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Cryopreservation of Mouse IVF Zygotes by Vitrification

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체외수정된 생쥐 1-세포기 배의 초자화 동결

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요 약

본 연구는 30% Ficoll과 0.3 M sucrose가 함유된 mDPBS 용액에 40%의 ethylene glycol을 첨가함으로서 제조된 EFS40을 이용하여 1-세포기의 생쥐수정란을 효율적으로 동결할 수 있는 적정 조건을 확립하기 위하여 실시하였다. 체외수정에 의해 생산된 생쥐수정란은 1 단계 동결법 혹은 2단계 동결법, 두 가지 동결 방법에 의해 각각 초자화 동결되었으며, 동결 후, 융해된 수정란의 생존율은 2-세포기로 분할율과 배양 5일째 탈출배반포기로의 발달율로 각각 검증하여 다음과 같은 결과를 얻었다. 1 단계 동결법에서, 수정란을 직접 초자동결액에 1분 동안 노출시켰을 때, 수정란의 생존율은 85.5%, 발달율은 31.9%였다. 수정란을 먼저 20% ethylene glycol에 1, 3, 5 분간 노출시킨 후, 1 분동안 EFS40 용액으로 옮겨 동결하는 2 단계 동결법에서는 1 단계 동결법보다 낮은 생존율을 보였다 (65.4, 53.6 및 29.6%). 가장 높은 생존율 (95.9%)은 EFS40 에서 30 초동안 노출한 1 단계 동결법에서 얻을 수 있었다. 이 조건하에서 2-세포기로 분할된 수정란의 63.8%가 탈출배반포기로 발달하였다. 또한, Differential labelling 기법을 이용하여 Total과 ICM (inner cell mass)의 세포수를 조사한 결과, 초자 동결 후 발달된 배반포의 Total과 ICM 세포수 (63.2±16.9, 13.5±4.0)와 대조군 세포수 (54.0±15.2, 12.3±4.6)간에는 각각 유의차가 인정되지 않았다. 이러한 결과는 동결로 인한 수정란의 발달율은 다소 낮았지만, 동결후 융해된 수정란은 정상적으로 배반포까지 발달함을 보여준다. 이러한 결과로 미루어 보아, 본 실험에 사용된 초자화 동결 방법은 1-세포기 생쥐 수정란을 성공적으로 동결시킬 수 있다는 것을 알 수 있었다.

Ⅰ. INTRODUCTION

Preservation of oocytes and embryos at various developmental stages has been established by cryopreservation methods for last two decades (Whittingham et al., 1972). In recent years, attention has been focused on vitrification as a simple and rapid alternative to the conventional freezing methods for the cryopreservation of mammalian embryos (Fahy et al., 1984).

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Vitrification could be avoid the potential risk of cellular injury caused by the formation of intracellular ice. In 1985, Rall and Fahy reported the first instance of successful vitrification of 8-cell mouse embryos. The vitrification solution they used (VS1) was based on dimethyl sulfoxide (DMSO) supplemented with acetamide, propylene glycol and polyethylene glycol as cryoprotectants. This method was applied to the cryopreservation mouse oocytes and embryos at various developmental stages (1-cell, 2-cell, 4-cell, 8-cell, blastocyst) (Hsu et al., 1986; Kono and Tsunoda, 1987; Matsumoto et al., 1987). However, the initial vitrification solution (VS1) was highly toxic and embryos must be exposed to this solution at 4℃.

Kasai et al. (1990) reported that mouse morula could be vitrified by a simple method. It was showed that a new vitrification solution, EFS40, based on ethylene glycol which permeates the cell rapidly and has low toxicity. This simple method developed for morula was examined to determine whether it was effective for mouse embryos at other developmental stages. It has been demonstrated suitable conditions for the vitrification of zona-intact mouse blastocysts using either an ethylene glycol-based solution (Zhu et al., 1993) or a glycerol-based solution (Zhu et al., 1994). Also, they achieved vitrification of hatched mouse blastocysts (Zhu et al., 1996). Indeed, modifications of the original vitrification technique or a simple vitrification solution were used in other species, pig or bovine embryos produced *in vitro* (Dobrinsky et al., 1994; Kuwayama et al., 1994).

However, it is not known whether this simple method is also effective for use on mouse zygotes, at stages other than the morula stage. Therefore, these experiments were conducted to determine the conditions for successful and efficient cryopreservation of mouse zygotes with 2 pronuclei, using simple vitrification procedures in an ethylene glycol-based solution.

Ⅱ. MATERIALS AND METHODS

1. Production of zygotes *in vitro*

Hybrid F_1 female mice (4~6 weeks old) from C57BL/6 × CBA/N were superovulated by intraperitoneal (i.p.) injection of 5 I.U. pregnant mare serum gonadotrophin (PMSG ; Sigma), followed by 5 I.U. human chorionic gonadotrophin (hCG ; Sigma) 50 h later. The cumulus-oocyte complexes (COCs) recovered by tearing the oviducts with medium M2 at 13.5 hr after hCG injection. After pre-incubation for 30 min., capacitated sperm were inseminated with COCs. After *in vitro* fertilization, the embryos were cultured in M16 medium for 4~12 hrs. Zygotes, 2 pronuclei and second polar body formed, were used in experiment for vitrification during *in vitro* culture (Fig. 1A).

2 Vitrification solutions

The vitrification solution, EFS40, described by Zhu et al. (1993) was used in the present experiments. This solution was prepared as 40% (v/v) ethylene glycol (Sigma) in modified Dulbecco's phosphate-buffered saline (mDPBS, Gibco BRL) containing 30% (w/v) Ficoll 70 (average molecular weight 70,000, Sigma) plus 0.5 M sucrose (Sigma). 20% (v/v) ethylene glycol (20% EG) in mDPBS was used as an equilibration solution.

3. Vitrification studies

The 0.25 ml french straws (I.M.V., L'aigle) were loaded vitrification solution and 0.5 M sucrose solution at room temperature (25℃) by suction with a syringe. All embryos were treated in a room at 25℃. Embryos were vitrified by modification of procedures outlined by Kim et

al. (1996 a,b). In the one-step method, zygotes were first suspended in EFS40 and then transferred to the straw for 1 min. In the two-step method, embryos were equilibrated with PBS medium containing 20% EG for 1, 3, 5 min. and exposed in EFS40 for 1min. For vitrification, the first part of the straw filled with sucrose (4 cm) was slowly immersed into liquid nitrogen(LN_2); the remaining part of the straw was then plunged into LN_2.

4. Thawing and assessment of survival

After a few days of storage in LN_2, the straws were placed in air for 3 sec. and then warmed rapidly in water bath at 25℃. The contents of each straw were expelled into dish containing 0.8 ml of DPBS containing 0.75 M sucrose (S-DPBS) by pushing the cotton plug and then put into fresh 0.75 M S-DPBS for 4 min. The embryos were transferred to 0.5 M, 0.25 M S-DPBS for 3 min., respectively. Finally, after recovery in fresh DPBS for 5 min., zygotes were cultured in a 50 μl droplet of M16 medium supplemented with 4 mg/ml BSA. In all experiments, dilution was performed at room temperature (25℃). Survival rate after warming was examined as cleavage rate to the 2-cell stage after 24 hours of culture (Fig. 1B). The development of 2-cell was assessed by their ability to develop into blastocysts at day 4 (Fig. 1C) and hatching blastocysts at day 5 culture (Fig. 1D).

5. Experimental design

Experiment 1. Production of 1-cell embryos with 2 pronuclei

To determine the optimal time for collection of zygotes with 2 pronuclei and second polar

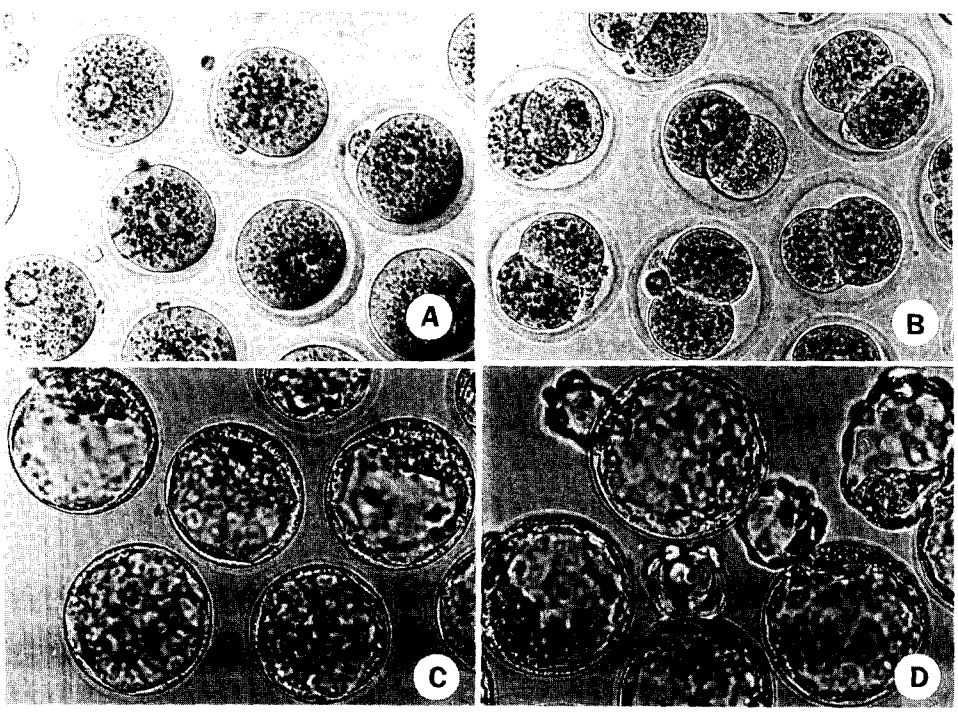

Fig. 1. Morphology of mouse embryos; A: Zygotes before vitrification, B: 2-cell at 24 h, C: Blastocysts at 4 day, D: Hatching blastocysts at 5 day culture after thawing (×300).

body, embryos were examined at 18 to 26 hr after hCG injection *in vivo* and *in vitro*. Zygotes of poor quality were discarded and these of good quality were used in individual experiments.

Experiment 2. Embryo development *in vitro* on various vitrification methods

In vitro produced zygotes were vitrified by two methods. First, zygotes were directly exposed to the vitrification solution (EFS40) at 25℃ for 1 min. Second, embryos were equilibrated with a dilute 20% EG for 1, 3, 5 min. before a 1 min. exposure to EFS40. After warming and dilution, the vitrified embryos were cultured in M16 up to the blastocyst stages.

Experiment 3. The effects of exposure time in EFS40

We assessed the effects of the exposure time (30, 60, 90, 120 sec.) to the vitrification solution (EFS40) to investigate whether greater permeation of the cryoprotectant would improve developmental rate.

Experiment 4. The Total and ICM cell counts of blastocysts developed in vitrified-thawed embryos.

Fresh and vitrified day-4 cultured embryos were stained as described by Kim et al. (1996). The cell numbers of Total and ICM in vitrified-thawed embryos by differential labelling of the nuclei with immunosurgery and polynuceotide-specific fluorochromes were compared to that of control.

6. Statistics

Survival rate of each treatment was compared with that in the control group, with Chi-square test using SAS Institute software and T-test.

Ⅲ. RESULTS

1. Production of 1-cell embryos with 2 pronuclei

To determine the optimal time for collection of zygotes with 2 pronuclei and second polar body, embyos were examined at 18~26 hr after hCG injection *in vivo* and *in vitro*. After hCG injection at 22~24 h, about over 85% of recovered zygotes by IVF were used in this serial experiments.

2. Embryo development *in vitro* on various vitrification methods

The survival of zygotes was assessed by the cleavage into 2-cell and 2-cell was cultured into blastocysts at day 4 and hatching blastocysts at day 5. As shown in Table 1, the survival of embryos that were vitrified with two methods was compared to that of control embryos. All of the vitrified groups displayed significantly reduced viability when compared to control (42.5%), except one-step method. In the one-step method, the survival rate was obtained 85.5 % and 31.9% of 2-cells were developed to hatching blastocysts. However, by the two-step method, the survival rate was 65.4~29.6%. Subsequently, when zygotes were only exposed in EFS40, the rates of cleavage and development were significantly higher than that of others.

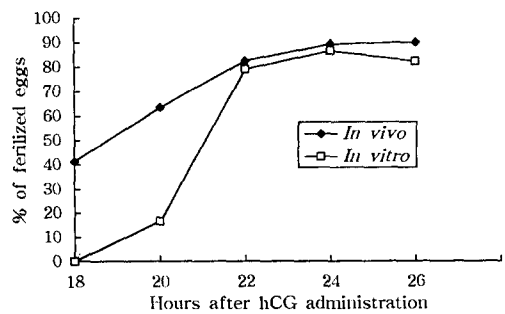

Fig. 2. Percentage of fertilized eggs with two pronuclei following time course after hCG administration.

3. The effects of exposure time in EFS40

To examine the effect of the exposure time on vitrification solution, zygotes were exposed for 30, 60, 90, 120 sec. in EFS 40. As shown in Table 2, the optimum exposure time was found to be 30 sec., giving 95.9% cleavage rate and an overall rate of 63.8% of vitrified zygotes developing to hatching blastocyst. A 90 and 120 sec. of exposure time resulted in dramatically reduced survival and developmental rates compared to the shorter exposure time.

4. The Total and ICM cell counts of blastocysts developed in vitrified-thawed embryos

To evaluate embryo qualities, blastocysts de-

Table 1. Survival *in vitro* of mouse zygote vitrified after various treatments

Treatment	Exposure time (min)		No. of zygotes	Development		
	20% EG	EFS40		2-cell (D1)	> BL (D4)	> Hing (D5)
Control	–	–	44	40^{a} (90.9)	27^{a} (67.5)	17^{a} (42.5)
Vitrified (one-step)	–	1	55	47^{a} (85.5)	25^{ab} (53.2)	15^{ab} (31.9)
Vitrified (two-step)	1	1	52	34^{b} (65.4)	15^{bc} (44.1)	6^{bc} (17.7)
	3	1	56	30^{bc} (53.6)	3^{d} (10.0)	1^{d} (3.3)
	5	1	54	16^{c} (29.6)	0^{d}	0^{d}

[a-d] Different superscripts within column were significantly different ($p<0.05$).

Table 2. Survival of mouse zygotes according to exposure time at EFS40

Treatment	Exposure time (sec.) EFS40	No. of zygotes	Development		
			2-cell (D1)	> BL (D4)	> Hing (D5)
Control	–	40	40^{a} (100.0)	37^{a} (92.5)	30^{a} (75.0)
Vitrified	30	49	47^{a} (95.9)	32^{b} (68.1)	30^{b} (63.8)
	60	49	37^{b} (72.4)	18^{b} (48.7)	9^{b} (24.3)
	90	43	20^{c} (46.5)	3^{c} (15.0)	1^{c} (5.0)
	120	33	11^{c} (33.3)	3^{c} (27.3)	1^{c} (9.1)

[a-d] Different superscripts within column were significantly different ($p<0.05$).

Table 3. Numbers of ICM and TE cell of vitrified-thawed blastocysts

Treatment	No. of zygotes	Development		Cell number		
		2-cell (D1)	Blastocyst (D4)	ICM		Total (M+SEM)
				M+SEM	Proportion(%)	
Control	54	50 (92.6)	49 (98.0)	12.3±4.6	22.7	54.0±15.2
Vitrified	73	58 (79.5)	29 (50.0)	13.5±4.0	21.4	63.2±16.9

veloped from vitrified-thawed embryos were stained by the differential labelling of Total and ICM nuclei. The counts of Total and ICM cell number using differential labelling are summarized in Table 3. There is no significantly difference in the number of total cell between day 4 fresh cultured blastocysts (54.0±15.2) and vitrified (63.2±16.9). This result demonstrates that vitrified zygote could be normal development into good quality blastocysts *in vitro*. However, in the proportion of ICM, control blastocysts were slightly higher than vitrified embryos.

Ⅳ. DISCUSSION

Zygotes produced by IVF were vitrified by a method of Kim et al.. (1996 a,b) with some modifications. Kim et al.. (1996 b) reported a higher survival rate (82.9~88.4%) of blastocyst by the two-step vitrification method which exposes blastocyst stage embryos in 20% EG for 5 min. and then EFS 40 for 1 min. at 25℃. However, the zygotes differ structurally from the blastocysts with a large blastocoel. In the vitrification of zygotes, the survival rate after two-step exposure (65.4~29.6%) was decreased following the pre-permeation time longer. The good survival rate was not obtained by a two-step method which enables pre-permeation, but by a one-step method (85.5%), which exposes zygotes in EFS40 for 1 min. (Table 1). The present result shows that mouse zygotes are more sensitive to cryopreservation than blastocysts.

The EFS40 solution is based on ethylene glycol as the permeating cryoprotectant and two non-permeating agents, Ficoll and sucrose. Although ethylene glycol is less commonly used for embryo freezing than DMSO and glycerol, the present study indicates that it has an advantage of lower toxicity (Miyake et al., 1993). For successful embryo preservation by vitrification, permeation of cryoprotectant is considered essential and its exposure time affects on the survival of embryos. Therefore, the exposure time of the embryo to the vitrification solution needs to be optimal to allow a sufficient amount of the cryoprotectant to permeate into the embryo and to avoid any toxic effects (Shaw et al., 1992; Ali and Shelton, 1993a,b). In the study reported here, we found that survival rate of embryos after vitrification decreased following to exposure time longer (90, 120 sec.). The maximum survival rate obtained in the one-step vitrification method was 95.9% with 30 sec. of exposure to EFS40 at 25℃ (Table 2). And developmental rates into hatching blastocysts of these embryos (63.8%) were significantly higher than those exposed for 60, 90, 120 sec. (24.3, 5.0, 9.1%), respectively. In various exposure time of vitrification methods, Nakagata (1989) suggested that exposure time requires the shortest period (only 5 to 15 sec.) for pretreatment of embryos before rapid cooling. However, his method is less practical than other, since survival rate drops sharply if pretreatment takes longer than 10 to 15 sec. In practice, 30 sec. is enough time to pretreat embryos for suspending them in vitrification solution and loading into a straw before plunging them into LN_2.

Although survival and developmental rates of embryos were reduced by vitrification compared to those of control, cell number of developed vitrified-thawed embryos was not significantly difference compared to that of control. In addition, cryopreservation of mammalian zygotes has potential value for producing transgenic mice or application of human embryos. Considering the simplicity of the procedure, the high levels of embryonic survival and development, this vitrification method with EFS solution should be suitable for zygotes stages. The present result shows that it is also possible cryopreservation of

any other species.

V. SUMMARY

This study was carried out to determine the optimal condition for successful and efficient cryopreservation of zygotes, 1-cell embryos, using EFS40 which was 40% (v/v) ethylene glycol diluted in DPBS medium containing 30% Ficoll (w/v) and 0.3 M sucrose. After mouse zygote produced by IVF was vitrified by two freezing methods, the post-warming survival rates of 1-cell zygotes were assessed as cleavage to the 2-cell stage and development into the hatching blastocysts at 5 day. In the one-step method, when embryos were directly exposed to the vitrification solution at 25℃ for 1 min., survival and development rates of zygotes were 85.5% and 31.9% In the two-step method, embryos were equilibrated with a dilute 20% EG for 1, 3, 5 min. before 1 min. exposure to EFS40, respectively. However, the rates of development (17.7, 3.3, 0%) were lower than that of one-step method. The highest survival rate (95.9%) was obtained by one-step method which exposes embryos in EFS40 for 30 sec. In this condition, 63.8% of cleaved 2-cell developed into hatching blastocysts. In the cell number of Total and ICM using differential labelling technique, there are no significant differences in the cell number of Total and ICM between blastocysts developed in vitrified-thawed embryos (63.2±16.9, 13.5±4.0) and control balstocysts (54.0±15.2, 12.3±4.6). Therefore, these results show that mouse zygotes can be successfully cryopreserved by a simple vitrification method although developmental rates of vitrified embryos were reduced. In conclusion, this proposed vitrification procedures can be useful in the cryopreservation of mouse IVF zygotes.

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