

Vitrification of human blastocysts using cryoloops: clinical outcome of 223 cycles*

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BACKGROUND: The need to cryopreserve human blastocysts is increasing. The successful birth has been reported of a baby from a blastocyst vitrified using the cryoloop technique. The present study expands on this earlier report to confirm the effectiveness of this vitrification procedure. **METHODS:** In patients undergoing IVF at one of three clinics, supernumerary blastocysts on day 5 or 6 at various stages of development were vitrified using cryoloops. **RESULTS:** Of 725 vitrified blastocysts, 583 (80.4%) survived. After the transfer of 493 blastocysts in 207 cycles, 76 women (37%) became clinically pregnant. Among these women, 21 pregnancies ended in miscarriage, 23 healthy babies were born in 18 deliveries, and 37 pregnancies are ongoing. The survival rate of day 5 blastocysts (87%) was higher than that of day 6 blastocysts (55%), but implantation rates and pregnancy rates were not statistically significantly different. **CONCLUSIONS:** Clinical outcomes with 725 blastocysts and 207 transfers showed that vitrification using cryoloops is effective and practical for the cryopreservation of human blastocysts. Early blastocysts on day 5 seem to be the most suitable in terms of stage and age for cryopreservation, but developed and day 6 blastocysts can also be cryopreserved.

Key words: blastocyst/cryoloop vitrification/cryopreservation/embryo/human

Introduction

In assisted reproductive technology, the cryopreservation of embryos has proved important for the best use of supernumerary embryos. In the cryopreservation of embryos, there is a risk of various types of injury (Kasai, 1996; Kasai *et al.*, 2002), among which the formation of intracellular ice appears to be the most damaging. The first strategy to prevent intracellular ice formation was to adopt a lower concentration of cryoprotectant and a long slow-cooling stage. This slow-freezing method has proven effective for embryos of a wide range of mammalian species. Unlike embryos of laboratory animals and domestic animals, in which dimethyl sulphoxide (DMSO), glycerol or ethylene glycol (EG) is commonly used as the cryoprotectant, human embryos at early cleavage stages have most often been frozen in a solution of propanediol supplemented with sucrose (Lassalle *et al.*, 1985), although those at the blastocyst stage have more frequently been frozen with glycerol (Fehilly *et al.*, 1985; Hartshorne *et al.*, 1991; Ménézé *et al.*, 1992). With slow freezing, however, it is

difficult to eliminate injuries from ice completely. Furthermore, the slow-freezing method requires a long period of time before the embryos can be stored in liquid nitrogen.

In 1985, the first report was made of an innovative approach called vitrification, in which injuries related to ice are minimized by using very high concentrations of cryoprotectant (Rall and Fahy, 1985). This approach simplifies the cooling process, because embryos can be cooled directly in liquid nitrogen. Although embryos subjected to vitrification are liable to be injured by the toxicity of the high concentration of cryoprotectant, the method has been refined and proven effective for the cryopreservation of embryos at various stages of development in laboratory and domestic species. In 1998, it was shown that vitrification using an EG-based vitrification solution (EFS40) (Kasai *et al.*, 1990) with conventional cryostraws was effective for human embryos at the 4- to 8-cell stage (Mukaida *et al.*, 1998). The effectiveness of vitrification was confirmed for human embryos at the 8- to 16-cell stage (Saito *et al.*, 2000) and the morula stage (Yokota *et al.*, 2001b), also using EG-based solutions.

Recent advances in culture systems with sequential media have made it possible to develop human IVF embryos into blastocysts quite easily. Because the blastocyst is better suited to the uterine environment, and because blastocyst formation is

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a form of selection for more viable embryos, blastocyst transfer has become a promising option to raise the pregnancy rate (Gardner *et al.*, 1998; Cruz *et al.*, 1999). Accordingly, the need to cryopreserve human blastocysts is increasing. One group (Ménézo *et al.*, 2000) cryopreserved human blastocysts which were developed in a co-culture system using the slow-freezing method with glycerol and obtained reasonable clinical results (27% pregnancy rate, 17% implantation rate). However, results reported by other clinics have not been consistent (Troup *et al.*, 1990; Nakayama *et al.*, 1995; Ludwig *et al.*, 1999). It has also been speculated that the outcome of cryopreservation is influenced by the culture conditions (Ménézo *et al.*, 2000).

Although a recent report described the successful vitrification of human blastocysts in straws (Yokota *et al.*, 2001a), our own attempts to repeat this approach resulted in only 45% survival (39/86, unpublished data). Others (Vanderzwalmen *et al.*, 1999) also reported a low pregnancy rate with human blastocysts vitrified in straws. Human blastocysts are much less permeable to cryoprotectant and water, and have been observed to shrink more slowly than mouse and bovine blastocysts when placed in the cryoprotectant solution; this suggests that they are more likely to be injured by intracellular ice.

Increased rates of cooling and warming can help circumvent the problem of intracellular ice formation in less permeable embryos. Faster rates of cooling and warming can be achieved by minimizing the volume of the solution with which embryos are vitrified, i.e. by using minute tools such as electron microscopic grids (Martino *et al.*, 1996), open pulled straws (Vajta *et al.*, 1998) or cryoloops (Lane *et al.*, 1999a) (for a review, see Kasai, 2002). It was shown that the transfer of human blastocysts vitrified with cryoloops leads to the successful birth of a baby (Mukaida *et al.*, 2001), and this method of vitrification has been used in the present authors' group of three clinics to cryopreserve blastocysts on days 5 and 6. The present report summarizes the results obtained with 223 warming cycles, and confirms the effectiveness of the cryoloop technique in the cryopreservation of human blastocysts.

Materials and methods

Patients and IVF

All patients who entered this blastocyst transfer programme had had previous multiple failures of conventional day 2 or day 3 embryo transfer, and agreed to use of the cryoloop vitrification method to cryopreserve their supernumerary blastocysts obtained at 5 or 6 days after oocyte retrieval. The mean age of the women was 34.2 (range 24–46) years.

Women were treated with GnRH agonist and hMG using either a long- or a short-treatment protocol. They were administered hCG when dominant follicles reached a diameter of ~18 mm. Oocytes were collected 36 h after hCG administration using a vaginal ultrasound-guided procedure. The oocytes were inseminated using either conventional IVF or ICSI, and incubated in human tubal fluid (hTF) medium containing 5 mg/ml human serum albumin (hSA) or G1.2 medium (Vitrolife, Gothenburg, Sweden) in a 4-well multi-dish under mineral oil in a CO₂ incubator at 37°C in an atmosphere of 6% CO₂, 5% O₂ and 89% N₂.

Embryo culture and grading of blastocysts

Fertilization was assessed at 15–18 h after insemination by the presence of two pronuclei. One-cell embryos were washed well and cultured in groups of two to three in 4-well dishes under oil in the CO₂ incubator. Embryos were cultured in G1.2 medium or Blast Assist Medium 1 (Medicult, Jyllinge, Denmark) for 48 h, and then placed in G2.2 medium (Vitrolife) or Blast Assist Medium 2 (Medicult) for 48–72 h. In some cases, when 4- to 8-cell embryos were removed for transfer to the patient after 24–48 h of culture in the first medium, only supernumerary embryos were cultured in the second medium.

After 48–54 h of culture in the second medium, 5 days after oocyte retrieval, embryos were examined for development into blastocysts. If all the embryos were at the morula stage or earlier, they were cultured until day 6 and then examined for development. On day 5 or 6, each embryo which had developed to the blastocyst stage was scored depending on the developmental stage, and graded according to quality using published criteria (Gardner and Schoolcraft, 1999; Gardner *et al.*, 2000) with slight modifications.

First, blastocysts were given a numerical score from 1 to 6 on the basis of their degree of development, as follows: 1, early blastocysts with a blastocoele <50% of the embryo volume; 2, early blastocysts with a blastocoele ≥50% of the embryo volume; 3, full blastocysts with a blastocoele completely filling the embryo but not expanded; 4, expanded blastocysts with a blastocoele volume larger than that of the early embryo, with a thinning zona; 5, hatching blastocysts with the trophectoderm starting to herniate through the zona; and 6, hatched blastocysts, in which the blastocyst has completely escaped from the zona.

Second, the blastocysts were graded in three ranks by quality. Early blastocysts (scored as 1–2) were assessed based on morphological appearance as A (many equal-shaped cells), B (many unequal-shaped cells) or C (few cells and degeneration). More advanced blastocysts (scored as 3–6) were classified on both the quality of the inner cell mass (A = many tightly packed cells; B = several loosely grouped cells; C = few cells), and the quality of the trophectoderm (A = many cells forming a cohesive epithelium; B = fewer cells forming a loose epithelium; C = very few large cells).

When the patient had their fresh embryos transferred on day 2–3, all the remaining embryos were cultured to allow those which developed into blastocysts to be vitrified. Patients who received transfers of fresh blastocysts had all their remaining supernumerary blastocysts vitrified. On day 5, if at least one supernumerary blastocyst was graded as A or B, all the blastocysts of the patient were vitrified regardless of the developmental stage and the grading. In a few cases, compacted morulae were also vitrified with the blastocysts. If all the blastocysts of the patient were graded C, they were not cryopreserved. On day 6, if at least one blastocyst had a large blastocoele (i.e. scored as 3–6) and was graded as A or B, all the developed blastocysts scored as 3–6 were vitrified.

Vitrification of blastocysts

The protocol for the cryoloop vitrification of blastocysts was adopted from previous reports (Lane *et al.*, 1999a,b), albeit with slight modifications, and had been described previously (Mukaida *et al.*, 2001). The cryoloop consisted of a nylon loop (20 µm wide; 0.5–0.7 mm diameter) mounted on a stainless steel pipe that was inserted into the lid of a cryovial (Hampton Research, Laguna Niguel, CA, USA). A metal insert on the lid enabled the use of a stainless steel handling rod with a small magnet (Crystalwand, Hampton Research) for manipulation of the loop at low temperature.

One to three blastocysts were vitrified in each cryoloop after a two-step procedure to load the blastocysts with cryoprotectants at ~35°C.

Table I. Survival and development of human blastocysts vitrified on day 5 or day 6

Parameter	Age of blastocyst		
	Day 5	Day 6	Total
Average age of patients (years)	34.2	34.0	34.2
No. of cycles for vitrification	164	51	215
No. of cycles for warming and transfer	172	51	223
No. of embryos vitrified and recovered	569	156	725
No. of embryos survived (%)	497 (87) ^a	86 (55) ^b	583 (80)
No. of embryos transferred	419	74	493
No. of embryos implanted (%)	86 (21)	13 (18)	99 (20)
No. of cycles transferred	165	42	207
No. of clinical pregnancies (%)	64 (39)	12 (29)	76 (37)
No. of miscarriages (%)	15 (23)	6 (50)	21 (28)
No. of deliveries	16	2	18
No. of ongoing pregnancies	33	4	37
No. of babies (male:female ratio)	21 (10:11)	2 (1:1)	23 (11:12)

^{a,b}Values are statistically significantly different ($P < 0.01$).

As the base medium, HEPES-buffered modified hTF medium containing 5 mg/ml hSA was used. Initially, blastocysts were placed in the base medium containing 7.5% (v/v) DMSO and 7.5% (v/v) EG (cryoprotectant solution I). After 2 min, the blastocysts were suspended in the base medium containing 15% (v/v) DMSO, 15% (v/v) EG, 10 mg/ml Ficoll 70 (average molecular weight 70 000; Pharmacia Biotech, Uppsala, Sweden) and 0.65 mol/l sucrose (cryoprotectant solution II). Both cryoprotectant solutions had been warmed briefly in an incubator at 37°C, and blastocysts were handled on the stage warmer of a dissecting microscope at 37°C.

While the embryos were suspended in cryoprotectant solution I, a cryoloop was dipped into cryoprotectant solution II in order to create a thin, filmy layer of solution, by surface tension, on the nylon loop. The blastocysts were then washed quickly in solution II and transferred onto the filmy layer on the nylon loop using a micropipette. Immediately after the loading of blastocysts, the cryoloop was plunged into liquid nitrogen. The time that blastocysts were exposed in solution II before cooling was limited to 25–30 s. Using the stainless steel rod, the loop containing the blastocysts was sealed in a cryovial, which was previously submerged in liquid nitrogen. The vials were attached in standard canes and stored in liquid nitrogen. The entire procedure was completed within 5 min.

Warming of blastocysts and assessment of survival

In a 4-well multi-dish, ~1 ml of base medium containing 0.33 mol/l sucrose, base medium containing 0.2 mol/l sucrose, and base medium were warmed briefly in an incubator at 37°C and then placed on the stage warmer of a dissecting microscope. With the cryovial submerged in liquid nitrogen, the vial was opened with the aid of the stainless steel rod, and the loop containing blastocysts was removed from the liquid nitrogen and placed directly and quickly into a well of the 0.33 mol/l sucrose solution. Blastocysts immediately fell from the loop into the solution. Thus blastocysts were warmed and diluted instantly at ~35°C. After 2 min, blastocysts were transferred to the 0.2 mol/l sucrose solution. After an additional 3 min, blastocysts were washed and kept in the base medium for 5 min, and then returned to G2.2 medium for further culture until transfer.

At ~2 h after warming, the appearance of the blastocysts was examined on an inverted microscope at ×400 magnification, and survival was assessed based on the morphological integrity of the blastomeres, inner cell mass and trophoctoderm, and re-expansion of the blastocoele. The surviving blastocysts were scored as to developmental stage and graded for quality as described above.

Transfer of blastocysts and assessment of pregnancy

All women received transdermal estradiol (Estrana, 0.4 mg/day; Hisamitsu, Tokyo, Japan) with GnRH agonists for preparation of the endometrium. Administration of progesterone (50 mg in oil, daily) was initiated when the endometrial thickness was >10 mm. At 5 days (in a few cases, 6 days) after the initiation of progesterone treatment, blastocysts were warmed and surviving blastocysts were transferred into the patient's uterus. Most patients received one to three blastocysts; occasionally, four to five blastocysts were transferred, depending on the patient's background (i.e. multiple failures of assisted reproduction). Chemical pregnancy was assessed based on serum hCG levels at 9–10 days after blastocyst transfer, after which implantation and clinical pregnancy was confirmed by the presence of fetal heart activity or a gestational sac at ~30 days after blastocyst transfer.

Statistical analysis

The data obtained were examined for differences using χ^2 analysis, unless the expected frequency was <5, in which case Fisher's exact probability test was used.

Results

The survival and development of human blastocysts vitrified by cryoloops are summarized in Table I. A total of 725 blastocysts originating from 215 cycles of oocyte collection from 193 patients was vitrified and warmed. After 223 cycles of warming for blastocyst transfer, 583 (80.4%) of the embryos survived. In 13 cycles for 13 patients, no blastocysts survived and embryo transfer was not conducted. In three cycles for three patients, viable embryos were obtained but embryo transfer was cancelled because the number and the quality of the embryos were low. A total of 493 blastocysts was transferred into 180 patients in 207 cycles, the mean number of blastocysts transferred per cycle being 2.4. Of the 493 transferred embryos, 99 (20%) were diagnosed as implanted about 30 days after transfer. Of 207 transfers, 76 resulted in clinical pregnancy; the pregnancy rate was 35% per vitrification cycle, 34% per warming cycle, and 37% per transfer. Although 21 of 76 transfers (28%) ended in miscarriage, 23 healthy babies were born in 18 deliveries, and 37 pregnancies are ongoing. No bias in the sex ratio was observed, as 11 babies

Table II. Survival rates of vitrified blastocysts classified by the most developed stage of the group of blastocysts of each patient before vitrification

Groups classified by development*	No. of blastocysts survived/no. of blastocysts vitrified (%)		
	Day 5	Day 6	Total
I	48/55 (87) ^{ab}	-	48/55 (87) ^{ab}
II	62/64 (97) ^a	-	62/64 (97) ^a
III	99/111 (89) ^{ab}	13/14 (93) ^a	112/125 (90) ^a
IV	215/250 (86) ^b	33/65 (51) ^b	248/315 (79) ^b
V	73/89 (82) ^b	40/77 (52) ^b	113/166 (68) ^c
Total	497/569 (87)	86/156 (55)	583/725 (80)

*First, embryos were classified by degree of development, as morulae, early blastocysts with a small blastocoele (score 1), early blastocysts with a larger blastocoele (score 2), unexpanded blastocysts with a fully developed blastocoele (score 3), expanded blastocysts (score 4), hatching blastocysts (score 5), or hatched blastocysts (score 6). Then a group of embryos for each attempted transfer was classified by the most developed stage of the embryos, as group I (with a score 1 embryo), II (with a score 2 embryo), III (with a score 3 embryo), IV (with a score 4 embryo), and V (with a score 5 or 6 embryo). Average proportions of score 1, 2, 3, 4 and 5–6 embryos included in group I, II, III, IV and V embryos were 80, 45, 52, 53 and 41% respectively.

^{a,b,c}Values with different letters within each column are significantly different ($P < 0.05$).

Table III. Pregnancy rates after transfer of vitrified blastocysts classified by the most developed stage of the group of blastocysts of each patient before vitrification

Groups classified by development*	No. of pregnancies/no. of transfers (%)		
	Day 5	Day 6	Total
I	8/19 (42)	-	8/19 (42)
II	9/19 (47)	-	9/19 (47)
III	18/42 (43)	2/5 (40)	20/47 (43)
IV	22/65 (34)	4/18 (22)	26/83 (31)
V	7/20 (35)	6/19 (32)	13/39 (33)
Total	64/165 (39)	12/42 (29)	76/207 (37)

*Embryos were classified as defined in Table II.

No significant difference was found among the rates in the same column.

Table IV. Pregnancy rates after transfer of vitrified blastocysts classified by the most developed stage of the group of blastocysts of each patient after warming and a short period of culture

Groups classified by development*	No. of pregnancies/no. of transfers (%)		
	Day 5	Day 6	Total
I	8/37 (22) ^a	0/11 (0) ^a	8/48 (17) ^a
II	17/50 (34) ^{ab}	6/8 (75) ^b	23/58 (40) ^{bc}
III	22/44 (50) ^{bc}	2/5 (40)	24/49 (49) ^b
IV	15/26 (58) ^c	1/8 (13)	16/34(47) ^{bc}
V	2/8 (25)	3/10 (30)	5/18 (28)
Total	64/165 (39)	12/42 (29)	76/207 (37)

*After warming and a short period of culture, surviving embryos were classified as defined in Table II.

Average proportions of score 1, 2, 3, 4 and 5–6 embryos included in group I, II, III, IV and V embryos were 81, 59, 52, 55 and 51% respectively.

^{a,b,c}Values with different letters within each column are significantly different ($P < 0.05$).

were boys and 12 were girls. Of 126 pregnancies established from fresh blastocyst transfers in the clinics during the same period, 27 (22%) resulted in miscarriages. There was no significant difference among the two miscarriage frequencies.

The effect of the age (day) of blastocysts on the survival after warming and clinical outcomes after transfer are shown in Table I. Of 725 vitrified blastocysts, 569 (78%) were obtained on day 5, and 156 (22%) on day 6. The survival rate of day 5

blastocysts (87%) was significantly higher than that of day 6 blastocysts (55%) ($P < 0.01$). However, implantation rates for transferred embryos were similar (21 versus 18%). Although the pregnancy rate with day 5 blastocysts (39%; 64/165) was higher than that with day 6 blastocysts (29%; 12/42), the difference was not statistically significant.

It was not possible to follow the survival and developmental potential of each blastocyst, because one to three blastocysts

Table V. Pregnancy rate after transferring various numbers of vitrified blastocysts into patients

No. of blastocysts transferred	No. of pregnancies/no. of transfers (%)		
	Day 5	Day 6	Total
1	7/24 (29) ^a	4/19 (21)	11/43 (26) ^a
2	22/64 (34)	5/16 (31)	27/80 (34) ^a
3	19/49 (39)	1/5 (20)	20/54 (37) ^a
4-5	16/28 (57) ^b	2/2 (100)	18/30 (60) ^b
Total	64/165 (39)	12/42 (29)	76/207 (37)

^{a,b}Values with different letters within each column are significantly different ($P < 0.05$).

Table VI. Survival of vitrified blastocysts and pregnancy after transfer of vitrified blastocysts as assessed by age of the women

Age of women (years)	No. of blastocysts survived/vitrified (%)	No. of blastocysts implanted/transferred (%)	No. of women pregnant/transferred (%)
24–34	286/360 (79)	56/239 (23)	40/99 (40)
35–46	297/365 (81)	43/254 (17)	36/108 (33)

No significant difference was found among rates in the same column.

Table VII. Survival of vitrified blastocysts and pregnancy after transfer of vitrified blastocysts as assessed by the cause of infertility

Cause of infertility*	No. of blastocysts survived/vitrified (%)	No. of blastocysts implanted/transferred (%)	No. of women pregnant/transferred (%)
Male	113/142 (80)	27/103 (26)	20/41 (49) ^a
Female	238/302 (79)	37/193 (19)	31/83 (37) ^{ab}
Unexplained	207/249 (83)	31/177 (18)	21/75 (28) ^b

*Data for eight patients with multiple causes were not included.

^{a,b}Values with different letters are significantly different ($P < 0.05$).

were vitrified on a cryoloop, embryos of each patient were cultured in a group after warming, and one or more embryos were transferred to each woman. Thus, a group of blastocysts for each attempted transfer was classified by the most developed stage (i.e. the highest score number) of the group of blastocysts for retrospective analysis: that is, as group I (with a stage 1 embryo); group II (with a stage 2 embryo); group III (with a stage 3 embryo); group IV (with a stage 4 embryo); and group V (with a stage 5–6 embryo). For example, if three blastocysts of a patient were scored as 4, 3 and 2, all the blastocysts in this group were classified as group IV. The survival rates of vitrified blastocysts classified by this criterion before vitrification are shown in Table II. Average proportions of score 1, 2, 3, 4 and 5–6 embryos included in group I, II, III, IV and V embryos were 80, 45, 52, 53 and 41% respectively. The survival rate of embryos in group II or III, which included unexpanded blastocysts (90–97%), was statistically significantly higher than the rates of blastocysts in group IV–V, including those at more developed stages (68–79%) ($P < 0.05$). Accordingly, pregnancy rates with earlier blastocysts in groups I–III (44%; 37/85 in total) tended to be higher than those with more developed blastocysts in groups IV–V (32%; 39/122 in total), although the difference was not statistically significant (Table III). By contrast, when the pregnancy rate was evaluated

according to the most developed stage of the group of embryos after warming and a short period of culture (Table IV), higher pregnancy rates (40–49%) were obtained with embryos in groups II–IV, which regained a larger blastocoele, than with those in group I, which remained at less developmental stages. Average proportions of score 1, 2, 3, 4 and 5–6 embryos included in group I, II, III, IV and V embryos were 81, 59, 52, 55 and 51% respectively.

The effect of the number of vitrified blastocysts transferred to the recipient on pregnancy rate is shown in Table V. The frequency of pregnancy increased as the number of transferred embryos increased, reaching 60% (18/30) when four to five embryos were transferred.

The survival and developmental potential of vitrified blastocysts were further analysed from non-embryonic factors retrospectively. As shown in Table VI, the age of women did not affect the post-warming survival rate of blastocysts (79–81%). As expected, both the implantation rate (17 versus 23%) and pregnancy rate (33 versus 40%) were slightly lower in older women than in younger women, although the difference was not statistically significant. When patients were classified depending on the cause of infertility, briefly by male factors, female factors and unexplained factors, the survival rate of vitrified

blastocysts was not influenced by these factors (79–83%) (Table VII). However, the pregnancy rate in patients with male factors (49%, 20/41) was statistically significantly higher than the rate in patients with unexplained factors (28%, 21/75) ($P < 0.05$).

Discussion

The data on cryoloop vitrification of human blastocysts presented in the present report were obtained by the HART Clinic group of Hiroshima, Osaka and Tokyo HART Clinics. Among a total of 725 vitrified blastocysts, high rates of survival (80%) and pregnancy (37%) were obtained. Because the aim of the report was to evaluate the effectiveness of the cryoloop technique with a large number of blastocysts, the results contained a small amount of data previously reported (Mukaida *et al.*, 2001).

The present authors have adopted blastocyst transfer since sequential culture systems first became available. Initially, to cryopreserve blastocysts, straw vitrification was attempted using EFS40 (which contains 40% EG), but post-warming survival was quite poor (45%, unpublished data). It has been speculated that blastocysts are much less permeable to water and cryoprotectant than embryos at early cleavage stages, as blastocysts respond to hypertonic solutions much more slowly than earlier embryos (Mukaida *et al.*, 2001). This suggests that intracellular ice is more likely to form in the blastocyst during cryopreservation.

An attempt has been made to vitrify human morulae and blastocysts in straws (Vanderzwalmen *et al.*, 2002), but the efficiency of vitrification was shown to decrease with expansion of the blastocoele. The subsequent strategy of these authors to cryopreserve human blastocysts was to make a small hole in the trophectoderm with a needle, thereby causing the embryo to shrink. The artificially shrunken blastocysts were vitrified in straws in EFS40, and this resulted in a dramatic increase in post-warming survival rate such that eight pregnancies resulted from 35 transfers. By contrast, others (Yokota *et al.*, 2001a) vitrified intact blastocysts in straws and reported six pregnancies from 18 transfers. Considering that these authors used a vitrification solution containing 25% EG + 25% DMSO, the use of a high concentration (50%) of cryoprotectant might be effective in preventing injury caused by intracellular ice. However, the risk of injury caused by chemical toxicity of the cryoprotectant would increase as the concentration increased. An alternative strategy to cryopreserve human blastocysts would be to adopt ultrarapid vitrification, in which embryos are vitrified extremely rapidly using a minimal amount of vitrification solution, as this reduces the chance of intracellular ice forming (Kasai, 2002). Using this approach, one group vitrified human blastocysts using electron microscopic grids, and reported five pregnancies from 20 transfers (Choi *et al.*, 2000).

The cryoloop is a sophisticated tool which enables handling of the loop with a magnet-attached stainless steel rod and preservation of embryos in a cryovial (Lane *et al.*, 1999a,b). Preliminary results of the cryoloop vitrification of human blastocysts have been reported in which six pregnancies with

one delivery were obtained (Mukaida *et al.*, 2001). The present data with 207 transfers resulting in 76 pregnancies demonstrate the effectiveness of the cryoloop technique for the cryopreservation of human blastocysts (Mukaida *et al.*, 2001). It has also been reported that cryoloop vitrification yields a superior survival of Rhesus monkey blastocysts (Yeoman *et al.*, 2001). These authors showed that while a vitrification solution containing 25% EG and 25% glycerol was effective, an alternative solution containing 20% EG and 20% DMSO was ineffective. This shows that permeation by the cryoprotectant and concentration by the embryo are important factors, even in the cryoloop vitrification of primate blastocysts. The vitrification solution used in the present study contained a lower concentration of permeating cryoprotectant (15% EG and 15% DMSO), which might be less effective in preventing the formation of intracellular ice. In order to promote dehydration and permeation of the cryoprotectant, however, blastocysts were loaded with cryoprotectants at a higher temperature (~35°C). Successful vitrification with a lower concentration of cryoprotectant suggests that this approach is actually effective at preventing the formation of intracellular ice in human blastocysts. The use of a lower concentration of cryoprotectant must be more effective in minimizing the risk of toxic injury.

Although the overall survival rate (80%, 583/725) with vitrified blastocysts was high, the rate was affected by embryonic factors, such as the age (day) and the developmental stage of blastocysts. Most embryos (78%) developed to the blastocyst stage on day 5 and were vitrified on that day, whereas retarded embryos were vitrified on day 6. The overall survival rate was higher for blastocysts vitrified on day 5 than day 6 (87 versus 55%; Table I). More precisely, however, the distribution of embryos in terms of developmental stage was different among the blastocysts of different ages. If the survival rates of blastocysts on day 5 and day 6 are compared in more homogeneous groups, i.e. only for blastocysts in the same groups with embryos scored as 3–5 (groups III–V) from the data shown in Table II, the survival rate of day 5 blastocysts was 86% (387/450), whereas that of day 6 blastocysts was 55% (86/156), and with a statistically significant difference ($P < 0.01$) between these subgroups.

The effect of developmental rate in vitrification outcome of blastocysts parallels that obtained with day 2–3 human embryos, in that more of the morphologically normal ‘on-time’ embryos (86%) survive than retarded embryos (58%) (Mukaida *et al.*, 1998). It is strange that both rates are quite similar to the survival rates for day 5 and day 6 blastocysts shown above, but it indicates that normally developing human embryos are more resistant to cryopreservation than retarded ones, not only in embryos at early cleavage stages but also in blastocysts, when the survival was assessed by the appearance. Vitrified human blastocysts could be damaged as a result of the chemical toxicity of the cryoprotectant, intracellular ice formation, or osmotic stress during the removal of the cryoprotectant. Because toxic injury is not reflected in the morphological survival of blastomeres (Kasai *et al.*, 2002), possible mechanisms for the difference in cryoresistance among normally developed and retarded blastocysts would

be intracellular ice formation or osmotic stress. Both injuries are closely related to the lower permeability of the membrane.

The implantation rates of the surviving blastocysts after transfer to the recipient were similar for both day 5 and day 6 blastocysts (21 and 18%; Table I). Although the pregnancy rate for day 6 blastocysts (29%) was lower than that of day 5 blastocysts (39%), the difference was not statistically significant. It should be noted that while day 5 blastocysts include early blastocysts, which tends to result in higher pregnancy rates (Table II), the day 6 blastocysts did not. In addition, the number of embryos per transfer was larger for day 5 blastocysts (2.5 in average) than day 6 blastocysts (1.8 in average), because the number of transferable embryos tended to be small on day 6; the present results also show that the pregnancy rate increases as the number of transferred embryos per recipient increases, as expected. Considering these factors, it seems that once they have survived vitrification, blastocysts on day 5 and 6 have comparable potential to develop further.

As one or more embryos of each patient were vitrified and cultured together, it was not possible to assess post-warming survival and development for each blastocyst. In an attempt to assess the effect of the developmental stage briefly, a group of blastocysts of each patient was classified retrospectively based on the most developed stage of the blastocysts. Hence, embryos classified as early blastocysts (groups I–II) did not include developed blastocysts, whereas embryos classified as developed blastocysts (groups III–V) included early blastocysts. Thus, this classification was not accurate. Nevertheless, analyses on the classification suggested that earlier blastocysts were more resistant to vitrification than more developed blastocysts (Tables II and III). This finding was in accord with the observation of others (Vanderzwalmen *et al.*, 1999, 2002)—that the efficiency of vitrification of human blastocysts was negatively correlated with the expansion of the blastocoele.

One reason for the lower survival rate in more developed blastocysts could be the presence of a large amount of blastocoele fluid, in which ice may form. A study on mouse blastocysts also reported that survival rates of vitrified blastocysts after a one-step exposure to EFS40 decreased as the volume of the blastocoele cavity increased (Miyake *et al.*, 1993). In mouse blastocysts, two-step loading of cryoprotectant was effective in preventing the decrease in post-warming survival of fully expanded blastocysts (Zhu *et al.*, 1993). Bovine blastocysts were also successfully vitrified after a two-step loading of cryoprotectant (Mahmoudzadeh *et al.*, 1995). However, human blastocysts seem to be different (unpublished results; Vanderzwalmen *et al.*, 2002), and thus must have specific characteristics related to the lower cryoresistance. The characteristics could be attributable to the permeability of the membrane, which would decrease as the blastocyst develops. Therefore, it would be preferable to cryopreserve human blastocysts on day 5, before the blastocoele is fully developed.

The pregnancy rate in human assisted reproduction is affected not only by embryonic factors but also by factors related to patients. It is well known that the age of the recipient has significant effects (Wang *et al.*, 2001). In the present study, the pregnancy rate was lower in older women (33%) than in

younger women (40%), although the difference was not statistically significant (Table VI). The cause of infertility affected the pregnancy rate significantly; when infertility was related to male factors, the rate was high (Table VII), probably because recipient women had no problems. However, the morphological survival of vitrified blastocysts was not affected either by the age of the woman (Table VI) or the cause of infertility (Table VII). This shows that these factors do not affect the quality of embryos, at least in terms of cryoresistance.

While 28% (21/76) of pregnancies ended in miscarriages, this outcome cannot be attributable to cryopreservation, because 22% (27/126) of pregnancies established from fresh blastocyst transfers in the authors' clinics during the same period also resulted in miscarriage. There was no statistically significant difference among the two miscarriage frequencies, though others (Ménézo *et al.*, 2000) reported that the overall miscarriage rate with frozen blastocysts was 20%.

In conclusion, the clinical outcome with 725 vitrified embryos and 207 transfers confirmed that vitrification using the cryoloop technique is effective and practical for the cryopreservation of human blastocysts. Early blastocysts on day 5 seem to be the most suitable in terms of stage and age for cryopreservation, but more developed blastocysts and even retarded ones on day 6 can also be cryopreserved, which would lead to the best use of valuable embryos.

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