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An improved protocol for dilution of cryoprotectants from vitrified human blastocysts*

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BACKGROUND: The purposes of this study were to compare the survival rate of human blastocysts thawed by two different methods after vitrification using electron microscopic (EM) grids, and to report the successful pregnancies and live births resulting from the transfer of blastocysts that had survived the established thawing method. METHODS: The blastocysts produced from three pronuclei zygotes in IVF cycles were vitrified on an EM grid. After dilution of cryoprotectant by either a 6- or 2-step method, the survival rate of the blastocysts was compared. RESULTS: The survival rate of blastocysts thawed using the 6-step method (82.6%, 100/121) was significantly higher than that of the 2-step method (50.6%, 87/172; $P < 0.01$). Therefore, we applied the 6-step method in our blastocyst cryopreservation programme. Overall, 34.1% (14/41) of clinical pregnancies and 11 live births were achieved. CONCLUSIONS: The present results indicated that a 6-step thawing method was more effective than a 2-step thawing method for thawing vitrified human blastocysts.

Key words: EM grid/human blastocyst/live birth/survival rate/vitrification

Introduction

Cryopreservation of human embryos has become a routine procedure to increase cumulative pregnancy rates, to help avoid the risk of multiple pregnancies after the transfer of many embryos and to avoid unnecessary additional stimulation procedures.

Recently, blastocyst transfer based on an improved culture system has been proven effective for increasing the pregnancy rate in assisted reproductive technology (Gardner *et al.*, 1998; Yoon *et al.*, 2001). Therefore, a reliable procedure for the cryopreservation of supernumerary blastocysts is needed because only a small number of blastocysts after transfer are likely to be available for cryopreservation. Freezing of human blastocysts has been carried out with the slow cooling method, but clinically satisfactory results have not been obtained (Ménézo *et al.*, 1992; Kaufman *et al.*, 1995). Therefore, it is essential to establish a simple, fast and reliable procedure to optimize clinical outcomes of cryopreservation at the blastocyst stage. Several investigators have reported the clinical usefulness of vitrification for the cryopreservation of human embryos (Mukaida *et al.*, 1998; Yokota *et al.*, 2000; Vanderzwalmen *et al.*, 2002).

Martino and colleagues observed a higher percentage of blastocyst formation from oocytes surviving vitrification on electron microscopic (EM) grids to obtain more rapid cooling and warming rates (Martino *et al.*, 1996). Since then, several researchers have applied EM grids successfully in the vitrification of human oocytes and blastocysts (Choi *et al.*, 2000; Yoon *et al.*, 2000). In addition, we reported previously that a higher survival rate of bovine blastocysts derived from in-vitro maturation (IVM) could be obtained by a simple 2-step vitrification method using EM grids and freezing solution (Park *et al.*, 1999). Several successful cases of human blastocyst vitrification have been reported (Choi *et al.*, 2000; Yokota *et al.*, 2000; Mukaida *et al.*, 2001). EM grids (Choi *et al.*, 2000) or cryo-loop (Mukaida *et al.*, 2001) have been used to substantially increase the cooling rate. In this study, we compared a new thawing protocol with a protocol used for bovine blastocysts (Park *et al.*, 1999) on the survival of vitrified human blastocysts. In addition, we attempted clinical application of the new thawing protocol of vitrified blastocysts.

Materials and methods

Approval for the study was obtained from the Institutional Review Board of the Maria Infertility Hospital.

IVF of oocytes

Women were treated with GnRH agonist and hMG in either a long or short treatment protocol. When more than two follicles reached 18 mm in diameter, 10 000 IU hCG (IVF-C; LG Chemical, Korea) was administered. Oocytes were retrieved transvaginally 36–38 h after hCG injection and the oocytes were inseminated by either conventional IVF or ICSI. Fertilization was examined 17–19 h after insemination for the presence and number of pronuclei (PN).

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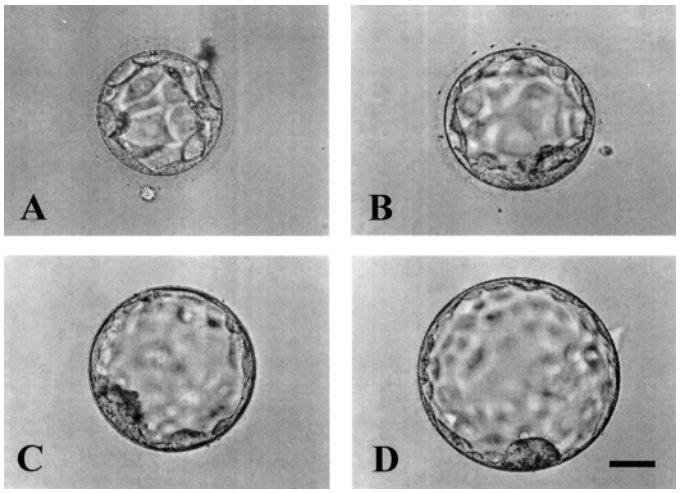

Figure 1. Developmental stage of human blastocysts cultured *in vitro* for 5 or 6 days from 2PN zygotes. (**A**) Early blastocyst (ErB), (**B**) early expanding blastocyst (EEB), (**C**) middle expanding blastocyst (MEB) and (**D**) expanded blastocyst (EdB). Scale bar = 50 μm.

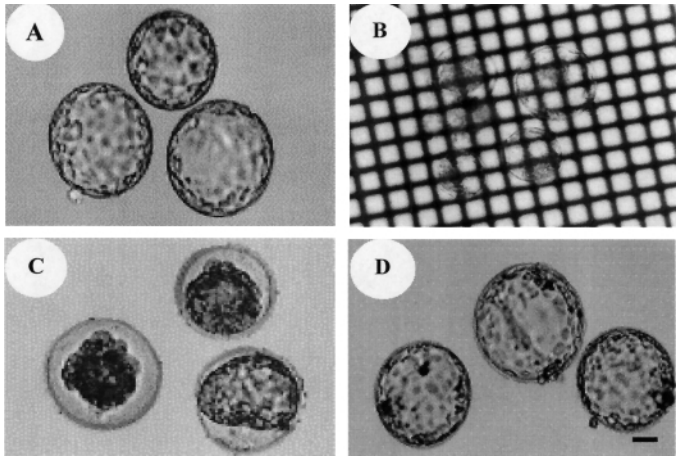

Figure 2. A series course of freezing and warming of human in-vitro cultured blastocysts on electron microscope (EM) grids. (**A**) Blastocysts before freezing, (**B**) loaded onto EM grids, (**C**) just after 6-step cryoprotectant dilution and (**D**) 18 h after 6-step cryoprotectant dilution. Scale bar = 50 μm.

Embryo culture

The procedure used to culture fertilized oocytes was the same as described in a previous study (Yoon *et al.*, 2001). All zygotes were co-cultured with cumulus cells in a 10 μl YS (Yoon Sanhyun) medium supplemented with 10% hFF for 5 or 6 days (Yoon *et al.*, 2001). The hFF was prepared using a previously reported method (Chi *et al.*, 1998). The cumulus cells for co-culture were prepared using the method reported by Yoon *et al.* (Yoon *et al.*, 2001). The developed blastocysts were classified according to their degree of expansion as follows (Figure 1): Early blastocyst (ErB), the blastocoele is less than half the volume of the embryo and diameter <140 μm; early expanding blastocyst (EEB), the blastocoele is greater than, or equal to, half of the volume of the embryo and diameter 140–160 μm; middle expanding blastocyst (MEB), the blastocoele completely fills the embryo and diameter 160–180 μm; expanded blastocyst (EdB), the blastocoele volume is larger than that of the early embryo with thinning zona and diameter >180 μm.

Vitrification of blastocysts using an EM grid

The freezing solution for vitrification, EFS40, was prepared according to a previously described method (Kasai *et al.*, 1990), and consisted of 40% (v/v) ethylene glycol (EG; Sigma Chemical Co., St Louis, USA), 18% (w/v) Ficoll (Ficoll 70, average MW 70 000 Da; Pharmacia Biotech, Uppsala, Sweden), 0.3 mol/l sucrose and 20% hFF in modified Dulbecco's phosphate-buffered saline (m-DPBS). A pretreatment solution, m-DPBS (EG20) containing 20% EG and 10% hFF, was prepared. The blastocysts were classified according to developmental stage and vitrified on an EM grid. The blastocysts were equilibrated in EG20 for 1.5 min before exposure to the vitrification solution at room temperature. The blastocysts were then incubated in EFS40 at room temperature, loaded onto the EM grid (IGC 400; Pelco International, CA, USA), had excess cryoprotectant removed using sterilized filter paper, and were directly plunged in LN_2 within 30 s. The EM grids containing the blastocysts were sealed in a cryovial that had previously been submerged under LN_2. The cryovials were attached in canes and stored in LN_2.

Thawing of blastocysts derived from 3PN zygotes

For thawing of vitrified blastocysts, EM grids containing blastocysts at the same developmental stage were divided randomly into two groups. For one group a 2-step cryoprotectant dilution method was used in which the EM grids stored in LN_2 were directly transferred into a 100 μl drop of 0.3 mol/l sucrose solution (prepared in DPBS containing 20% hFF) as soon as possible, and then quickly transferred into fresh 0.3 mol/l sucrose and incubated for 1.5 min at room temperature. Recovered blastocysts were transferred into DPBS containing 20% hFF at room temperature. After 1.5 min, the embryos were washed three times in culture medium and co-cultured with cumulus cells in 10 μl YS medium containing 10% hFF.

For the other group a 6-step cryoprotectant dilution method was used in which the EM grids containing blastocysts were transferred to a 100 μl drop of 0.5 mol/l sucrose. After 3 min, the blastocysts were transferred sequentially to 100 μl drops containing 10% hFF in DPBS supplemented with 0.4, 0.3, 0.2, 0.1 and 0 mol/l sucrose at intervals of 1.5 min at room temperature. The blastocysts were then washed three times in culture medium and co-cultured with cumulus cells in 10 μl YS medium containing 10% hFF. The post-thawing survival of blastocysts was observed ~18–20 h after warming under a microscope, and blastocysts with a morphologically intact inner cell mass, trophectoderm and re-expanding blastocoele were judged to have survived.

Thawing of blastocysts derived from 2PN zygotes (clinical use)

A total of 806 blastocysts from 258 patients were cryopreserved by vitrification. The patients consented to have their supernumerary embryos vitrified after fresh embryo transfer. The 6-step cryoprotectant dilution method and blastocyst transfer after thawing was conducted in 41 patients between September 1999 and July 2000. The blastocysts had been stored for 3–12 months in LN_2. The age and duration of infertility of the 41 patients (mean ± SD) were 32.6 ± 3.7 and 3.1 ± 2.7 years respectively. The causes of infertility were male factor in 11, tubal factor in 19, unexplained infertility in two, anovulation in three and mixed causes in six patients. Vitrified day 5 and 6 blastocysts were thawed in the afternoon of the day before embryo transfer. Embryo transfer was scheduled on days 4–5 after ovulation in the spontaneous cycles of 38 patients, or on days 19–20 in artificial cycles prepared with exogenous estrogen and progesterone for three anovulatory patients. One to three surviving blastocysts were transferred into the patient's uterus. Pregnancy was first assessed by serum β-hCG 9 days after blastocyst transfer, and

Table I. The survival rate of blastocysts after thawing with the two dilution methods at each blastocyst stage vitrified

Thawing method	Type of blastocyst				Total
	ErB	EEB	MEB	EdB	
2-step					
No. of blastocyst thawed	42	51	42	37	172
No. of blastocysts survived (%)	22 (52.4)	32 (62.7)	18 (42.9)	15 (40.5)	87 (50.6)[a]
6-step					
No. of blastocyst thawed	37	24	33	27	121
No. of blastocysts survived (%)	32 (86.5)	22 (91.7)	25 (75.8)	21 (77.8)	100 (82.6)[a]

[a]$P < 0.01$.
ErB = early blastocyst; EEB = early expanding blastocyst; MEB = middle expanding blastocyst; EdB = expanded blastocyst.

Table II. Clinical results for patients receiving blastocysts survived after 6-step cryoprotectant dilution in vitrification using EM grids

Variable	Value
No. of patients receiving blastocysts	41
No. of blastocysts thawed	120
No. of surviving blastocysts (%)	101 (84.2)
No. of blastocysts transferred (%)	92 (76.7)
Mean no. of blastocysts per transfer	2.2 (92/41)
No. of blastocysts implanted (%)	19 (20.7)
No. of clinical pregnancies (%)	14 (34.1)
No. of deliveries (%)	11 (29.3)

then clinical pregnancy was determined by the presence of fetal heart activity 30 days after blastocyst transfer.

Statistical analysis

Differences between treatments groups in each experiment were compared with the χ^2-test using the Statistical Analysis System (SAS Institute, Cary, NC, USA) software package.

Results

In the preliminary experiment, 293 out of 930 3PN zygotes developed to blastocyst stage (31.5%) and were cryopreserved by vitrification. Blastocysts were thawed with either the 2- or 6-step cryoprotectant dilution method. Figure 2 shows the morphology of human blastocysts before and after cryopreservation. Table I shows the survival rate of blastocysts after thawing. As shown in Table I, the survival rate of blastocysts following the 6-step dilution method was significantly higher than that of the 2-step dilution method ($P < 0.01$). There was a tendency towards higher survival in the early blastocyst stages (ErB, EEB) than in the late (MEB, EdB) in both thawing methods, but the difference was not significant.

Based on the above result, we applied the 6-step thawing method clinically. Table II shows the clinical results. A total of 41 patients and 120 blastocysts were included in this study. There were 90 day 5 and 30 day 6 frozen blastocysts. There was no difference in survival rates between day 5 (84.4%, 76/90) and day 6 (83.3%, 25/30) frozen blastocysts. After thawing of day 5 frozen blastocysts, 90.9% of ErB (20/22), 91.9% of EEB (34/37), 72.2% of MEB (13/18) and 69.2% of EdB (9/13) became re-expanded. After thawing of day 6 frozen blastocysts, 100% of ErB (6/6), 90% of EEB (9/10), 66.7% of MEB (4/6), and 75% of EdB (6/8) became re-expanded. Out of 101 survived blastocysts, 15 (14.6%) hatched at the time of embryo transfer. Before transfer, all embryos for each patient were pooled and selected for transfer. All 41 patients underwent embryo transfer. After transfer, 14 clinical pregnancies (34.1%) were achieved. Nine male and six female infants (four sets of twins and seven singletons) from 11 patients were born and three others ended in miscarriage. Birth weights of the infants were within the range of 1850–3750 g, and all delivered infants had normal physical profiles up to the present.

Discussion

In this study, for the first time, we examined the survival rate of vitrified human blastocysts after different cryoprotectant dilution methods. Acceptable pregnancy and delivery were obtained after clinical use of vitrification and 6-step cryoprotectant dilution protocols. This study indicates that vitrification on EM grids and 6-step cryoprotectant dilution can be applied to human blastocyst cryopreservation.

Cryopreserved blastocyst survival depends in part on the freezing–thawing procedure. Previously, Park *et al.* reported that higher survival and hatching rates of vitrified–thawed bovine blastocysts could be obtained using EM grids and a 2-step cryoprotectant dilution (Park *et al.*, 1999). In our hospital, a few investigations into various concentrations and times of cryoprotectant dilution after thawing to improve the efficiency of human blastocysts vitrified on EM grids resulted in disappointing survival rates (data not shown). However, we had obtained a good survival rate by changing the first concentration of sucrose to 0.5 mol/l for 5 min in cryoprotectant dilution after thawing. Thus, we compared a new thawing protocol of 6-step dilution with a 2-step dilution used for bovine blastocysts (Park *et al.*, 1999) on the survival of vitrified human blastocysts derived from 3PN. We obtained a low survival rate (50.6%) of vitrified–thawed human blastocysts using a 2-step cryoprotectant dilution. In contrast, a higher survival rate (82.6%) with a 6-step thawing method was

obtained. When we observed microscopically the blastocysts during the thawing process of a 2-step cryoprotectant dilution, thawed human blastocysts were morphologically normal in first 0.3 mol/l sucrose solution, but degenerated abruptly in second 0 mol/l sucrose solution.

Thus, the low percentage of blastocysts that survived after thawing in a 2-step dilution method might be due to dramatic osmotic shock. So, it could be speculated that the higher survival of human blastocysts in a 6-step cryoprotectant dilution may result from decreasing osmotic shock. Another author (Kobayashi *et al.*, 1998) also demonstrated that stepwise dilution of the cryoprotectant after warming appears to reduce or perhaps eliminate osmotic injury to cells of vitrified porcine embryos.

It was observed that the survival rate of blastocysts at late stage (MEB, EdB) was lower than that of blastocysts at early stage (ErB, EEB) in both 2- and 6-step dilution methods, although the numbers in this study were too small to tell any possible significant difference. The explanation for this observation could be that late blastocysts consist of a full-filled blastocoele, which may disturb cryopreservative potential due to ice crystal formation caused by inadequate permeation of the cryoprotectants during the cooling step. Actually, we have observed that late human blastocysts are less permeable than earlier stage embryos, suggesting that intracellular ice is more likely to form as has been previously asserted (Vanderzwalmen *et al.*, 2002). Therefore, further study is necessary to avoid mechanical damage, which may be caused by ice crystal formation, and this possibility is under investigation.

We applied the 6-step thawing method clinically in vitrified human blastocysts in our cryopreservation programme. After thawing of the vitrified blastocysts by the 6-step method, 84.2% of thawed blastocysts survived. Several investigators have reported high survival and pregnancy rates by vitrification of human blastocysts (Mukaida *et al.*, 1998; Yokota *et al.*, 2000). Mukaida *et al.* showed 63% survival and a 32% clinical pregnancy rate in human blastocysts vitrified on nylon loops (Mukaida *et al.*, 2001). Yokota *et al.* reported 80% survival and a 33.3% clinical pregnancy rate after vitrification that used a modified vitrification solution and straw (Yokota *et al.*, 2000). These studies are similar to our results. In contrast, Choi and colleagues observed that 51.6% of human blastocysts survived after thawing (Choi *et al*., 2000). It could be implied that even in vitrification performed using the same EM grids as this study, survival of blastocysts after thawing might depend on the concentrations and times they are exposed to cryoprotectant solutions. Another possible explanation could be the different culture conditions used for producing the blastocysts. We obtained a reasonable clinical pregnancy (34.1%) rate following transfer, but this pregnancy outcome was lower than that obtained from the transfer of fresh blastocysts in our hospital (Yoon *et al.*, 2001). Therefore, further studies are necessary to determine the optimal method for vitrification of human blastocysts in order to improve viability after thawing.

A disadvantage of vitrification on EM grids or loops is the risk of contamination for pathogens such as virusus, prions and bacteria caused when the vitrification solution comes into direct contact with LN_2 during cooling or storage (Bielanski *et al.*, 2000). Although there are no cases of contamination occurring via LN_2 in our vitrification system until now, the development of safety strategies for reducing the risk of contamination by larger pathogens is necessary.

In this report, we have shown that a 6-step thawing method was more effective than a 2-step method after vitrification of human blastocysts on EM grids. In addition, we achieved acceptable pregnancy rates clinically by combining vitrification on an EM grid and 6-step thawing. Therefore, vitrification of human embryos at the blastocyst stage will be a reliable approach in the near future.

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