburg, Sweden) and BlastAssist System 1 medium (BAS1; MediCult, Jyllinge, Denmark) in a 4-well multidish under mineral oil at 37°C in an atmosphere of 6% CO₂, 5% O₂, and 89% N₂.

Embryo Culture and Grading of Blastocysts

Fertilization was assessed at 16–18 h after insemination by the presence of two pronuclei. Embryos were cultured in G1.2 or BAS1 for 48 h and then placed in G2.2 medium (Vitrolife) or BAS2 (MediCult) for 48–72 h. In cases of conventional day 3 transfer, only supernumerary embryos were cultured in the second medium.

After 48–54 h of culture in the second medium, which was on day 5 after the removal of the oocyte, embryos were examined for development into blastocysts. If all the embryos were at the morula stage or earlier, they were cultured until day 6 and then examined for development. On day 5 or day 6, each embryo, which had developed to the blastocyst stage, was scored depending on the developmental stage and was graded according to quality by using the criteria of Gardner and Schoolcraft (1, 9).

When the patient had their fresh embryos transferred on day 2–3, all the blastocysts were vitrified. On the other hand, when patients were on the blastocyst transfer program, 1–3 fresh blastocysts were transferred into each patient. Only good quality supernumerary blastocysts, which were blastocyst stage and more on day 5 except CC grades of ICM and trophectoderm (Gardner's score ≥ 1) and expanded blastocyst ($\geq 4BB$) on day 6, were vitrified.

Vitrification of Blastocysts

The protocol for the cryoloop vitrification of blastocysts was described previously (8, 9). The cryoloop consisted of a nylon loop (20 μm wide; 0.5–0.7 mm in diameter) mounted on a stainless steel pipe inserted into the lid of a cryovial (Hampton Research, Laguna Niguel, CA). A metal insert on the lid enables the use of a stainless-steel rod with a small magnet (Crystalwand; Hampton Research) for manipulation of the loop at low temperature.

One to three blastocysts were vitrified in a cryoloop after a two-step loading with cryoprotectants at 37°C. Initially, blastocysts were placed in cryoprotectant solution I, which is the base medium (Hepes-buffered modified HTF containing 5 mg/mL of HSA) containing 7.5% (v/v) dimethyl sulfoxide (DMSO) and 7.5% (v/v) ethylene glycol (EG). After 2 min, the blastocysts were suspended in cryoprotectant solution II, which is the base medium containing 15% (v/v) DMSO, 15% (v/v) EG, 10 mg/mL of Ficoll 70 (average molecular weight 70,000; Pharmacia Biotech, Uppsala, Sweden), and

0.65 mol/L sucrose. Both cryoprotectant solutions had been warmed briefly in an incubator at 37°C and blastocysts were handled on the stage warmer of a dissecting microscope at 37°C.

While the embryos were suspended in solution I, a cryoloop was dipped into cryoprotectant solution II to create a thin filmy layer of solution, by surface tension, on the nylon loop. Then the blastocysts were washed quickly in solution II and transferred onto the filmy layer on the nylon loop using a micropipette. Immediately after the loading of blastocysts, the cryoloop was plunged into liquid nitrogen. The time blastocysts were exposed in solution II before cooling was limited to 25–30 sec. Using the stainless rod, the loop containing the blastocysts was sealed in a cryovial, which was previously submerged in liquid nitrogen. The vials were attached in standard canes and stored in liquid nitrogen. The whole procedure was completed within 5 min.

Warming of Blastocysts and Assessment of Survival

In a 4-well multidish, approximately 1 mL of base medium containing 0.33 mol/L sucrose, base medium containing 0.2 mol/L sucrose, and base medium were warmed briefly in an incubator at 37°C and then placed on the stage warmer of a dissecting microscope. With the cryovial submerged in liquid nitrogen, the vial was opened with the aid of the stainless rod, and the loop containing blastocysts was removed from the liquid nitrogen and placed directly and quickly into a well of the 0.33 M sucrose solution. Blastocysts immediately fell from the loop into the solution. Thus blastocysts were warmed and diluted instantly at 37°C. After 2 min, blastocysts were transferred to the 0.2 mol/L sucrose solution. After an additional 3 min, blastocysts were washed and kept in the base medium for 5 min and then returned to the second medium for further culture until transfer.

About 2 h after warming, the appearance of the blastocysts was examined on an inverted microscope at 400 \times magnification, and survival was assessed based on the morphologic integrity of the blastomeres, inner cell mass, and trophectoderm, and reexpansion of the blastocoele. The blastocysts judged to have survived were scored as to developmental stage and were graded for quality as described above.

Transfer of Blastocysts and Assessment of Pregnancy

All women received transdermal E₂ (Estrana 0.4 mg/d; Hisamitsu, Tokyo, Japan) with GnRH agonists for preparation of the endometrium. Administration of progesterone (50 mg in oil, daily) was initiated when the endometrial thickness became more than 10 mm. Basically, 1–3 blastocysts that survived vitrification were transferred into the patient's uterus 5 days (in a few cases, 6 days) after the initiation of progesterone treatment

Clinical pregnancy was confirmed by the presence of a gestational sac.

Neonatal Outcome and Congenital Birth Defects

We performed retrospective analysis of data obtained from postal questionnaire of parents after delivery and/or from referred physicians in case of abnormal outcome. We obtained 108 responses out of 142 parents (76.1%). Of 108 deliveries, we evaluated 147 live-born infants and compared with 205 live-born infants born from 153 out of 196 (78.1%) responding parents who conceived after fresh blastocyst transfer during the same period.

Statistics

The data were examined for differences by χ^2 analysis, unless the expected frequency was less than 5, in which case Fisher's exact probability test was used.

RESULTS

Table 1 shows the mean age and clinical outcome of patients who completed fresh and vitrified blastocyst transfer program. Many patients in both groups were identical because they received vitrified blastocyst transfer owing to failure of conception or having the second baby after a successful birth after fresh blastocyst transfer. The mean age of the women was 34.5 ± 4.2 (25 – 40) years in the vitrified group and 35.7 ± 4.4 (24 – 43) years in the fresh group. The vitrified group was significantly younger ($P < .05$), indicating that younger women had a high number of good quality of supernumerary blastocysts vitrified. The number of lost follow-up (either no responses or changed address) was 34 patients in vitrification (23.9%) and 46 in the fresh transfer (23.1%).

The survival rate of vitrified blastocysts was 85.7% (967/1,129). We transferred 716 warmed-vitrified blastocyst in 413 cycles out of 435 attempted cycles (mean number of embryo transfer: 1.7). One hundred eighty-two were pregnant (44.1%, per transfer) and implantation rate was 29.0%. Of those, 40 resulted in abortion (22.0%) and 108 deliveries were reported.

In fresh blastocyst transfer, of 662 cycles, transfer could be carried out in 602 cycles with 1,252 blastocysts (mean number of embryo transfer: 2.1), and 267 were pregnant (44.4%, per transfer) and implantation rate was 23.4%. Of those, 68 resulted in abortion (25.5%) and 153 deliveries were reported. There were no statistical difference between groups.

Table 2 shows perinatal outcome of children born in this program. Out of 108 deliveries with vitrified blastocyst, 147 babies (74 boys and 73 girls) were born. The mean gestational age was 38.1 weeks and birth weight was $2,601 \pm 709$ g. There were 20 preterm deliveries (18.5%). There were 40 sets of twins (27.8%) and 4 sets of triplets (5.1%).

Out of 153 deliveries with fresh transfer, 205 babies (120 boys and 85 girls) were born. Male ratio was significantly higher ($P < .05$). The mean gestational age was 37.9 weeks and birth weight was $2,593 \pm 629$ g. There were 19 preterm

TABLE 1

Clinical outcome of vitrified and fresh blastocyst transfer between 2000 and 2003.

Cycles	Fresh	Vitrification
Patients' age (y)	35.7 ± 4.4^a	34.5 ± 4.2
No. of cycles	662	435
No. of cycles transferred	602	413
No. of embryos transferred	1,252	716
Mean	2.1	1.7
No. of implantation (%)	293 (23.4)	208 (29.0)
No. of pregnancy % per ET	267 44.4	182 44.1
Ongoing pregnancy (%)	199 (74.5)	142 (78.0)
No. of deliveries	153	108
No. of lost follow-up	46	34

^a $P < .05$.

Takahashi. Perinatal outcome of vitrified blastocyst. *Fertil Steril* 2005.

deliveries (12.4%). There were 40 sets of twins (26.1%) and 6 of triplets (3.9%). No statistical difference was seen between groups except for sex ratio.

Table 3 shows cases of congenital birth defects. Two were observed in vitrification (1.4%) and four in fresh transfer (2.0%). In vitrification, one of dizygotic twins had Treacher-Collins syndrome or mandibulofacial dystosis and the other had patent ductus arterioses (PDA). In fresh transfer, a baby from a 39-year-old mother died with 21 trisomy at 2 days of age and three minor defects included anal atresia, PDA, and defect of fourth lumbar spine.

DISCUSSION

Introduction of newly developed sequential media has made it possible to extend culture of human embryos to blastocyst stage easily. Since the blastocyst transfer is more physiological than conventional ET, this has become a promising way to increase pregnancy and implantation rates (10). Furthermore, in blastocyst formation it is easier for us to select more viable embryos, which allowed us to reduce the number of transfer embryos to reduce the rate of multiple pregnancy without adversely affecting pregnancy rate (3).

Accordingly, the need to cryopreserve human blastocysts has increased. Although several methodologies of cryopreservation had been introduced, including slow freezing and vitrification (4–6, 11), their clinical results were not consistent, as we described previously (9). Therefore, we used a new ultrarapid vitrification method using a cryoloop, which can increase rates of cooling and warming by mini-

TABLE 2**Comparison of mode of delivery and characteristics of infants conceived after fresh and vitrified blastocyst transfer.**

	Fresh blastocyst transfer	%	Vitrified blastocyst transfer	%
Live born	205		147	
Male	120	58.5 ^a	74	50.3
Female	85	41.5 ^a	73	49.7
Vaginal delivery	70	45.8	44	40.7
Cesarean section	83	54.2	64	59.3
Mean gestational age (wk)	37.9 ± 2.5		38.1 ± 2.8	
Preterm (<37 wk)	19	12.4	20	18.5
Mean birth weight (g)	2,593 ± 629		2,601 ± 709	
<1,500 g	9		8	
1,500–2,500 g	66		56	
>2,500 g	130		83	
Twins	40	26.1	30	27.8
Triplets	6	3.9	4	5.1

^a *P* < .05.Takahashi. Perinatal outcome of vitrified blastocyst. *Fertil Steril* 2005.

mizing the volume of the solutions of vitrification (12, 13). In addition, this method is simple and fast, suitable for freezing small numbers of blastocysts in practice.

The results of the present study—29.0% implantation rate and 44.1% pregnancy rate after 87.5% survival rate—were comparable with that of fresh blastocyst transfer. Considering that patients in the blastocyst transfer program in our clinics had experienced at least one failure of pregnancy after conventional IVF/ICSI, these results indicated that this method is effective in clinical use.

There were two major concerns for this method. First is using relatively high concentration of cryoprotectants, which might affect embryos and subsequent growth in the uterus. We compared the perinatal outcome of blastocyst transfer using this method with that of fresh blastocyst transfer. There were no statistical differences in the mean gestational age, birth weight, preterm birth rate, or congenital birth defect rate. Male sex ratio was 50.3%, compared with 58.5%

in fresh transfer. These results proved that our vitrification method of blastocyst using cryoloop is safe for clinical use.

The second concern is the possibility of viral contamination during storage in liquid nitrogen (LN), because embryos in cryoloop are directly exposed to LN at vitrification and the cryovial is not completely sealed. The transmission of hepatitis B virus to patients by transplanted bone marrow cells stored in contaminated LN has been reported (14). Viral contamination was found in zona pellucida of bovine embryos in unsealed containers stored in LN with high concentration of bovine virus equivalent viremic stage (15). The possibility of viral contamination in our method can be thought to occur only by embryos from patients with viremic stage. However, the concentration of virus in LN from repeatedly washed contaminated blastocyst can be expected to be far lower than reported previously. We perform routine screening tests for viral infections, including HIV and hepatitis B and C, on all couples undergoing IVF and do not

TABLE 3**Prevalence of major and minor birth defects in infants.**

	Fresh blastocyst transfer	Vitrified blastocyst transfer
Major defects	1 (21 trisomy, deceased)	1 (Treacher-Collins syndrome ^a)
Minor defects	3 (anal atresia, PDA, defect of 4 th lumbar spine)	1 (PDA)
Total (%)	4 (2.0%)	2 (1.4%)

^a Mandibulofacial dysostosis.Takahashi. Perinatal outcome of vitrified blastocyst. *Fertil Steril* 2005.

vitrify blastocysts from those with positive results, even though it is very unlikely storage tank would be contaminated by their embryos.

In conclusion, the vitrification of blastocyst using cryoloop is effective and safe in clinical use. Combined with the fresh blastocyst transfer program, this method will allow us to perform single blastocyst transfer because of high implantation rate.

REFERENCES

1. Gardner DK, Schoolcraft WB. In-vitro culture of human blastocyst. In: Jansen R, Mortimer D, eds. *Towards reproductive certainty: infertility and genetics beyond 1999*. Carnforth, New York: Parthenon, 1999. p. 378–88.
2. Cruz JR, Dubey AK, Patel J, Peak D, Hartog B, Gindoff PR. Is blastocyst transfer useful as an alternative treatment for patients with multiple in vitro fertilization failures? *Fertil Steril* 1999;72:218–20.
3. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril* 2000;73:1155–8.
4. Menezo Y, Nicollet B, Herbaut N, Andre D. Freezing cocultured human blastocysts. *Fertil Steril* 1992;58:977–80.
5. Menezo Y, Sakkas D, Veiga A. Cryopreservation of blastocysts. In: Gardner DK, Lane M, eds. *ART and the human blastocyst*. New York: Springer-Verlag, 2000. p. 188–95.
6. Ludwig M, Al-Hasani S, Felberbaum R, Diedrich K. New aspects of cryopreservation of oocytes and embryos in assisted reproduction and future perspectives. *Hum Reprod* 1999;14(Suppl 1):162–85.
7. Gardner DK, Lane M, Stevens J, Schoolcraft WB. Changing the start temperature and cooling rate in a slow-freezing protocol increases human blastocyst viability. *Fertil Steril* 2003;79:407–10.
8. 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.
9. Mukaida T, Nakamura S, Tomiyama T, Wada S, Oka C, Kasai M, et al. Vitrification of human blastocysts using cryoloop: clinical outcome of 223 cycles. *Hum Reprod* 2003;18:384–91.
10. Gardner DK, Schoolcraft WB, Wagley L, Schlenker T, Stevens J, Hesla J. A prospective randomized trial of blastocyst culture and transfer in in vitro fertilization. *Hum Reprod* 1998;13:3434–40.
11. Yokota Y, Sato S, Yokota M, Yokota H, Araki Y. Birth of a healthy baby following vitrification of human blastocysts. *Fertil Steril* 2001;75:1027–9.
12. Lane M, Bavister BD, Lyons FA, Forest KT. Containerless vitrification of mammalian oocytes and embryos. *Nat Biotechnol* 1999;17:1234–6.
13. Lane M, Schoolcraft WB, Gardner DK. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil Steril* 1999;72:1073–8.
14. Tedder RS, Zuckerman MA, Goldstone AH, Hawkins AE, Fielding A, Briggs EM, et al. Hepatitis B transmission from contaminated cryopreservation tank. *Lancet* 1995;346:137–40.
15. Bielanski A, Nadin-Davis S, Sapp T, Lutze-Wallace C. Viral contamination of embryos cryopreserved in liquid nitrogen. *Cryobiol* 2000;40:110–6.