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In Vivo Development of Vitrified Mouse Expanding /Hatching/Hatched Blastocysts

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초자화 동결된 생쥐 팽창/탈출/완전탈출 배반포기배의 체내 발달

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요 약

본 연구는 초자화 동결된 생쥐 팽창, 탈출, 완전탈출 배반포기배의 체내 발달율을 조사하기 위해 실시하였다. 체외수정하여 얻어진 생쥐 배반포기배는 EFS40(40% ethylene glycol, 30% Ficoll, 0.3M sucrose)으로 초자화 동결하였다. 팽창, 탈출 배반포기배는 20% ethylene glycol에 5 분동안 평형시킨 다음, EFS40 용액에 1분간 노출후 액체질소에 침지하여 초자화 동결하였다. 완전탈출 배반포기배는 0.4% BSA가 첨가된 m-CR1 배양액에서 5일동안 배양하여 얻었으며, 10% EG에 5분, EFS40에 30초동안 노출하여 초자화 동결시켰다. 융해후 재팽창이 이루어진 배반포기배는 가임신 3일된 대리모의 한쪽 또는 양쪽 자궁각에(6~8개 /자궁각) 이식하였다. 대리모의 임신율과 착상율은 임신 15일째 외과적 해부로 판정하였다. 그 결과를 요약하면 다음과 같다. 1) 임신율과 정상 산자율은 초자화 동결된 팽창 배반포기배의 경우 77.8과 25.0%이었고, 탈출 배반포기배의 경우는 77.8과 26.4%로서 각각의 대조군에 있어서 66.7과 42.9%, 83.3과 40.4%에 비해 유의차가 없었다. 2) 완전탈출 배반포기배의 체외 발달율은 34.0%였고, 3) 체내 발달율은 33.3%였다. 이러한 결과는 본 실험에 사용된 EFS40 동결액을 이용한 초자화 동결방법이 생쥐 팽창, 탈출 배반포기배의 초자화 동결은 물론, 완전탈출 배반포기의 초자화 동결에도 유용하게 이용될 수 있다는 가능성을 시사하였다.

(Key words : Vitrification, EFS40, Chromosomal normality, Recipient, Live fetus)

I. INTRODUCTION

Vitrification is a powerful cryopreservation method of embryos. Rall and Fahy (1985) showed that mouse embryos can be successfully cryopreserved by vitrification. Scheffen et al. (1986) reported survival of mouse blastocysts after vitrification using a simple vitrification solution consisting of 25% glycerol and 25% 1, 2-propanediol. In recently, the method using vitrification solution based on ethylene glycol, which permeates the cell rapidly and has low toxicity, was firstly introduced by Kasai et al.

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(1990) at mouse morula stage. Since these days, many investigators also examined to determine whether it was effective for mouse embryos at other developmental stages(Ali et al., 1993; Miyake et al., 1993; Zhu et al., 1993).

To date, a few attempts have been made to cryopreserve expanded blastocysts. Various stages frozen in oocytes and embryos of mouse serve as useful models of the cryopreservation of cattle, pig, goat and of human oocytes/embryos for the *in vitro* fertilization system. In livestock embryos, the blastocysts is a stage of great importance. Recently, it was possible to vitrify of expanded cattle blastocysts through an process of *in vitro* maturation, fertilization and development(Massip et al., 1993; Tachikawa et al., 1993).

In previous study, we showed that *in vitro* produced mouse blastocysts could be successfully vitrified in EFS40, after equilibration to 20%(v/v) ethylene glycol (Kim et al., 1996a). Therefore, in the present study, we vitrified *in vitro* produced mouse blastocysts as a previously noted method and compared *in vivo* survival rates between vitrified expanding and hatching blastocysts. And experiments were also performed to determine whether hatched blastocysts could be vitrified using EFS40 and produced offspring.

Ⅱ. MATERIALS AND METHODS

1. Collection of blastocysts

Hybrid F1 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 hr later. At 13.5 hr after hCG injection, recovered oocytes were fertilized *in vitro* by epididymal sperm of hybird F1 male mice(C57BL/6 × CBA/N).

Then, mouse embryos were cultured in M16 medium upto the blastocysts or hatching blastocyst stage at 37℃, 5% CO_2 in air for 4 days. Also, some 4 day blastocysts were cultured in m-CR1 medium(Park et al., 1995) upto the hatched blastocysts stage for 5 days.

2. Vitrification

Vitrification solutions were designated EFS40 of which ethylene glycol was diluted to 40% (v/v) in modified Dulbecco's phosphate-buffered saline(mDPBS, Gibco BRL) medium containing 30%(w/v) Ficoll 70(average molecular weight 70,000, Sigma) and 0.5 M sucrose(Sigma). Ten or 20%(v/v) ethylene glycol in mDPBS was prepared as an equilibration solution. Vitrification of expanding/hatching blastocysts has been performed as a method of Kim et al. (1996b). And hatched blastocysts were vitrified according to the procedures described by Zhu et al.(1996).

3. Thawing and assessment of survival *in vitro*

The straws thawed rapidly in water bath at 25℃. The contents of each straw were expelled into dish containing 0.8 ml of DPBS containing 0.3 M sucrose (S-DPBS) by pushing the cotton plug and then put into fresh 0.3 M S-DPBS for 5 min. The embryos were transferred to fresh DPBS. Finally, after recovery in fresh DPBS for 5 min., they were cultured in a 50 μl droplet of M16 medium supplemented with 4 mg/ml bovine serum albumin. Survival rate after warming was assessed by examining re-expanded of blastocoel after 18 hr of culture.

4. *In vivo* development of blastocysts vitrified in EFS40

After thawing, recovered embryos were cultured in M16 or m-CR1 medium for 18 hr.

Re-expanded blastocysts were transferred surgically to one or both uterine horns(6~8 embryos per horn) of recipient female(ICR mice) on day 3 of pseudopregnancy. The day on which a copulation plug was found was designated day 1 of pseudopregnancy. Embryos recipients were autopsied on day 15 or day 16 of gestation. When autopsy was performed on day 15 or day 16, the number of normal fetuses and resorbing implantation was recorded.

5. Statistics

In vivo and *in vitro* survival rate of vitrified group was compared with that in the control group, with Chi-square test using SAS institute software.

Ⅲ. RESULTS AND DISCUSSION

1. *In vivo* development of vitrified mouse expanding and hatching blastocysts in EFS40

To examine *in vivo* development of vitrified expanding and hatching blastocyst (BL) respectively, re-expanded blastocysts after thawing at 18 hr were transferred into recipients on day 3 of pseudopregnancy.

As shown in Table 1, a total of 126 vitrified embryos(expanding BL : 60, hatching BL : 66) were transferred to 9 pseudopregnant recipients, respectively. Each 7 recipient became pregnant (77.8 and 77.8%). On day 15 gestation, 12 and 14 normal fetuses were observed and total implantation rates were 66.7 and 69.8% in vitrified group when expressed on the basis of the number of embryos transferred to recipients that became pregnant. In each control group, when 55 and 53 embryos were transferred to 6 recipients, each 4 and 5 were pregnant(66.7 and 83.3%). In pregnancy recipients, normal fetus rates(42.9 and 40.4%) and total implantation rates (66.7 and 63.8%) were observed on 15 day gestation. This result showed that there was no significantly difference *in vivo* development between control and vitrified group.

The high *in vitro* survival rates of vitrified mouse blastocysts(early, middle, hatching) in EFS40 were shown in Kim et al.(1996a). The survival rates of vitrified embryos *in vitro* were dependent on embryo stage(Kono and Tsunoda, 1987; Zhu et al., 1993). In blastocysts at various stages of development, survival rates decreased as the blastocoel enlarged(Miyake et al., 1993). However, Ali et al.(1993) reported that there are no clear difference between morula and early blastocysts *in vitro* survival but significantly difference *in vivo* offspring rates. Kim et al. (1996a) reported that cryopreservation of delayed embryos(early blastocyst stage) was much less successful than that of normally developed embryos(middle and hatching blastocyst stage). However, vitrified embryos in this experiment were normally developed to fetus and there was no significantly difference *in vivo* development between expanding and hatching stages.

2. *In vitro* survival of hatched blastocysts vitrified in EFS40

Cultured blastocysts in M16 medium for 4 days were transferred into m-CR1 medium to induce hatched stage from zona pellucida. About 50% of day 4 transferred blastocysts were hatched from the zona pellucida in m-CR1 medium at day 5 or day 6. Experimented blastocysts which were obtained at day 5, were vitrified by a method of Zhu et al.(1996). Embryos were equilibrated with a 10% (v/v) ethylene glycol for 5 min. before exposure to EFS40 for 30 sec. As indicated in Table 2, *in vitro* survival rate of vitrified hatched blastocysts was 34.0%. There was significantly difference compared to control($P<0.01$).

Table 1. *In vivo* development of expanding/hatching mouse blastocysts after vitrification

Treatment	Stage of blastocyst	No. of		*No. (P, T%)** of	
		Pregnant recipient(%)	Transferred embryos(PR/T)	Implantation	Live fetus
Control	Expanding	4/6(66.7)	42/55	28(66.7, 50.9)	18(42.9, 32.7)
	Hatching	5/6(83.3)	47/53	30(63.8, 56.6)	19(40.4, 35.9)
Vitrified	Expanding	7/9(77.8)	48/60	32(66.7, 53.3)	12(25.0, 20.0)
	Hatching	7/9(77.8)	63/66	37(69.8, 56.1)	14(26.4, 21.2)

PR : No. of transferred embryos on pregnancy recipient.

T : No. of transferred embryos to total recipient.

* : Number of implantations on day 15 of pregnancy.

** : Percentage of embryos transferred to recipients that became pregnant (P) and in total (T).

Table 2. *In vitro* development of hatched mouse blastocysts after vitrification

Treatment	No. of hatched blastocysts	No. (%) of embryos survived after 18 hr of culture
Control	50	49(98.0)
Vitrified	106	36(34.0)[a*]

[a] $P<0.01$, significantly different from control.

* No. of embryos survived after thawing 18 hr.

3. *In vivo* development of hatched blastocysts vitrified in EFS40

In vivo developmental potential of hatched blastocysts was examined by equilibrating embryos with 10%(v/v) ethylene glycol for 5 min. at 25℃ and vitrifying them after exposure for 30 sec. in EFS40. In total, 60 vitrified embryos were transferred to 11 recipients, of which only three became pregnant. On 15 day gestation, three normal fetuses were observed.

We also tested vitrification of day 6 hatched blastocysts, but survival rate was much less than that of day 5(data not shown). The viability of delayed embryos will be low and may be reduced further by the stress of cryoprotectant and cryopreservation(Zhu et al., 1996). Early hatched blastocyst has more resistant to cryopreservation than that hatched later. In bovine blastocysts produced through *in vitro* maturation and *in vitro* fertilization, it is known that blastocysts that developed early have more cells and fewer chromosomal anomalies than those developed later(Goto et al., 1992).

In the vitrification of day 5 hatched blastocysts in EFS40, the maximal survival rate obtained was only 34.0%. The low rate of development of *in vivo* and *in vitro* in this experiments could be due to restriction by either intracelluar ice formation by insufficient permeation of ethylene glycol or the chemical toxicity of ethylene glycol during exposure(Shaw et al., 1992). Another reason is due to embryo quality. For the efficient cryopreservation of *in vitro* produced embryos, it will also be important to produce embryos of high quality(Iwasaki et al., 1990).

Zhu et al.(1996) demonstrated that ethylene glycol permeates into hatched mouse blastocysts too rapidly and reduces survival due to its toxic action. In zona-intact mouse blastocysts, a high survival rate(92~95%) has been obtained after vitrification in EFS40 both by a one-step

Table 3. *In vivo* development of hatched mouse blastocysts after vitrification

Treatment	No. of		*No. (P, T%)** of		
	Pregnant recipient	Transferred embryos(PR/T)	Resorption	Live fetus	Total implantation
Control	3/8	17/43	9	2	11(64.7, 25.6)
Vitrified	3/11	18/60	3	3	6(33.3, 10.0)[a]

PR : No. of transferred embryos on pregnancy recipient.

T: No. of transferred embryos to total recipient.

* : Number of implantations on day 15 of pregnancy.

** : Percentage of embryos transferred to recipients that became pregnant (P) and in total (T).

[a] $P<0.01$, significantly different from control.

method(Kasai et al., 1990) and by a two-step method(Zhu et al., 1993). Considering that the permeability increases as development proceeds(Mazur et al., 1976), together with the shedding of the zona pellucida, the permeation of ethylene glycol into hatched blastocysts is expected to be more rapid than that into zona-intact blastocysts. This also suggested that hatched blastocysts are more likely to be injured by the toxicity of ethylene glycol after rapid permeation.

In conclusion, high survival of vitrified blastocysts was obtained in day 4 expanding and hatching stage blastocysts using EFS40. In hatched blastocysts, although a little normal fetus were produced, the survival rates were much less. However, the present study showed that mouse blastocysts after being hatched from the zona pellucida can be cryopreserved by vitrification.

The these results indicated that it is possible to vitrify expanding and hatching mouse blastocysts without significant loss of viability *in vivo*. However, It was necessary to further study for successful vitrification of hatched blastocysts.

Ⅳ. SUMMARY

This study was carried out to investigate the *in vivo* development rates of vitrified-thawed mouse expanding, hatching and hatched blastocysts(BL). *In vitro* fertilization produced blastocysts were vitrified in EFS40(40% ethylene glycol, 30% Ficoll and 0.3 M sucrose in phosphate buffer saline containing 10% FBS). Expanding and hatching blastocysts were equilibrated in 20% ethylene glycol(EG) for 5 min. before exposure to EFS40 at 25℃ for 1 min., they were then vitrified in liquid nitrogen. Hatched blastocysts which cultured in m-CR1 medium supplemented 0.4% bovine serum albumin on day 5, were equilibrated in 10% EG for 5 min. and then vitrified in EFS40 for 30 sec. After thawing, re-expanding blastocysts were transferred to recipients(3 day of pseudopregnant) on one or both uterine horns(6~8 embryos per a horn). Pregnancy rates of recipients and implantation were asscessed by autopsy on 15 gestation. The results obtained in these experiments were summarized as follows; 1) The pregnancy and live fetus rates, for vitrified expanding BL(77.8 and 25.0%) and hatching BL(77.8 and 26.4%) were not significantly difference in those of control BL (66.7 and 42.9%; 83.3 and 40.4%), respectively, 2) *in vitro* development of vitrified hatched BL was 34.0%, and 3) *in vivo* developmental rate of vitrified hatched BL was only 33.3%. These results suggested that proposed rapid vitrification procedures used EFS40 cryoprotectant can be

effectively performed in mouse expanding/hatching blastocysts and that mouse blastocysts after being hatched from zona pellucida can be successfully cryopreserved.

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