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Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele

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BACKGROUND: The purpose of this study was to examine the effects on blastocyst survival and subsequent pregnancy rate of 'artificial shrinkage' (i.e. induced collapse of the blastocoele) before vitrification of human blastocysts. METHODS: After embryo transfer in IVF cycles, surplus embryos that developed to the expanded blastocyst stage were cryopreserved. Before vitrification on electron microscopy (EM) grids, artificial shrinkage was induced in expanded blastocysts using a 29-gauge needle. After thawing, transfers were performed on 25 couples. Post-thaw survival rates and clinical outcome after the transfer of vitrified blastocysts were examined. RESULTS: Of 90 expanded blastocysts vitrified from 25 patients, 81 survived (90.0%) and 40 of them were hatched (49.4%) at the time of transfer. The implantation rate was 29.0% (20/69), and the pregnancy rate was 48.0% (12/25). Nine patients delivered 15 infants, two pregnancies are ongoing and one ended in miscarriage. CONCLUSIONS: The results suggest that artificial shrinkage is a useful technique for vitrification of expanded blastocysts on EM grids.

Key words: artificial shrinkage/cryopreservation/electron microscopy grid/human blastocyst/vitrification

Introduction

Recently, blastocyst transfer based on the improved culture system has been proven effective for increasing the pregnancy rate in assisted reproductive treatment, while minimizing multiple gestations (Gardner *et al*., 1998; Yoon *et al*., 2001). Therefore, a reliable procedure for the cryopreservation of supernumerary blastocysts is needed.

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). In addition, the slow-freezing method requires expensive equipment and is time-consuming. Therefore, it is essential to establish a simple, fast and reliable procedure to optimize clinical outcomes of cryopreservation at the blastocyst stage. The successes of human blastocyst vitrification procedures have been recently increased by techniques using either electron microscopy (EM) grids (Choi *et al*., 2000) or cryoloop (Yokota *et al*., 2000; Mukaida *et al*., 2001) that substantially increase the cooling rate.

We also established a vitrification system using EM grids and a six-step thawing method, and have reported the clinical usefulness of the system for the cryopreservation of human blastocysts (Cho *et al*., 2002). However, a relatively poor survival rate for the expanded blastocysts after vitrification was obtained (Cho *et al*., 2002). As previously suggested (Vanderzwalmen *et al*., 2002), the loss of viability after vitrification of blastocysts could be attributed to physical damage resulting from ice formation during the cooling procedure. In fact, in contrast to early blastocyst stage embryos, expanded blastocysts consist of a blastocoele, which may disturb cryopreservative potential due to ice crystal formation during the cooling step. Recently, Vanderzwalmen *et al*. (2002) reported, after vitrification of blastocyst inside a 0.25 ml straw, an increase in the survival rate of the blastocyst by artificially reducing the volume of the blastocoele.

Therefore, we attempted clinical application of artificial shrinkage before vitrification on EM grids of human blastocysts at expanded stage in our cryopreservation programme.

Materials and methods

Patients

Women were treated with GnRH agonist and hMG in either a long- or a short-treatment protocol. When two and more follicles reached 18 mm in diameter, a dose of 10 000 IU hCG (IVF-C; LG Chemical, Seoul, 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 for fertilization 17–19 h after insemination for the presence and number of pronuclei.

Embryo culture

Culture of fertilized oocytes was the same as described in a previous study (Yoon *et al*., 2001). Embryos having two pronuclei were washed

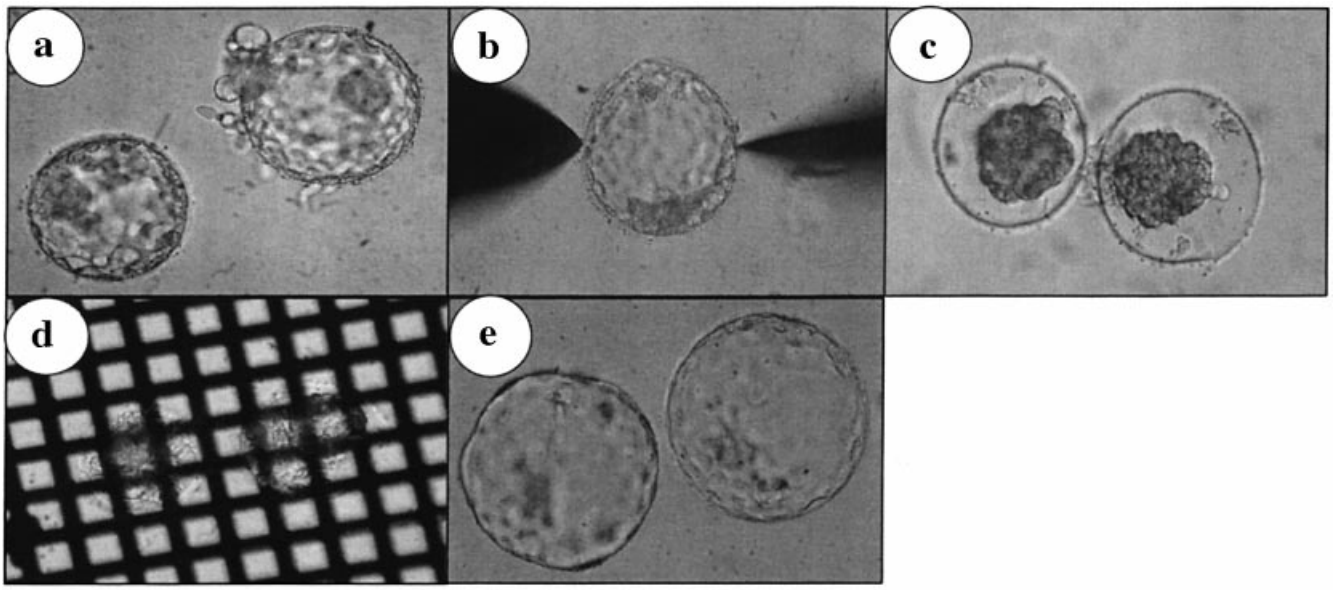

Figure 1. Freezing and warming of human expanded blastocysts on electron microscopy (EM) grids after artificial shrinkage. Human expanded blastocysts (**a**) before artificial shrinkage, (**b**) during artificial shrinkage, (**c**) after artificial shrinkage, (**d**) loaded onto EM grids, and (**e**) hatched blastocysts 18 h after thawing. Original magnification ×100.

well and co-cultured with cumulus cells in a 10 µl YS (Yoon Sanhyun) medium supplemented with 20% human follicular fluid (hFF) (Yoon *et al*., 2001) 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 2 day embryos according to published criteria (Yoon *et al*., 2001). After the embryo transfer, regardless of the embryo transfer date, surplus embryos were cultured until day 6, and embryos that developed to the expanded blastocyst stage (diameter ⩾160 µm) were cryopreserved with either slow freezing or vitrification. In all, 293 blastocysts from 121 patients were vitrified on EM grids after artificial shrinkage (see below) between February 2001 and October 2001. Of these, 25 patients who had blastocysts transferred after thawing were studied.

Preparation of vitrification solution

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), 18% (w/v) Ficoll 70 (~70 kDa), 0.3 mol/l sucrose and 20% hFF in Dulbecco's phosphate-buffered saline (DPBS). A pretreatment solution, DPBS (EG20) containing 20% ethylene glycol and 20% hFF, was prepared.

Artificial shrinkage of expanded blastocysts and vitrification

Artificial shrinkage of expanded blastocysts was performed with two 29-gauge needles. 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 trough the trophectoderm cell into the blastocoele cavity until it shrank (Figure 1). Contraction of the blastocysts was then observed after 30 s to 1 min. After complete shrinkage of the blastocoele, the blastocysts were equilibrated in EG20 for 1.5 min before exposure to the vitrification solution. The blastocysts were then incubated in EFS40, loaded onto the EM grid (IGC 400; Pelco International, CA, USA) and directly plunged in liquid nitrogen within ~30 s. The EM grids containing the blastocysts were sealed in a cryovial that had previously been submerged under liquid nitrogen. The cryovials were attached in canes and stored in liquid nitrogen.

Thawing of blastocysts

Blastocysts were warmed using a six-step dilution with sucrose (Cho *et al*., 2002). The EM grids containing blastocysts were transferred to 100 µl drops of 0.5 mol/l sucrose. 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 and 0 mol/l of 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 20% hFF for further culture until transfer. The post-thawing survival of blastocysts was observed ~18–20 h after warming under a microscope, and blastocysts with a morphologically intact ICM, trophectoderm and a re-expanding blastocoele were judged to have survived. Embryo transfer was scheduled on day 4–5 after ovulation in the spontaneous cycles. 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 then clinical pregnancy was determined by the presence of fetal heart activity 30 days after blastocyst transfer.

Results

In a preliminary experiment, we evaluated the effect of artificial shrinkage on blastocyst survival and the further development after thawing of poor quality vitrified expanded blastocysts, which had been donated by consenting patients. The rates of survival (90.6%, 48/53) and hatching (56.3%, 27/48) in the artificial shrinkage group were significantly higher than those of the control group (71.2%, 37/52; 10.8%, 4/37). No harmful effect of the procedure was noted.

Based on the above result, we applied the artificial shrinkage technique clinically. Figure 1 shows the morphology of human blastocysts before and after cryopreservation. Table I shows the clinical results of the human blastocyst vitrification after artificial shrinkage. A total of 90 expanded blastocysts was vitrified and warmed from 25 patients. Eighty-one blastocysts (90.0%) were re-expanded after warming. Of the 81 blastocysts that survived, 40 had hatched (49.4%) at the time of

Table I. Results of human blastocyst vitrification after artificial shrinkage

Variables	Value
No. of patients (no. of treatment cycles)	25 (25)
No. of expanded blastocysts vitrified	90
No. of blastocysts survived (%)	81 (90.0)
No. of blastocysts hatched at ET (%)	40 (49.4)
Average no. of blastocysts transferred	2.8
Implantation rate (%)	20/69 (29.0)
Pregnancy rate (%)	12/25 (48.0)

ET = embryo transfer.

transfer. A total of 69 blastocysts was transferred into 25 patients. The implantation rate was 29.0% (20/69) and the pregnancy rate was 48.0% (12/25). Eight male and seven female infants (six sets of twins and three singletons) from nine patients were born, one cycle had a spontaneous abortion at 6 weeks of gestation, and the other two pregnancies are ongoing. Birthweights of the infants were within the range of 1950–3550 g, and all delivered infants had normal physical profile up to the present.

Discussion

This study demonstrates that artificial shrinkage of human blastocysts before vitrification on EM grids shows very encouraging clinical results.

Vitrification would be a very attractive alternative to the conventional slow-freezing protocol with advantages of the lack of ice crystal formation and ease of operation. We have already reported a vitrification method for human blastocysts on EM grids and the success of vitrification procedures has been increased by use of a six-step thawing technique that substantially reduces osmotic shock (Cho *et al*., 2002). In the report, acceptable pregnancy and delivery were obtained after clinical application of vitrification and six-step thawing protocols, implying that vitrification on EM grids combined with a six-step thawing protocol could be applied to human blastocyst cryopreservation. However, we have observed that the survival rate of blastocysts at the expanded stage (71%) was lower than that of blastocysts at early stage (92%) with the established vitrification methods (Cho *et al*., 2002). An explanation for this was that late blastocysts consist of a fluid-filled blastocoele, which may disturb cryopreservative potential due to ice crystal formation during the cooling step. Actually, we have observed that expanded human blastocysts are dehydrated and concentrated more slowly than earlier stage embryos, suggesting that intracellular ice is more likely to form.

Recently, it has been suggested that mechanical damage caused by ice crystal formation could be avoided by reducing the fluid content of the blastocoele in expanded blastocysts using a glass micro-needle (Vanderzwalmen *et al*., 2002). These authors also reported that the rates of pregnancy and implantation were improved after artificial shrinkage compared with the control, intact blastocyst, group.

Similarly, we applied clinically the artificial shrinkage technique in our vitrification system, using EM grids and six-step thawing procedure, and examined the effect on survival and hatching of vitrified human blastocysts. From the high survival rate (90.0%) that we obtained with this approach in our vitrification system, we can confirm that this method is a useful technique for the vitrification of expanded human blastocysts. Furthermore, the high percentage of hatching (49.4%) seen at the time of embryo transfer might be due to the effect of assisted hatching caused by the formation of a large hole in the zona pellucida produced by using a 29-gauge needle.

We achieved higher implantation and clinical pregnancy rates (29.0 and 48.0%) by application of the artificial shrinkage in our vitrification programme, and the pregnancy outcome was similar to that obtained from the transfer of fresh blastocysts in our hospital (Yoon *et al*., 2001). This result was compared with the report of Vanderzwalmen *et al*. (2002), although the numbers in this study were too small to detect any difference. The explanation for this observation could be that even through vitrification was performed using the same artificial shrinkage technique, clinical success might depend not only on the vitrification process, but also on the apparatus which is used in the vitrification, such as EM grid, and also on the apparatus which is used for artificial shrinkage, such as a 29-gauge needle. This is probably due to the substantially increased cooling rate achieved by EM grids compared with classical vitrification using 0.25 ml straws, which were used by Vanderzwalmen *et al*. (2002). Another possible explanation could be the different culture conditions used for producing the blastocysts and the quality of expanded blastocysts cryopreserved. We cryopreserved only the good quality expanded blastocysts in our hospital.

In conclusion, this study showed that vitrification of human blastocysts at the expanded blastocyst stage using EM grids and artificial shrinkage technique is a clinically useful cryopreservation method.

References

Cho, H.J., Son, W.Y., Yoon, S.H., Lee, S.W. and Lim, J.H. (2002) An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. *Hum. Reprod.*, **17**, 2419–2422.

Choi, D.H., Chung, H.M., Lim, J.M., Ko, J.J., Yoon, T.K. and Cha, K.Y. (2000) Pregnancy and delivery of healthy infants developed from vitrified blastocysts in an IVF–ET program. *Fertil. Steril.*, **74**, 838–844.

Gardner, D.K., Schoolcraft, W.B., Wagley, L., Schlenker, T., Stevens, J. and Hesla, J. (1998) A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. *Hum. Reprod.*, **13**, 3434–3440.

Kasai, M., Komi, J.H., Takakamo, A., Tsudera, H., Sakurai, T. and Machida, T. (1990) A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *J. Reprod. Fertil.*, **89**, 91–97.

Kaufman, R.A., Ménézo, Y., Hazout, A., Nicollet, B., DuMont, M. and Servy, E.J. (1995) Cocultured blastocyst cryopreservation: experience of more than 500 transfer cycles. *Fertil. Steril.*, **64**, 1125–1129.

Ménézo, Y., Nicollet, B., Herbaut, N. and Andre, D. (1992) Freezing cocultured human blastocysts. *Fertil. Steril.*, **58**, 977–980.

Mukaida, T., Nakamura, S., Tomiyama, T., Wada, S., Kasai, M. and Takahashi, K. (2001) Successful birth after transfer of vitrified human blastocysts with use of a cryoloop containerless technique. *Fertil. Steril.*, **76**, 618–620.

Vanderzwalmen, P., Bertin, G., Debauche, C.H., Standaert, V., van Roosendaal, E., Vandervorst, M., Bollen, N., Zech, H., Mukaida, T., Takahashi, K. *et al*. (2002) Birth after vitrification at morula and blastocyst stage: effect of artificial reduction of the blastocoeleic cavity before vitrification. *Hum. Reprod.*, **17**, 744–751.

Yokota, Y., Sato, S., Yokota, M., Ishikawa, Y., Makita, M., Asada, T. and Araki, Y. (2000) Successful pregnancy following blastocyst vitrification. *Hum. Reprod.*, **15**, 1802–1803.

Yoon, H.G., Yoon, S.H., Son, W.Y., Kim, J.G., Im, K.S. and Lim, J.H. (2001) Alternative embryo transfer of day 3 or day 5 for reducing the risk of multiple gestations. *J. Assist. Reprod. Genet.*, **18**, 262–267.

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