Re-cryopreservation by Vitrification of Human Blastocysts Developed from Frozen-Cleaved Embryos: A Report of 15 Cycles[a]

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Abstract: *Human blastocysts have been successfully cryopreserved and many live births occur each year using blastocysts that have been cryopreserved once. However, there is a paucity of information on re-cryopreservation of human blastocysts developed from frozen embryos. Therefore, we document the data for 27 blastocysts from 15 cycles (12 patients) of warming to review the efficacy of re-cryopreservation by vitrification of human blastocysts developed from frozen-cleaved embryos. A total of 27 surplus human blastocysts developed from frozen-cleaved embryos, obtained from 12 healthy infertile women undergoing IVF treatment between February 2004 and December 2006, were re-cryopreserved by vitrification. Of these, 26 (96%) re-expanded after warming and were transferred into 12 patients (15 cycles). Following transfer, the implantation rate was 35% (9/26) and the pregnancy rate was 47% (7/15). Two pregnancies ended in miscarriage, 5 healthy babies were born in 4 deliveries, and 1 pregnancy is ongoing. Our results suggest that re-cryopreservation by vitrification of human blastocysts developed from frozen-cleaved embryos can be performed without impairing their implantation ability after warming.*

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Key words: *Blastocyst, Frozen embryo, Human, Re-cryopreservation, Vitrification*

Introduction

With the introduction of the sequential culture system, blastocyst culture is being adopted by many IVF clinics as a means of increasing pregnancy rates, while minimizing multiple gestations [1–3]. Therefore, a reliable procedure for cryopreservation of supernumerary blastocysts is needed. Human blastocysts have been successfully cryopreserved using either slow-freezing methods [4, 5] or vitrification [6–27]. Many live births occur each year using blastocysts that have been cryopreserved once. However, there have been at least 7 reports of live births of healthy babies after the transfer of human blastocysts which had been re-cryopreserved after cryopreservation and thawing at the pronuclear [28, 29], cleaved [30–32] and blastocyst [33, 34] stages. All the reports are of single cases. Therefore, there is a paucity of information on re-cryopreservation of human blastocysts. To date, we have re-cryopreserved 12 cohorts of blastocysts developed from frozen-cleaved embryos, all of which had been warmed. In the present report, we document the data for 27 re-cryopreserved blastocysts from 15 cycles of warming (12 patients) and review the efficacy of re-cryopreservation by vitrification of human day 5 and day 6 blastocysts developed from frozen-cleaved embryos.

Case Report

Twelve couples enrolled in our re-cryopreserved blastocyst transfer program. They had different types of infertility and had had previous multiple failures of conventional day 2 or day 3 embryo transfers. Informed consent was obtained from the all couples prior to the use of re-cryopreserved blastocysts. The mean age of the women at the transfer of re-cryopreserved blastocyst was 29.6 ± 3.1 (range, 25–35) years.

Women were treated with GnRH agonist and hMG in either a long or a short treatment protocol. They were administered hCG when dominant follicles reached a diameter of 18 mm. Oocytes were collected 35 h after hCG administration using a vaginal ultrasound-guided procedure. The oocytes were inseminated using conventional IVF or intracytoplasmic sperm injection, and incubated in HTF medium (Irvine Scientific, CA) containing 10% (v/v) serum substitute supplement (SSS; Irvine). The day of oocyte retrieval was considered as day 0. Fertilized oocytes were cultured in P-1™ Medium (Irvine) containing 10% (v/v) SSS (Irvine) until day 3. After the transfer of fresh cleaved embryos, surplus day 2 or day 3 embryos were cryopreserved by the slow-freezing method as described by Chi [35], with slight modifications. Briefly, cryopreservation of the embryos was performed using Dulbecco's phosphate-buffered saline supplemented with 20% SSS (Irvine), 0.1 mol/l sucrose and 1.4 mol/l ethylene glycol.

For all the couples, after failure of frozen-cleaved embryo transfer, the remaining frozen day 2 or day 3 embryos were thawed and cultured for better embryo selection before embryo transfer. Following thawing, thawed day 2 (the day of thawing was considered as day 2) embryos were placed in Blast Assist Medium 1 (Medicult, Denmark), in Blast Assist Medium 2 (Medicult) on day 3 and cultured until day 6; thawed day 3 (the day of thawing was considered as day 3) embryos were placed in Blast Assist Medium 2 (Medicult) and cultured until day 6. In all cases one to two blastocysts developed from the frozen-cleaved embryos were transferred on day 5 or day 6 in natural cycles or hormone replacement cycles [36]. After blastocyst transfer, the surplus embryos that had developed to the expanded blastocyst stage were re-cryopreserved on day 5 or day 6. Re-cryopreservation of the expanded blastocysts was performed by the method developed by Kuwayama [8] using a cryotop (Kitazato Supply Co., Fujinomiya, Japan), with slight modifications as described previously [12]. The cryotop consists of a 0.4 mm wide × 20 mm long × 0.1 mm thick polyethylene strip attached to a plastic handle and equipped with a cover straw. One blastocyst was vitrified in each cryotop. As the base medium, Modified HTF Medium-HEPES (Irvine) plus 20% (v/v) SSS (Irvine) was used. The equilibration solution contained 7.5% (v/v) ethylene glycol (EG) (Sigma Chemical Co., MO) and 7.5% (v/v) dimethyl sulfoxide (DMSO) (Kanto Chemical Co., Tokyo, Japan). The vitrification solution was composed of 15% (v/v) EG, 15% (v/v) DMSO and 0.5 mol/l of sucrose (Nacalai Tesque, Inc., Kyoto, Japan). Before starting the vitrification procedure, artificial shrinkage of expanded blastocysts was performed in the equilibration solution. First, pipetting of the expanded blastocyst was started immediately after placing the embryo in 1 ml of 30°C equilibration solution with a glass pipette slightly smaller in diameter (~140 μm) than the expanded blastocyst. After confirmation of slight shrinkage of the blastocoele, pipetting was performed with a pipette slightly smaller in diameter than the first one (~100–120 μm). This procedure was repeated 2–3 times until the blastocoele had collapsed completely. After blastocoele contraction, the blastocysts were equilibrated in the same equilibration solution for another 2 min before exposure to the vitrification solution. The blastocysts were then incubated in 1 ml of 30°C vitrification solution and loaded, within 45 s, onto the tip of the cryotop with ~1 μl of cryoprotectant solution. Then the cryotop was immediately plunged into liquid nitrogen which had been filter sterilized through a 0.22 μm filter (MILLIPORE, Cork, Ireland) [37]. Under the liquid nitrogen the plastic cover was placed over the strip to provide protection during storage.

The warming procedure was done as follows. The protective cover was removed in the liquid nitrogen and the end of the polypropylene strip was immersed directly into 1 ml of 37°C 1.0 mol/l sucrose solution for 1 min. The blastocysts were then transferred into 1 ml of 37°C 0.5 mol/l sucrose solution for 3 min and washed twice in the base medium for 5 min. After warming, assisted hatching was performed for all warmed blastocysts as described previously [13]. Briefly, as soon as warming of the blastocysts had been completed, a 35- to 40-μm hole was created in the zona pellucida using acid Tyrode's solution while the warmed blastocyst remained collapsed. Then, the warmed blastocyst was cultured in Blastocyst medium (Irvine) containing 10% SSS (v/v) (Irvine) until transfer. The post-warming survival of the blastocysts was observed 3 h after warming under a microscope, and a re-expanded blastocyst was judged to have survived.

Table 1. Results of human blastocysts re-cryopreserved by vitrification on day 5 or day 6

Parameter	Age of blastocyst		
	Day 5	Day 6	Total
Average age of patients (years)	29.7 ± 3.0	29.5 ± 3.6	29.6 ± 3.1
No. of cycles for vitrification	7	5	12
No. of cycles for warming	9	6	15
No. of blastocysts vitrified	16	11	27
No. of blastocysts re-expanded after warming (%)	15 (94)	11 (100)	26 (96)
No. of embryos transferred	15	11	26
Average no. of blastocysts transferred	1.7 ± 0.5	1.8 ± 0.8	1.7 ± 0.6
No. of embryos implanted (%)	4 (27)	5 (45)	9 (35)
No. of transfer cycles	9	6	15
No. of clinical pregnancies (%)	4 (44)	3 (50)	7 (47)
No. of miscarriages	2	0	2
No. of deliveries	2	2	4
No. of ongoing pregnancies	0	1	1
No. of babies (male:female ratio)	2 (0:2)	3 (2:1)	5 (2:3)

Embryo transfer was performed in the natural cycle or hormone replacement cycle [36]. One to three surviving blastocysts were transferred into the patient's uterus. Pregnancy was first assessed by urinary hCG 9 days after blastocyst transfer; then clinical pregnancy was confirmed by presence of fetal heart activity 30 days after blastocyst transfer.

In order to assess if the cryopreservation process induced hardening of the zona pellucida, the time for zona pellucida lysis was recorded after enzymatic treatment of fresh (n=10), vitrified (n=10) or re-cryopreserved blastocysts (n=10), which had been donated by consenting patients. Re-cryopreserved blastocysts were embryos re-cryopreserved by the vitrification method at the blastocyst stage which had previously been cryopreserved at the cleavage stage by the slow-freezing method. The zona pellucida was enzymatically removed according to the method of Fong *et al.* [38]. The blastocysts were transferred into 1 ml of 37°C 10 IU/ml pronase solution (Sigma, St. Louis, MO) in Sperm Washing Medium (Irvine). After transferring the blastocysts into the pronase solution, the embryos were not moved until the zonae had been completely dissolved. The time for complete dissolution of the zonae was recorded at ×400 magnification under an inverted Hoffman modulation contrast microscope with a 37°C stage warmer. The results revealed a significant difference ($P<0.05$) between fresh (90±9.8 s; mean ± SD), vitrified (120 ± 10.2 s) and re-cryopreserved (135 ± 9.4 s) blastocysts.

In all, 113 frozen-cleaved embryos were thawed and cultured for blastocyst transfer. Of these, 45 (40%) grew to expanded blastocysts on day 5 and 61 (54%) on day 6 (days 5 and 6). After blastocyst transfer, 27 surplus blastocysts from 12 patients were re-cryopreserved by vitrification on day 5 or day 6 and transferred in 15 warming cycles between February 2004 and December 2006. The results of the re-cryopreserved human blastocysts are summarized in Table 1. Twenty-six blastocysts (96%) re-expanded after warming. A total of 26 blastocysts were transferred to 12 patients in 15 cycles. The implantation rate was 35% (9/26) and the pregnancy rate was 47% (7/15) (two sets of twins and five singletons). Two male and three female infants (one set of twins and three singletons) from four patients have been born (Table 2). Two pregnancies (two singletons) ended in spontaneous abortion at 7 and 9 weeks of gestation, and another pregnancy (one set of twins) is ongoing at 26 weeks of gestation. To date, all delivered infants have had normal physical profiles.

The effect of the day of vitrification is shown in Table 1. Of 27 vitrified blastocysts, 16 (59%) were re-cryopreserved on day 5, and 11 (41%) on day 6. The re-expansion and pregnancy rates of day 5 blastocysts (94%, 15/16; 44%, 4/9) were similar to those of day 6 blastocysts (100%, 11/11; 50%, 3/6). The implantation rate with day 6 blastocysts (45%, 5/11) was higher than that with day 5 blastocysts (27%, 4/15), but the difference is not statistically significant.

Discussion

We previously reported a single case of successful pregnancy following the transfer of re-cryopreserved human day 6 blastocysts [32]. This current case series

Table 2. Birth outcome for pregnancies following re-cryopreservation by vitrification of human blastocysts developed from frozen-cleaved embryos on day 5 or day 6

Patient age (age)	ET details	Gestation length (days)	Birth weight (g)	Apgar score 1 min	5 min	Sex
31	2 × day 6	255	2155	9	10	M
			2590	9	10	F
29	2 × day 5	264	2400	9	10	F
29	2 × day 5	290	3112	9	10	F
27	2 × day 6	266	3006	9	10	M

ET details: e.g. 2 × day 6 = transfer of two day 6 blastocysts. M = male; F = female.

report includes the pregnancy previously reported and demonstrates high implantation and pregnancy outcomes following re-cryopreservation of human day 5 and day 6 blastocyst in 15 transfers.

The re-expansion, implantation and pregnancy rates of re-cryopreserved human blastocysts in the present report (96%, 26/27; 35%, 9/26; 47%, 7/15) were similar to those of human blastocysts vitrified and warmed once (98%, 48/49; 33%, 16/48; 50%, 14/28) in our previous report [12]. These results indicate that re-cryopreserved human blastocysts developed from frozen-cleaved embryos have similar implantation potential to blastocysts cryopreserved once after warming. Therefore, re-cryopreservation of blastocysts assists the selection of the highest quality blastocysts from frozen-cleaved embryos and eliminates concerns about the disposal of the rest of the good quality embryos. However, this report is limited to 15 cycles of warming and so our information and experience of re-cryopreservation of blastocysts are still incomplete. Further data is needed on re-cryopreservation of blastocysts to determine the risk of spontaneous abortions, the live birth rate and birth defects. Until these data are collected and the safety of this procedure is confirmed, re-cryopreservation of embryos should be approached with caution and couples must be informed of the benefits and the risks of its procedure regarding birth defects.

Assisted hatching was performed for all re-cryopreserved blastocyst because, as was reported by Vanderzwalmen *et al*. [26], we previously demonstrated that the rates of pregnancy and implantation of vitrified human blastocyst significantly improved after assisted hatching compared with the control, zona intact blastocyst, group [13]. In addition, the resistance to enzymatic removal of zona pellucida by pronase significantly increased after vitrification as compared with fresh blastocysts, and also significantly increased after re-cryopreservation (slow-freezing and vitrification) as compared with vitrified blastocysts. These observations suggest that hatching deficiencies can result from hardening of the zona pellucida, which is due to the cryopreservation process of slow-freezing and vitrification. Accordingly, for the re-cryopreserved blastocysts described in the present report, hardening of the zona pellucida might have occurred by two cycles of cryopreservation (slow-freezing and vitrification). Consequently, we suggest that assisted hatching might be a critical factor for helping twice-cryopreserved embryos to divest themselves of their zona pellucida, thus improving their potential for implantation.

In conclusion, re-cryopreservation by vitrification of human blastocysts developed from frozen-cleaved embryos can be performed without impairing their implantation ability after warming. This could contribute to increasing cumulative pregnancy rates in human assisted reproductive technology.

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