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Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos

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Experiments were conducted to find a suitable cryoprotectant and suitable procedure for vitrification of 8-cell mouse embryos. The method was then applied clinically to the cryopreservation of human embryos in our assisted reproduction programme. Mouse embryos were vitrified with 30 or 40% 1,2-propanediol (PROH), dimethylsulphoxide (DMSO), ethylene glycol, glycerol, or acetamide, each diluted with a solution containing 30% Ficoll plus 0.5 M sucrose. Embryos were exposed to the solutions for 0.5 or 2 min at 20 or 25°C, cooled in liquid nitrogen and warmed rapidly. Embryo survival was assessed by in-vitro development. In PROH-, DMSO- and acetamide-based solutions, higher survival rates (29–82%) were obtained with less permeating conditions, suggesting that these cryoprotectants are considerably toxic. In glycerol- and ethylene glycol-based solutions, however, higher survival rates (74 and 92% respectively) were obtained with more permeating conditions, suggesting that these cryoprotectants are less toxic. Human embryos on days 2–3 were vitrified in an ethylene glycol-based solution (EFS40). Survival, assessed by the morphology, was higher in 4-cell embryos on day 2 and 8-cell embryos on day 3 than in 2–3-cell embryos on day 2 or 2–7-cell embryos on day 3. From 18 transfers, one ended with the delivery of healthy twin babies.

Key words: embryo/human/in-vitro fertilization/mouse/vitrification

Introduction

In assisted reproductive technology (ART), cryopreservation of embryos has several advantages. By storing embryos in liquid nitrogen, it is possible to transfer a limited number of embryos on successive occasions. This makes the best use of the embryos produced, reduces the number of attempts needed to retrieve oocytes, and reduces the chance of multiple pregnancies. Storage in liquid nitrogen also makes it possible to eliminate the risk of ovarian hyperstimulation syndrome because embryos can be transferred to patients in unstimulated cycles.

The first human pregnancy resulting from cryopreserved embryos was reported by Trounson and Mohr (1983). They equilibrated 4–8-cell embryos in 1.5 M dimethylsulphoxide (DMSO), cooled the samples slowly (at 0.3°C/min) to –80°C, and then plunged them into liquid nitrogen. Later, Zeilmaker *et al*. (1984) reported the birth of monozygotic twins who originated from a frozen–thawed 8-cell embryo. The embryo had been suspended in 1.45 M DMSO and frozen slowly to –40°C before rapid cooling in liquid nitrogen. Lassalle *et al*. (1985) reduced the slow cooling stage further; embryos were equilibrated in a solution containing 1.5 M 1,2-propanediol (PROH) + 0.1 M sucrose, and the slow cooling stage was terminated at –30°C. Since then, many in-vitro fertilization (IVF) centres have adopted the 'interrupted slow freezing' method of Lassalle *et al*. (1985) for the cryopreservation of human embryos, although the original long slow cooling method of Trounson and Mohr (1983) using DMSO has also proven to be effective (Van der Elst *et al*., 1995). Even in the interrupted slow method, however, the whole procedure takes ⩾2 h, and an expensive programmable freezer is necessary to control the slow cooling stage. Moreover, in clinical ART programmes, only a small number of embryos can generally be cryopreserved in each case. Therefore, a faster and simpler method is desirable.

To eliminate the slow cooling process, an ultrarapid freezing method was developed, in which human embryos suspended in a higher concentration of DMSO + sucrose were plunged directly into liquid nitrogen (Trounson *et al*., 1988). Although successful pregnancies were obtained from embryos preserved by this method (Barg *et al*., 1990; Gordts *et al*., 1990), more attention has been focused on vitrification, in which embryos suspended in a more concentrated solution are preserved in liquid nitrogen without ice formation (Rall and Fahy, 1985). However, the first attempt to vitrify human embryos was unsuccessful (Quinn and Kerin, 1986), probably because the original vitrification solution, containing DMSO, PROH and acetamide, was highly toxic. Thereafter, few trials have been reported on the vitrification of human embryos. For other mammalian embryos, on the other hand, various solutions have been developed (Kasai, 1996), and very high survival rates have been reported in some cases, e.g. mouse 8-cell embryos (Rall, 1987), mouse morulae (Kasai *et al*., 1990a) and rabbit morulae (Kasai *et al*., 1992). In vitrification, high survival rates are expected because the absence of the extracellular ice should reduce the chances of cell injury.

In vitrification, the selection of a cryoprotectant requires care because its concentration can be as high as 8 M. For freezing human embryos, PROH and DMSO have been the dominant cryoprotectants, although glycerol is used when embryos are frozen at the blastocyst stage (Cohen *et al*., 1985;

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Table I. Vitrification solutions

Permeating cryoprotectant	Concentration (v/v)	
	30%	40%
1,2 Propanediol	PFS30	PFS40
Dimethylsulphoxide	DFS30	DFS40
Glycerol	GFS30	GFS40
Ethylene glycol	EFS30	EFS40
Acetamide	AFS30	AFS40

All the concentrations were diluted with FS solution (PB1 medium containing 30% Ficoll 70 + 0.5 M sucrose).

Ménézo *et al*., 1992). On the other hand, ethylene glycol is now widely used for vitrification of various mammalian embryos (Kasai, 1996). In a few recent trials for vitrification of human embryos, ethylene glycol-based solutions have also been used (Ohta *et al*., 1996; Vanderzwalmen *et al*., 1997). However, few comparative studies have examined the effect of the cryoprotectant on the survival of vitrified embryos. In the present study, experiments were conducted to find a suitable cryoprotectant and suitable conditions for exposing embryos to the vitrification solution using 8-cell mouse embryos as a model. Based on these conditions, human embryos were subsequently vitrified. After thawing, viable embryos were transferred to women in our ART programme.

Materials and methods

Mouse embryos

Female ICR mice (CLEA Japan Inc, Tokyo, Japan) aged 6–12 weeks were stimulated to ovulate using i.p. injections of 5 IU of pregnant mare's serum gonadotrophin (PMSG, Serotropin; Teikokuzoki, Tokyo, Japan) and 5 IU of human chorionic gonadotrophin (HCG, Puberogen; Sankyozoki, Tokyo, Japan) given 48 h apart. They were then mated with male ICR mice. At 67–68 h after HCG injection, the females were killed by cervical dislocation and 8-cell embryos were flushed from the removed oviducts with modified phosphate-buffered saline (PB1) (Whittingham, 1971). Only embryos with eight distinct blastomeres were used, and those with compacted blastomeres were not included.

Vitrification solution

As cryoprotectants, five permeating agents (PROH, DMSO, glycerol, ethylene glycol, and acetamide) were used. They were mixed with two non-permeating agents, Ficoll 70 (average molecular weight 70 000, Pharmacia, Uppsala, Sweden) as a macromolecule to facilitate vitrification of the solution, and sucrose as a low molecular weight compound which causes cells to shrink. Each permeating cryoprotectant was diluted to 30 or 40% (v/v) in FS solution (PB1 medium containing 30% w/v Ficoll plus 0.5 M sucrose). Thus, 10 solutions were prepared, and were named after the initials of the components with the concentration of the cryoprotectant (Table I). The final concentrations of Ficoll for 30 and 40% cryoprotectant solutions were 21 and 18%, and the final concentrations of sucrose for the 30 and 40% cryoprotectant solutions were 0.35 and 0.3 M respectively.

Vitrification of mouse embryos

The room temperature was adjusted in order to equilibrate the instruments and all the solutions at 20 or 25°C. 8-cell embryos were treated and loaded in a 0.25 ml straw essentially by the simple method described elsewhere (Kasai *et al*., 1990). Briefly, embryos were directly suspended in a vitrification solution at room temperature, and about 10 embryos were loaded in a straw. The configuration of the straw was described previously (Kasai *et al*., 1990). At 30 s or 2 min after exposure of the embryos to a vitrification solution, the straws were plunged into liquid nitrogen. When embryos were exposed for only 0.5 min, they were introduced into the vitrification solution in the straw directly, and when the embryos were exposed to the solution for 2 min, they were first suspended in the vitrification solution in a watch glass, washed in the solution twice, and then transferred into the vitrification solution in the straw.

Straws were taken out of liquid nitrogen and immediately plunged into water at 20 or 25°C, depending on the room temperature at which the embryos had been treated. After ~5 s, the straws were removed from the water, quickly wiped dry and the contents of the straw were expelled into a watch glass containing 0.8 ml of PB1 medium containing 0.5 M sucrose (S-PB1 medium), by pushing the cotton plug with a steel rod. The embryos were then pipetted into fresh S-PB1 medium. Approximately 5 min after being flushed out, the embryos were transferred to a drop of fresh PB1 medium prepared under paraffin oil in a culture dish.

Assessment of survival of vitrified mouse embryos

Embryos recovered after vitrification were washed and cultured in 0.2 ml modified Krebs–Ringer bicarbonate medium (mKRB; Toyoda and Chang, 1974) under paraffin oil in a culture dish in an incubator at 37°C in an atmosphere of 5% CO_2 in air. Within a few hours, the embryos were assessed for their morphological appearance under a dissecting microscope. Then the survival of embryos was assessed by their ability to develop to expanded blastocysts in culture.

Human embryos

The patients in this study (both male and female) were undergoing treatment in our IVF programme and agreed to use the vitrification method to cryopreserve some of their viable embryos. The women were treated with gonadotrophin-releasing hormone (GnRH) agonist (buserelin acetate, Suprecure; Hoechst Japan, Tokyo, Japan) and human menopausal gonadotrophin (HMG, Humegon; Organon Japan, Tokyo) in either a long- or a short-treatment protocol. Oocytes were collected using the vaginal ultrasound-guided procedure. The oocytes were inseminated by either conventional IVF or by intracytoplasmic sperm injection (ICSI); in a few cases, testicular spermatozoa were used for ICSI. The oocytes were then cultured in HTF medium (Quinn *et al*., 1985) supplemented with 0.5% human serum albumin in a CO_2 incubator. On day 2 and day 3 after oocyte retrieval, 1–4 normally fertilized and cleaved embryos were transferred into the uterus, and the remaining embryos at the 2–8-cell stage were cryopreserved by vitrification.

Vitrification of human embryos

Embryos were treated in a room at 25–27°C. From the results of experiments on mouse embryos, an ethylene glycol-based solution, EFS40, was selected as the vitrification solution for human embryos. The toxicity of EFS40 was quite low (Figure 1) and high survival rates were obtained with all the exposure conditions used (Table II). However, in the vitrification of mouse embryos, it is known that the survival of 2–4-cell embryos is lower than that of 8-cell embryos (Miyake *et al*., 1993), and a two-step method is recommended for the vitrification of 2-cell mouse embryos; that is, pretreatment of embryos in a solution with 20% ethylene glycol (EFS20) followed by a brief exposure to EFS40 (Kasai, 1997). Because we vitrified human embryos at the 2–8-cell stage, which is the most commonly used stage, the 2-step method was adopted for the vitrification of

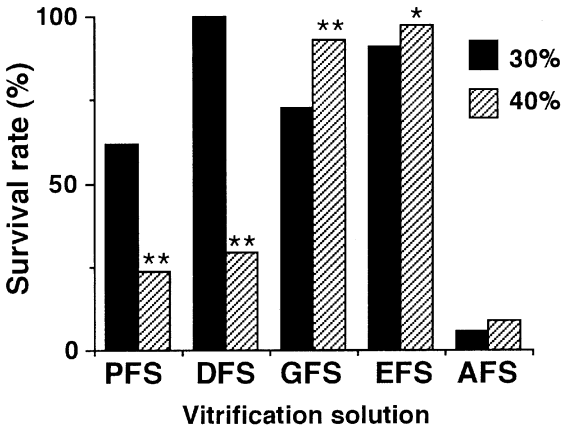

Figure 1. Survival of vitrified 8-cell mouse embryos, assessed by their ability to develop to expanded blastocysts, expressed as the percentage of morphologically normal embryos at recovery for each vitrification solution, regardless of the time and temperature of exposure. Vitrification solutions contain 30% (closed bars) or 40% (hatched bars) permeating cryoprotectant. ** $P<0.01$, *$P<0.05$; significantly different within solutions containing the same permeating cryoprotectant. AFS = acetamide-based solution; PFS = propanediol-based solution; DFS = dimethylsulphoxide-based solution; GFS = glycerol-based solution; EFS = ethylene-based solution.

human embryos. For equilibration, embryos were first suspended for 2 min in EFS20, followed by 1 min of exposure to EFS40 at room temperature. In addition, since it has recently been reported that fracture damage can be prevented by including moderate cooling and warming steps (Kasai *et al.*, 1996), each straw was loaded with between one and five embryos; the straws were then positioned horizontally in the vapour phase above the liquid nitrogen for 3 min before being plunged into liquid nitrogen.

Embryos were thawed and recovered as described above for mouse embryos, except that the straws were kept in air for 10 s before immersion in water at 27–28°C. By including the moderate cooling and warming steps, fracture damage can be prevented (Kasai *et al.*, 1996). For manipulating embryos, sterile culture dishes were used instead of watch glasses. The recovered embryos were assessed for the apparent integrity of each blastomere under a dissecting microscope, and morphological scores were calculated from the number of normal blastomeres per total number of blastomeres for each treated embryo. The embryos were cultured, and between one and four embryos with ⩾50% morphologically normal blastomeres were transferred to each recipient woman.

Transfer of human embryos and luteal support

Embryos with 50% or more intact blastomeres were transferred into the donor women on the day of thawing. Patients with controlled endometrial cycles were treated with 50 mg progesterone per day for 14 days, while patients receiving embryos during stimulated cycles were treated with 50 mg progesterone on days 3, 8 and 11, and 3000 IU HCG on day 5 after transfer. Pregnancy was assumed when the serum and urine β-HCG concentrations were positive on day 12 after embryo transfer.

Statistical analysis

The survival rate of mouse embryos after each treatment was compared using the χ^2 test, unless the expected frequency was <5, in which case Fisher's exact probability test was used. The morphological score of human embryos was compared with Wilcoxon's test.

Results

Vitrification of mouse embryos

The survival rates of 8-cell embryos vitrified in various solutions after exposure to the solutions for 0.5 or 2 min at 20 and 25°C are summarized in Table II. In total, 95.3% (1606 out of 1685) of vitrified embryos were recovered, and only 1.6% (27 out of 1685) of recovered embryos were found to be devoid of the zona pellucida. Survival, assessed by the developmental potential *in vitro*, was affected by the vitrification solution and by the time and temperature of exposure.

When embryos were vitrified in PROH-based solutions, the highest survival rate (82%) was obtained when embryos were treated with 30% PROH (PFS30) for 2 min at 20°C. However, when the temperature was raised to 25°C or the concentration of PROH was increased (PFS40), the survival rate was low (0–63%), even when high proportions of morphologically normal cells were recovered.

With DMSO-based solutions, the highest survival rate was only 53% with 0.5 min of exposure to DFS30 at 25°C, and survival further decreased when embryos were vitrified with DFS40 (0–33%).

In glycerol-based solutions, very low survival rates, 0–22 and 3–32%, were obtained when the embryos were treated with the lower concentration of glycerol (GFS30) at 20 and 25°C respectively. Survival rates increased (74%) when embryos were treated with a higher concentration of glycerol (GFS40), especially for a longer exposure time (2 min), and at a higher temperature (25°C).

The highest levels of survival were obtained with ethylene glycol-based solutions. Although none of the vitrified embryos were morphologically normal when embryos were vitrified after 0.5 min exposure to EFS30, the survival rate was over 90% when embryos were treated for a longer time (2 min) at a higher temperature (25°C), or when embryos were treated with a higher concentration of ethylene glycol (EFS40) at a higher temperature (25°C).

Acetamide-based solutions were more toxic than other solutions, since only 0–10% of the recovered embryos developed in culture even when they were morphologically normal at recovery.

Vitrification of human embryos

The survival of human embryos after vitrification with EFS40 is shown in Table III. In total, 52 embryos were vitrified, and all of them were recovered; none of the embryos had damaged zonae pellucidae, suggesting that the moderate cooling and warming methods adopted may have been effective in preventing fracture damage. However, large numbers of embryos need to be tested before this can be proven.

In normally developing embryos, i.e. in 4-cell embryos on day 2 and 8-cell embryos on day 3, 85–86% of the blastomeres were morphologically intact. On the other hand, in retarded embryos, i.e. in 2–3-cell embryos on day 2 and 2–7-cell embryos on day 3, only 58% of the blastomeres were morphologically normal. In all the embryos, the morphological score was higher with embryos fertilized by conventional IVF (75.0%, $n = 28$) than with those fertilized by ICSI (53.2%,

Table II. Survival of mouse 8-cell embryos vitrified in various solutions after exposure for 0.5 or 2 min at 20 or 25°C

Vitrification solutions	Exposure		No. of embryos				
	Temperature (°C)	time (min)	vitrified	recovered (%)	without zona (%)	with normal morphology (%)*	survived[b] (%)**
PFS30	20	0.5	40	37 (93)	0 (0)	12 (32)	3 (8)[c]
		2	40	39 (98)	0 (0)	33 (85)	32 (82)[a]
	25	0.5	45	37 (82)	0 (0)	15 (41)	10 (27)[c]
		2	40	36 (90)	2 (6)	27 (75)	8 (22)[c]
PFS40	20	0.5	40	38 (95)	0 (0)	28 (74)	24 (63)[a]
		2	40	36 (90)	2 (6)	12 (33)	0 (0)[c]
	25	0.5	40	38 (95)	1 (3)	36 (95)	0 (0)[c]
		2	40	38 (95)	1 (3)	27 (71)	0 (0)[c]
DFS30	20	0.5	40	38 (95)	0 (0)	11 (29)	15 (39)
		2	38	38 (100)	0 (0)	17 (45)	14 (37)
	25	0.5	40	40 (100)	0 (0)	22 (55)	21 (53)
		2	40	38 (95)	0 (0)	13 (34)	16 (42)
DFS40	20	0.5	40	40 (100)	0 (0)	29 (73)	13 (33)[a]
		2	40	37 (93)	2 (5)	10 (27)	0 (0)[c]
	25	0.5	40	40 (100)	1 (3)	20 (50)	2 (5)[c]
		2	40	40 (100)	1 (3)	10 (25)	5 (13)[b]
GFS30	20	0.5	40	40 (100)	0 (0)	0 (0)	0 (0)
		2	40	37 (93)	0 (0)	12 (32)	8 (22)
	25	0.5	40	39 (98)	0 (0)	1 (3)	1 (3)
		2	40	37 (93)	1 (3)	16 (43)	12 (32)
GFS40	20	0.5	40	39 (98)	5 (13)	18 (46)	17 (44)[c]
		2	50	42 (84)	2 (5)	27 (64)	23 (55)
	25	0.5	52	50 (96)	0 (0)	25 (50)	23 (46)[c]
		2	46	43 (93)	2 (5)	32 (74)	32 (74)[a]
EFS30	20	0.5	40	39 (98)	1 (3)	0 (0)	0 (0)[c]
		2	40	39 (98)	0 (0)	26 (67)	20 (51)[c]
	25	0.5	40	39 (98)	0 (0)	0 (0)	0 (0)[c]
		2	53	52 (98)	1 (2)	51 (98)	50 (96)[a]
EFS40	20	0.5	40	40 (100)	0 (0)	31 (78)	28 (70)[b]
		2	50	50 (100)	1 (2)	36 (72)	39 (78)[a,b]
	25	0.5	50	47 (94)	2 (4)	43 (91)	43 (91)[a]
		2	50	48 (96)	0 (0)	48 (100)	44 (92)[a]
AFS30	20	0.5	40	37 (93)	2 (5)	8 (22)	2 (5)
		2	40	39 (98)	0 (0)	23 (59)	0 (0)
	25	0.5	48	44 (92)	0 (0)	3 (7)	0 (0)
		2	40	35 (88)	0 (0)	0 (0)	0 (0)
AFS40	20	0.5	40	39 (98)	0 (0)	20 (51)	4 (10)
		2	40	39 (98)	0 (0)	22 (56)	0 (0)
	25	0.5	41	40 (98)	0 (0)	4 (15)	0 (0)
		2	42	42 (100)	0 (0)	0 (0)	0 (0)

*Embryos with all the blastomeres intact were considered morphologically normal.
**Survival was assessed by the ability to develop to expanded blastocysts in culture.
Values with different superscripts are significantly different within the same vitrification solution ($^{a,b}P < 0.05$; $^{a,c}P < 0.01$).

$n = 24$). This difference was statistically significant ($P < 0.05$; Wilcoxon's test).

In total, 41 embryos had 50% or more morphologically normal blastomeres. These embryos were transferred to 18 recipient women, and one of them who received four 4-cell embryos on day 2 became pregnant, with twin fetuses. She delivered healthy twin babies in the 34th week of gestation.

Discussion

In order to find suitable conditions for vitrification of mouse and human embryos, we first examined the effects of the cryoprotectant, its concentration, and the temperature and time of exposure of embryos to the vitrification solution before cooling, on the post-warming survival of vitrified mouse 8-cell embryos. Survival rates, assessed by the developmental potential *in vitro*, were highly variable, ranging from 0 to 96%, depending on the conditions used. The survival rates of vitrified embryos depend on several mechanisms of cell injury, such as the chemical toxicity of the cryoprotectant, intracellular ice formation, fracture damage, and osmotic swelling during the removal of the cryoprotectant.

For deducing the mechanism by which vitrified embryos are injured, morphological observation of recovered embryos under a dissection microscope is helpful. Fracture damage can be detected by zona injury. In this study, the frequency of fracture damage was quite low (1.6%) for all the treatments (Table II). If cells are injured by intracellular ice formation or osmotic swelling, the injuries must be physical, which can be distinguished by the morphology of the blastomeres. However, if cells are injured by the chemical toxicity of the cryoprotectant, the appearance of the blastomeres may be damaged, even when they lose developmental ability. Therefore, morphologically normal cells which do not develop in culture are most likely to have been injured by the chemical toxicity.

As an index of toxicity, survival rates assessed by in-vitro

Table III. Survival of human embryos ($n = 52$) after vitrification in EFS40

Development of embryos			No. of vitrified	Morphological score (%)[a]	No. of embryos	
	Age	Stage			Transferred	Implanted
Normal	Day 2	4-cell	7	85.7	6	2 (33)
	Day 3	8-cell	6	85.4	6	0 (0)
	Total		13	85.6[b]	12	2 (17)
Retarded	Day 2	2–3-cell	8	58.3	7	0 (0)
	Day 3	2–7-cell	31	58.0	22	0 (0)
	Total		39	58.0[c]	29	0 (0)

[a]Morphological scores of vitrified embryos were calculated from the proportion of normal blastomeres per each vitrified embryo.
[b,c]Values are significantly different ($P < 0.01$).

development were expressed as the percentage of morphologically normal embryos for each vitrification solution, regardless of the time and temperature of exposure (Figure 1). First, the graph shows that acetamide-based solutions (AFS30 and AFS40) are relatively toxic, because most of the morphologically normal embryos after vitrification in the solutions did not develop in culture. Secondly, in PROH- and DMSO-based solutions, the toxicity increased as the concentration of the cryoprotectant increased from 30% (PFS30 and DFS30) to 40% (PFS40 and DFS40), showing that PROH and DMSO are considerably toxic. On the other hand, glycerol based- and ethylene glycol-based solutions are less toxic, because most (96–98%) of the morphologically normal embryos could develop even after vitrification with solutions containing a higher concentration of cryoprotectant (GFS40, EFS40). These results are in agreement with the results of a previous study (Kasai, 1994), in which mouse morulae were exposed to these cryoprotectants without vitrification. In that study, the survival rate was highest with ethylene glycol, followed by glycerol, then DMSO, PROH, and finally acetamide.

If we consider the exposure conditions, the toxicity would be expected to be higher when the exposure time was longer or the exposure temperature was higher (Kasai *et al*., 1992b). For instance, 63 and 33% of vitrified embryos survived with 0.5 min of exposure to PFS40 and DFS40 at 20°C, respectively, but the survival rates dropped to 0–5% when the exposure time was increased to 2 min or exposure temperature was elevated to 25°C. These decreases can be attributed to the toxicity of the cryoprotectant.

In contrast to the toxicity, intracellular ice is more likely to be formed when the permeation of the cryoprotectant and its concentration in the cytoplasm are insufficient. That is, when the concentration of the cryoprotectant is lower, the exposure time is shorter, and the exposure temperature is lower. For instance, the proportions of morphologically normal embryos were consistently low when vitrified in solutions with 30% cryoprotectant after exposure for 0.5 min at 20°C (0–39%). In glycerol-based solutions and ethylene glycol-based solutions, however, the survival increased with increasing concentration of the cryoprotectant, with increasing exposure time, and with increasing temperature. These results suggest that embryos vitrified in less permeating conditions were injured by the formation of intracellular ice.

In our ART programme, normal embryos were first assigned for immediate transfer and then for conventional slow freezing, and the rest of the embryos were subjected to vitrification in the present study. Therefore, the quality of the vitrified embryos was variable, and the results in Table III show that the survival, assessed by the morphology of the blastomeres, of delayed embryos was lower than that of normally developed embryos. In delayed embryos, membrane properties, such as the permeability to cryoprotectants, might have been altered. In addition, cycle stage may be important in terms of cryosensitivity. In vitrification of zona-hatched mouse blastocysts, post-warming survival rates were higher in embryos that had developed earlier than in those that had developed later (Zhu *et al*., 1996). So, the viability of delayed embryos may be low, and may be reduced further by the stress of cryopreservation.

As a cryoprotectant for conventional freezing of human embryos, PROH has been widely used, although DMSO (Van der Elst *et al*., 1995) and glycerol (Cohen *et al*., 1985; Ménézo *et al*., 1992) have also proven effective. The results of the present study suggest that ethylene glycol is also a good candidate for human embryos. Actually, 8-cell human embryos have been successfully vitrified with an ethylene glycol-based solution quite similar to EFS40, in which sucrose was replaced with trehalose (Ohta *et al*., 1996), although the procedure for the vitrification was less practical, since the embryos were treated at 4°C before vitrification and diluted in five steps after warming. In another preliminary study (Vanderzwalmen *et al*., 1997), human morulae-blastocysts were vitrified with the EFS40, and after transfer, resulted in pregnancies.

Of a total of 18 transfers, one pregnancy was obtained in the present study. The low pregnancy rate probably reflects the fact that most of the embryos (29 out of 41) had delayed development, and many recipient women who received vitrified embryos had failed to become pregnant with previous transfers using fresh or conventionally frozen embryos. Thus, our results show that the simple vitrification method using an ethylene glycol-based solution is effective for the cryopreservation of human embryos. In a chromosomal analysis of human embryos vitrified with 5.5 M ethylene glycol plus 1 M sucrose, it has been shown that the incidence of anomalies in vitrified embryos is comparable to that in fresh embryos (Ali *et al*., 1995).

So far, no increase in congenital anomalies has been reported in babies born from frozen embryos in ART programmes (Society for Assisted Reproductive Technology, 1996). In North America, ~7000 frozen embryo transfer procedures

were reported in 1994 (Society for Assisted Reproductive Technology, 1996). Although this is a relatively substantial number compared with ~30 000 fresh cycles, a simple vitrification method may increase the use of cryopreserved embryos further, because the vitrification method greatly simplifies the cooling process and eliminates the use of elaborate equipment to control the cooling rate. Furthermore, vitrification has another advantage in that it may improve survival if the procedure is further optimized for human embryos, since all the physical and chemical injuries caused by extracellular ice are eliminated in vitrification.

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