大韓不妊學會雜誌: 第 23 卷 第 1 號 1996
Kor. J. Fertil. Steril., Vol. 23, No. 1, 1996, 4

Cryopreservation of Mouse IVF/IVC Blastocysts by Vitrification

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체외수정된 생쥐 배반포기배의 초자화 동결

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= 국문초록 =

본 연구는 체외수정에 의해 생산된 생쥐 배반포기배를 vitrification 방법으로 동결보존하였을때 높은 생존율을 얻기 위한 적정조건을 검토하고자 실시하였다. 배반포기배를 생산하기 위하여, B6CBA F1 (C57BL/6, ♀ × CBA/N, ♂) 계통의 생쥐 미수정란에 1×10^6 spermatozoa/ml 농도의 정자로서 수정을 유도하였으며, 이후 37℃, 5% CO_2배양기내에서 96시간동안 체외배양하였다. 배양 4일째의 배반포기배는 발달상태에 따라 early, middle 그리고 hatching blastocysts로 구분하였다. 본 실험에 사용된 동결보존액은 30% Ficoll과 0.5mol의 sucrose가 첨가된 mDPBS 용액에 40%의 ethylene glycol를 첨가한 EFS 40 (Zhu *et al.*, 1993) 이었고, 수정란은 25℃의 상온에서 먼저 20% ethylene glycol에 노출된 후 EFS 40 용액으로 옮겨 액체질소에 침지하는 2단계 동결법에 의해 동결보존되었으며, 급속융해하여 다음과 같은 결과를 얻었다.

1. 체외수정율과 배양 4일째 배반포기까지의 배발달율은 각각 89.4%와 86.1%였다.

2. 20% ethylene glycol에서 5분간 평형된 후 EFS 40 용액에 냉동보존된 후 융해된 난자의 생존율은 20% ethylene glycol에 0, 1, 3분간 평형된 난자의 생존율에 비해 유의하게 높았다.

3. 배반포기배를 20% ethylene glycol에서 5분간, EFS 40 용액에 1분간 차례로 노출한 다음 체외배양하였던 바, 배양 24시간째 생존율은 82.9%~88.4% 였다.

본 연구 결과, 체외수정, 배양된 생쥐 배반포기배는 20% ethylene glycol과 EFS40에 대한 노출만으로는 난자의 생존성에 나쁜 영향을 미치지 않는 것으로 미루어 보아 배반포기배의 초자화 동결이 가능함을 시사하였다. 따라서 동결 융해 후 높은 생존율은 상온에서 난자를 2단계 즉, 20% ethylene glycol에 5분간 평형시킨 후 EFS 40 용액에 노출하여 1분내에 LN2에 직접 침지하는 간편한 동결방법으로 얻을 수 있었다.

INTRODUCTION

Attention has focused on vitrification as a rapid and efficient method for cryopreservation of biological systems (Fahy *et al.*, 1984). Vitrification involves the rapid cooling of liquid medium in the absence of ice crystal formation and the solution forms an amorphous glass as a result of rapid cooling by direct submersion of the embryo in a plastic straw into liquid nitrogen (LN2). In this state, the glass is devoid of all ice crystals, which is a major cause of cell death, and embryos are not subjected to the physical damage that is associated with ice crystal formation, but cryoprotectant toxicity and osmotic injury to

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embryos can occur (Dobrinsky and Johnson, 1994). These effects depend on the choice of cryoprotectant, concentration of cryoprotectant, duration of exposure and temperature of exposure (Ali and Shelton, 1993).

The first successful vitrification of mouse embryos was accomplished by using a vitrification solution (VS1) composed of 4 agents : dimethyl sulfoxide (DMSO), acetamide, propylene glycol and polyethylene glycol and stepwise exposure of the embryos at a temperature of 4℃ (Rall and Fahy, 1985). Since then, many researchers have studied cryopreservation of embryos by vitrification. Scheffen *et al.*, (1986) reported that mouse embryos could be vitrified in a solution containing two permeating agents (glycerol and propylene glycol) in two step equilibration at room temperature. Using this method, bovine embryos at morula to approximately early blastocyst stage were successfully vitrified for the first time, resulting in pregnancy (Massip *et al.*, 1986) and the birth of young (Massip *et al.*, 1987). However, their methods were not adequate for the vitrification of bovine blastocysts (Ishimori *et al.*, 1993; Tachikawa *et al.*, 1993).

Kasai *et al.*, (1990) obtained excellent results with mouse morulae. This method using a new vitrification solution based on ethylene glycol, which permeates the cell rapidly and has low toxicity, requires embryo treatment for only 2 min. at 20℃ before the sample is plunged into liquid nitrogen. EFS (Ethylene glycol, Ficoll, Sucrose), which was first described by Kasai *et al.*, (1990) for mouse morulae, has also been used successfully for the vitrification of rabbit embryos (Kasai *et al.*, 1992), bovine morulae produced *in vivo* (Mahmoudzadeh *et al.*, 1993), bovine blastocysts produced *in vitro* (Tachikawa *et al.*, 1993) and mouse expanded blastocysts (Zhu *et al.*, 1993). Many factors contribute to the chilling sensitivity of *in vitro* derived embryos. To date, there have been no reports on the successful vitrification of *in vitro* produced mouse blastocysts.

On the basis of these reports, the present study was conducted to fine optimal conditions for obtaining high survival of *in vitro* produced mouse blastocysts after vitrification in solution based on ethylene glycol.

MATERIALS AND METHODS

1. Animals

Hybrid F1 mice from C57BL/6, ♀ × CBA/N, ♂ were used for all experiments. All mice of this study were maintained on 14h light and 10h dark (lights on at 0600 h). Four to six weeks old, F1 female mice were superovulated by intraperitoneal (i.p.) injection of 5 i.u. pregnant mare serum gonadotrophin (PMSG ; Sigma, St. Louis, MO) at 2000 h, followed by 5 i. u. human chorionic gonadotrophin (hCG ; Sigma) 50h later.

2. Production of Blastocysts *In Vitro*

Sperm suspension was squeezed out of caudae epididymis and dispersed into 200μl drops of M16 containing 4mg/ml BSA (Fraction V, Sigma) under mineral oil. The sperm were capacitated for 2h by incubating them at 5% CO_2 in air at 37℃. The females were sacrificed at 13h after hCG injection to obtain unfertilized eggs. The embryos were recovered by tearing the oviducts with medium M2 containing 4 mg/ml BSA and then transferred into 50μl drops of M16 medium under mineral oil for insemination. The cumulus-oocyte complexes were induced into each sperm suspension. Sperm concentration at the insemination was 1 x 10^6 cells/ml. The eggs were collected from the insemination drops at 6h after insemination and then washed five times in fresh M2 medium before introducing into culture drop. Fertilization was assessed as the presence of 2-cell stage embryos at 24h later. Embryos were cultured in 50μl drops of M16 medium under mineral oil at 5% CO_2 in air at 37℃. The IVF/IVC blastocysts were divided

into three stages of early, middle and hatching at 96h after insemination, individually (Fig. 1).

3. Vitrification Solutions

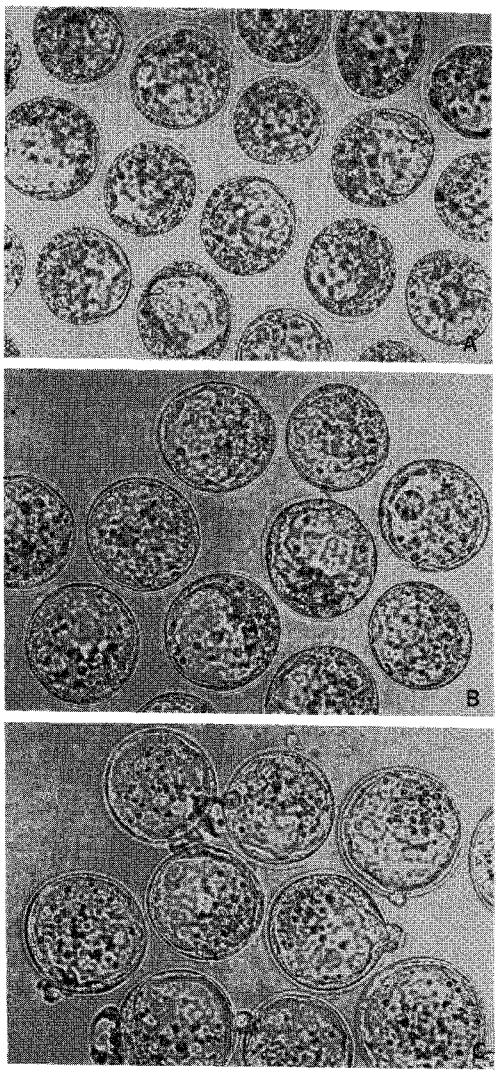

Fig. 1. Morphology of mouse IVF/IVC blastocysts ; **a**) Early blastocysts (blastocoele is smaller than 2/3 of the whole embryonic cell), (×300) **b**) Middle blastocysts (blastocoele is larger than 2/3 of the whole embryonic cell and thinning of the zona pellucida), (×300) **c**) Hatching blastocysts (×300)

The vitrification solution, EFS 40, described by Zhu et al., (1993) was used in the present experiments. Ethylene glycol (Sigma) 40% (v/v) 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 mol sucrose (Sigma). 20% ethylene glycol in mDPBS (20% EG) was prepared as an equilibration solution. When the solution was aspirated in a 0.25ml French straw (IMV, L'Aigle) and transferred into liquid nitrogen, it remained transparent; on being transferred to 25℃ water, EFS 40 remained transparent, which was a sign of vitrification.

4. Vitrification of Blastocysts

Embryos were vitrified by using a method of Mahmoudzadeh *et al.*(1995) with some modifications. Embryos were equilibrated in 20% EG for 0, 1, 3 and 5min., individually.

The 0.25ml french straws were loaded as follows: a 4cm length was filled with 0.5mol sucrose (prepared in mDPBS containing 10% FBS) followed by a 1cm air bubble, 0.5cm EFS 40, an air bubble (0.5cm), 2cm EFS 40 containing the embryos, an air bubble (0.5cm), 0.5cm EFS 40, and an air bubble (1cm). The remaining part of the straw was filled with 0.5 mol sucrose and the straw was sealed with powder (Fig 2). About ten embryos were loaded in each straw. For vitrification, the first part of the straw filled with sucrose (4cm) was solwly immersed into liquid nitrogen; the remaining part of the straw was then plunged in.

5. Thawing and Assessment of Survival

After a few days of storage in liquid nitrogen, the straws were warmed rapidly in water at 25℃ The contents of each straw were ex-

CP	0.5MS	AB	EFS	AB	00000	AB	EFS	AB	0.5MS	SP

Fig. 2. Configuration of a 0.25ml straw loaded with embryos before vitrification. Embryos were pipetted into a 2cm EFS 40 column in a straw. CP: cotton plug, 0.5MS: dilution solution (0.5 mol sucrose in mDPBS), AB: air bubble, EFS: vitrification solution (EFS 40), SP: sealing powder

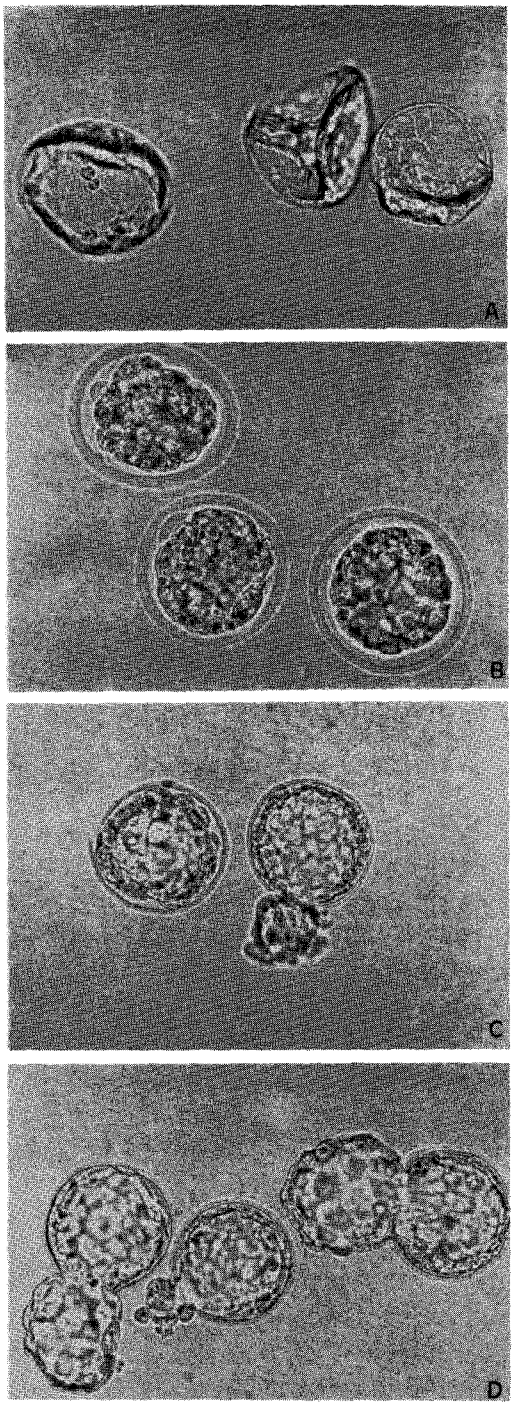

Fig. 3. A series of course from vitrification to survival after thawing; a) Blastocysts exposed to EFS 40, (×300) b) Blastocysts diluted in 0.5mol sucrose solution after thawing, (×300) c) Re-expanding blastocysts at 24h after thawing, (×300) d) Developing blastocysts to hatching at 48h after thawing (×300)

pelled into a well of a sterile four-well multidish plate containing 0.8ml of 0.3 mol sucrose and then put into fresh 0.3 mol sucrose for 5min. The embryos were transferred into a well containing 0.8ml of mDPBS medium with 10% FBS. After 5min., embryos at each developmental stage of each group were cultured in a 50μl droplet of M16 medium supplemented with 4mg/ml BSA. In all experiments, dilution was performed at room temperature (25℃). Survival after warming was defined as the percentage of vitrified embryos that re-expanded at 24h and hatching at 48h of culture (Fig. 3).

Experiment 1. *In Vitro* Fertilization Rate of Mouse Eggs

In vitro fertilization rate of eggs was assessed at 24h later by the presence of 2-cell stage embryos. Also, the developmental rates to expanded blastocysts and hatching blastocysts were defined at 96h and 144h after insemination, respectively.

Experiment 2. Effect of Equilibration Time

Influence of equilibration time in 20% EG on the viability of the vitrified embryos according to blastocyst stage was investigated. Embryos of each stages were exposed to 20% EG for 0, 1, 3 or 5min. and then to EFS 40 for 1min. at room temperature. The straws were cooled by plunging into LN2.

Experiment 3. Test of Cryoprotectant Toxicity to Embryos

To examine cryoprotectant-induced injury during exposure, approximately ten embryos were exposed to 20% EG for 5min. and EFS 40 for 1min. Exposure of embryos to EFS 40 was carried out in a 2cm EFS 40 column in a 0.25ml French straw. Without being cooled, the contents of each straw were expelled into a well containing 0.8ml of 0.3mol sucrose and then pipetted into fresh 0.3mol sucrose. After 5min., the embryos were transferred into a

Table 1. The rates of fertilization and development in mouse IVF

No. of oocytes	No. of 2-cell (%)	Development to Bla. at 96h (%)			Development to Hatching Bla. at 144h (%)		
		> Bla.	Bla.	Hing	**> Hing**	Hing	Hed.
226	**202**	**174**	102	72	**156**	78	78
	(89.4)	**(85.1)**	(50.5)	(35.6)	**(77.2)**	(38.6)	(38.6)

Table 2. *In vitro* survival rates of *in vitro* produced mouse blastocysts after various exposure times to 20% ethylene glycol

Blastocyst stage	Development to re-expanded Bla. after 24h (%)				Development to Hatching Bla. after 48h (%)			
	0 min.	1 min.	3 min.	5 min.	0 min.	1 min.	3 min.	5 min.
Early	$23/55^{a}$	$21/51^{a}$	$18/48^{a}$	$26/48^{a}$	$6/55^{a}$	$8/51^{a}$	$8/48^{a}$	$18/48^{b}$
	(41.8)	(41.2)	(37.5)	(54.2)	(10.9)	(15.7)	(16.7)	(37.5)
Middle	$4/40^{a}$	$12/22^{b}$	$18/26^{b}$	$46/64^{b}$	$2/40^{a}$	$4/22^{a,b}$	$7/26^{b}$	$38/64^{c}$
	(10.0)	(54.5)	(69.2)	(71.9)	(5.0)	(18.2)	(26.9)	(58.5)
Hatching	$27/64^{a}$	$14/21^{b}$	$49/64^{b}$	$77/86^{c}$	$10/64^{a}$	$12/21^{b,c}$	$32/64^{b}$	$59/86^{c}$
	(42.2)	(66.7)	(76.6)	(89.5)	(15.6)	(57.1)	(50.6)	(68.6)
Total	$54/159^{a}$	$47/94^{b}$	$85/138^{b}$	$149/198^{c}$	$18/159^{a}$	$24/94^{b}$	$47/138^{b}$	$115/198^{c}$
	(34.0)	(50.0)	(61.6)	(75.3)	(11.3)	(25.5)	(34.1)	(58.1)

$^{a, b, \& c}$; Different superscripts in the same row were significantly different ($p < 0.05$)

well containing mDPBS medium with 10% FBS.

6. Statistical Analysis

Survival rate of each treatment was compared with that in the control group, with Chi-squire test using SAS Institute software package (SAS Institute Inc., 1985).

RESULTS AND DISCUSSION

Experiment 1. *In Vitro* Fertilization Rate of Mouse Eggs

As shown in Table 1, *in vitro* fertilization rate of mouse eggs was 89.4%. Also, the rates of development to blastocyst stage at 96h and hatching blastocyst stage at 144h of the eggs were 86.1% and 77.2%, respectively. The fertilization rate (89.4%) was higher than those of others (77% of Lee *et al.*, 1989; 79.3% of Tournaye *et al.*, 1994) and the developmental rate (86.1%) to blastocyst stage was similar with the result (85.2%) of Tournaye *et al.* (1994). This result shows that mouse IVF system in this lab. is stable.

Experiment 2. Effect of Equilibration Time

The *in vitro* survival rates of the vitrified mouse embryos are shown in Table 2. High survival rates (54.2, 71.9 and 89.5%) and developmental rates (37.5, 58.5 and 68.6%) of each group (early, middle and hatching blastocyst stage) of vitrified embryos were observed after 5min. equilibrations in 20% EG. (Table 2).

Kasai *et al.* (1990) reported a higher survival rate (98%) of morulae when the embryos were vitrified in the one step vitrification method. However, the blastocyst differs structurally from the morula in that it has a fluid-filled blastocoel cavity. It has been shown that inclusion of a macromolecule in a solution facilitates vitrification (Fahy *et al.*, 1984). In the blastocoel cavity there must be few macromolecules, and, possibly, sufficient permeation of ethylene glycol is required for the

Table 3. Survival of mouse blastocysts exposed to or vitrified in vitrification solution (EFS40)

Blastocyst stage	Embryo treatment	No. of embryos	Development to re-expanded Bla. after 24h (%)	Development to Hatching Bla. after 48h (%)
Early	Control	18	18 (100)	15 (83.3)
	Exposed	32	27 (84.4)	19 (59.4)
	Vitrified	48	26 (54.2)[d]	18 (37.5)[c]
Middle	Control	23	23 (100)	21 (91.3)
	Exposed	43	38 (88.4)	38 (88.4)
	Vitrified	64	46 (71.9)[c]	38 (58.5)[b]
Hatching	Control	26	26 (100)	26 (100)
	Exposed	35	29 (82.9)[a]	28 (80.0)[a]
	Vitrified	86	77 (89.5)	59 (68.6)[c]

[a] $P < 0.05$ compared with controls, [b] $P < 0.01$ compared with controls, [c] $P < 0.005$ compared with controls, [d] $P < 0.001$ compared with controls.

vitrification of the cavity; insufficient permeation is likely to lead to the formation of ice crystals in the cavity, which must reduce the post-warming survival. For the permeation, however, ethylene glycol must first penetrate into the cells and then into the cavity, which may require a certain time. Yet, the toxic effects of the cryoprotectant to the embryos must be avoided during this exposure time (Zhu et al., 1993).

Ethylene glycol is the only permeable component in the vitrification solution used in this report. At a high concentration during short periods of exposure, the intracellular concentration of ethylene glycol with low molecular mass increases more rapidly than that of glycerol, considering the molecular weights of the agents. (Szell et al., 1989; Tachikawa *et al.*, 1993). 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 of longer exposure than necessary (Tachikawa *et al.*, 1993). The possible method for reducing solution effect injury during vitrification is a stepwise exposure embryos to a successively increasing concentration of cryoprotectant, combined with a subsequently decreasing exposure time. With this procedure, *in vivo* produced embryos of different species (Rall and Fahy, 1985; Massip *et al.*, 1986; Smorag *et al.*, 1989; Rall, 1992; Zhu *et al.*, 1993) and also *in vitro* derived bovine embryos (Kuwayama *et al.*, 1992; Agca *et al.*, 1994; Dinnyes *et al.*, 1994; Mahmoudzadeh *et al.*, 1995) have been successfully cryopreserved. In the study reported here, survival rate of the embryos after vitrification was increased from 34.0% to 75.3% by adding the vitrification solution in two steps. Equally high survival rates were obtained by Zhu *et al.* (1993) for mouse expanded blastocysts produced *in vivo* and vitrified after exposure to 10 or 20% ethylene

glycol for 5min., and then exposed to EFS for 30s.

The present study, embryos suspended in 20% ethylene glycol regained their isotonic volumes within 5min. at 25℃, indicates that ethylene glycol permeated fully into the blastocoel cavity during the pretreatment period. These results strongly support that blastocysts can survive after vitrification if sufficient ethylene glycol permeates into the embryo, possibly into the blastocoel cavity (Zhu *et al.*, 1993).

Experiment 3. Test of Cryoprotectant Toxicity to Embryos

To investigate the toxicity of vitrification solution, when embryos were exposed to EFS 40, the survival rates after 24h of culture were 82.9~88.4% and the developmental rates to hatching stage after 48h were 59.4~88.4%.

Kasai *et al.* (1990) has shown that a combination of ethylene glycol, Ficoll, and sucrose; Ficoll as a macromolecule would tend to promote the formation of stable glasses (Fahy *et al.*, 1984), whereas sucrose would be tend to reduce the toxic effect of the permeating agent by promoting dehydration of the embryos by osmosis (Kasai *et al.*, 1990). EFS solution, which permits direct exposure of the embryos for sufficient permeation, appears to have the advantage of low toxicity (Zhu *et al.*, 1993).

This present results showed that exposure to the vitrification solutions at the early and middle stage had no detrimental effect on subsequent development to hatching blastocysts, but development of the hatching stage exposed was significantly reduced. This injury must be have been caused by the osmotic stress; the intracellular concentration of ethylene glycol in the hatching blastocyst would be expected to increase more rapidly than in the early and middle blastocyst, and this might promote the toxic effect.

When the expanded and hatching blastocysts were vitrified in EFS 40 and assessed by the re-expansion of the blastocoel cavity after 24 h, the postwarming survival rates of them (71.9% and 89.5%) were significantly higher than that of the early blastocysts (54.2%). This was very pronounced for embryos at the hatching blatocyst stage, but less pronounced for embryos at the middle blastocysts stage. It is also had a positive effect on the rate of hatching of the surviving embryos, particulary at the hatching blastocyst stage. It shows that cryopreservation of delayed embryos (early blastocyst stage) was much less successful than that of normally developed embryos (middle and hatching blastocyst stage).

For the efficient cryopreservation of *in vitro* produced embryos, it will also be important to produce embryos of high quality (Kennedy *et al.*, 1983; Iwasaki *et al.*, 1990). The increased sensitivity of embryos produced *in vitro* was not only due to the vitrification procedure, but also to a greater extent to embryos quality. (Mahmoudzadeh *et al.*, 1995).

CONCLUSIONS

This experimant was carried out to fine optimal conditions for obtaining high survival of mouse IVF/IVC blastocysts after vitrification. To produce blastocysts, B6CBA F1 (C57BL/6, ♀ × CBA/N, ♂) mouse eggs were inseminated with 1×10^6 spermatozoa/ml and cultured at 37℃ in 5% CO_2 in air for 96 h. The IVF/IVC blastocysts were divided into three stages of early, middle and hatching at day 4, individually. The vitrification solution used was EFS 40, contained 40% ethylene glycol diluted in mDPBS medium containing 30% Ficoll plus 0.5 mol sucrose (Zhu *et al.*, 1993). The embryos were exposed to 20% ethylene glycol and EFS 40 in two steps at 25℃, vitrified in liquid nitrogen and warmed rapidly.

The results obtained in these experiments were summarized as follows: 1) The rates of fertilization and development to blastocyst stage at day 4 of mouse eggs were 89.4% and

86.1%, respectively. 2) The postwarming survival rates of blastocysts equilibrated to 20% ethylene glycol for 5min. were significantly higher than those of them for 0, 1 and 3 min. 3) The rates of re-expanded blastocysts exposed to 20% ethylene glycol for 5min. and EFS 40 for 1min. were 82.9~88.4% after 24h of culture.

In conclusion, the present results show that exposure of the embryos produced *in vitro* to 20% ethylene glycol and EFS 40 has not a detrimental effect on their survival and it is possible to vitrify mouse blastocysts at 96h after IVF, especially middle and hatching, without significantly loss of viability *in vitro*. For high survival, embryos have to be exposed to cryoprotectants in steps; the 5min. pretreatment followed by 1min. exposure to the vitrification solution at room temperature is a simple procedure.

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