Improved Outcome of Frozen-Thawed Blastocyst Transfer with Menezo's two-step Thawing Compared to the Stepwise Thawing Protocol¹

Jianchi Ding,^{2,3} Michelle Pry,² Nasir Rana,² and W. Paul Dmowski²

Submitted October 2, 2003; accepted April 14, 2004

Purpose: To compare the effects of two different blastocyst thawing protocols on implantation, pregnancy, and live birth rates.

Methods: Ninety four consecutive frozen-thawed blastocyst transfers from 1996 to 2002 were retrospectively analyzed. Blastocysts were cryopreserved using Menezo's two-step slow-freezing protocol. Frozen blastocysts were thawed for transfer according to Menezo's stepwise or two-step protocol. Immediately after thawing, assisted hatching was performed and all embryos were cultured for 3 to 5 h before transfer. Only viable embryos were selected for transfer.

Results: Implantation, pregnancy (determined by the presence of fetal cardiac activity), and live birth rates were significantly higher with two-step (25, 45.7, and 42.9%) than with stepwise thawing protocol (9.2, 18.6, and 16.9%, $P < 0.01$). The percentage of gestational sacs resulting in live babies was higher with two-step thawing (76.6%) than with stepwise thawing (50%, $P = 0.04$).

Conclusions: We conclude that the two-step thawing protocol offers advantages over the stepwise method. The two-step thawing protocol dramatically increased embryo implantation potential, resulting in higher pregnancy rate, and subsequent live birth rate, after frozen blastocyst transfer.

KEY WORDS: Blastocyst; embryo cryopreservation; implantation rate; live birth; pregnancy rate.

INTRODUCTION

Blastocyst transfer has evolved owing to the advancements of embryo culture systems during the past decade (1–2). Advantages of blastocyst transfer include the following: 1) embryos of highest viability are selected for transfer; and 2) uterine environment may be better suitable for blastocysts rather than cleaved embryos. It is therefore agreed that fewer embryos

at the blastocyst stage are required for transfer to achieve a reasonable pregnancy rate. Subsequently, the risk of high-order multiple pregnancies are reduced with blastocyst transfer (1).

To implement a successful blastocyst transfer program, an effective blastocyst cryopreservation technique must be established. In 1992, Menezo *et al.* (3) compared several freezing and thawing protocols. In their two-step freezing and stepwise thawing protocol, blastocysts were equilibrated in freezing solution using a two-step method with the final step containing 9% glycerol and 0.2 M sucrose, followed by a slow-freezing process (Menezo's two-step freezing protocol). Blastocysts were then thawed and the glycerol was removed from embryonic cells with a six-step dilution in which the embryos are rinsed in decreasing concentration of glycerol (stepwise thawing). Using

¹ Part of the data was presented at 59th Annual Meeting of the American Society for Reproductive Medicine, October 11–15, 2003, San Antonio, Texas, and the abstract was published in *Fertil. Steril.* 2003; **80** (Suppl 3):S147.

² Oak Brook Fertility Center, 2425 W. 22nd Street, Suite 102, Oak Brook, Illinois 60523.

³ To whom correspondence should be addressed; e-mail: jding@oakbrookfertility.com.

this method, they achieved a survival rate of 74.7% and a pregnancy rate (PR) of 21%. In 1997, Menezo and Veiga (4) further reported that when the stepwise thawing was replaced with a simplified, two-step thawing protocol, they achieved a similar pregnancy rate. Using the same two-step freezing and two-step thawing protocols, Behr *et al.* (5) recently reported a PR of 36%, with a live birth rate of 27%. We used a similar (two-step) blastocyst freezing protocol since 1996. Blastocysts were thawed for transfer using stepwise thawing before June 2001 and two-step thawing since June 2001. The objective of this study was to compare the effect of two different thawing protocols on embryo recovery, implantation, pregnancy, and live birth rates with frozen blastocyst transfer (FBT).

MATERIALS AND METHODS

All FBT cycles from 1996 to 2002 were analyzed. The same physicians and embryologists managed all patient cycles. All blastocysts were cryopreserved using the Menezo's two-step protocol with slight modification (3) (Table I). The base medium was mHTF (Irvine Scientific, Santa Ana, CA) with 10% patient serum, and the freezing media were made at our facility. The seeding temperature was -7.5°C instead of -6°C . Embryos were thawed using the stepwise method before June 2001 and the two-step method thereafter. The stepwise and two-step thawing protocols were the same as described by Menezo *et al.* (3) and Menezo and Veiga (4) (Table II). The stepwise thawing solution was made at our facility and the two-step thawing solution was purchased from Irvine Scientific. Immediately after thawing, assisted hatching (Fig. 1A and B) was performed with acid-

ified Tyrodes Solution (Irvine Scientific, Santa Ana, CA). Embryos were then cultured in blastocyst culture medium for 3 to 6 h prior to transfer. At this time, they were classified into 4 categories: 1) Reexpanded: blastocysts recovered $>80\%$ of its original size with a single blastocoele (Fig. 1C and D); 2) Reexpanding: blastocysts recovered $<80\%$ of the original size with a clear blastocoele (Fig. 1E and F); 3) Nonexpanding: having more than 50% live cells, but no evidence of reexpansion (Fig. 1, G); and 4) Nonviable: with less than 50% live cells and with no signs of reexpansion (Fig. 1H).

Blastocysts were transferred during GnRHa-suppressed, and estradiol- and progesterone-stimulated cycles. Briefly, Lupron 1 mg daily was started during the midluteal phase and Estradiol Valerate 2 to 4 mg every other day was administered intramuscularly (I.M.) from day 5 of the following menstrual cycle. When endometrial thickness reached >8 mm (10 to 14 mm preferred), Lupron was discontinued, and progesterone in oil 100 mg/day was administered I.M. together with Estradiol Valerate. Embryos were transferred on the 7th day of progesterone administration. After embryo transfer, patients continued to receive Estradiol Valerate and progesterone until the β -hCG testing 10 to 12 days after FBT.

Patients with a β -hCG elevation were defined as β -hCG positive and continued to receive estradiol and progesterone support. Patients with increasing β -hCG levels underwent ultrasonographic examination. Those with intrauterine gestational sac(s) were defined as clinical pregnancies (Clinical PR), those with fetal cardiac activity as ongoing pregnancies (Ongoing PR), and those who delivered live babies as live births. Patients who had β -hCG elevation but no gestational sac were considered biochemical pregnancies (Biochem PR).

Table I. Blastocyst Cryopreservation Procedure

Menezo's two-step freezing protocol (3) with slight modification

1. Place blastocysts in solution 1 (5% glycerol in mHTF with 10% patient serum) for 10 min.
2. Transfer blastocysts from solution 1 to solution 2 (9% glycerol, 0.2 M sucrose in mHTF with 10% patient serum) for 10 min.
3. Load embryos into freezing straws in solution 2 during the above step. (All above steps were carried out at room temperature, 20 to 25°C .)
4. Proceed with the slow cooling procedure:
  - 1) Start temperature at 20°C .
  - 2) Decrease temperature from 20 to -7.5°C at $-2^{\circ}\text{C}/\text{min}$.
  - 3) Soak for 2 min.
  - 4) Seeding.
  - 5) Hold for 10 min.
  - 6) Decrease temperature from -7.5 to -37°C at $-0.3^{\circ}\text{C}/\text{min}$.
  - 7) Decrease temperature from -37 to -140°C at $-40^{\circ}\text{C}/\text{min}$.
  - 8) Plug into liquid nitrogen.

Table II. Comparison of Menezo's Stepwise and Two-Step Thawing Protocols

	Stepwise ^a			Two-step ^b		
	Thaw at room temperature (20–25°C) for 2 min			Thaw at room temperature (20–25°C) for 30 s, then in 30°C water for 45 s		
	Glycerol conc. (%)	Sucrose conc. (M)	Duration (min)	Glycerol conc. (%)	Sucrose conc. (M)	Duration (min)
Steps of glycerol removal						
1	5	0.4	5	0	0.5	10
2	4	0.2	6	0	0.2	10
3	3	0.2	7			
4	2	0.2	7			
5	1	0.2	6			
6	0	0.2	2			

^a The base solution is mHTF containing 10% patient sera.

^b The base solution is mHTF containing 12 mg/ml human serum albumin (from Irvine Scientific, Santa Anna, CA).

The data was analyzed using χ^2 or Student's *t* test where appropriate. A *p* value of ≤ 0.05 was considered significant.

RESULTS

Patient Characteristics

A total of 94 consecutive FBT cycles were performed between 1996 and 2002. Fifty-nine of these were thawed using stepwise thawing protocol, and 35 cycles were thawed with the two-step thawing protocol. There was no difference between the two groups in the patients' mean age (34.2 ± 4.43 vs 34.2 ± 4.3 , $P > 0.05$) and the proportion of FBT cycles with frozen embryos from oocyte donation cycles were similar between the stepwise thawing and the two-step thawing methods (18.6 vs 22.9%, $P > 0.05$). Main diagnoses of infertility in autologous FBT cycles (frozen embryos from autologous oocytes) were comparable in both groups: tubal factor, 22.9 vs 22.2%; male factor, 12.5 vs 22.2%; endometriosis, 31.3 vs 40.7%; and ovarian, 18.8 vs 14.8% in the stepwise thawing and in the two-step thawing methods, respectively. Endometrial thickness at the time of the first progesterone injection was similar in the stepwise thawing and two-step thawing methods (11.7 ± 1.8 vs 11.5 ± 1.3 , $P > 0.05$; Table III).

Embryo Survival Rate and Morphology After Thawing

In total, 336 frozen blastocysts were thawed. Two hundred and ten blastocysts were thawed with the

stepwise thawing, resulting in 171 (81.4%) survived embryos. One hundred and sixteen blastocysts were thawed with the two-step thawing method, resulting in 105 (90.5%) survived embryos. The survival rate was significantly higher in the two-step thawing than in the stepwise thawing ($P < 0.05$). The percentage of reexpanded blastocysts was similar for two-step and stepwise thawing (38.8 vs 38.1%, $P > 0.05$; Fig. 2). The proportion of reexpanding blastocysts was slightly lower in the two-step thawing (24.1%) than in stepwise thawing (28.1%), but the difference was not statistically significant ($P > 0.05$). The percentage of nonexpanding blastocysts was significantly higher in two-step thawing (27.6%) than in stepwise thawing (15.2%, $P < 0.05$, see Fig. 2).

Implantation and Pregnancy Outcome

Embryo transfers were performed in all 35 FBT cycles after two-step thawing and in 57 of 59 FBT cycles after stepwise thawing (100 vs 96.6%, $P > 0.05$). Mean number of embryos transferred per cycle was similar in both groups (2.74 and 2.88 for two-step and stepwise thawing, respectively; $P > 0.05$, Table III). The mean number of reexpanded blastocysts per transfer was also similar (1.3 vs 1.4 for two-step and stepwise thawing, respectively, $P > 0.05$). The percentage of β -hCG elevation, clinical PR, ongoing PR, and live birth rate were all significantly higher in the two-step thawing (60.0, 51.4, 45.7, and 42.9%, respectively) than in the stepwise thawing protocol (33.9, 25.4, 18.6, and 16.9%, respectively, $P < 0.05$; Table III). Clinical and ongoing implantation rates and live babies/embryo were also significantly higher in the two-step thawing (31.3, 25.0,

Fig. 1. Microphotographs of thawed blastocysts (400X). Immediately after thawing, the blastocysts were at the shrunken state and assisted hatching was performed with acidified Tyrodes solution (A and B); after 3 to 5 h culture, the blastocysts were classified as Reexpanded (C and D) (blastocysts recovered >80% of their original size with a single blastocoele), Reexpanding (E and F) (blastocysts recovered <80% of the original size with clear blastocoele), Nonexpanding (G) (having >50% live cells, but no evidence of reexpansion) and Nonviable (H) (with <50% live cells with no signs of reexpansion) embryos.

Table III. Influence of the Two Blastocyst Thawing Protocols on Blastocyst Survival Rate and Pregnancy Outcomes

	Stepwise	Two-step	P value*
# Thawing cycles	59	35	
Mean age (years old)	35.7 ± 6.0	36.4 ± 6.3	>0.05
Mean endometrial thickness (mm)	11.7 ± 1.8	11.5 ± 1.3	>0.05
# Blastocysts thawed	210	116	
# Blastocysts survived	171 (81.4%)	105 (90.5%)	0.043
# Transfers	57	35	
Mean # embryos/transfer	2.88 ± 0.83	2.74 ± 0.61	>0.05
Mean # of reexpanded/transfer	1.4 ± 1.3	1.3 ± 1.0	>0.05
# β -hCG elevation (%)	20 (33.9%)	21 (60.0%)	0.02
# Clinical PR (%)	15 (25.4%)	18 (51.4%)	0.02
# Ongoing PR (%)	11 (18.6%)	16 (45.7%)	0.01
# Live births (%)	10 (16.9%)	15 (42.9%)	0.006
# Live birth for day-5 blastocysts	4 (16.7%)	9 (42.8%)	0.09 (Fisher's)
# Live birth for day-6 blastocysts	6 (17.1%)	6 (42.8%)	0.08 (Fisher's)
Clinical implantation/embryo	24 (14.6%)	30 (31.3%)	0.002
Ongoing implantation/embryo	15 (9.2%)	24 (25.0%)	0.002
Live births (+Ongoing)/embryo	12 (7.3%)	23 (23.9%)	0.0003

Note. Values are expressed as mean ± SD.

*Data were analyzed using *t* test for means, χ^2 or Fisher's exact test for percentage data.

and 23.9%, respectively) than in the stepwise thawing group (14.6, 9.2, and 7.3%, respectively; $P < 0.01$; Table III). The percentage of patients with β -hCG elevation who failed to develop intrauterine gestational sac(s) (Biochem PR) and the percentage of clinical pregnancy without cardiac activity (Sac only) in the

two-step thawing (14.3 and 9.5%) were half of that in the stepwise thawing protocol (25 and 20%, Fig. 3), although the miscarriage rates after cardiac activity were similar in both groups (5 vs 4.8%). In total, 50% of pregnancies miscarried (including Biochem PR) in stepwise thawing, which was greater than in

Fig. 2. Comparison of embryo status before transfer between stepwise and two-step thawing protocols: ■ Reexpanded, ▨ Reexpanding, ▤ Nonexpanding, and ▧ Nonviable. * $P < 0.05$.

Fig. 3. Effect of thawing protocols on pregnancy losses: ■ Biochemical pregnancies, ▨ with Intrauterine gestational sac(s) but with no cardiac activity, and ▧ Miscarried after detection of Cardiac Activities. † Total pregnancy loss.

Fig. 4. Comparison of the proportions of gestational sacs without viable fetuses between stepwise and two-step thawing protocols. * $P = 0.04$.

the two-step thawing protocol (28.6%), though not statistically significant ($P > 0.05$; Fig. 3). The proportion of total intrauterine gestational sacs with no viable fetuses was significantly greater in stepwise thawing (50%) than in two-step thawing (23.3%, $P = 0.04$; Fig. 4).

There was no difference in live birth rate between blastocysts frozen on day 5 (day-5 blastocysts) and on day 6 (day-6 blastocysts) (Table III). In two-step thawing, the live birth rate was 42.8% for both day-5 and day-6 blastocysts. In stepwise thawing, the live birth rate was 16.7% for day-5 blastocysts and 17.1% for day-6 blastocysts.

DISCUSSION

The success rate of blastocyst freezing programs has been variable in the literature. Nakayama *et al.* (7) reported a low implantation rate of 1.7% out of 119 frozen-thawed blastocysts. Pantos *et al.* (8) reported a low survival rate of 56% and a low pregnancy rate of 12.8% for frozen blastocysts, and Alper *et al.* (9) reported a similar low pregnancy rate of 10.3%. Therefore, question has been raised whether blastocyst culture is worthwhile owing to the low success rate of

the blastocyst freezing program (9). Menezo's group (3,6) described their first successful freezing and thawing protocol for cocultured human blastocysts, resulting in an implantation rate of 13.4%, clinical PR of 21.7%, and ongoing PR of 19%. In this protocol, the blastocysts were equilibrated in the freezing media containing glycerol using the two-step method in order to prepare embryos for freezing. Embryos were frozen using a slow-cooling protocol. Cryopreserved blastocysts were thawed using a stepwise thawing protocol to remove glycerol (stepwise thawing). In 1997, Menezo and Veiga (4) replaced the stepwise thawing with a much simplified, two-step thawing protocol, achieving improved implantation rate, clinical PR, and ongoing PR (17, 29, and 22.1%, respectively). Behr *et al.* (6) recently reported a similar implantation rate and clinical PR (16 and 36%, respectively) when the same two-step freezing and two-step thawing protocols were used. With a slightly modified freezing and thawing protocol, Virant-Klun *et al.* (10) achieved a 29.5% clinical PR with an average of 1.9 embryos per transfer.

We began freezing blastocysts in 1996 with a modified Menezo's two-step freezing method (Table I). Embryos were thawed for transfer with the stepwise thawing method before June 2001 and the two-step thawing method thereafter. The overall embryo survival rate was higher with the two-step thawing than with stepwise thawing, primarily because of a higher percentage of viable but nonexpanding embryos with the two-step thawing method (Fig. 2). However, the proportion of well-recovered embryos (reexpanded and reexpanding blastocysts) after 3 to 5 h of culture was similar with both thawing methods. Therefore, with respect to morphological criteria, two-step and stepwise thawing methods were very comparable. The advantage of two-step thawing protocol was simplicity and less time required.

Our most intriguing findings in this study were significant increases in the implantation, clinical pregnancy, and live birth rates of embryos thawed with the two-step protocol when compared to the stepwise protocol (31.3, 51.4, and 42.9% vs 14.6, 25.4%, and 16.9%, respectively). Our results for the stepwise thawing were comparable to those reported by Menezo's group (3,6), whereas the results for the two-step thawing protocol were greater than those reported by Menezo and Veiga (4) and Behr *et al.* (5). The reason for this finding is not entirely clear. Considering that there were no major morphological differences between embryos thawed with two-step

and stepwise methods, and that our study was a retrospective analysis of the data, it is possible that factors other than the thawing techniques were involved. However, the patient characteristics and quality of embryos at the time of freezing (data not shown) were comparable and the procedures of embryo transfer were not changed. Furthermore, the live birth rate during 23 FBT cycles in which embryos were frozen before June 2001 and thawed for transfer after June 2001 using the two-step method was 39.1% (9/23, data not shown), similar to the overall live birth rate of 42.9% with the two-step method. It appears therefore that increased implantation and live birth rates with the two-step thawing were not due to changes in the patient population, original embryo quality, or change in the embryo-transfer technique.

Approximately 50% of the β -hCG positive cycles in the stepwise thawing FBT group did not develop into viable pregnancies. Half of these were chemical pregnancies and the other half miscarried after detection of intrauterine gestational sac(s) (Table III). In contrast, in the two-step thawing group, only 28.6% were failing pregnancies (14.3% chemical pregnancies and 14.3% miscarried after detection of intrauterine gestational sacs), significantly lower than with the stepwise thawing protocol. This could suggest that the frozen blastocysts thawed with the stepwise method had reduced developmental potential when compared to those thawed with the two-step protocol.

A major difference between the stepwise thawing and the two-step thawing is the speed of glycerol removal from thawed blastocysts (Table II). With stepwise thawing, thawing solutions contain reduced concentration of glycerol in a stepwise (6-step) fashion (Table II). The protocol emphasizes slow removal of glycerol from embryonic cells to avoid osmotic shock. Such protocol, although capable of maintaining the overall high survival rate and recovery rate at the initial stage, may cause prolonged adverse effects of the cryoprotectant (glycerol) on embryonic cells. In the two-step thawing protocol, glycerol is removed from the embryonic cells much faster than with the stepwise protocol because the thawing solution used contained no glycerol. Therefore, adverse effects of glycerol during the thawing process can be minimized. Our data seem to suggest that rapid removal of glycerol during thawing in the two-step protocol offers advantages for the long-term survival of thawed embryos and may result in improved implantation and reduced miscarriage rates.

Simple, efficient, and nontoxic freezing and thawing techniques are crucial for a successful FBT program. Our data clearly indicate that Menezo's two-step freezing protocol combined with the two-step thawing method is simple and efficient, and results in greater survival rate and live birth rate after transfer. Further modification of the present two-step freezing and two-step thawing protocols such as equilibration in glycerol freezing solution at 37°C instead of room temperature as described by Virant-Klun *et al.* (10) may further improve the survival and implantation rates. In a recent report (11), Gardner *et al.* were able to dramatically increase blastocyst recovery (69%) and implantation rates (30%) by modifying the starting temperature from 20 to -6°C and by increasing the cooling rate from -0.3 to -0.5°C/min during the temperature decrease from -6 to -32°C. Although more studies are needed, Menezo's two-step freezing and two-step thawing protocol currently offers the best choice for human blastocysts.

We conclude that human blastocysts can be successfully cryopreserved without a dramatic loss of viability with slow-freezing protocols and can yield an acceptable pregnancy rate and subsequent live birth rate.

REFERENCES

1. Gardner DK, Vella P, Lane M, Wagley L, Schlenker T, Schoolcraft WB: Culture and transfer of human blastocysts increases implantation rates and reduces the need for multiple embryo transfers. *Fertil Steril* 1998;69:84-88
2. Gardner DK, Lane M: Culture of viable human blastocysts in defined sequential serum-free media. *Hum Reprod* 1998;13 (Suppl 3):148-159 (Discussion 160)
3. Menezo Y, Nicollet B, Herbaut N, Andre D: Freezing cocultured human blastocysts. *Fertil Steril* 1992;58:977-980
4. Menezo Y, Veiga A: Cryopreservation of blastocysts. In *Proceedings of the 10th World Congress on In Vitro Fertilization and Assisted Reproduction*, Vancouver, Canada, 1997, pp. 49-53
5. Behr B, Gebhardt J, Lyon J, Milki AA: Factors relating to a successful cryopreserved blastocyst transfer program. *Fertil Steril* 2002;77:697-699
6. Kaufmann RA, Menezo Y, Hazout A, Nicollet B, DuMont M, Servy EJ: Cocultured blastocyst cryopreservation: experience of more than 500 transfer cycles. *Fertil Steril* 1995;64:1125-1129
7. Nakayama T, Goto Y, Kanzaki H, Takabatake K, Himeno T, Takakura K, Mori T, Noda Y: Developmental potential of frozen-thawed human blastocysts. *J Assist Reprod Genet* 1995;12:239-243

8. Pantos K, Stefanidis K, Pappas K, Kokkinopoulos P, Petroutsou K, Kokkali G, Stavrou D, Tzigounis V: Cryopreservation of embryos, blastocysts, and pregnancy rates of blastocysts derived from frozen-thawed embryos and frozen-thawed blastocysts. *J Assist Reprod Genet* 2001;18:579–582
9. Alper MM, Brinsden P, Fischer R, Wikland M: To blastocyst or not to blastocyst? That is the question. *Hum Reprod* 2001;16:617–619
10. Virant-Klun I, Tomazevic T, Bacer-Kermavner L, Mivsek J, Valentincic-Gruden B, Meden-Vrtovec H: Successful freezing and thawing of blastocysts cultured in sequential media using a modified method. *Fertil Steril* 2003;79:1428–1433
11. 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;79:407–410