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In Vitro/In Vivo Development of Vitrified Mouse Zygotes and Chromosome Analysis of Offspring

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초자화 동결된 생쥐 1-세포기배의 체외/체내 발달과 산자의 염색체 분석

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요 약

본 연구는 체외생산된 생쥐 1-세포기배의 초자화동결이 체외/체내 발달율과 수정란 이식후 태어난 산자의 염색체에 미치는 영향을 검토하기 위해 실시하였다. 체외수정하여 얻어진 생쥐 1-세포기배는 EFS40 (40% ethlyene glycol, 30% Ficoll, 0.3 M sucrose) 동결액을 이용하여, 상온 (25℃)에서 30 초 동안 노출한후, 액체질소에 침지하여 초자화동결하였다. 동결후 융해된 생쥐 1-세포기 수정란은 M16 배양액에서 4일동안 배반포기까지 배양하였고, 이때 배반포기까지의 체외배 발달율은 71.5%였으며, 배양된 배반포기배는 가임신 3일된 대리모의 한쪽 또는 양쪽 자궁각에 (6~8개/자궁각) 이식하였다. 모든 대리모는 분만을 유기하였으며, 그 결과를 요약하면 다음과 같다. 임신율과 체내 생존율 즉, 산자 생산 (80.0, 39.6%)에 있어서 대조군 (77.8, 50.0%)과 유의차가 없었다. 또한 수정란 이식후 태어난 모든 산자의 염색체 (n=40)는 정상이었다. 이상의 결과로 미루어볼때 본 실험에서 이용된 초자화동결방법은 생쥐 1-세포기배의 동결에 효과적으로 이용될 수 있음을 시사한다.

(Key words : Vitrification, Mouse zygote, Chromosone, EFS4O)

I. INTRODUCTION

Vitrification is a simple and rapid freezing method for the preservation of mammalian embryos (Fahy et al., 1984; Rall and Fahy, 1985). Last many investigators were applied vitrification to various developmental stage embryos (Ali et al., 1993; Miyake et al., 1993; Zhu et al., 1993; Zhu et al., 1996). Kasai et al. (1990) demonstrated that mouse morulae have been vitrified by a simple method which based on ethylene glycol (EFS40). Kim et al. (1996c) were successfully vitrified mouse blastocysts using EFS40. It has been reported that the cryosurvival rates of mouse 1-cell embryos were relatively low in vitrification techniques (Hsu et al., 1986). However, Norihiro et al. (1993) obtained high cryosurvival rate (80%) in 1-cell embryos using DPS (DMSO + propylene glycol

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+ sucrose). In recent, we also have established conditions for the vitrification of mouse 1-cell zygotes, using EFS40 cryoprotectant (Kim et al., 1996b).

However, it has been known that embryos derived from vitrified had an increased incidence of aneuploid and development poorly to fetus (Kola et al., 1988; Bouquet et al., 1995). Moreover, our previous results were showed that vitrification or exposure to EFS40 of mouse zygotes induced increase of sister chromatid exchanges (SCE) frequency (Kim et al., 1996a). But it is not known whether our vitrification method is directly harmful for production of offspring after embryo transfer.

Therefore, it would seem necessary to further study the development of embryos obtained from vitrification, particularly after embryo transfer and the production of descendants. In this paper, we reported on the developmental potential of vitrified mouse zygotes after uterus embryo transfer and the incidence of chromosome abnormalities in delivered live young.

Ⅱ. MATERIALS AND METHODS

1. Production of zygotes *in vitro*

Hybrid F1 female mice (4~5 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 inseminated *in vitro*. After IVF at 20~22 hr, zygotes, 2 pronuclei and second polar body formed, were collected.

2. Vitrification of zygotes

Zygotes were vitrified by using a method of Kim et al. (1996a). The vitrification solution, EFS40, consisted of 40% (v/v) ethylene glycol (Sigma), 30% (w/v) Ficoll 70 (average molecular weight 70,000, Sigma) and 0.3 M sucrose (Sigma) dissolved in modified Dulbecco's phosphate-buffered saline (mDPBS, Gibco BRL). Zygotes were exposed to EFS40 at 25℃ and loaded into 0.25 ml straw for 30 sec. The plug end of each straws was powder and heat sealed and slowly immersed into liquid nitrogen (LN_2).

3. Thawing and assessment of *in vitro* survival

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 *ul* droplet of M16 medium supplemented with 4 mg/ml BSA. Survival rate after thawing was examined as cleavage rate to the 2-cell stage after 24 hr of culture. The development of 2-cell was assessed by their ability to develop into blastocysts at day 4 culture.

5. *In vivo* development of embryos

Fresh and vitrified day 4 embryos that developed to the blastocyst stage 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. All recipients were allowed to produce litters. The number and sex of normal pups of produced litters were recorded.

6. Chromosome analysis

3-week-old produced mice by ET were used in

chromosome analysis. After colcemid (0.2 ml) injects i.p. for 1.5~2 hr, isolated femurs from mouse plushed two times with phosphate buffer saline. Bone marrow cells were placed in a hypotonic solution of potassium chloride (0.075M) for 25 min. and then fixed methanol : acetic acid = 3 : 1. After the slides were warmed in slide warmer for at least 24 hr, the slides were stained with 8% Giemsa at pH 6.8.

7. Statistics

Survival rate of vitrified group was compared with that in the control group, with Chi-square test using SAS institute software.

Ⅲ. RESULTS

In vitro produced zygotes (by *in vitro* fertilization) were vitrified in EFS40 for 30 sec. at 25 ℃ and recovered after warming as a method of Kim et al. (1996b). As shown in Table 1, over 90% of vitrified zygotes were developed into 2-cell during culture at M16. And about 70% of 2-cells was cultured upto blastocysts for culture 4 days. In Table 2, to test *in vivo* viability of vitrified embryos, cultured blastocysts after thawing were transferred to the uterine horns of recipients. Total 71 vitrified embryos were transferred to 10 recipients, of which 8 (80.0%) became pregnant: eight of these animals delivered 16 live young and 5 stillborn fetuses on days 21. In control, seventy-two fresh embryos (day 4 blastocysts) were transferred to 9 recipients. Seven of them became pregnant and they delivered 27 live young and 3 stillborn fetuses. Also, of the young, eleven were females and 7 were males (3 were not identified) in vitrified group, and 17 were females and 11 were males (2 were not identified) in control group. Especially, pregnancy rates of recipients and the *in vivo* survival rate after vitrification (80.0, 39.6%) were not significantly different in those of control group (77.8, 50,0%), although total fetus rates in vitrified group were slightly lower compared to those in control group.

Ten of produced live male young and 10 of female were tested chromosome analysis (control : ♂6, ♀4, vitrified : ♂4, ♀6) (Fig. 1). All of examined young were chromosomally normal. The number of chromosome was 40.

Table 1. *In vitro* development of vitrified mouse zygotes

Treatment	No. of zygotes	Development (%)	
		2-cell (D1)	Blastocyst (D4)
Control	87	84 (96.6)	76 (90.5)
Vitrified	150	137 (91.3)	98 (71.5)*

* $P<0.05$, significantly different from control.

Table 2. *In vivo* development of blastocysts *in vitro* cultured after vitrified-thawed zygotes

Treatment	No. of (%)			No. of embryos transferred to PR[a]	No. of young (%)		
	Recipient	Pregnant recipients	Embryos transferred		Still births[b]	Alive	Total
Control	9	7 (77.8)	72	60	3	27	30 (50.0)
Vitrified	10	8 (80.0)	71	53	5	16	21 (39.6)

[a] Recipients that became pregnant.

[b] Dead fetus day 21~23 after delivery

Fig. 1. The mouse chromosome of female live-born young after vitrification. Metaphase spread showing normal chromosome complement of 40 (×1,000).

Ⅳ. DISCUSSION

It has been shown rapid and efficient cryopreservation of mouse preimplantation at zygotes by the vitrification method using EFS40 in our previous experiment (Kim et al., 1996b). In this study, we were obtained 71.5% *in vitro* survival rates at vitrified 1-cell mouse zygotes. Although *in vitro* development rates into blastocysts were slightly decreased compared to control, there was no different in total cell number between control and vitrified group (Kim et al., 1996b).

However, Bouquet et al. (1993, 1995) demonstrated that the increased frequency of polyploidy was observed in embryos after cryopreservation of oocytes. Indeed, embryos obtained from frozen oocytes or oocytes exposed to prefreezing manipulations showed an increase in the frequency of SCE. We also obtained the same results of which the vitrification and exposure to EFS40 increased frequencies of SCE (Kim et al., 1996a). Some investigators reported that the incidence of early postimplantation loss in vitrified embryos compared to that in control embryos was relatively high, but malformed fetuses were not reported. (Kono et al., 1987; Nakagata et al., 1989). Dinnyés et al. reported that the cryopreservation did not influence *in vitro* embryos (8- to 16-cell mouse embryos) survival after thawing but the *in vivo* development of vitrified embryos was significantly influenced by cryopreservation method (vitrification vs slow freezing) and genotype. In addition, it has been known that viability of vitrified embryos depends on developmental stage (Norihiro et al., 1993). It has been reported that 1-cell embryos may be more sensitive to the vitrification process. Thus, it is not known whether our proposed vitrified method really influence to development of offspring in mouse 1-cell zygotes.

To examine *in vivo* developmental rates after vitrification, we performed uterus embryo transfer at blastocyst stages. The results of embryo transfer showed that the viabilities of vitrified mouse (39.6%) were comparable with those in control (50.0%). Also, there were no significantly difference in pregnancy rates between vitrified and control (80.0 vs 77.8%).

On the other hand, all of delivered live young in both group were chromosomally normal (n=40). In our preliminary test, it was shown that mice derived 1-cell zygotes vitrified with EFS40 could reproduce (data not shown).

Therefore, it can be concluded that mouse 1-cell zygotes could be effectively vitified our proposed methods using EFS40 and this method did not make malformed fetus in chromosome.

Ⅳ. SUMMARY

The objective of this study was to investigate the *in vitro*/*in vivo* embryonic development after vitrification of mouse zygotes and the chromosomal normality of delivered live young after

embryo transfer. Mouse IVF zygotes were cryopreserved by vitrification using vitrification solution, EFS40 (40% ethylene glycol, 30% Ficoll and 0.3 M sucrose in phosphate buffer saline containing 10% FBS). After mouse zygotes were exposed to EFS40 at 25℃ for 30 sec., they were immediately plunged into LN_2. Vitrified-thawed mouse zygotes were cultured upto blastocysts in M16 for 4 days. The rates of *in vitro* development were 71.5% under this condition. Cultured blastocysts were transferred to recipients (3 day of pseudopregnant) on one or both uterus horns (6~8 embryos per a uterus horn). And all recipients were allowed to produce litters. The results obtained in these experiments were summarized as follows; The pregnancy rates and *in vivo* survival rates, live fetus rates, for vitrified zygotes (80.0, 39.6%) were not significantly difference in those of control zygotes (77.8%, 50.0%). Also, all of live-born young mice were chromosomally normal (n=40). This results suggested that proposed rapid vitrification procedures can be effectively use in 1-cell mouse zygotes cryopreservation.

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