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In Vivo Development of Vitrified Rat Embryos: Effects of Timing and Sites of Transfer to Recipient Females[1]

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ABSTRACT

In cryopreserved rat embryos, survival rates obtained in vitro are not always consistent with the rates obtained in vivo. To determine the optimal conditions for in vivo development to term, rat embryos at the 4-cell, 8-cell, and morula stages were vitrified in EFS40 by a one-step method and transferred into oviducts or uterine horns of recipients at various times during pseudopregnancy. Vitrified and fresh 4-cell embryos only developed after transfer into oviducts of asynchronous recipients on Days −1 to −2 of synchrony (i.e., at a point in pseudopregnancy 1–2 days earlier than the embryos). Approximately half the vitrified embryos transferred into oviducts on Day −1 developed to term, but only a minority of embryos, whether vitrified (10%–34%) or fresh (24%–33%), transferred at later times did so, suggesting that this may not be the most suitable stage for cryopreservation. Very few 8-cell embryos, either vitrified or fresh, developed when transferred into oviducts on Day 0 to −0.5. However, when transferred into uterine horns, high proportions of vitrified 8-cell embryos (~63%) developed to term in reasonably synchronous recipients (Day 0 to −0.5) but not in more asynchronous ones (6%; Day −1). A majority of vitrified morulae also developed to term (52%–68%) in a wider range of recipients (Days 0 to −1), the greatest success occurring in recipients on Day −0.5. Similar proportions of vitrified and fresh 4-cell embryos, 8-cell embryos, and morulae developed to term when appropriate synchronization existed between embryo and recipient. Thus, vitrification of preimplantation-stage rat embryos does not appear to impair their developmental potential in vivo.

early development, embryo, oviduct, pregnancy, uterus

INTRODUCTION

Transfer of embryos to recipient females has become a valuable experimental tool, particularly in the fields of embryology and genetics [1]. Since Heape [2] performed the first successful embryo transfer in the rabbit, many studies have been carried out on the transfer of mammalian embryos. In rats, the first transfer experiment constituted an embryo viability test and established the importance of synchrony between donors and recipients [3]. Later studies demonstrated that the development of transferred embryos is dependent on close synchronization between embryonic development and endometrial preparation in a number of mammalian species, such as rabbits [4, 5], mice [6], sheep [7], rats [8], cattle [9], and ferrets [10]. It has also been shown that asynchrony is more tolerated when embryos are at a more progressed stage than the recipient uteri [6, 8, 10].

Rat embryos have been successfully cryopreserved at various developmental stages, such as the 1-cell [11, 12], 2-cell [13–15], 4-cell [13], 8-cell [13, 16–18], morula [19], and late-morula to early blastocyst [14] stages. In many cases, survival of the embryos was assessed by transfer to recipients, probably because the in vitro culture system for rat embryos was not as effective as that for mouse embryos. Consequently, reported survival rates have been variable but generally low.

In a recent study [20], we compared the survival of vitrified rat embryos, ranging from the 1-cell to the blastocyst stage, using an efficient culture system (in vitro) and a successful embryo-transfer technique (in vivo). Because very high proportions (94%–100%) of vitrified embryos developed in vitro, we concluded that the 4-cell, 8-cell, and morula stages are suitable for embryo cryopreservation; furthermore, the good developmental potential in vitro of these embryos led us to expect they would develop equally well in vivo. However, the in vivo survival rate of vitrified 4-cell embryos was relatively low (40%) and that of 8-cell embryos extremely low (4%), although similar poor results were obtained with fresh embryos (29% and 5%, respectively). In contrast, the in vivo survival rate of vitrified morulae (61%) was high and very similar to that of fresh embryos (70%). In that study, both fresh and vitrified 4- and 8-cell embryos were transferred into oviducts of pseudopregnant recipients that were at a point in pseudopregnancy 1 day earlier than the embryos (Day −1 of synchrony), whereas morulae were transferred to uterine horns of synchronous recipients (Day 0).

We hypothesized that it might be possible to improve the in vivo survival of vitrified embryos at all stages by adjusting the synchrony/asynchrony with respect to the recipient females and by altering the site of transfer. The present study was undertaken to test this hypothesis using rat embryos vitrified at the 4-cell, 8-cell, and morula stages.

MATERIALS AND METHODS

All experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction.

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TABLE 1. In vivo development of vitrified 4-cell rat embryos transferred into oviducts of recipients at various times during pseudopregnancy.

Embryo	Time of transfer: Day of pseudo-pregnancy	Time of transfer: Time (h)	Time of transfer: Day of synchrony	No. of recipients (pregnant/total)	No. of embryos transferred	No. (%) of implantation sites	No. (%) of young
Vitrified	1	0900	−2.5	0/7	98	15 (15)[c]	0
		2100	−2.0	4/6	83	56 (67)[d]	8[a] (10)[c]
	2	0900	−1.5	5/5	70	32 (46)[e]	24[ab] (34)[d]
		2100	−1.0	3/3	42	23 (55)[e]	22[a] (52)[e]
	3	0900	−0.5	0/5	61	1 (2)[f]	0
		2100	0	0/5	67	0	0
Fresh	1	2100	−2.0	6/6	83	62 (75)[c]	20 (24)[g]
	2	2100	−1.0	4/4	48	20 (42)[d]	16 (33)[g]
	3	2100	0	0/5	68	0	0

[a] Including one dead neonate.
[b] Including one dead fetus on Day 25.
[c–f] Values with different superscripts within each column in each group of embryos differ significantly ($P < 0.05$).
[g] Values differ significantly from those in vitrified embryos at the same time of transfer ($P < 0.05$).

Collection of Embryos

Outbred Wistar rats were bred in-house and kept in a room under a 14L:10D photoperiod (lights-on, 0600–2000 h; lights-off, 2000–0600 h). Mature female rats (age, 2–3 mo) at proestrus, as assessed from vaginal smears, were placed overnight with mature males. On the following morning (Day 1 of pregnancy), the females were examined for mating by the presence of a vaginal plug or spermatozoa in the vagina. Mated females were humanely killed by cervical dislocation at 2100–2200 h on Day 3 of pregnancy for 4-cell embryos, at 0800–0900 h on Day 4 for 8-cell embryos, and at 1800–2000 h on Day 4 for morulae. Embryos were recovered by flushing the excised oviducts and/or uterine horns with modified phosphate-buffered saline (PB1) [21].

Vitrification of Embryos

For vitrification, EFS40 was used [22]; this solution was 40% (v/v) ethylene glycol and 60% (v/v) PB1 medium containing 30% (w/v) Ficoll 70 (average molecular weight, 70 000; Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and 0.5 M sucrose. Thus, the final concentrations of Ficoll 70 and sucrose in EFS40 were 18% (w/v) and 0.3 M, respectively.

Embryos were vitrified in EFS40 in 0.25-ml Cassou straws (IMV, L'Aigle, France) following the procedure described by Kasai et al. [22]. All the procedures were conducted in a room at 25°C. Before freezing, PB1 medium containing 0.5 M sucrose (S-PB1) was drawn up into a straw to a depth of ~60 mm, followed by air (25–30 mm), EFS40 (~5 mm), another volume of air (~5 mm), and finally, more EFS40 (~12 mm). Twelve to 14 embryos were transferred directly from PB1 medium into the larger volume of EFS40 in the straw, and the straw was sealed. After exposure of embryos to EFS40 for 30 sec, the straw was positioned in the liquid nitrogen vapor phase by placing it horizontally on a styrofoam boat (thickness, ~1 cm) floating on the surface of the liquid nitrogen for at least 3 min in a Dewar vessel (inner diameter, 140 mm). The straw was then immersed in liquid nitrogen.

After being stored in liquid nitrogen for at least 1 day, each straw was kept in air for 10 sec and then immersed in water at 25°C. When the crystallized S-PB1 medium in the straw began to melt (after ~7 sec), the straw was removed from the water and quickly wiped dry, and the contents of the straw were then expelled into a watch glass by flushing the straw with 0.8 ml of S-PB1 medium. After gently agitating the watch glass to promote mixing of the contents, the embryos were pipetted into fresh S-PB1 medium. Approximately 5 min after being flushed out, the embryos were transferred to fresh PB1 medium.

Embryo Transfer

The females were stimulated by inserting a glass rod connected to an electric vibrator into the vagina at 1930–2000 h on the day of proestrus (Day 0 of pregnancy) to induce pseudopregnancy. Transfers were then carried out on specified days of pseudopregnancy. Because we did not have access to animal rooms with different lighting schedules, it was only possible to adjust the synchrony by intervals of 24 h for investigations using fresh embryos. However, with vitrified embryos (the focus of the present study), it was possible to adjust the synchrony by smaller intervals. Morphologically normal vitrified embryos were recovered in PB1 medium as described above and transferred to pseudopregnant females without further culture.

Vitrified 4-cell embryos were transferred into oviducts at 0900 and 2100 h on Day 1, 2, or 3 of pseudopregnancy. The time of 2100 h on Day 3 of pseudopregnancy was designated as Day 0 of synchrony, because 4-cell embryos were collected at the same hour on Day 3 of pregnancy. As a control, uncultured fresh 4-cell embryos were transferred into oviducts at 2100 h on Day 1, 2, or 3 of pseudopregnancy. Vitrified 8-cell embryos were transferred into oviducts or uterine horns at 0800 and 2000 h on Day 3 or at 0800 h on Day 4 of pseudopregnancy (the latter time was Day 0 of synchrony for 8-cell embryos). As a control, uncultured fresh 8-cell embryos were transferred into oviducts or uterine horns at 0800 h on Day 3 or 4 of pseudopregnancy. Because the results in our previous study [20] were poor when either fresh or vitrified 8-cell embryos were transferred into oviducts on Day −1 of synchrony, those treatment conditions were not repeated in the present study. Vitrified morulae were transferred into uterine horns at 0600 and 1800 h on Day 3 or 4 of pseudopregnancy; the time of 1800 h on Day 4 of pseudopregnancy was designated as Day 0 of synchrony. As a control, uncultured fresh morulae were transferred into uterine horns at 1800 h on Day 3 or 4 of pseudopregnancy.

Six to seven embryos were transferred to each oviduct or uterine horn. Transfer of embryos into oviducts was conducted as described by Toyoda and Chang [23], except that a small drop of 0.1% epinephrine solution was put on the surface of the bursal membrane before cutting the membrane to prevent bleeding. Transfer into uterine horns was conducted as described by Miyoshi et al. [24]. After transfer, vaginal smears from the recipients were examined daily. Recipients showing proestrus or estrus were killed, and their uterine horns were examined for implantation sites. However, recipients that showed proestrus or estrus on the fourth or fifth day were considered to be nonpseudopregnant and were excluded from the present study. Pregnant females were allowed to give birth and were then killed. Females that had not delivered by Day 25 of pregnancy were killed, and their uterine horns were examined for resorption sites, implantation sites, and fetuses.

Statistical Analyses

The data were analyzed using chi-square tests unless the expected frequency was less than five, in which case the Fisher exact probability test was used.

RESULTS

In Vivo Development of 4-Cell Embryos

As shown in Table 1, when 4-cell embryos were transferred into oviducts of synchronous recipients (Day 0 of synchrony), none of the females became pregnant. Similarly, no recipients became pregnant after receiving vitrified embryos either on Day −0.5 (nearly synchronous) or on Day −2.5 (extremely asynchronous). However, when 4-cell embryos were transferred on Days −1 to −2 of synchrony, most females became pregnant. Although implantation rates

TABLE 2. In vivo development of vitrified 8-cell rat embryos transferred into either oviducts or uterine horns of recipients at various times during pseudopregnancy.

Embryo	Time of transfer: Day of pseudo-pregnancy	Time of transfer: Time (h)	Time of transfer: Day of synchrony	Transfer site	No. of recipients (pregnant/total)	No. of embryos transferred	No. (%) of implantation sites	No. (%) of young
Vitrified	3	0800	−1.0	Uterus	1/6	79	10 (13)[c]	5 (6)[c]
		2000	−0.5	Oviduct	2/5	68	3 (4)[c]	3[a] (4)[c]
				Uterus	7/7	98	79 (81)[d]	62 (63)[d]
	4	0800	0	Oviduct	2/5	67	4 (6)[c]	3[b] (4)[c]
				Uterus	6/6	77	70 (91)[d]	48 (62)[d]
Fresh	3	0800	−1.0	Uterus	4/6	84	26 (31)[cf]	18[ab] (21)[cf]
	4	0800	0	Oviduct	1/5	66	3 (5)[d]	2 (3)[d]
				Uterus	6/6	80	68 (85)[e]	53[b] (66)[e]

[a] Including one to three dead fetuses on Day 25.
[b] Including one to two dead neonates.
[c–e] Values with different superscripts within each column in each group of embryos differ significantly ($P < 0.05$).
[f] Values differ significantly from those in vitrified embryos at the same time of transfer ($P < 0.05$).

in recipients on Day −2 were relatively high with both vitrified and fresh embryos, less than a quarter of the transferred embryos developed to term, and of these, significantly more ($P < 0.05$) fresh embryos than vitrified embryos completed development. In contrast, when transferred on Day −1, significantly more ($P < 0.05$) vitrified embryos than fresh embryos developed to term. Although more vitrified embryos developed to term when transferred on Day −1 to −1.5 than on Day −2, fresh embryos developed to term equally well when transferred on either Day −1 or Day −2.

In Vivo Development of 8-Cell Embryos

As shown in Table 2, when 8-cell embryos were transferred to oviducts of recipients on Day 0 or Day −0.5, the pregnancy rates were low with both vitrified and fresh embryos. In contrast, when embryos were transferred to uterine horns on either Day 0 or Day −0.5, all the recipients became pregnant; in more asynchronous females (Day −1), fewer pregnancies occurred irrespective of embryo type.

Thus, transfers to recipients on Day 0 to −0.5 were much more successful when vitrified embryos were transferred into uterine horns than into oviducts; transfers into more asynchronous recipients (Day −1) gave very poor results. Similarly, when fresh embryos were transferred into uterine horns of asynchronous recipients (Day −1), less than a third implanted and developed to term. In synchronous recipients, most embryos implanted, and approximately two thirds developed to term. Transfer into oviducts of synchronous recipients resulted in poor development.

In Vivo Development of Morulae

As shown in Table 3, when morulae were transferred into uterine horns on Days 0 to −1 of synchrony, all recipients became pregnant, and high rates of implantation and full-term development were obtained with both vitrified and fresh embryos. The highest rate of full-term development with vitrified embryos was obtained with transfer to slightly asynchronous females (Day −0.5); those results were significantly ($P < 0.05$) higher than those obtained after synchronous transfer and equivalent to those obtained from transfer of fresh embryos on Day −1. In contrast, when vitrified embryos were transferred to females on Day −1.5, two females failed to get pregnant, and implantation and full-term development was very poor in the remaining five females.

DISCUSSION

Several decades ago, Noyes and Dickmann [1] reported that fresh rat embryos obtained 3 days after mating (possibly at the 2- to 4-cell stage) developed poorly after transfer into uterine horns of synchronous recipients. Greater success was obtained when such embryos were transferred into oviducts of recipients on Day −1 or Day −2 of synchrony. Although there has been general interest regarding cryopreservation of mammalian embryos, very few studies using cryopreserved rat embryos at similar developmental stages have been published. In the first such study, Whittingham [13] transferred frozen-thawed 4-cell embryos into oviducts of recipients on Day −2 of synchrony but obtained

TABLE 3. In vitro development of vitrified rat morulae transferred into uterine horns of recipients at various times during pseudopregnancy.

Embryo	Time of transfer: Day of pseudo-pregnancy	Time of transfer: Time (h)	Time of transfer: Day of synchrony	No. of recipients (pregnant/total)	No. of embryos transferred	No. (%) of implantation sites	No. (%) of young
Vitrified	3	0600	−1.5	5/7	93	17 (18)[b]	9[a] (10)[b]
		1800	−1.0	6/6	80	54 (68)[c]	46[a] (58)[cd]
	4	0600	−0.5	8/8	110	81 (74)[c]	75 (68)[c]
		1800	0	8/8	112	78 (70)[c]	58 (52)[d]
Fresh	3	1800	−1.0	6/6	75	67 (89)[e]	53 (71)
	4	1800	0	5/5	67	53 (79)	42 (63)

[a] Including one dead neonate.
[b–d] Values with different superscripts within each column in each group of embryos differ significantly ($P < 0.05$).
[e] Values differ significantly from those in vitrified embryos at the same time of transfer ($P < 0.001$).

no live fetuses. Subsequently, rat embryos ranging from the 1-cell to the blastocyst stage, but not at the 4-cell stage, have been cryopreserved. To our knowledge, our investigations are the first to obtain live young from cryopreserved 4-cell rat embryos [20; present study].

In the present study, when we varied the degree of synchrony between embryo and pseudopregnant recipient, reasonable results with vitrified 4-cell embryos were obtained only with asynchronous recipients (Day −1), with 52% developing to term. However, we think that this result may be slightly misleading, because only three recipients on Day −1 were used in the present study. In our earlier study [20], six recipients at this stage were used. Of those, five got pregnant, and 40% of embryos developed to term. Taken together, these results suggest that vitrified 4-cell embryos are only moderately able to develop to term. Surprisingly, the best results with fresh embryos (24%–33%) were either lower than or only just comparable to those obtained with vitrified embryos. This may reflect the fact that we could not adjust lighting conditions in the animal unit to vary the degree of synchrony for recipients of fresh embryos; thus, we could only adjust the synchrony by intervals of 24 h (Days −1 and −2) in experiments using fresh embryos. In contrast, with vitrified embryos we were able to assess developmental potential following transfer to recipients on Days −0.5, −1.5, and −2.5, as well as −1 and −2, of synchrony. Thus, survival of 4-cell rat embryos does not decrease significantly after vitrification, which is consistent with our recent demonstration that as much as 94% of 4-cell embryos vitrified by our method could develop into blastocysts in culture [20].

The proportion of vitrified 4-cell embryos that developed to term (34%–52%) (Table 1) may be adequate for practical use, but even higher success rates were obtained with vitrified 8-cell embryos (62%–63%) (Table 2) and morulae (58%–68%) (Table 3). For 8-cell embryos, transfer into oviducts was not effective, but transfer into uterine horns (especially of more synchronous recipients) was very effective. These results led us to evaluate transfer of vitrified 4-cell embryos into uterine horns of recipients on Day 0 to −0.5, but the in vivo survival rate was very low (unpublished observations). Therefore, we conclude that the lower in vivo survival of 4-cell embryos, whether cryopreserved or fresh, reflects something unusual about this developmental stage in the rat.

Higher proportions of transferred embryos can develop in vivo when recipients are either synchronous with or at a slightly earlier stage than the embryos. In both the present study and that of Han et al. [20], few (<15%) 8-cell embryos, either vitrified or fresh, developed to term when transferred into oviducts, regardless of the day of synchrony, indicating that the site of transfer is very important. Consistent with our results, Noyes and Dickmann [1] found that in vivo development of fresh embryos obtained 4 days after mating (possibly at the 8-cell to morula stage) was well supported when transferred into uterine horns, but not oviducts, of synchronous recipients. However, comparatively low rates (0–38%) of in vivo development of cryopreserved 8-cell rat embryos have been reported even after synchronous transfer into uterine horns [16–18]. In contrast to those earlier investigations, in the present study the majority (~63%) of vitrified 8-cell embryos developed to term when transferred into uterine horns of recipients on Day 0 to −0.5 (Table 2), which is very similar to the results with fresh embryos (66%). Thus, the in vivo developmental potential of 8-cell embryos is not markedly reduced by vitrification using EFS40; indeed, 100% of vitrified and thawed 8-cell embryos had normal morphology and, thus, could be transferred [20]. When transferred into uterine horns of more asynchronous recipients (Day −1), however, only a minority of both vitrified and fresh 8-cell embryos developed to term (6%–21%). This may indicate that earlier in the cycle, the uterine environment is hostile to such embryos; results in the various earlier studies cited above suggest that in the uterine cycle, there are times when conditions within the uterus can either kill or prevent implantation of early embryos. In contrast, the majority of morulae (Table 3) developed to term following transfer to both synchronous (Day 0 to −0.5) and asynchronous (Day −1) recipients, suggesting that these older embryos are more flexible in their requirements for implantation and subsequent development.

Kasai et al. [19] reported that 0%–50% of rat morulae frozen-thawed in the presence of various cryoprotectants developed to term after transfer to synchronous recipients. In our previous study [20], when rat morulae were transferred to synchronous recipients, 61% of vitrified embryos developed to term, which is very similar to the 70% rate obtained with fresh embryos. In the present study, high proportions of vitrified morulae developed to term when transferred to either asynchronous (Day −0.5 to −1) or synchronous (Day 0) recipients, but the best results were obtained following transfer into slightly asynchronous recipients (Day −0.5). Thus, vitrified 8-cell embryos and morulae appear to have similar developmental potential. However, the morula may be a more convenient stage for embryo cryopreservation, because the synchrony requirements of the recipients appear to be less exacting.

In conclusion, although approximately half the vitrified 4-cell rat embryos developed to term in asynchronous recipients (Day −1), this does not appear to be the optimal stage for cryopreservation; even fresh embryos had a similarly modest developmental potential. On the other hand, high proportions (>60%) of vitrified 8-cell embryos and morulae developed to term when transferred into uterine horns of more synchronous recipients (Day 0 to −0.5) or slightly asynchronous recipients (Day −0.5 to −1), respectively. The proportions of vitrified 4-cell, 8-cell, and morula-stage embryos developing to term were all comparable with the results for fresh embryos as long as the recipient was at the appropriate stage of synchrony. We therefore conclude that the full-term developmental potential of rat embryos at these stages is not damaged by vitrification, although further experiments are needed to determine whether similar results can be obtained with other strains of rats.

Because the rat is a very important model for the study of human disease and a traditional experimental model system, it is vital to have genetic integrity and transportability of strains. The present data suggest that vitrified rat embryos may provide a valuable experimental tool that will allow investigators using the rat model system to achieve these requirements.

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Symposium: Cryopreservation and assisted human conception

Cryopreservation of animal and human embryos by vitrification

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Abstract

Vitrification is a method in which not only cells but also the whole solution is solidified without the crystallization of ice. For embryo cryopreservation, the vitrification method has advantages over the slow freezing method. For example, injuries related to ice is less likely to occur, embryo survival is more likely if the embryo treatment is optimized, and embryos can be cryopreserved by a simple method in a short period without a programmed freezer. However, solutions for vitrification must include a high concentration of permeating cryoprotectants, which may cause injury through the toxicity of the agents. Since the development of the first vitrification solution, which contained dimethylsulphoxide, acetamide, and propylene glycol, numerous solutions have been composed and reported to be effective. However, ethylene glycol is now most widely used as the permeating component. As supplements, a macromolecule and/or a small saccharide are frequently added. Embryos of various species, including humans, can be cryopreserved by conventional vitrification using insemination straws or by ultrarapid vitrification using minute tools such as electron microscopic grids, thin capillaries, minute loops, or minute sticks, or as microdrops. In the ultrarapid method, solutions with a lower concentration of permeating cryoprotectants, thus having a lower toxicity, can be used, because ultrarapid cooling/warming helps to prevent ice formation.

Keywords: *cryopreservation, embryo, ethylene glycol, human, vitrification*

Introduction

In cryopreservation, cells are suspended in a suitable solution, cooled, stored in liquid nitrogen, warmed to room temperature, and returned to a physiological solution. During each step of this process, cells are at risk for various types of injuries. The primary injury is that caused by the formation of intracellular ice during cooling and warming. To prevent this injury, inclusion of a cryoprotectant is essential for large cells like mammalian embryos. However, the cryoprotectant brings other causes of injuries, i.e. chemical toxicity of the agent and osmotic over-swelling of the cells during removal of the permeated cryoprotectant. During the removal, embryos are usually exposed to a hypertonic solution with sucrose, and embryos can be injured by osmotic over-shrinkage in some cases. In addition, embryos can be dissected physically by a fracture plane if such a plane is formed in the medium and traverses the embryos during passage through the glass transition temperature. Furthermore, certain types of embryos are injured just by chilling at 20–0°C. In order for embryos to survive cryopreservation, the effect of each of these injuries must be minimized (Kasai *et al.*, 2002).

Vitrification is a reasonable and effective strategy for preventing the primary cause of injury, that is, intracellular ice formation. Fracture damage and chilling injury may also be minimized in vitrification. Vitrification also has advantages over slow freezing in that survival of embryos is more likely if the embryo treatment is optimized, and embryos can be cryopreserved by a simple method in a short period without a programmed freezer. This study provides a brief outline of cryopreservation of animal and human embryos by vitrification.

Principle of vitrification

Vitrification in slowly frozen samples

Cryopreservation of mammalian embryos became possible in 1972, when Whittingham, Leibo, and Mazur reported the successful deep freezing of mouse embryos, resulting in the production of viable young. Their method was the slow freezing method, in which embryos are loaded with cryoprotectant by being suspended in a solution supplemented with 1–1.5 mol/l cryoprotectant, ice-seeded, and cooled very slowly (0.3–0.5°C/min) so that the cellular contents become concentrated by gradual dehydration in response to the concentration of the extracellular unfrozen fraction during the growth of extracellular ice, before the sample is placed in liquid nitrogen. The slow cooling stage is necessary to prevent the formation of intracellular ice in large cells.

When samples are cooled in liquid nitrogen after sufficient dehydration, the cytoplasm of the embryos, together with the extracellular concentrated fraction in which embryos are suspended, will turn to a glassy solid without ice formation, that is, they will vitrify (**Figure 1**). Vitrification can be defined as the solidification of a solution by an extreme elevation in viscosity without crystallization. Therefore, in an aqueous solution, vitrification means ice-free solidification, while freezing means ice formation. Even in slow freezing, therefore, embryos must be vitrified, because intracellular ice is fatal. The cytoplasm is vitrified at the glass transition temperature, which is around –130°C.

The slow freezing method was proven effective for embryos of various mammalian species, and is routinely used for the cryopreservation of mammalian embryos, mainly in mice, cattle, and humans. The disadvantage of this method is that it requires a long time for cooling and a programmed freezer to control the cooling rate.

Vitrification method

If embryos are suspended, at 0°C or above, in a highly concentrated solution, similar to the extracellular unfrozen fraction in the slowly cooled sample, and the cellular contents are concentrated, embryos should be able to survive direct cooling into liquid nitrogen, with both extracellular solution and cytoplasm being vitrified. Based on this idea, Rall and Fahy (1985) devised a vitrification method using mouse embryos. With this method, embryos suspended in a vitrification solution can be cooled in liquid nitrogen in a few seconds (**Figure 1**). This approach eliminated the slow cooling process and the use of elaborate equipment to control it.

An additional advantage of this approach is that a high level of embryo viability may be maintained. This is primarily attributed to the smaller chance of intracellular ice forming, although intracellular ice can form even in vitrification if the

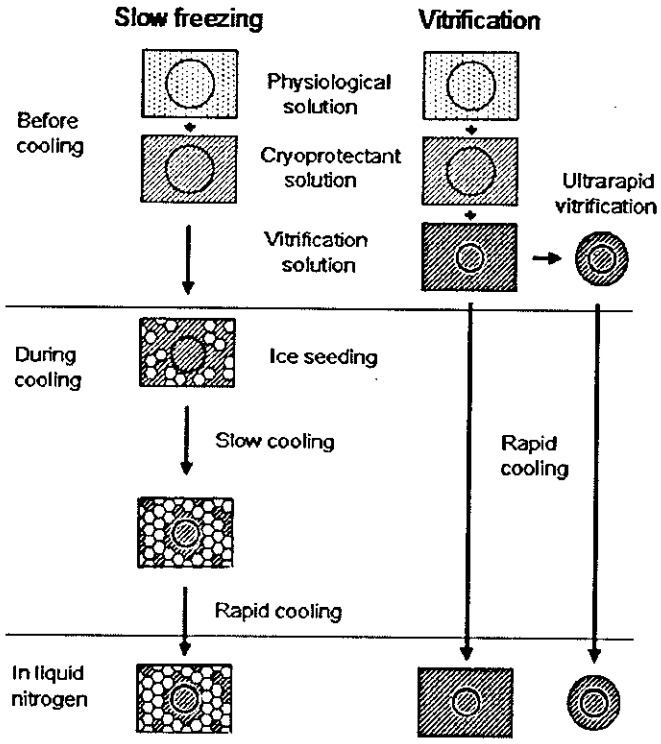

Figure 1. Schematic presentation of an embryo (circle) before cooling, during cooling and in liquid nitrogen in slow freezing, conventional straw vitrification, and ultrarapid vitrification. White hexagons represent ice crystals.

concentration of cryoprotectant in the cell is not high enough. The biggest obstacle to this approach is the toxicity of the solution, because 4–8 mol/l of cryoprotectant is used to prevent extracellular ice from forming. Various improvements have been made to this approach, and it is now possible to cryopreserve many types of embryos with a minimal loss of viability, although more delicate handling of embryos is required than with the slow freezing method.

Ultrarapid vitrification

Unfortunately, consistently high survival has not been obtained after cryopreservation either by slow freezing or by vitrification. The first reason for the low survival would be the sensitivity of embryos to chilling; examples are bovine embryos at early cleavage stages (Leibo *et al.*, 1996) and pig embryos before the peri-hatching stage (Hayashi *et al.*, 1989). The second reason would be lower permeability of the cell membrane, which would lead to the formation of intracellular ice and also osmotic over-swelling. An example is the human blastocyst, which must be dehydrated and concentrated more slowly than earlier stage embryos (Mukaida *et al.*, 2001). The third reason would be the toxicity of the cryoprotectant during exposure of cells to the concentrated vitrification solution. In blastocysts with a large blastocoel, for instance, longer exposure would be necessary to make this space concentrated. Furthermore, some types of embryos (e.g. hamster embryos) are quite sensitive just to exposure to an in-vitro environment.

In an attempt to overcome these injuries, modified vitrification methods have been devised in which the cooling and warming rate is markedly increased by minimizing the volume of the solution and the container (**Figure 1**). The modified methods employ electron microscope (EM) grids (Martino *et al.*, 1996), thin capillaries called open pulled straws (OPS) (Vajta *et al.*, 1998), minute nylon loops called cryoloops (Lane *et al.*, 1999a,b), tiny plastic tools called hemi-straws (Vanderzwalmen, 2003) or cryotops (Hiraoka *et al.*, 2004), or containerless microdrops (Le Gal and Massip, 1999). By this approach, critical temperatures at which cells are injured by chilling could be passed through quickly enough to minimize the injury, intracellular ice might be prevented from forming even in less concentrated cells, and the use of a vitrification solution with a lower concentration of the cryoprotectant, thus a less toxic solution, may be possible. The effectiveness of this approach has been approved by many reports (Kasai, 2002).

Vitrification solutions

Permeating cryoprotectant, the essential component

To prevent the injury caused by the formation of intracellular ice in mammalian embryos, cryoprotectant must permeate into the cell. Therefore, a permeating agent is an essential component in all vitrification solutions. In their pioneering report, Rall and Fahy (1985) used a solution called VS1, which contains dimethylsulphoxide (DMSO), acetamide, and propylene glycol as permeating components. Thereafter, numerous vitrification solutions have been devised not only with these three agents but also with glycerol and ethylene glycol, in combination or as a single permeating component. So far, these five agents have almost exclusively been used as the permeating components for the vitrification of mammalian embryos. The mechanism of the protective properties of the agents is considered the same, but the toxicity and the permeating properties of the agents are quite different.

Among the five agents, ethylene glycol and glycerol are less toxic than DMSO and propylene glycol, and acetamide is the most toxic, at least to mouse morulae (Kasai, 1994). Ethylene glycol is the most permeating for mouse morulae, and glycerol is the least permeating for one-cell mouse embryos (Kasai, 1996). However, the permeating properties of the cryoprotectants are different in embryos at other stages. Rapidly permeating agents are generally favourable, because exposure time before cooling can be shortened, and because they are more likely to diffuse out of the cell rapidly, which helps prevent osmotic swelling.

Macromolecule, a supplementary component

In addition to permeating cryoprotectants, a macromolecule is frequently incorporated in the vitrification solution, though not an essential component. The incorporation of a macromolecule may promote vitrification of a solution. If so, it would be possible to reduce the toxicity of the solution by decreasing the concentration of permeating agents required for vitrification of the solution. Rall and Fahy (1985) first used polyethylene glycol. Although other macromolecules such as polyvinylpyrrolidone (Leibo and Oda, 1993) and bovine serum albumin (Rall, 1987) are used thereafter, Ficoll 70 seems to have advantages of lower toxicity, higher solubility, and lower viscosity (Kasai *et al.*, 1990). Macromolecules are non-permeating to the cell and much less toxic than permeating agents.

Small saccharide, a supplmentary component

Macromolecules contribute to the osmolality of the solution only a little, while mono- or disaccharides exert considerable osmotic effects as smaller non-permeating molecules. Incorporation of a small saccharide therefore promotes dehydration and thus intracellular vitrification, and it reduces the amount of intracellular cryoprotectant, which will help reduce the toxic effect of the cryoprotectant. In fact, it was found that incorporation of sucrose reduced the toxicity of a vitrification solution significantly (Kasai *et al.*, 1990). Inclusion of a small saccharide will also help prevent over-swelling of the cell during removal of the permeated cryoprotectant.

As a small saccharide, sucrose is most commonly incorporated in the vitrification solution, although other saccharides such as trehalose, glucose, and galactose have also been shown to be effective (Kasai, 1997). It has been shown that sucrose, and probably other saccharides, are virtually non-toxic when used at a refrigerated temperature (Kasai, 1986), although they can be harmful at higher temperatures (Kasai *et al.*, 1992a).

Solutions for vitrification

Considering that the essential component is the permeating agent, vitrification solutions can be classified into four categories: those with only a permeating agents(s), those with a permeating agent(s) plus a macromolecule, those with a permeating agent(s) plus a small saccharide, and those with all three components. On the other hand, vitrification solutions can also be classified by the permeating agent, which is the primary component. In the following sections, some major solutions classified by this criterion are described.

Before the sample is vitrified in liquid nitrogen, some types of embryos are directly suspended in the vitrification solution in one step. Other types of embryo need a two-step procedure for the exposure: embryos are first suspended in a dilute solution containing a lower concentration of a cryoprotectant at room temperature for permeation without causing toxic injury, and then they are exposed to a vitrification solution for a short period or at low temperature. For the dilute solution, only a permeating agent(s) is incorporated; ethylene glycol or DMSO is most frequently used.

Vitrification of animal embryos

Vitrification with solutions containing DMSO, acetamide, and propylene glycol (DAP)

The original vitrification solution of Rall and Fahy (VS1) was composed of 2.6 mol/l DMSO, 2.6 mol/l acetamide, 1.3 mol/l propylene glycol and 6% polyethylene glycol. However, because the toxicity of this solution is quite high, embryos were treated stepwise at a refrigerated temperature. The efficacy of this solution (and of a slightly dilute solution, 90% VS1) was proven for mouse (Rall *et al.*, 1987) and bovine (Lopez-Gatius and Camon-Urgel, 1989) embryos, but this solution is not now used. Nakagata (1993) modified VS1 and created a solution with 2 mol/l DMSO, 1 mol/l acetamide, and 3 mol/l propylene glycol, naming it DAP213. This solution is used for the cryopreservation of mouse embryos (Nakao *et al.*, 1997), although embryos still have to be exposed to the solution at 0°C. As the container of embryos, insemination straws (0.25 ml) are most commonly used, but Nakagata's group uses cryotubes.

Vitrification with a solution containing ethylene glycol, Ficoll, and sucrose (EFS)

Considering the cryoprotective properties of the three categories of agents, Kasai *et al.* (1990) created a low-toxicity vitrification solution named EFS40. This solution comprises representatives of three different categories of agents, 40% (7.2 mol/l) ethylene glycol as a low toxicity permeating agent, 18% Ficoll 70 as a highly soluble macromolecule, and 0.3 mol/l sucrose as a small saccharide. The solution was first composed for vitrifying mouse morulae; a quite high proportion of them survived vitrification after brief exposure to the solution at room temperature. Then it was confirmed that EFS40 is effective not only for mouse embryos at other stages (Miyake *et al.*, 1993) but also for rabbit morulae (Kasai *et al.*, 1992b), bovine blastocysts (Tachikawa *et al.*, 1993), horse blastocysts (Hochi *et al.*, 1994), and sheep embryos (Martinez and Matkovic, 1998), if the embryo treatment is optimized. Embryos of mastomys (Mochida *et al.*, 1998) and Mongolian gerbil (Mochida *et al.*, 1999) have been successfully cryopreserved only by vitrification with EFS40. Recently, it has been shown that high proportions of rat embryos at various stages can develop after vitrification with this solution (Han *et al.*, 2003, 2004).

Vitrification with solutions containing ethylene glycol and DMSO

Ishimori *et al.* (1992) reported that a solution with 25% (4.5 mol/l) ethylene glycol and 25% (3.5 mol/l) DMSO was effective for vitrifying mouse embryos. This solution was also proven effective for bovine embryos (Ishimori *et al.*, 1993). A solution with 20% (3.6 mol/l) ethylene glycol and 20% (2.7 mol/l) DMSO is reported effective for rabbit morulae (Vicente and Garcia-Ximenez, 1996) and goat blastocysts (El-Gayar *et al.*, 2001).

More recently, a solution with 15–20% (2.7–3.6 mol/l) ethylene glycol, 15–20% (2.1–2.8 mol/l) DMSO, 1% Ficoll, and 0.65 mol/l sucrose was used for an ultrarapid approach. Like EFS40, this solution contains all three components, permeating agents, a macromolecule, and a small saccharide. Using cryoloops, hamster embryos were successfully cryopreserved for the first time with this solution (Lane *et al.*, 1999a). Porcine embryos, which are sensitive to chilling, are also successfully vitrified using OPS with a similar solution that does not contain Ficoll (Berthelot *et al.*, 2000).

Vitrification with solutions containing ethylene glycol and glycerol

In the study cited above, Ishimori *et al.* (1992) also created a vitrification solution with 25% (4.5 mol/l) ethylene glycol and 25% (3.4 mol/l) glycerol. Although this solution was not more effective than a solution with ethylene glycol plus DMSO for mouse embryos (Ishimori *et al.*, 1992), with ethylene glycol plus glycerol, it has been shown to be effective for vitrification of sheep (Naitana *et al.*, 1995) and bovine (Agca *et al.*, 1998) embryos with straws and monkey blastocysts with cryoloops (Yeoman *et al.*, 2001). A solution with 20% (3.6 mol/l) ethylene glycol and 20% (2.7 mol/l) glycerol, supplemented with small saccharides and polyethylene glycol, was used for vitrifying llama embryos (Aller *et al.*, 2002).

Vitrification of human embryos

Vitrification of early stage embryos with straws in DMSO-based solutions

Soon after the first report of Rall and Fahy (1985) on the vitrification of mouse embryos with VS1 (containing 6.6 mol/l permeating cryoprotectants), Quinn and Kerin (1986) tried to vitrify human embryos using the same protocol. Unfortunately, however, only one of 11 embryos developed in culture after warming. In a solution with a lower concentration of cryoprotectant (4.5 mol/l DMSO with 0.3 mol/l sucrose), 2- to 16-cell human embryos survived cryopreservation by direct plunging into liquid nitrogen, resulting in four pregnancies out of 38 transfers (Barg *et al.*, 1990) and nine pregnancies out of 92 transfers (Feichtinger *et al.*, 1991). Although they did not

describe the method as vitrification, the solution containing over 30% (4.2 mol/l), DMSO would actually vitrify in liquid nitrogen. Using a solution containing 4.5 mol/l cryoprotectant (2.25 mol/l DMSO and 2.25 mol/l propylene glycol), one-cell zygotes were also successfully cryopreserved with four pregnancies out of 54 transfers (Van den Abbeel *et al.*, 1997).

Vitrification of early stage embryos with straws in ethylene glycol-based solutions

In 1998, Mukaida *et al.* reported that the protocol for vitrification of 8-cell mouse embryos using EFS40 is effective for day 2–3 human embryos, resulting in a twin pregnancy. Using a vitrification solution with 40% (7.2 mol/l) ethylene glycol, 18% Ficoll, and 0.3 mol/l trehalose (EFT) (Yoshino *et al.*, 1993), which is quite similar to EFS40, Ohta *et al.* (1996) vitrified 8-cell human embryos, which resulted in the birth of twin babies. Later, their group reported five pregnancies out of 31 transfers with 8- to 16-cell embryos vitrified by the same protocol (Saito *et al.*, 2000).

Vitrification of blastocysts in straws

Since sequential culture media enabled the production of blastocysts, the need for cryopreservation of blastocysts has increased. Yokota *et al.* (2001) used a vitrification solution containing 25% (4.5 mol/l) ethylene glycol and 25% (3.5 mol/l) DMSO (50% in total) and succeeded in the cryopreservation of human blastocysts (six pregnancies out of 18 transfers). However, vitrification of blastocysts with straws in EFS40 (containing 40% ethylene glycol) did not result in high survival (unpublished observation by Mukaida *et al.*). This is probably because human blastocysts are less permeable to water, and ice is more likely to form in the blastocoelic cavity during cooling/warming. In 2002, Vanderzwalmen *et al.* showed that straw vitrification with EFS40 is effective if the blastocoelic cavity of blastocysts had been artificially collapsed before vitrification; they reported eight pregnancies out of 35 transfers.

Vitrification of embryos with minute tools

Lane *et al.* (1999b) suggested that the ultrarapid vitrification method using cryoloops would be effective for cryopreservation of human blastocysts, because the chance for intracellular ice formation must be minimized. Then, Mukaida *et al.* (2001) showed, using a solution with 15% (2.7 mol/l) ethylene glycol, 15% (2.1 mol/l) DMSO, 1% Ficoll, and 0.65 mol/l sucrose, that the cryoloop method is actually effective; they reported six pregnancies out of 19 transfers with vitrified embryos. Later, Mukaida *et al.* (2003a) reported that this method resulted in consistently high rates of pregnancy with 76 pregnancies of 207 transfer cycles (37%). Reed *et al.* (2002) also reported the effectiveness of this method (two pregnancies of four transfers). The precise procedure for the cryoloop method has been described (Mukaida *et al.*, 2003b).

At the same time, other minute tools for ultrarapid vitrification have been shown to be effective for human embryos. Choi *et al.* (2000) vitrified blastocysts with EM grids in a solution containing 5.5 mol/l ethylene glycol and 1 mol/l sucrose, and reported five pregnancies out of 20 transfers. Cho and his group also vitrified blastocysts with EM grids in EFS40, and obtained a twin pregnancy (Son *et al.*, 2002) and 14 pregnancies (of 41 transfers) (Cho *et al.*, 2002). They also reported that artificial reduction of the blastocoel may improve the pregnancy rate (12 pregnancies of 25 transfers) after vitrification even with ultrarapid cooling/warming (Son *et al.*, 2003). As another minute tool, the OPS was proven effective for zygotes (two pregnancies out of four transfers by Selman and El-Danasouri, 2002; four pregnancies out of 10 transfers by Isachenko *et al.*, 2003) and embryos at early cleavage stages (11 pregnancies out of 36 transfers by El-Danasouri and Selman, 2001).

Conclusion

Vitrification is an effective method for embryo cryopreservation. Although a solution with DMSO, acetamide, and propylene glycol is still available, solutions with ethylene glycol are more widely used, because ethylene glycol is less toxic and more permeable to embryos (especially at later stages). For chilling-sensitive embryos like pig embryos, and for less permeating embryos like human blastocysts, ultrarapid vitrification using minute tools would be useful.

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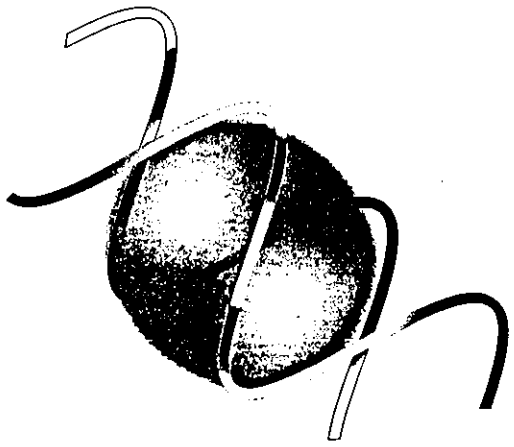

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卵子の凍結保存

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はじめに

ヒト不妊治療のためのARTの進歩とともに，凍結保存技術の果たす役割は増大している．体外受精で得られる余剰胚の凍結保存は，妊娠率の向上，多胎の防止，患者の負担軽減に役立てられている．さらに，受精前の卵子を凍結保存する試みが増加してきた．卵子の凍結保存は，未婚女性が放射線療法や化学療法によって卵巣内卵子に傷害を受ける場合や，卵子採取時に精子が得にくい場合などに利用できる．また，卵子提供が認められる場合にも有効な手段となる．さらに，胚に比べて取り扱いに倫理的制約が少ない利点もある．

1977年に，マウスにおいて，胚と同様の方法を用いて第2成熟分裂中期（MII期）の卵子を凍結保存できることが示された．また，1989年にはウサギで，1992年にはウシで，凍結保存したMII期卵子由来の産子が得られた．ヒトにおいては凍結胚による初の出産例が報告されたわずか2年後の1986年に，凍結保存したMII期卵子を用いた妊娠例が報告されている．しかし動物，ヒトのいずれにおいても，凍結保存した卵子の生存率や受精後の着床・発生率は凍結胚に比べてかなり低く，保存技術は実用的に普及するレベルには達していない．

卵巣中には，減数分裂前期の卵核胞期（GV期）にある未成熟な卵子が多数存在しており，ウシやブタの体外受精には，発育したGV期卵子が用いられている．一方，通常ヒト卵子は受精直前のMII期に回収されるが，GV期で回収されるケースも想定される．GV期卵子の凍結保存も試みられ，マウスにおいては1994年に，ウシにおいては1996年に，そしてヒトでは1998年に，それぞれ凍結保存したGV期卵子由来の産子が得られている．これらの成功を同じ種におけるMII期卵子の最初の成功と比較すると，マウスでは17年，ウシでは4年，ヒトでは12年遅れている．このことは，GV期卵子はMII期卵子よりもさらに凍結保存が困難なことを示している．

卵巣内の未成熟な卵子を凍結保存して利用する手段として，卵巣ごと凍結する手段が考えられる．メン羊，マウス，ラットでは，凍結保存した卵巣組織由来の産子が得られている．ヒトにおいても最近，卵巣組織の凍結保存の試みが多数報告されるようになってきた．卵巣の凍結は，凍結保存技術とともに，卵巣移植や未熟な卵子の体外発育技術が進めば，卵子の有効な保存法として期待できる．

本稿では卵子の凍結保存について，技術的側面から概説する．

1. 卵子の低温生物学的特性

哺乳動物卵子は特別大型の細胞で，細胞質の約85％は水分である．生理的な溶液に浸した卵子をそのまま氷点下まで冷却すると，細胞内の水分も凍結し氷晶が生じる．細胞内の氷晶は細胞の構造を物理的に破壊し，致命的な損傷を与える．卵細胞内に氷晶を形成させないためには，保存液に耐凍剤を加える必要がある．ジメチルスルフォキシド（DMSO），グリセロール，プロピレングリコール，エチレングリコール，アセトアミドなどの耐凍剤は，いずれも分子量100以下の荷電していない物質で，保存液と細胞質の浸透圧を高めることによって細胞内氷晶を防止する働きがある．

卵子の凍結保存においては，たとえ耐凍剤が存在していても，細胞内氷晶は依然として主要な傷害要因である．さらに，耐凍剤に由来する傷害（耐凍剤毒性，浸透圧的膨張，浸透圧的収縮）や，低温による傷害（低温傷害，フラクチャー）が生じる可能性もある（表1）．卵子を生存させたまま凍結保存するためには，これらの要因による致命的な影響を回避しなければならない．卵子では，胚と異なり，凍結保存後に正常な受精能を維持される必要がある．しかし，たとえこのハンディを考慮しても，卵子の耐凍性は，胚と比べると明らかに低い．このことは，上記の傷害要因と関連した卵子の低温生物学的特性，すなわち，形態，低温感受性，細胞膜の透過性，耐凍剤感受性，低張圧・高張圧に対する耐性などが，胚と異なるためと考えられる．幸いヒト卵子は低温傷害をもたらす低温感受性を有していない．また，フラクチャー傷害は，保存液に生じる物理的な亀裂によるもので，保存する細胞の種類とは関係がない．したがってここでは，形態，細胞膜の透過性，耐凍剤毒性，および低張圧・高張圧に対する耐性について考察する．

1）形　態

細胞内氷晶を防ぐためには，耐凍剤の透過と水分の流出による細胞質の濃縮が必要である．細胞膜を介した水や耐凍剤の出入りは，体積に対する表面積の割合が大きいほど速やかに進む．卵子が受精後，細胞質の体積が一定のまま球形の割球に卵割すると仮定すると，体積に対する表面積の割合は，2細胞期では卵子の126％，4細胞期では159％，8細胞期胚では200％まで増加する．このことは，卵子は，胚に比べて水分や耐凍剤の流入・流出に時間を要し，濃縮されにくいことを示している．

ところが，同じ1細胞の前核期胚では，体積に対する表面積の割合は卵子とほぼ同じであるにもかかわらず，耐凍性は卵子より高いことが知られている．卵子と前核期胚の違いの一つに表層粒がある．MII期の卵子では，細胞膜近くの細胞質内に多数の表層粒が存在しているのに対し，受精卵では精子の刺激によりすでに表層粒が囲卵腔に開裂している．表層粒の内容物の放出によって透明帯反応が誘起されると同時に，表層粒を構成していた膜成分は細胞膜に取り込まれる．したがって，受精卵の細胞膜は余剰な膜成分を多量に含むことになる．表層粒に由来する細胞膜成分の量は25％に達するとの試算もある．このことは，後述するように，浸透圧的膨張に対する耐性の違いを生じると考えられる．

胚の細胞は分裂期にある時間が短いのに対し，MII期卵子は核膜を持たず，染色体は赤道板にならんで紡錘体が付着した状態にとどまっている．したがって，もし凍結保存によって卵子の紡錘体に傷害が生じれば，第二減数分裂における染色体

表1　卵子凍結保存における主な細胞傷害の要因

原因	種類	模式図
	凍結前	
氷晶	細胞内氷晶（緩慢法）	
	細胞の氷晶（ガラス化法）	
耐凍剤	耐凍剤毒性	
	浸透圧的膨張	
	浸透圧的収縮	
低温	低温傷害	
	フラクチャー（緩慢法）	
	フラクチャー（ガラス化法）	

分離に異常をきたし，正常な発育は望めない．紡錘体を構成する微小管は，チューブリンが重合した構造を持つ．チューブリンは冷却によって脱重合することが知られており，さらに，耐凍剤によっても脱重合する可能性もある．したがって，MII 期卵子を凍結保存すると，染色体異常の割合が高まるのではないかと考えられる．一方，脱重合したチューブリンは加温すると再重合することが知られており，凍結することによって染色体異常が増加することはないとする報告もみられる．しかしいずれにしても，凍結融解卵子は，正常な再重合に必要な時間を考慮して，授精前に数時間培養することが必要である．また，凍結融解直後の細胞では，細胞内小胞輸送系が停止していると考えられており，この機能の回復のためにも，短時間の培養は必要である[1]．

GV 期卵子は，MII 期卵子とは異なり，紡錘体を有していない．そのため，MII 期卵子より凍結保存後の生存性が高いのではないかと期待されたが，実際は明らかに GV 期卵子の方が耐凍性は低い．GV 期の卵細胞は，透明帯を通して卵細胞と卵丘細胞と結合しており，凍結保存によって卵丘細胞が死滅あるいは剥離して失われやすいことも，発育能低下の一因になるであろう．

2）細胞膜の透過性

耐凍剤透過と水分の流出による卵細胞の濃縮をスムーズに進行させるためには，水と耐凍剤の速やかな透過が重要である．また，耐凍剤透過が遅いと長時間耐凍剤にさらす必要があり，耐凍剤の毒性による影響を受けやすい．さらに，耐凍剤透過性が低いと融解後の耐凍剤の除去過程に膨張による傷害も受けやすい．耐凍剤透過性は，耐凍剤の種類や卵子・胚の発育ステージによって大きく異なる．マウス，ウシ，ウサギの MII 期卵子について調べた結果，プロピレングリコール，DMSO，エチレングリコール，アセトアミドは徐々に透過するのに対しグリセロールの透過は極めて遅いという，共通した特性がみられた．したがって，グリセロールは卵子の耐凍剤としては適していないといえる．ヒト卵子の水や耐凍剤に対する透過性は，マウス卵子と類似していると思われるが，ヒトでは個々の卵子の透過性に差異が大きいと考えられており，このことも，ヒト卵子の凍結保存後の生存率が安定しない一因かもしれない．

細胞内外への水や耐凍剤の透過は，従来，脂質二重膜を通した拡散によると考えられていた．しかし近年，細胞膜上に発現する水チャンネルが次々と同定され，チャンネルを介した水の促進拡散の機構が明らかとなってきた．水チャンネル発見の業績に対しては，2003 年にノーベル化学賞が授与されている．水チャンネルは，アクアポリンと名付けられ，現在 0 から 10 までの，11 のタイプのアクアポリンが同定されている．さらにこれらのなかには，水だけではなく，中性低分子物質も透過させるタイプがあることも明らかとなった．たとえば，アクアポリン 3，7，9 は，グリセロールなどの耐凍剤を透過させる．マウスの卵子には，これらのタイプのアクアポリンの mRNA が存在することが判明したが，その発現量は非常に少ないと推測される．したがって，卵子における水や耐凍剤の透過は，主に脂質二重膜を介した経路で行われるため透過性が低いと考えられる．

3）耐凍剤の毒性

哺乳動物卵子凍結保存の耐凍剤としては，DMSO，アセトアミド，プロピレングリコール，エチレングリコールが用いられている．卵子に対する耐凍剤毒性を比較したデータは少ないが，胚に対して毒性の高い物質が卵子に対しても毒性が高いことは予想できる．筆者らが，アセトアミドを含む溶液によってマウス MII 期卵子をガラス化凍結したところ，融解後に正常な形態を維持した卵子の割合は高く，受精能も有していたが，移植後に産子まで発育できる割合は極めて低かった．マウス卵子の凍結には，アセトアミドを含む溶液も使用されているが，この耐凍剤のマウス胚に対する毒性は極めて高く，ヒト卵子に対しても何らかの毒性の影響を及ぼす可能性がある．

卵子に対する傷害のひとつに，透明帯の硬化が

ある．DMSOなどの耐凍剤は，表層粒を開裂させて透明帯反応を誘起し，精子を侵入させにくくさせることが知られている．DMSOによる表層粒の開裂は，保存液に血清アルブミンを加えることによって軽減できるといわれている．しかし，ヒトの体外受精では顕微授精（ICSI）が普及したことから，透明帯の硬化と関係なく容易に受精させることができるようになった．しかし，受精した卵子を透明帯からスムーズにハッチさせるためには人為的アシストが有効となるかもしれない．

卵子が耐凍剤によって活性化され，単為発生的に卵割する可能性が指摘されている．単為発生は，耐凍剤濃度が高く，処理温度が高く，処理時間が長いほど生じやすいことが報告されており，耐凍剤の処理条件を調整することによって，耐凍剤による単為発生はほとんど抑制することができる．単為発生は，卵子の成熟後の時間（エイジング）による影響が大きいため，MII期に達した卵子はできるだけ早く受精させることが重要であろう．

4）浸透圧的膨張

融解した卵子は内部に耐凍剤を含んでおり，これを除去する必要がある．しかし，もし卵子をいきなりPBSなどの等張液に戻すと，細胞が膨張して傷害を受ける可能性がある（表1）．これは，細胞内外の浸透圧が等しく保たれる過程で，耐凍剤が細胞外へ拡散するよりも速く水分が細胞内へ流入するためである．水分の流入を防ぐために，シュクロースを添加した希釈液が広く用いられている．シュクロース（分子量342）は細胞内に透過せず，細胞外の浸透圧を高めることによって細胞の膨張を緩和する働きがある．シュクロースは凍結保存液に添加されることも多いが，これは凍結時の濃縮の促進だけでなく，融解後の膨張の防止にも効果があると考えられる．

卵子は，表面積/体積比が小さく，耐凍剤透過性は高くないため，融解後の耐凍剤除去過程では，耐凍剤の拡散・流出に時間を要する．したがって，浸透圧的膨張による傷害を受けやすい．細胞膜の膨張に対する耐性は，卵子の発育ステージによって大きく異なる．マウスでは，MII期卵子の耐性は低く，受精直後の前核期胚で急激に高くなる（図1）．その後，卵割とともに低下し，桑実胚で最も低くなる[2]．これは，受精後は，上に述べたように，表層粒の開裂に由来する細胞膜成分の増加によって膨張に対する耐性が上昇し，卵割によって卵子の体積に対する表面積の割合が増加するために膨張に弱くなるためではないかと推測される．しかし，凍結・融解直後の卵子の膨張に対する耐性は，いずれの発育段階においても，新鮮な卵子に比べて低い．とくに卵子は，膨張に弱いことから，希釈直後の膨張は最小限にとどめなければならない．

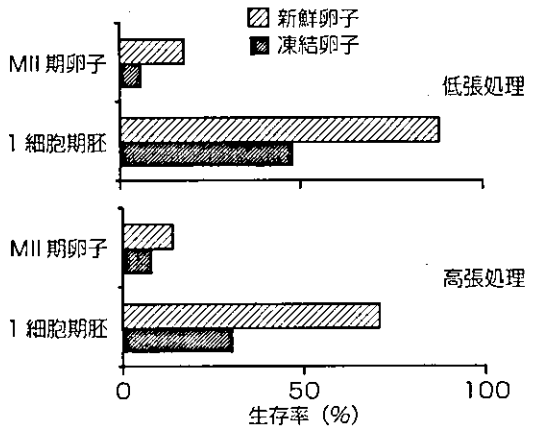

図1　25℃の低張液（0.2×PB1液）あるいは高張液（1.0Mシュクロース添加PB1液）で30分間処理したマウス卵子の生存性（形態）

ヒト胚の緩慢凍結には，1.5Mプロピレングリコールと0.1Mシュクロースを添加した溶液が広く用いられているが，ヒト卵子の凍結においては，シュクロース濃度が高い（0.3M）方が適していることが報告されている．また筆者らは，胚のガラス化保存液としてEFS液を用いているが，この液にも0.3Mシュクロースが含まれている．さらに，マウスMII期卵子をEFS40でガラス化凍結した場合，融解直後の希釈液のシュクロース濃度を1.5～2.0Mまで高めることによって生存率

が大きく改善されることを確認している．いずれも，膨張の防止に効果があると考えられる．

5）浸透圧的収縮

一方，細胞内の耐凍剤が外部に拡散した後には，細胞はシュクロースの影響によって収縮したままとなる．とくに室温においては，過度な収縮状態は傷害の原因となる（**表1**）．細胞膜の収縮に対する耐性も，発育ステージによって異なる[3]．マウスMII期卵子は，受精後の胚と比べて，収縮による傷害を受けやすい．また，膨張の場合と同様に，凍結・融解直後の卵子は，新鮮な卵子に比べて収縮に対する耐性が低い（**図1**）．MII期卵子が収縮によって傷害を受けやすい原因は不明であるが，融解後の耐凍剤除去過程においては，高濃度のシュクロースがもたらす過度な収縮状態に放置しないようにするべきである．

2．ヒト卵子の凍結保存

1986～87年に，凍結保存したヒトMII期卵子の体外受精による妊娠・出産例が報告されたが，その後約10年間は，卵子の凍結保存に関する報告数はわずかであった．しかし，1998年以降，ICSI技術の普及とともに，関連した試みは急激に増加している．凍結ヒト卵子を用いた体外受精の主な成功例を，**表2**に示した．データはPubMedで検索できる情報をもとにまとめたものであるが，他にも，学会等で報告された例もあり，また我が国でも宮城県（2001年）や長野県（2003年）のクリニックによって出産例が報告されている．しかし，現在までに誕生した子供の数は依然として少ない．

ヒト卵子の凍結保存法は，基本的に胚と同じ方法である．すなわち，保存液に氷晶形成させてゆっくり冷却する緩慢法と，保存液全体に氷晶を形成させずに急速に冷却するガラス化法に大別される（**図2**）．いずれの方法においても，細胞内氷晶形成を防ぐことが最大の課題である．緩慢法では，比較的低濃度（1～1.5M）の耐凍剤を卵子に十分透過させ，毎分約0.3℃の緩慢な速度で冷却したのち液体窒素（－196℃）に投入する．当初は－70℃以下まで緩慢冷却したのち液体窒素に投入する方法が用いられたが，現在では，緩慢冷却過程を－30℃前後で中止して液体窒素に浸す方法が普及している．緩慢法では，緩慢冷却の過程に細胞内の水分の流出を促し，細胞内を徐々に濃縮させることで細胞内氷晶を防止する．一方，ガラス化法（Vitrification法）では，5Mを越える高濃度の耐凍剤を含む保存液に浸して，直接液体窒素で冷却し，保存液ごと氷晶形成させずにガラス化する．一般的なガラス化法としては，緩慢凍結法と同じストロー（0.25ml）を用いた方法が普及しているが，最近では，微細な道具（電子顕微鏡用グリッド，キャピラリー，ループ，スティックなど）を用いる超急速ガラス化法が考案され用いられている[4]．この新しい手法は，冷却・加温速度を早めることによって氷晶形成を抑制することをねらいとしている．保存液が微量なために卵子をガラス化溶液で処理する温度，濃度，時間などの条件の影響を受けやすく，安定したテクニックが必要であるが，とくにヒト卵子ではその有効性が注目されている．

卵巣内の卵子は胎児期に形成され，その後減少し続けるが，卵巣組織内には未熟な卵母細胞が多数存在している．この卵巣内の未熟な卵子を体外で発育させる技術が開発されつつある[5]．マウスでは原始卵胞内の卵子を，ウシでは発育途上の卵胞内卵子を，それぞれ体外で発育させたのち産子が得られている．未熟卵子の体外発育は最初の成功例の報告にとどまっているが，ヒトにおいても，将来卵胞内の未熟な卵子を利用することができるようになるかもしれない．したがって，小さい卵胞内の未熟な卵子を卵胞ごと凍結保存することも考えられる．しかし，卵巣から取り出した卵胞をそのまま凍結保存する方法による産子は，動物においてもまだ得られていない．

3．ヒト卵巣の凍結保存

卵子を卵巣組織ごと凍結保存する試みもなされ

ている．ヒトにおいては，卵巣を摘出する場合に保存しておくケースが想定される[6]．卵巣組織はさまざまな種類の細胞から構成されており，各細胞の機能が保持されなければならない．卵巣凍結は古くから試みられており，1960年に－79℃に凍結保存したラット卵巣から産子が得られてい

表２　ヒト卵子凍結保存の成功例

Stage	年	著者	文献	凍結法	耐凍剤	受精	妊娠数	分娩数
MII	1986	Chen	Lancet i : 884	Slow	1.5 D	IVF	1 (2)	?
	1987	van Uem et al.	Lancet i : 752	Slow *	1.5 D	IVF	1	1
	1987	Al-Hasani et al.	HR 2 : 695	Slow	1.5 D/1.5 P＋0.1 S	IVF	2	?
	1988	Diedrich et al.	ANYAS 541 : 562	Slow	1.5 D	IVF	1	?
	1989	Siebzehnrubl et al.	HR 4 : 312	Slow	1.5 D	IVF	1	1
	1996	Tucker et al.	HR 11 : 1513	Slow	1.5 P＋0.1 S	ICSI	3	0
	1997	Porcu et al.	FAS 68 : 724	Slow	1.5 P＋0.2 S	ICSI	1	1
	1998	Tucker et al.	HR 13 : 3156	Slow	1.5 P＋0.1 S	ICSI	5	1 (2)＋1?
	1998	Polak de Fried et al.	FAS 69 : 555	Slow	1.5 P＋0.1 S	ICSI	1	?
	1998	Young et al.	FAS 70 : 360	Slow	1.5 P＋0.2 S	ICSI	1 (3)	?
	1998	Nawroth & Kissing	AOGS 77 : 462	Slow	1.5 P＋0.1 S	ICSI	1	0
	1999	Porcu et al.	JARG 16 : 283	Slow	1.5 P＋0.2 S	ICSI	1 (2)	1 (2)
	1999	Kuleshova et al.	HR 14 : 3077	V (OPS)	7.2 E＋0.6 S	ICSI	1	1
	1999	Porcu et al.	AJOG 180 : 1044	Slow	1.5 P＋0.1S	ICSI	1	?
	2000	Yoon et al.	FAS 74 : 180	V (EMG)	5.5 E＋1.0 S	ICSI	3	2＋?
	2000	Porcu et al.	MCE 169 : 33	Slow	1.5 P＋0.2 S	ICSI	20	11 (13)
	2002	Quintans et al.	HR 17 : 3149	Slow	1.5 P＋0.1 S	ICSI	6	2
	2002	Chen et al.	HR 17 : 1412	Slow	1.5 P＋0.3 S	ICSI	1 (2)	?
	2003	Yoon et al.	FAS 79 : 1323	V (EMG)	5.5 E＋1.0 S	ICSI	6	6 (7)
	2003	Boldt et al.	HR 18 : 1250	Slow	1.5 P＋0.2 S	ICSI	4	4 (5)
	2003	Fosas et al.	HR 18 : 1417	Slow	1.5 P＋0.3 S	ICSI	4	4 (5)
	2003	Katayama et al.	FAS 80 : 223	V (CT)	2.7 E＋2.1 D＋0.5 S	ICSI	2	?
	2003	Huttelova et al.	JARG 20 : 293	Slow	1.5 P＋0.1S	ICSI	1	1
GV	1998	Tucker et al.	FAS 70 : 578	Slow	1.5 P＋0.1S	ICSI	1	1
	1998	Tucker et al.	HR 13 : 3156	Slow	1.5 P＋0.1S	ICSI	1	1
	2001	Wu et al.	Reprod 121 : 389	V (EMG)	5.5 E＋1.0 S	IVF	1	?

文　献：HR = Hum Reprod, ANYAS = Ann NY Acad Sci, FAS = Fertil Steril, AOGS = Acta Obstet Gynecol Scand, JARG = J Assist Reprod Genet, AJOG = Am J Obstet Gynecol, MCE = Mol Cell Endocrinol, Reprod = Reproduction.

凍結法：Slow =緩慢法（*は最初の緩慢法），V = vitrification, OPS = open pulled straw, EMG = electron microscopic grid, CT = cryotop.

耐凍剤：D = DMSO, P = propylene glycol, S = sucrose. 数値はモル濃度を表す.

妊娠・分娩数：（ ）内は着床・出産数を，?は不明を表す.