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Study on the Usability of Vitrified Mouse Hatched Blastocysts in Embryo Transfer

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초자화 동결된 생쥐 완전탈출 배반포기배 이식에 관한 유용성 검토

이봉경 · 김은영 · 남화경 · 이금실 · 윤산현* · 박세필 · 임진호*

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요 약

본 연구는 체외에서 배양된 생쥐 완전탈출 배반포기배를 동결보존액 EFS35를 이용하여 초자화 동결하였을 때 체내발달의 적합성 여부를 조사하기 위해 실시하였다. 공시된 완전탈출 배반포기배($\theta \leq 130$ μm)는 체내에서 생산된 전핵기 수정란을 5일 동안 체외배양하여 얻었으며, 10% ethylene glycol(EG)에 5분 노출한 후 EFS35(35% EG, 18% Ficoll, 0.3 M sucrose)에 30초 동안 노출하거나, 초자화 동결하였다. 융해 후, 재팽창이 이루어진 완전탈출 배반포기배는 가임신 3일된 대리모의 한쪽 또는 양쪽 자궁각(4~6개 /자궁각)에 이식하였다. 대리모의 임신율과 착상율은 임신 15일째 외과적 해부로 판정하였다. 그 결과를 요약하면 다음과 같다. 융해 30분 후, 완전탈출 배반포기배의 체외생존율은 노출군(65.5%)과 동결군(54.5%)간에 유의한 차이는 없었다. 또한, 체내발달율을 조사하였던 바, 착상율에 있어서 동결군(41.0%)과 대조군(58.5%)간에 유의한 차이는 없었지만, 정상산자율에서는 동결군(24.0%)의 결과가 대조군(58.3%)보다 유의하게 낮게 나타났다($p<0.05$). 이러한 결과는 EFS35를 이용한 완전탈출 배반포기배의 초자화 동결은 정상산자율은 감소하였지만, 완전탈출 배반포기배의 이용 효율성을 넓히는데 이용될 수 있다는 것을 알 수 있었다.

(Key words : Mouse, Hatched blastocysts, EFS35, *In vivo* development)

I. INTRODUCTION

Recently, attention has focused on vitrification as rapid and efficient method for cryopreservation of mammalian embryos. Vitrification has advantages over conventional methods of cryopreservation in that it takes only a few seconds to complete cooling, and there is no extracellular ice, which is one of the major causes of injury to cells (Mazur, 1984). Many investigators reported that mouse embryos, at stages from the one-cell to the zona-intact blastocyst, can be deeply frozen (Whittingham et al., 1972; Ali and Shelton, 1993; Miyake et al., 1993; Zhu et al., 1993). However, it is

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known that the sensitivity of embryos to cryopreservation is variable, and there have been only a few reports on the cryopreservation of mouse embryos at the stage of hatched blastocysts (HBs). Shaw et al. (1995) showed that high proportion of mouse HBs can survive after conventional slow freezing, but the survival rate of HBs by a rapid method was lower than that of embryos at earlier stages of development (Shaw et al., 1991). Recently, Zhu et al. (1996) reported successful and efficient vitrification freezing method of mouse HBs. Based on simple method, we tested to confirm the effects on survival and *in vivo* development of mouse HBs vitrified by the cryoprotectant EFS35.

II. MATERIALS AND METHODS

1. Production of mouse hatched blastocysts (HBs)

Hybrid F_1 female mice (4~6 weeks old) from C57BL/6 × CBA/N were superovulated by intraperitoneal (i.p.) injection with 7.5 IU pregnant mare's serum gonadotrophin, followed by i. p. injection with 7.5 IU human chorionic gonadotrophin (hCG) 50 hr later. The mice were mated with adult male mice of the same strain immediately after the injection of hCG and checked for mating the following morning. Zygotes produced *in vivo* were collected at 20 hr post hCG and they were cultured in mCR1aa (Park et al., 1995) until the formation of HBs for 5 days.

2. Vitrification and thawing

Embryos were vitrified in EFS35 by the procedures described by Kim et al. (1997), with some modifications. In this study, only more than middle size HBs ($\theta \geq 130$ μm) were examined on the basis of results in previous study (Yi et al., 1998). The embryos were manipulated at 25℃ and were equilibrated in 10% ethylene glycol (EG) based on Dulbecco's phosphate-buffered saline (DPBS) medium containing 10% FBS for 5 min and transferred to EFS35 (35% EG, 18% Ficoll, 0.3 M sucrose) in the straw for 30 sec before being plunged into LN_2. The exposed group was put through the same procedure as vitrification except being plunged into LN_2. For embryo recovery, straw was warmed rapidly at 25℃ and expelled into dish containing 0.8 ml of DPBS containing 0.5 M sucrose (S-DPBS) by pushing the cotton plug and then put into fresh 0.5 M S-DPBS for 5 min. And then, the embryos were placed to fresh DPBS.

3. Assessment of *in vitro* survival

After recovery from vitrification, embryos were washed and pooled in M16 at 37℃. And then, their survival was determined as re-expansion of the blastocoel within 30 min after recovery (Fig. 1).

4. *In vivo* development of vitrified HBs

To examine the developmental potential *in vivo*, control and survived embryos from the exposed and vitrified group were transferred to each uterine horn of recipient on day 3 pseudopregnancy that had been mated with vasectomized ICR males. All recipients were examined on day 15 of gestation to score the total number of fetuses including the resorption sites.

5. Statistical analysis

In each groups, *in vitro* survival and *in vivo* development rate were compared using the chi-square (χ^2) test.

III. RESULTS

1. *In vitro* survival of mouse HBs exposed or vitrified in EFS35

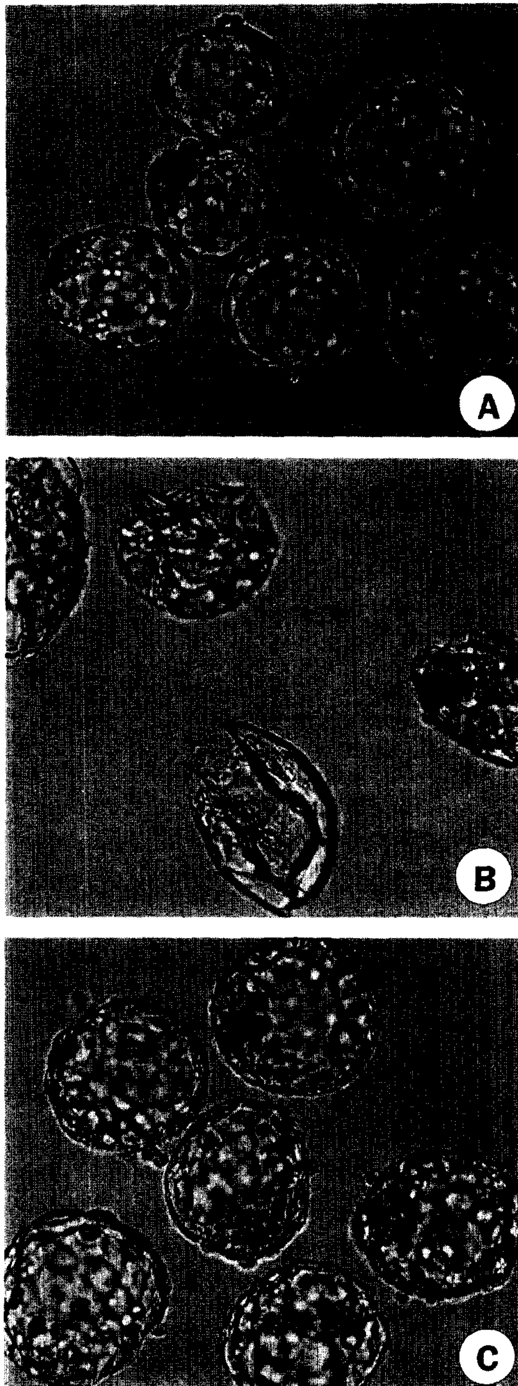

Fig. 1. Mouse hatched blastocyts on day 5. (A) before vitrification, (B) exposed to EF-S35, (C) survived embryos (×200)

The survived embryo *in vitro* was assessed as re-expansion of the blastocoel within 30 min after thawing. As shown in Table 1, a total of 55 exposed and 116 vitrified-thawed embryos were examined and *in vitro* survival rates of exposed and vitrified-thawed HBs were 65.5% and 54.5%, respectively. There was no significant difference.

2. *In vivo* development of vitrified mouse HBs

To examine the developmental potential *in vivo*, survived embryos in each group were transferred to day 3 pseudopregnant recipients. As shown in Table 2, in pregnancy rates, there were no significant differences among control (86.7%), exposed (83.3%) and vitrified (75.0%) groups, respectively ($p<0.05$). Also, total implantation against transferred embryos was no significant differences in each groups (58.5, 40.4 and 41.0%), respectively, although the total implantation against pregnant embryos was significant differences between control (72.7%) and vitrified (44.6%) groups ($p<0.05$). However, in implanted embryo, live fetuses and resorption sites were no significant differences between exposed (47.6 and 52.4%) and vitrified (24.0 and 76.0%) groups, although there were significant differences between control (58.3 and 41.7%) and vitrified (24.0 and 76.0%) groups ($p<0.05$).

Ⅳ. DISCUSSION

Many investigators have been reported successfully on cryopreservation of various embryo stages from the one-cell to the zona-intact blastocysts (Whittingham et al., 1972; Rall and Fashy, 1985; Ali and Shelton, 1993; Miyake et al., 1993; Zhu et al., 1993, 1996). Although the cryopreservation of mouse HBs has been only a few reports, if it can be cryopreserved, it will be

Table 1. *In vitro* survival of mouse HBs vitrified in EFS35

Treatment	No. of embryo examined	No. of embryos		
		Vitrified	Recoverd (%)	Survived (%)*
Exposed	55	–	55 (100.0)	36 (65.5)
Vitrified	116	116	112 (96.6)	61 (54.5)

* No. of embryos re-expanded within 30 min after thawing.
No significant difference from each other.

Table 2. *In vivo* development of mouse HBs after vitrified in EFS35

Treatment	No. of		No. of*		
	Pregnant recipient (%)	Transferred embryos (PR/T)	Total implantation (PR, T)**	Resorption sites	Live fetus
Control	6/7 (86.7)	33/41	24 (72.7^{a}, 58.5^{a})	10 $(41.7)^{b}$	14 $(58.3)^{a}$
Exposed	5/6 (83.3)	36/52	21 (58.3^{ab}, 40.4^{a})	11 $(52.4)^{ab}$	10 $(47.6)^{ab}$
Vitrified	6/8 (75.0)	56/61	25 (44.6^{b}, 41.0^{a})	19 $(76.0)^{a}$	6 $(24.0)^{b}$

PR : No. of transferred embryos on pregnant recipient.
T : No. of transferred embryos to total recipient.
* : No. of implantations on day 15 of pregnancy.
** : Percentage of embryos transferred to recipients that became pregnant (P) and in total (T).
a,b Means in the same column without common superscripts are significantly different ($p<0.05$).

passible to make wide use of embryonic resources. Our previous study (Yi et al., 1998) demonstrated that the classification of size in addition to morphological healthy level of HBs is important to improve the implantation rate and formation of live fetus. In addition, in our earlier study (Kim et al., 1997) reported that the lower survival rate and live fetus formation in HBs than in expanding or hatching blastocysts by vitrification may be due to the zona removed state which resulted in higher sensitivity to cryoinjury including cryoprotectant. Also, these results were obtained from EFS40 vitrification solution. In the present study, we investigated the *in vivo* developmental potential of mouse HBs using EFS35 vitrification solution. As shown in Table 2, in control group total implantation and live fetus rates were 58.5 and 58.3%, respectively. In vitrified group, 54.5% of HBs was survived and total implantation and live fetus rates were 41.0% and 24.0%, respectively. These results were higher than those of Kim et al. (1997). Kim et al. (1997) demonstrated that the maximal survival rate of HBs vitrified in EFS40 was only 34.0% and the proportion of total implantation and live fetuses were presented to 10.0 and 50.0%, respectively. Those differences may be originated from another factor. Generally, *in vitro* culture time for survival was given during 2~7 hr after thawing (Shaw et al., 1995; Zhu et al., 1996; Kim et al., 1997). It has known that HBs are more sensitive to culture

condition and poor tolerance to external stress. Thus, in this study, survival of thawed HBs was transferred to uterine immediately after being assessed within 30 min. On the other hands, Zhu et al. (1996) showed that high survival rate of HBs (92~95%) has been obtained after being vitrified-thawed based on glycerol (GFS40) and the proportion of total implantation and live fetus formation were 44.0 and 88.9%, respectively. In our study using EFS35, we obtained that the pregnancy rate and total implantation of HBs were not different between control and vitrified group, although live fetus rates were significantly different between two group ($p<0.05$). Therefore, this study suggested that vitrification freezing method of mouse HBs using EFS35 can be used to make wide the utility of embryo transfer of the more embryos produced *in vitro*.

V. SUMMARY

This study was carried out to confirm whether the *in vivo* developmental potential of mouse hatched blastocysts (HBs) can be obtained by vitrification method using the cryoprotectant EFS35. The HBs ($\theta \geq 130$ μm) were cultured *in vitro* until day 5 from zygotes produced *in vivo* and were equilibrated in 10% ethylene glycol (EG) for 5 min, and then were exposed or vitrified in EFS35 (35% EG, 18% Ficoll and 0.3 M sucrose). After 30 min thawing, re-expanding HBs were transferred into one or both uterine horns of pseudopregnant recipients on day 3 (4~6 embryos/horn). Pregnancy rates of recipients and implantation were assessed by autopsy on day 15 of gestation. The results obtained in these experiments were summarized as follows: After thawing, *in vitro* survival of HBs was not significantly different between exposed (65.5%) and vitrified (54.5%) group. Also, when the *in vivo* development potential was examined, total implantation was not different between control (58.5%) and vitrified (41.0%) group, although the live fetus formation of vitrified group (24.0%) was significantly lower than that of control (58.3%) group ($p<0.05$). These results suggested that vitrification freezing method of mouse HBs using EFS35 can be used to make wide the utility of embryo transfer of the more embryos produced *in vitro*.

VI. REFERENCES

1. Ali, J. and J. N. Shelton. 1993. Vitrification of preimplantation stages of mouse embryos. J. Reprod. Fertil., 98:459-465.
2. Kim, M. K., E. Y. Kim, H. S. Lee, B. K. Yi, S. H. Yoon, S. P. Park, K. S. Chung and J. H. Lim. 1997. *In vivo* development of vitrified mouse expanding/hatching/hatched blastocysts. Kor. J. Anim. Reprod., 21(2):131-137.
3. Mazur, P. 1984. Freezing of living cells: mechanisms and implications. Amer. J. Physiol., 247:C125-C142.
4. Miyake, T., M. Kasai, S. E. Zhu, T. Sakurai and T. Machida. 1993. Vitrification of mouse oocytes and embryos at various stages of development in an ethylene glycol-based solution by a simple method. Theriogenology, 40:121-134.
5. Park, S. P., S. E. Kim, S. J. Uhm, E. Y. Kim, T. Kim, S. H. Yoon, K. S. Chung and J. H. Lim. 1995. Effect of a simple serum-free medium, CR1, on the development of IVM/IVF bovine embryos. Kor. J. Fertil. Steril., 22(2):105-108.
6. Rall, W. F. and G. M. Fashy. 1985. Ice-free cryopreservation of mouse embryos at −196 ℃ by vitrification. Nature, 313:573-575.
7. Shaw, J. M., L. Diotallevi and A. O. Troun-

son. 1991. A simple rapid 4.5 M dimethylsulfoxide freezing technique for the cryopreservation of one-cell to blastocyst stage preimplantation mouse embryos. Reprod. Fertil. Develop., 3:621-626.

8. Shaw, J. M., C. Ward and A. O. Trounson. 1995. Survival of mouse blastocysts slow cooled in propanediol or ethylene glycol is influenced by the thawing procedure, sucrose and antifreeze proteins. Theriogenology, 43:1289-1300.

9. Whittingham, D. G., S. P. Leibo and P. Mazur. 1972. Survival of mouse embryos frozen to −196℃ and −269℃. Science, 178:411-414.

10. Yi, B. K., E. Y., Kim, H. K. Nam, K. S. Lee, S. H. Yoon, S. P. Park and J. H. Lim. 1998. Study on the usability of mouse hatched blastocysts in embryo transfer. Kor. J. Anim. Reprod., 22(1):11-17.

11. Zhu, S. E., M. Kasai, H. Otoge, T. Sakurai and T. Machida. 1993. Cryopreservation of expanded mouse blastocysts by vitrification in ethylene glycol-solutions. J. Reprod. Fertil., 98:139-145.

12. Zhu, S. E., T. Sakurai, K. Edashige, T. Machida and M. Kasai. 1996. Cryopreservation of zona-hatched mouse blastocysts, J. Reprod. Fertil., 107:37-42.

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