Comparative Medicine
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Vol 52, No 4
August 2002
Pages 342-346

Optimal Equilibration Conditions for Practical Vitrification of Two-Cell Mouse Embryos

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The objective of the study reported here was to elucidate the optimal equilibration conditions for carrying out vitrification of two-cell mouse embryos, using a solution containing 2*M* dimethyl sulfoxide, 1*M* acetamide, and 3*M* propylene glycol (DAP213) as a cryoprotectant.

Embryos were subjected to an equilibration process under 20 conditions of a combination of different temperatures (10 to 37°C) and times (5 to 90 sec), and viability of the embryos was assessed by the rate of development into blastocysts and into live fetuses. As a result, these rates of development into blastocysts did not differ from those for unfrozen embryos. The rate of development of frozen-thawed embryos into live fetuses under conditions of 30 sec. at 20°C, which was selected as having by highest operability, was 55.2%, comparable to the value (65.0%) for unfrozen embryos. Thus, the optimal equilibration condition for vitrification of two-cell mouse embryos, using DAP213 solution, was 30 sec at 20°C, under which embryo viability was maximized, and this equilibration process was considered useful as a practical two-cell embryo freezing process in the vitrification method.

Currently, the technology for preserving frozen embryos is indispensable in facilities that produce and maintain laboratory animals, particularly, mice. Application of embryo cryopreservation methods is useful in preventing genotypic mutation, reducing cost and labor of animal husbandry, and reducing the cost of animal maintenance; in addition, its application has recently been explored as a means of transportation of animals (1) and of production of genetically modified animals, such as transgenic and knockout animals (2-5). The technology for embryo cryopreservation currently in wide use is mainly classified into two methods, vitrification and slow freezing. The vitrification method, which was developed by Rall and Fahy (6), is less expensive, time consuming, and space occupying than is the conventional slow-freezing method, in that it does not require use of expensive equipment/apparatus and can lead to complete freezing in a short time.

To attain high viability of frozen-thawed embryos, however, some measures have to be taken to implement vitrification of embryos under conditions that ensure sufficient permeation of cryoprotectants and generate an environment able to avoid toxic and/or osmotic injury (6, 7). Such being the case, various established procedures that were established through extensive investigations are now applied in carrying out vitrification under different conditions, in terms of kinds and concentrations of cryoprotectants (8-11), pre-freezing embryo equilibration conditions (12-15), and vitrification containers (16, 17).

Nakagata (18-21) developed a vitrification method in which the embryo freezing/thawing process could be implemented according to a simple procedure at room temperature. Advantages of this method include feasibility of one-step equilibration of pre-freezing embryos at room temperature, convenience of the thawing process, and high viability, whereas its disadvantage was the need for a certain degree of skill in manipulating pre-freezing embryos because of rapid completion of the embryo equilibration process (within 10 to 15 sec) (22).

For an embryo freezing method to be widely practicable, the requirements include feasibility of each freezing and thawing process without implementing troublesome manipulations, completion of freezing within a short time, and ease of manipulation so as to be practicable by any operator. Another requirement of the embryo freezing method is feasibility of the procedures, such as freezing/thawing and embryo equilibration at an ambient temperature, so as to be readily performed.

Taking account of these circumstances, we conducted a study to elucidate the optimal embryo equilibration condition by examining in detail the relation between temperature and time in the equilibration process, according to the method of Nakagata (18, 19) in an attempt to develop a practical vitrification method suitable as a routine procedure for embryo cryopreservation. As a result, we have established an embryo freezing method with high practicability, by identifying a condition under which the embryo freezing process can be implemented, taking a reasonably longer period without losing the advantages of Nakagata's method of one-step equilibration process at room temperature as reported.

Materials and Methods

Animals. Specific-pathogen-free mice—Crj:B6C3F1 mice (Charles River Japan, Inc., Kanagawa, Japan) and Jcl:MCH mice (CLEA Japan, Inc., Tokyo, Japan)—were purchased at the age of six weeks for use in the study. The Crj:B6C3F1 mice were used for collecting oocytes and spermatozoa, and the Jcl:MCH mice were used as transfer recipients and vasectomized male animals. Mice were kept in a room maintained under conventional conditions and were allowed access to MM-3 pellets (Funabashi Farm Co., Ltd., Chiba, Japan) and tap water ad libitum. The animal room was maintained at a temperature of 24 ± 2°C, humidity of

Received: 12/28/01. Revision requested: 4/08/02. Accepted: 5/28/02.
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55 ± 10%, and lighting conditions of 12-h light:12-h dark (lights on from 8:30 a.m. to 8:30 p.m.). In carrying out the animal experiments, the guidelines established by Yakult Research Institute Animal Care Ethics Committee were followed.

Oocytes and spermatozoa were collected from animals after sacrifice by use of cervical dislocation as an euthanasia technique, and embryos were transferred to recipient animals under anesthesia with Pentobarbital Sodium (Abbott Laboratories, North Chicago, Ill.).

Source of two-cell embryos. Female B6C3F1 mice (6 to 12 weeks old) were injected with 5 IU of pregnant mares serum globulin (PMSG; Serotropin, Teikokuzoki Co. Ltd., Tokyo, Japan) and 5 IU of human chorionic gonadotropin (hCG; Gonatropin, Teikokuzoki Co. Ltd.), each given at an interval of 48 h, and oocytes were collected from the excised oviducts 15 to 17 h after administration of hCG. Spermatozoa were collected from the caudal part of the epididymis of male B6C3F1 mice (12 to 16 weeks old), and were suspended in 200 μl of TYH medium (23) and covered with mineral oil (M-8410, Sigma Chemical Co., St. Louis, Mo.). After pre-incubation for 1.5 h in 5% CO_2 in air at 37°C, the spermatozoal suspension was added to the medium containing the collected oocytes at a final spermatozoal concentration of 15×10^4 cells/ml. The two-cell embryos obtained 24 h after fertilization were used for the freezing process.

Freezing and thawing. As vitrification solution, DAP213 (1) solution containing 2*M* dimethyl sulfoxide, 1*M* acetamide, and 3*M* propylene glycol (Sigma Chemical Co.) was used. The embryo equilibration process was carried out under the conditions of each of 20 combinations of four temperatures of 10, 20, 25 and 37°C and five times of 5, 10, 30, 60, and 90 sec. A 30-μl aliquot of vitrification solution was placed in a cryotube (No. 377224; Nunc, Roskilde, Denmark), and its temperature was maintained constantly at a given setting (10, 20, 25, or 37°C) on a block heater (CHT-100; IWAKI GLASS Co., Ltd., Tokyo, Japan).

The embryo equilibration process was performed by placing 10 to 20 embryos in each of the tubes with a small amount of PB1 medium, followed by an equilibration process for a given time (5, 10, 30, 60, or 90 sec). Immediately after completion of the equilibration process, cryotubes containing equilibrated embryos were plunged into liquid nitrogen after being fixed to a cane (No. 5015; Nalgene Company, Rochester, N.Y.). Frozen embryos were stored in a liquid nitrogen tank for a period of one to seven days.

The thawing process was performed by transferring cryotubes from liquid nitrogen into warm water at 37 to 40°C, followed by addition of 300 μl of 0.3*M* sucrose (Wako Pure Chemical Industries, Ltd., Osaka, Japan)-containing PB1 medium warmed to 37°C into the cryotubes. Embryos were transferred from a cryotube to a culture dish (No. 1008, Becton Dickinson and Company, Franklin Lakes, N.J.) together with vitrification solution, followed by three runs of sequential transfer, using fresh PB1 medium (each 100 μl). The embryo equilibration process was repeated three times or more under each of the equilibration conditions.

Assessment of viability of embryos kept by cryopreservation. For in vitro culture, thawed embryos were subjected to counting of the number of recovered embryos and the number of morphologically normal embryos by macroscopic examination. Embryos were defined as morphologically normal if the cell had an intact plasma membrane, the cytoplasm was refractive, there were no cytoplasmic fragments inside the zona pellucida, and the zona pellucida was undamaged.

Morphologically normal two-cell embryos were washed three times with Whitten's medium (24), then were placed in mineral oil-overlaid Whitten's medium (100 μl), followed by culture for approximately 72 h under the conditions of 37°C and 5% CO_2/air. Viability of the embryos was assessed by the rate of development into expanded blastocysts in comparison with that of unfrozen embryos prepared by in vitro culture of two-cell embryos obtained by in vitro fertilization. In vitro culture of unfrozen two-cell embryos was repeated three times, using approximately 30 embryos in one-run culture.

To assess in vivo viability of the embryos, a particular embryo specimen was procured by use of a freezing process under the optimal condition selected through in vitro studies, and female recipient mice were housed in a cage with vasectomized male mice for mating in advance. Frozen-thawed two-cell embryos were transferred into the oviducts of pseudopregnant recipient mice on the day on which a vaginal plug was recognized (day 1). Recipient mice were subjected to necropsy 19 days after transfer, to count the number of normal live fetuses and implantation sites in comparison with the rate of development into live fetuses and the implantation rate observed for transferred unfrozen two-cell embryos.

Statistical analysis. The rate of morphologically normal embryos, and the rate of development of embryos into blastocysts were statistically analyzed, using Fisher's exact probability test (two-tailed) with the Bonferroni correction, respectively. Statistical analysis of the data on rate of development into fetuses was performed by use of the χ^2-test.

Results

In vitro assessment of viability of embryos. Recovery rates of the embryos manipulated by freezing and thawing by the vitrification method under each of the equilibration conditions were found to be 80% or higher in every combination of conditions of temperature and time. The rates of morphologically normal embryos by macroscopic examination were high in each of the combinations of 30 to 90 sec at 10°C, 30 to 60 sec at 20°C, and 5 to 10 sec at 37°C, whereas those for each of the other combinations were significantly lower (Table 1). The rate of development of the unfrozen embryos into blastocysts was 88.8% (79/89).

High rates of development into blastocysts equal to those of unfrozen embryos were attained in each of the combinations of 5 to 10 sec at 37°C, 30 to 90 sec at 10 and 20°C, and 30 sec at 25°C, whereas those in each of the other combinations were significantly lower (Fig. 1).

In vivo assessment of viability of embryos. In an experiment performed with the particular specimen of embryos frozen after equilibration at 20°C for 30 sec, the condition selected for optimal equilibration established through in vitro investigations, the implantation rate and rate of development into live fetuses were found to be 70.4 and 55.2%, respectively. These values did not differ significantly from the implantation rate and the rate of development into live fetuses of 75.6 and 65.0%, respectively, obtained as the transfer outcomes for unfrozen two-cell embryos (Table 2).

Discussion

The temperature for the pre-freezing embryo equilibration

Table 1. Percentage of morphologically normal embryos among those subjected to vitrification under various equilibration conditions

Temperature (°C)	Equilibration time (sec)				
	5	10	30	60	90
10	37.3 (31/83)*,a	37.9 (25/66)*	98.5 (67/68)	98.6 (69/70)	94.1 (64/68)
20	54.5 (42/77)	84.5 (60/71)*	96.3 (103/107)	96.6 (86/89)	87.5 (56/64)†
25	44.8 (26/58)*	86.0 (43/50)†	87.5 (42/48)†	81.5 (44/54)*	89.6 (60/67)‡
37	95.9 (94/98)	94.4 (85/90)	89.1 (98/110)†	77.6 (45/58)*	55.7 (34/61)*

[a]No. of morphologically normal embryos/No. of embryos recovered.
Significant at *$P < 0.001$, †$P < 0.01$, and ‡$P < 0.05$ (compared with unfrozen embryos).

Table 2. In vivo viability of two-cell embryos subjected to vitrification under the optimal equilibration condition (20°C, 30 sec)

Group	No. of recipients	Percentage of implantation sites	Percentage of live fetuses
Vitrified	17	70.4 (176/250)[a]	55.2 (138/250)[b]
Unfrozen	12	75.6 (93/123)	65.0 (80/123)

[a]No. of implantation sites/No. of embryos transferred.
[b]No. of live fetuses/No. of embryos transferred.

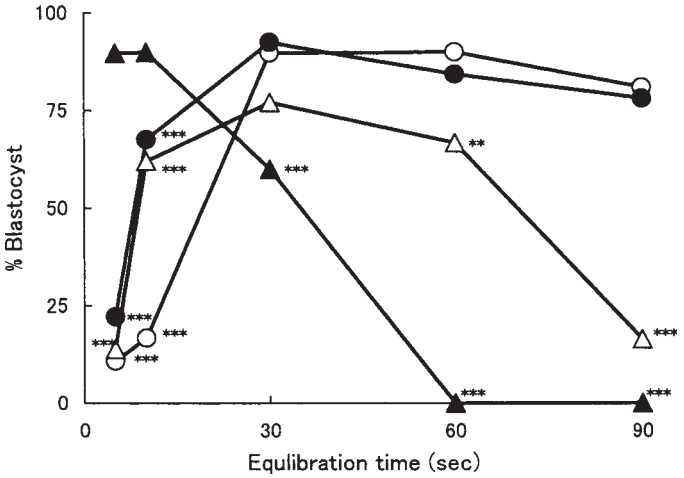

Figure 1. In vitro development of two-cell embryos after vitrification under various equilibration conditions. ○, ●, △, and ▲ indicate 10, 20, 25, and 37°C, respectively. Significant at **$P < 0.01$ and ***$P < 0.001$ (compared with unfrozen embryos).

process is an important factor in controlling permeability of the cryoprotectant; thus, the equilibration of embryos at various stages of development has been studied under various temperatures, including lower temperatures (25-27) and room temperature (12, 13, 28). We examined the optimal conditions for the pre-freezing two-cell embryo equilibration process at near-room temperature, using DAP213 solution. It has been reported that the permeation of cryoprotectants into the embryo is faster at higher temperatures (29) and slower at lower temperatures (6, 7). Insufficient permeation of cryoprotectants is known to induce intracellular ice formation (7, 30, 31), and, in agreement with these findings, the results of our study indicated that viability of embryos was lower under some conditions, including 5 to 10 sec at 10°C and 5 sec at 20 or 25°C, allowing us to speculate that the embryos might be damaged by intracellular ice formation possibly due to insufficient permeation of cryoprotectant, resulting in unsatisfactory vitrification under these particular equilibration conditions.

Another interesting finding was that the viability of embryos was extremely low under the equilibration conditions of 60 and 90 sec at 37°C, allowing us to speculate that excessively enhanced permeability of cryoprotectants into the cell body might damage the embryos through a toxic action of the cryoprotectants and/or osmotic pressure-induced damage in association with the high osmotic pressure of the vitrification solution. Our finding that the timespan within which high viability of embryos could be achieved was wider at 20 than at 25°C agreed well with that of other studies (12, 32) indicating that, within a range of room temperatures, the optimal equilibration timespan varied depending on the temperature at which the embryos were subjected to equilibration. Latitude in selecting equilibration time is one of the determinant factors for ready performance of the pre-freezing process, in that a wider latitude provides better operability. The results of our study clearly indicated that, in the two-cell embryo vitrification process, using DAP213 solution as a cryoprotectant, the optimal equilibration temperature is 20°C, at which high viability of embryos can be expected over a wide range of equilibration times of 30 to 90 sec, and the optimal equilibration time combined with 20°C is 30 sec, during which operators have sufficient time to carry out the pre-freezing equilibration process. More precisely, during an equilibration time of 30 sec, a series of operations to transfer embryos into a cryotube and plunge the tube into liquid nitrogen after fixation of embryos to a cane can be implemented at a reasonably lower speed and without accompaniment of excess leisure time, enabling continuous implementation of the embryo freezing process in an efficient manner.

Another important factor that cannot be ignored in developing a practicable embryo freezing process for routine work is its capacity to manipulate as many embryos as possible without any difficulty within a limited time. As indicated by findings of this study, the rate of development into live fetuses from embryos subjected to the freezing-thawing process under the optimal equilibration condition (20°C, 30 sec), 55.2%, was comparable to the 65.0% obtained for unfrozen two-cell embryos. Furthermore, our results indicated that in the vivo survival rate of frozen-thawed embryos compared favorably with the findings obtained in other studies of vitrification of two-cell embryos (19, 33, 34).

Freezing tolerance of mammalian eggs is generally known to vary depending on stage of development (35, 36); thus, viability of two-cell embryos was found to be lower than that of morulae and eight-cell embryos after being subjected to vitrification under the same conditions (33, 37-39). Stepwise procedures in the pre-freezing embryo equilibration process and the dilution process during thawing have been documented to be effective for attaining high viability of embryos in cryopreservation (37, 40); thus, equilibration of pre-freezing embryos and dilution during thawing have been performed under conditions of longer times and frequent repetitions in many studies of vitrification of two-cell embryos (13, 33).

The method having been established here by us, however, does not include any troublesome procedures in each freezing and thawing process, and can readily be carried out at room temperature. Taking into consideration the situation at most laboratories where embryos are manipulated is to maintain a temperature within the range of 20 to 25°C, an embryo equilibration process able to be performed at room temperature is believed to be suited for practical application.

As mentioned previously, we have elucidated how modification of the conventional Nakagata's method by selecting 20°C as the optimal equilibration temperature bestows on the embryo vitrification method some advantages, such as ready operability and wider latitude in selecting the equilibration time, without sacrificing the useful features of feasibility at room temperature and a one-step equilibration procedure.

In conclusion, the method of vitrification of two-cell embryos under the optimal pre-freezing equilibration conditions established through our study is considered to be suited to application for routine work, owing to the fact that each freezing and thawing process is capable of being readily practiced at room temperature in association with resultant high viability of embryos; thus, it is useful as a practical vitrification method for mouse two-cell embryos.

Acknowledgments

We thank H. Nagata of Yakult Central Institute for Microbiological Research for advice on statistical analysis.

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