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SHORT COMMUNICATION

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Optimization of a Dilution Method for Human Expanded Blastocysts Vitrified Using EM Grids After Artificial Shrinkage

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Purpose: To verify a more effective dilution method that can be applied to human expanded blastocysts that are vitrified after artificial shrinkage.
Methods: Surplus expanded blastocysts that remained after embryo transfer (ET) in in vitro fertilization (IVF) cycles, were cryopreserved. The blastocysts were vitrified on EM grids following artificial shrinkage. After thawing the blastocysts, cryoprotectants were diluted using either a 6- or 2-step method. We examined the survival rate and clinical outcome of blastocysts of 151 patients in our ET program after thawing.
Results: The survival rate of blastocysts that were thawed using a 2-step method (91.6%, 239/261) was comparable with that of the 6-step method (89%, 186/209). The clinical pregnancy rate (45.9%, 39/85) and implantation rate (24.1%, 53/220) were slightly higher in the 2-step method than in the 6-step method (40.9%, 27/66; 19.4%, 33/170).
Conclusions: Our data indicate that the 2-step dilution method could be a simpler and more effective protocol for human expanded blastocysts that are vitrified using EM-grid following artificial shrinkage.

KEY WORDS: Artificial shrinkage; EM grid; human blastocyst; 2-step method; vitrification.

INTRODUCTION

Cryopreservation of human embryos has generally been carried out using the conventional (slow) freezing method (1,2). Recently, there have been several reports of successful cryopreservation of human embryos by direct immersion into liquid nitrogen (vitrification). This method is now considered to be an attractive alternative to the conventional slow-freezing protocol, and has two major benefits: it takes only minutes to freeze embryos; and, it does not require specialized equipment. After the first pregnancy using human blastocysts that were vitrified with cryostraws was reported (3), attention has focused on this new and simple method. Now, human embryos at the blastocyst stage can be cryopreserved successfully by vitrification using cryotop (4), cryoloop (5–7), electron microscope (EM) grids (8–10), or hemistraw (11). In our hospital, we have established a vitrification system using EM grids and a 6-step dilution method, and have reported the clinical usefulness of this system for the cryopreservation of human blastocysts (9). In this study, we were able to increase the success rate for human blastocyst vitrification by increasing the postthaw removal step of permeable cryoprotectant. The gradual dilution of cryoprotectants was supposed to reduce the osmotic shock to the vitrified human blastocyst during rehydration after thawing. However, we found that there was a relatively poor survival of the expanded blastocysts after vitrification. It was thought that late blastocysts consist of a well-developed blastocoele, which may disturb cryopreservative potential due to ice crystal formation, and that this may be caused by inadequate permeation of the cryoprotectants during the cooling step. Recently, we introduced an artificial shrinkage technique into our vitrification system, which was slightly modified from the method of Vanderzwalmen *et al.* (12), and we have noted a dramatic increase in the survival rate of human expanded blastocysts (10). In this current study, we examined if the stepwise dilution of cryoprotectants after thawing, which we originally adopted to reduce osmotic pressure during the dilution step, was still needed for human blastocysts that were vitrified after artificial shrinkage. We compared the survival of vitrified human blastocysts, as well as the clinical results, between the stepwise dilution and the 2-step dilution method. We believed that this study would help optimize the dilution method appropriate for our vitrification system using artificial shrinkage and an EM grid.

MATERIALS AND METHODS

Approval for this study was obtained from the Institutional Review Board of the Maria Infertility Hospital. Women were treated with gonadotropin releasing hormone (GnRH) agonist and human menopausal gonadotropin (hMG) in either a long or a short treatment protocol. When two or more

follicles reached 18 mm in diameter, a dose of 10 000 IU of human chorionic gonadotrophin (hCG) (IVF-C, LG Chemical, Korea) was administered. Oocytes were retrieved transvaginally 36–38 h after hCG injection, and then inseminated by either conventional IVF or intracytoplasmic sperm injection (ICSI). The results of fertilization were examined 17–19 h after insemination, looking for the presence and number of pronuclei (PN). The procedure used for culturing fertilized oocytes was the same as that described in a previous study (13). Embryos having two pronuclei were cocultured with cumulus cells in a 10 μL YS (Yoon Sanhyun) medium supplemented with 20% human follicular fluid (hFF) (13) in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The embryos were transferred on either day 3 or day 5. The date of the embryo transfer was determined by the number of zygotes and the quality of the embryo on day 2, according to established criteria (13). After transferring the embryos, surplus embryos were further cultured until day 6, regardless of the embryo transfer date, and embryos which had developed to the expanded blastocyst stage (diameter $\geq$160 μm) were vitrified. A total of 1260 blastocysts from 383 patients were vitrified on EM grids following artificial shrinkage during the 13 months from March 2002 to March 2003. Among these, 151 patients who had received blastocysts after thawing were studied.

Artificial shrinkage of the expanded blastocysts was performed using two 29-gauge needles, as described in a previous paper (10). Briefly, after holding the expanded blastocyst with the flat side of a needle and placing the inner cell mass (ICM) at the 12 or 6 o'clock position, a needle was pushed through the trophectoderm into the blastocoele cavity until it shrank. Contraction of the blastocysts was subsequently observed 30 s to 1 min later. Following shrinkage of the blastocoele, the blastocysts were equilibrated in EG20 (20% ethylene glycol and 20% hFF in Dulbecco's phosphate-buffered saline [DPBS]) for 90 s at room temperature before exposure to the vitrification solution. The blastocysts were incubated in EFS40 [40% (v/v) ethylene glycol, 18% (w/v) Ficoll, 0.3 mol/L sucrose, and 20% hFF in DPBS] at room temperature, and then loaded onto the EM grid (IGC 400; Pelco International, CA, USA (14)). Excess vitrification solution was removed using sterilized filter paper, and the blastocysts were plunged directly into liquid nitrogen within 30 s. The EM grid containing the blastocysts was sealed in a cryovial, which was attached to cane and stored in liquid nitrogen. The vitrified blastocysts were warmed using either the 6-step (9) or the 2-step dilution method. For the first 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 (in DPBS containing 20% hFF). After 3 min, the blastocysts were transferred sequentially to 100 μL drops containing 20% hFF in DPBS supplemented with 0.4, 0.3, 0.2, 0.1, or 0 mol/L sucrose at room temperature at intervals of 90 s. The blastocysts were then washed three times in culture medium and cocultured with cumulus cells in 10 μL YS medium containing 20% hFF. For the second group, a 2-step cryoprotectant dilution method was used, in which the EM grids stored in LN_2 were directly transferred into 500 μL of 0.5 mol/L sucrose (in DPBS containing 20% hFF) as soon as possible, and then quickly transferred into fresh 0.5 mol/L sucrose and incubated for 5 min at room temperature. Recovered blastocysts were transferred into DPBS containing 20% hFF at room temperature. After another 5 min, the embryos were washed three times in culture medium and cocultured with cumulus cells in 10 μL YS medium containing 20% hFF. The postthawing survival of blastocysts was observed under the microscope 18–20 h after warming, and blastocysts with a morphologically intact inner cell mass, trophectoderm, and re-expanding blastocoele were judged to have survived. Embryo transfer was conducted on day 4–5 after ovulation in a spontaneous cycle. One to three surviving blastocysts were transferred into the patient's uterus. Pregnancy was first assessed by serum β-hCG on the 9th day after blastocyst transfer, and clinical pregnancy was determined by the presence of fetal heart activity on the 30th day following blastocyst transfer. Differences between the treatment groups in each experimental condition were compared by the χ^2-test, using the Statistical Analysis System (SAS Institute, Cary, NC, USA) software package.

RESULTS

In our previous study, we reported that the survival of human blastocysts that were vitrified on EM grid was improved by changing the concentration of the first sucrose solution to 0.5 mol/L in the first cryoprotectant dilution after thawing (9). In addition, in

our preliminary experiments, we tried to determine the optimal exposure time of cryoprotectant dilution in 0.5 mol/L sucrose, after thawing of poor quality expanded blastocysts, which had been donated by consenting patients and then vitrified. As a result, we observed the highest survival rate when blastocysts were treated with 0.5 mol/L sucrose for 5 min (86.7%, 26/30), a significantly higher survival than the groups that were treated for 3 min (60.9%, 14/23), 4 min (79.2%, 19/24), or 6 min (76.0%, 19/25) ($P < 0.01$). In this study, we compared the new 2-step dilution method, in which blastocysts were treated with 0.5 mol/L sucrose for 5 min, with the 6-step dilution method. We found no difference in survival rates of blastocysts between the 2-step method (85.6%, 77/90) and the 6-step method (87.4%, 76/87).

Based on these results, we applied this 2-step dilution method to clinical treatment. For 13 months, from March 2002 to March 2003, we randomly diluted blastocysts with either the 6-step or 2-step cryoprotectant dilution method. Table I shows the clinical results of human blastocyst vitrification after artificial shrinkage, followed by dilution with either the 6-step or 2-step dilution method. In the 6-step method group, 209 blastocysts were obtained from 66 patients, and then vitrified and warmed. One hundred eighty-six blastocysts (89%) were re-expanded after warming, among which 142 blastocysts had hatched (76.3%) at the time of transfer. A total of 170 blastocysts were transferred into 66 patients. The implantation rate was 19.4% (33/170) and the pregnancy rate was 40.9% (27/66). In the 2-step method group, 261 blastocysts from 85 patients were vitrified and warmed. The survival rate of these blastocysts was 91.6% (239/261), and the hatching rate was 87% (208/239). A total of 220 blastocysts were transferred into 85 patients. The implantation rate was 24.1% (53/220) and the pregnancy rate was 45.9% (39/85). There was a slight increase in the survival, hatching, and implantation rates, as well as the clinical pregnancy rate, in the 2-step group compared to the 6-step group, but the differences were not statistically significant.

Table I. Clinical Results of Vitrified Human Blastocysts that Were Diluted Following Either a 6-Step or a 2-Step Dilution Method

	6-step	2-step
No. of cycles	66	85
No. of blastocysts thawed	209	261
No. (%) of blastocysts surviving	186 (89.0)	239 (91.6)
No. (%) of blastocysts hatched at ET	142 (76.3)	208 (87)
No. (mean) of blastocysts transferred	170 (2.6)	220 (2.6)
No. (%) of clinical pregnancies	27 (40.9)	39 (45.9)
No. (%) of blastocysts implanted	33 (19.4)	53 (24.1)

Note. After ET, surplus expanded blastocysts were vitrified on an EM-grid following artificial shrinkage. Vitrified blastocysts were warmed using either a 6-step or a 2-step dilution method with sucrose for cryoprotectant removal. Data reflect the postthaw survival, hatching, implantation, and clinical pregnancy rates in patients who participated in our thawing-ET program.

During the period of this study, 27 infants (six sets of twins and 15 singletons) and 40 infants (eight sets of twins and 24 singletons) were born from the 6-step method group and the 2-step method group, respectively, and all delivered infants had a normal physical profile. Birthweights of the infants were within the range of 1950–3550 g. Until now, we have vitrified and warmed 2158 blastocysts from 738 cycles using the 2-step dilution method, and among those, 1967 blastocysts (91.1%) survived and 1716 (87.2%) hatched at the time of transfer. A total of 1918 blastocysts survived and were then transferred into 731 patients (seven cases of no ET). The implantation rate was 25.2% (484/1918) and the pregnancy rate was 49.7% (363/731).

DISCUSSION

In this study, we have examined a more effective method for postthaw cryoprotectant dilution to adopt human blastocysts that were vitrified after artificial shrinkage. Using 2-step cryoprotectant dilution method, we obtained good survival rates for the embryos and acceptable clinical outcomes in patients. This study indicates that the 2-step dilution method is appropriate for diluting human blastocysts that were vitrified on an EM grid after artificial shrinkage. In addition, the 2-step method is simple and effective. After being thawed, the vitrified human embryos must be separated from the permeable cryoprotectants, which were used in the cooling process. This is usually achieved by immersing embryos in a graded series of sucrose solutions until isotonic conditions are met. This gradual replacement of cryoprotectants with sucrose solution serves to re-hydrate the thawed embryos, and helps to reduce osmotic pressure during rehydration (15,16). Generally, 3- or 4-step dilution procedures have been used in this process (4,6,11,12,17). We previously improved the success rate of human blastocyst vitrification by increasing the number of cryoprotectant dilution

steps after thawing by using a 6-step dilution method (9).

Recently, we vitrified expanded blastocysts after artificially reducing the fluid content of the blastocoele, and as a result the survival rate was dramatically increased (10). The expanded blastocyst has a fluid-filled blastocoele, which is known to retard the permeation of cryoprotectants and may be followed by intrablastocoelic ice formation during freezing, resulting in damaged embryos. After this barrier is removed by such an artificial shrinkage technique, the small volume of blastomere, which forms the expanded blastocyst, can equilibrate more rapidly during the vitrification procedure and thus be less sensitive to osmotic stress. As a result, less osmotic injury occurs during the postthaw cryoprotectant removal (12). Given this improvement, we questioned the necessity of a 6-step dilution method, which was originally adopted to reduce the osmotic stress during rehydration before applying an artificial shrinkage technique. Based on the high survival rate (91%) and hatching rate (87%) in the 2-step dilution method, we conclude that direct rehydration (the 2-step dilution) method is effective and useful, just as is the stepwise (6-step dilution) method in human blastocyst vitrification after artificial shrinkage. Furthermore, we achieved high implantation and clinical pregnancy rates (24 and 45%) using this 2-step dilution method. These results suggest that, in the case of blastocysts vitrified after artificial shrinkage, we have greatly reduced the osmotic stress that occurs during removal of cryoprotectants. If a blastocyst is vitrified with a well-developed blastocoele, the embryo might be damaged by intrablastocoelic ice crystal formation as well as by extreme swelling due to sudden water influx during the postthaw removal of the cryoprotectants. When the blastocyst is vitrified after artificial shrinkage, there seems to be no physical damage from intrablastocoelic ice crystals. In addition, the fast exchange of cryoprotectant seems to be more important than reducing the osmotic stress during the cryoprotectant dilution step. Further study will be needed to elucidate the osmotic process that occurs during vitrification and postthaw rehydration of human blastocyst following artificial shrinkage.

In conclusion, we have found that the 2-step dilution method is simple and effective for diluting cryoprotectant following vitrification of human blastocysts at the expanded blastocyst stage, performed in a standard system using EM grids and an artificial shrinkage technique. In addition, we have achieved clinically acceptable pregnancy rates using this cryopreservation method.

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