韓國家畜繁殖學會誌 23(2) : 133~139(1999)
Korean J. Animal Reprod.

In Vitro/In Vivo Development of Vitrified Immature Mouse Oocytes

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초자화 동결된 생쥐 미성숙란의 체외/체내 발달

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요 약

본 연구는 생쥐 미성숙란을 초자화 동결-융해하였을 때, 체외/체내 배발달능을 검토하고자 실시하였다. 생쥐 미성숙란은 동해제인 EFS40(40% ethylene glycol, 18% ficoll, 0.5 M sucrose)으로 초자화 동결되었으며, 융해 후 16 시간동안 체외성숙을 유도하여, 제 1극체가 나타난 성숙된 난자를 1~2×10^6/ml 농도의 정자로 체외수정시킨 다음, 난할율(≥2-세포기)과 체외/체내 발달율을 조사하였다. 생쥐 미성숙란을 초자화 동결-융해하였던 군(63.1%)의 체외성숙율은 동해제 노출군(67.5%)과 대조군(66.3%)에 유사하게 나타났으나, 초자화 동결군의 난할율과 배반포형성율 (64.9, 59.0%)은 동해제노출군(83.7, 74.7%)과 대조군(90.7, 83.7%)에 비해 유의하게 감소하였다($p<0.05$). 그러나, 초자화 동결-융해하였던 생쥐 미성숙란으로부터 얻어진 배반포기배를 가임신 생쥐에 이식하였을 때, 체내발달율인 전체착상율(31.3%)과 착상된 배로부터 발달된 산자형성율 (66.7%)은 대조군의 결과(40.8%, 58.1%)와 각각 비교하였을 때 유의차가 인정되지 않았다. 따라서, 생쥐 미성숙란을 초자화 동결-융해하였을 때, 체외발달율은 유의하게 감소하였지만 생성된 배반포기배로부터의 산자발달율은 대조군과 유사하게 나타나, EFS40을 이용한 초자화 동결 방법은 생쥐 미성숙란 동결에 유용하게 이용될 수 있다는 것을 알 수 있었다.

(Key words : Mouse immature oocytes, Vitrification, EFS40, *In vivo* development)

I. INTRODUCTION

Several studies have been suggested that immature germinal vesicle (GV)-stage oocytes may be more suitable than mature oocytes for cryopreservation(van der Elst et al., 1993; Candy et al., 1994; Toth et al., 1994). Because the chromosomes of GV-stage oocytes are decondensed in early prophase and not arranged on the temperature-sensitive meiotic spindle, it is assumed to be less prone to microtubular and chromosomal damage. So, it is well known that these characteristics of GV-stage oocytes could be reduced detrimental changes on mature oocytes such as a decreased fertilizability, with incomplete reversibility of the depolymerization of the microtubules of the spindle, and hence with increased risk of chromosomal disorder, which might result in aneuploid embryos

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(Glenister et al., 1987; Pickering et al., 1990; van der Elst et al., 1992; Candy et al., 1994). However, in our previous study(Kim et al., 1998), we reported that the mature mouse oocytes could be cryopreserved successfully using a vitrification solution, EFS based on ethylene glycol(EG) and their developmental potential *in vivo* was not decreased, when vitrified embryos were thawed and transferred into a pseudopregnant recipient. Also, we confirmed that our vitrification method for mature oocytes did not induce the alteration of cytoskeleton and chromosomal constitution (Yi et al., 1998). In this study, we investigated the *in vitro* maturation and *in vitro /in vivo* development of immature mouse oocytes by previous freezing method used for mature mouse oocytes.

Ⅱ. MATERIALS AND METHODS

1. Collection of GV-stage mouse oocytes

Oocytes collected from 3 to 5 weeks old female mice (C57BL/CBA) F_1 hybrid were primed with an intraperitoneal injection of 7.5 IU pregnant mare's serum gonadotrophin (PMSG, Sigma). Between 48~52 hr after PMSG injection, the animals were killed. The ovaries were removed after rupture of the periovarian sac and incubated subsequently in M2 medium supplemented with 10% fetal bovine serum (FBS, Gibco) and containing 0.25 mM the meiotic inhibitor dibutyryl cAMP (dbcAMP, Sigma) at 37℃ chamber for about 30 min. Fully grown GV-stage oocytes (70~80 μm) were selected (Fig. 1. A). Oocytes were washed and divided into three groups according to experimental purpose; control, exposure to cryoprotectant and vitrification. The exposed group was put through the same procedure as vitrified group except being plunged into liquid nitrogen (LN_2).

2. Vitrification and thawing

The procedures was modified from that described by Kim et al. (1998). All manipulations were carried out using M2 medium containing 10% FBS and dbcAMP at 25℃. To find the optimal vitrification solution, in this study, EFS35 or EFS40 (35% or 40% ethylene glycol (EG), 18% ficoll, 0.5 M sucrose and 10% FBS added in M2) was used. Before freezing, the cumulus -free oocytes were equilibrated with exposure in a 10% EG (in case of EFS35) or 20% EG (in case of EFS40) for 5 min, respectively (Fig. 1. B). Then, oocytes were exposed in EFS consisting of 35% EG or 40% EG for 30 sec. before being plunged into LN_2, respectively. Thawing was achieved by agitating the straws in a 25℃ water until ice crystals disappeared. Oocytes were released and equilibrated in a M2 medium containing 0.5 M sucrose for 5 min, and then in a M2 medium for 10 min. (Fig. 1. C).

3. *In vitro* maturation (IVM), fertilization (IVF) and development (IVD)

Morphologically normal oocytes after thawing or exposure were assessed as survived embryos. In this study, cumulus cell monolayered drop was used in all procedures including IVM /IVF-/IVD to serve the more improved *in vitro* environment. Cumulus cell monolayered drop was prepared with 10 μl in 30 φ dish (#1007, Sigma) and then covered with mineral oil (Sigma). Immature oocytes from each treatment group were washed three times in M2 medium and then were transferred into cell drop containing mCR-1aa (Kim et al., 1997) medium supplemented with 10% FBS. After 16 hr, the oocytes were assessed for extrusion of the first polar body (Fig 1. D). *In vitro* matured oocytes from each treatment were placed into cell drop containing M16 medium and capacitated sperm was added ($1\sim2\times10^6$ cells /ml). After 6 to 8 hr of coincub-

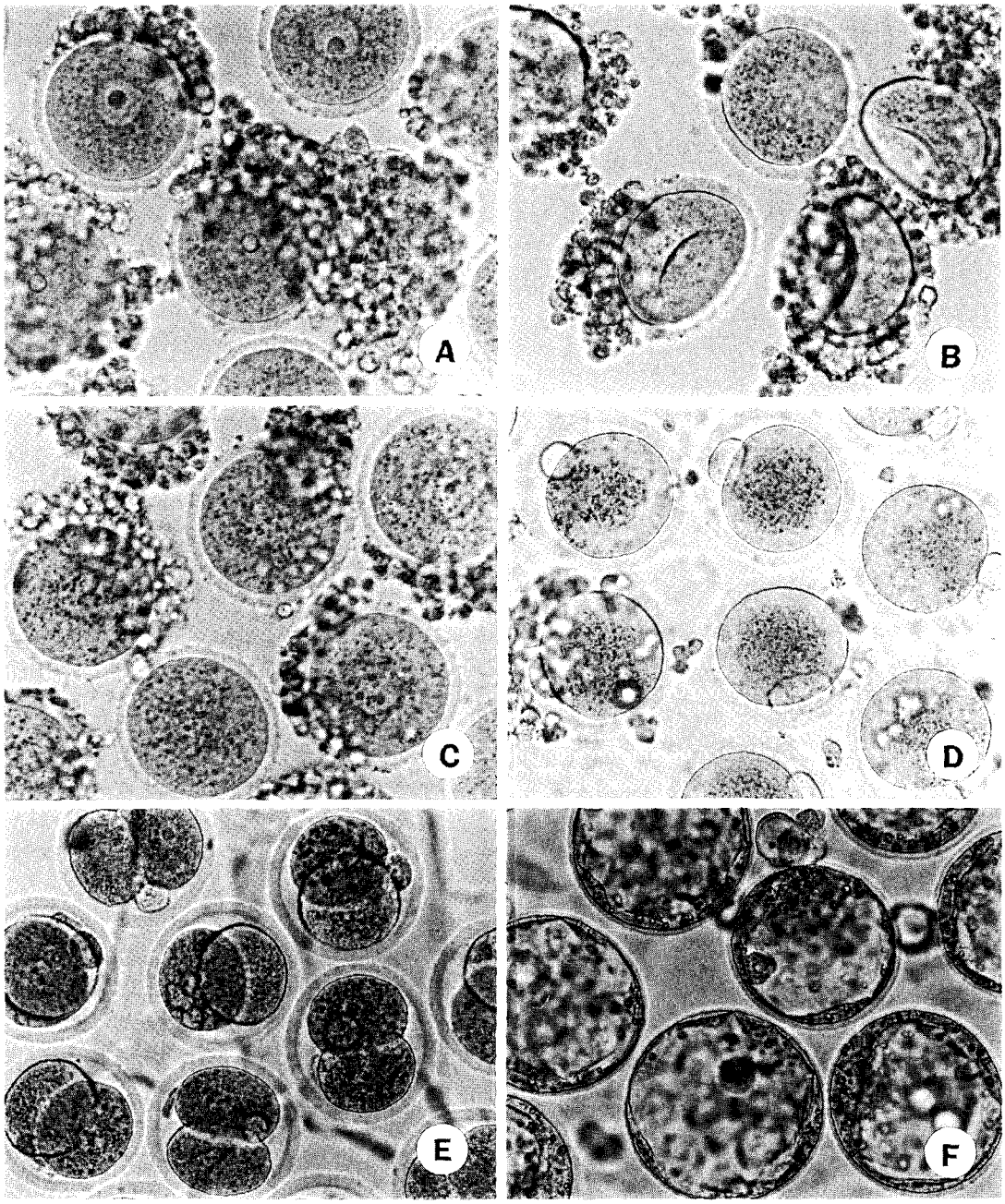

Fig. 1. A series of development *in vitro* of mouse immature oocytes vitrified using EFS40. (A) a group of partly attached by cumulus cells (fresh), (B) exposed in EFS40, (C) recovered, (D) matured ; mature oocytes demonstrating the first polar body extruded after maturation *in vitro*, (E) cleaved ; a cleaved embryo at 24 hr post insemination sperm, (F) day 4 blastocysts. ×250.

ation, inseminated oocytes were washed and transferred into cell drop containing mCR1aa medium (20 oocytes/50 μl). Embryo development to 2-cell and blastocyst was assessed at day 1 and 4 after insemination, respectively (Figs. 1E and F).

4. *In vivo* development

To examine the developmental potential *in vivo*, control and survived blastocysts developed from vitrified group were transferred to one uterine horn of recipient ICR mice on day 3 of pseudopregnancy (6~8 embryos/uterine horn). All recipients were examined on day 15 of gestation to score the total number of implanted fetuses including the resorption sites.

5. Statistical analysis

In each groups, the results were compared using the chi-square (χ^2) test.

Ⅲ. RESULTS

To select the optimal vitrification solution for immature mouse oocytes, we examined the *in vitro* maturation, cleavage and blastocyst formation of immature oocytes vitrified using EFS35 and EFS40 (Table 1). The blastocyst formation rate in EFS40 (68.7%) was significantly higher than that in EFS35 (35.3%), although the rates of survival (88.1 and 92.1%), maturation (64.9 and 58.5%) and cleavage (75.0 and 70.8%) were similar between two groups. When we investigated the toxicity of EFS40 solution to mouse im-

Table 1. *In vitro* developmental capacity of immature mouse oocytes vitrified using EFS35 or EFS40

Treatment	No. of oocytes vitrified	No. (%) of oocytes			No. (%) of	
		Survived.	Matured	Insem.*	2-cell	Blastocyst
EFS35	89	82 (92.1)	48 (58.5)	48	34 (70.8)	12 (35.3)[a]
EFS40	84	74 (88.1)	48 (64.9)	48	36 (75.0)	24 (68.7)[b]

[a,b] Superscripts are significantly different within the same column ($p<0.05$).

* insem.: inseminated.

Table 2. *In vitro* development of immature mouse oocytes vitrified using EFS40

Treatment	No. of oocytes	No. of oocytes			No. (%) of	
		Survived	Matured	Insem.*	2-cell	Day 4 blastocyst
Control	163	–	108 (66.3)	108	98 (90.7)[a]	82 (83.7)[a]
Exposed	175	154 (88.0)	104 (67.5)	104	87 (83.7)[a]	65 (74.7)[a]
Vitrified	165	149 (90.3)	94 (63.1)	94	61 (64.9)[b]	36 (59.0)[b]

[a,b] Superscripts are significantly different within the same column ($p<0.05$).

* insem.: inseminated.

Table 3. *In vivo* development of immature mouse oocytes vitrified using EFS40

Treatment	No. (%) of		No. (%) of *		
	Pregnant recipient	Transferred embryos (PR/T)	Total implantation (PR,T)**	Resorption sites	Live fetus
Control	6/9 (66.7)	45/76	31 (68.9, 40.8)	13 (41.9)	18 (58.1)
Vitrified	5/10 (50.0)	34/67	21 (61.8, 31.3)	7 (33.3)	14 (66.7)

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).

mature oocytes, as shown in Table 2, the cryoprotectant has not detrimental effect on IVM/IVF/IVD rates of exposed group. However, the cleavage (64.9%) and embryonic development (59.0%) rates of vitrified group were significantly lower than those of exposed (83.7 and 74.7%) and control group (90.7 and 83.7%). However, to examine the developmental potential *in vivo*, when the blastocysts produced at day 4 after IVF from control and vitrified-thawed immature oocytes were transferred to day 3 pseudopregnant recipient, there was no difference between two groups. As indicated in Table 3, the pregnancy rate of vitrified group (50.0%) was slightly lower than that of control (66.7%). Also, total implantation against transferred embryos in vitrified group (31.3%) was slightly lower than that in control (40.8%), but live fetus formation (66.7%) rate on day 15 was higher than that in control (58.1%). In contrast, the resorption rate of vitrified group (33.3%) was lower than that of control (41.9%).

Ⅳ. DISCUSSION

Cryopreservation of immature oocytes has been performed in several mammalian species that immature oocytes can be matured and fertilized *in vitro* and the resulting embryos develop to term (van der Elst et al., 1992, 1993; Cooper et al., 1998; Cha et al., 1991; Hunter et al., 1995). In this study, we applied a vitrification method used for the freezing of mature mouse oocytes in previous study (Kim et al., 1998) to that of immature mouse oocytes. Also, investigation on *in vitro* maturation and *in vitro/in vivo* development of vitrified immature mouse oocytes was undertaken. *In vitro* maturation rates of all group were similar. However, our data (63~67%) was somewhat lower than that (71 to 99%) reported by other researchers (van der Elst et al., 1993; Candy et al., 1994; Cooper et al., 1998).

To select the optimal vitrification solution for immature mouse oocytes, we compared the data from oocytes vitrified with EFS35 and EFS40. As shown in Table 1, the blastocyst formation rate in EFS40 (68.7%) was significantly higher than that in EFS35 (35.3%). It suggested that the vitrification using EFS40 is available for cryopreservation of immature oocytes. When the toxicity of EFS40 solution to immature mouse oocytes was examined, the cryoprotectant has not detrimental effect on fertilization and em-

bryonic development of exposed group (Table 2). However, the cleavage rate (63.1%) and embryonic development (59.0%) in frozen-thawed immature oocytes were significantly lower than those of exposed (83.7 and 74.7%) group and control (90.7 and 83.7%) ($p<0.05$). These data suggest that immature mouse oocytes were damaged by freezing and thawing. Van Blerkom et al. (1989) reported that reduced *in vitro* development rates of vitrified oocytes may be due to a certain subtle nuclear and cytoplasmic disorders.

When embryos derived from vitrified-thawed oocytes were transferred into recipient on day 3 pseudopregnancy, the pregnancy rate was similar to that of control (Table 3). This result indicates that embryos derived from vitrified immature oocytes can obtain a developmental potential *in vivo*. Although total implantation rate of vitrified group was lower than that of control, live fetuses were slightly higher than that of control,. but there was no significant difference between two groups. Candy et al. (1994) demonstrated cryopreserved GV-stage mammalian oocytes can produced a normal offspring, but they transferred 2-cell embryos and formed fetuses was 26%. Additionally, to date, no transfers have been performed with embryos resulting from oocytes frozen or very few pregnancies were observed (Cooper et al., 1998). However, in the present study, when the *in vitro* produced blastocysts of vitrified group were transferred into recipient, regardless of lower development *in vitro*, *in vivo* development was similar to that of control group. It is considered that immature mouse oocytes can be successfully cryopreserved using vitrification solution, EFS40.

V. SUMMARY

This study was carried out to investigate *in vitro /in vivo* development of vitrified-thawed immature mouse oocytes. Immature mouse oocytes were vitrified with EFS40 (40% ethylene glycol, 18% ficoll and 0.5 M sucrose). Thawed oocytes were matured for 16 hr *in vitro*. Matured oocytes with the first polar body were fertilized with the concentration of $1\sim2\times10^6$/ml of epididymal sperm. After fertilization, cleavage ($\geq$ 2-cell) and *in vitro /in vivo* development rates were examined. The results were summarized as follows: *in vitro* maturation rate of immature mouse oocytes in vitrified-thawed group was similar to that in exposed group (67.5%) and control (66.3%), but cleavage rate of vitrified-thawed oocytes (64.9 %) and blastocyst formation rate (59.0%) were significantly different compared to those of exposed group (83.7 and 74.7%) and control (90.7 and 83.7%) ($p<0.05$). However, when the blastocysts derived from immature mouse oocytes vitrified-thawed were transferred to pseudopregnant mouse, total implantation (31.3%) was slightly lower than that in control (40.8%), but live fetus formation rate (66.7%) was slightly higher than that in control (58.1%), there was not significantly different. Therefore, when the blastocyts produced *in vitro* were transferred into recipients, although the development *in vitro* of oocytes vitrified-thawed was decreased, live fetus formation rate was similar to that of control group. The present results indicate that immature mouse oocytes can be frozen successfully by vitrification with EFS40.

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(접수일자 : 1999. 4. 15. /채택일자 : 1999. 6. 4.)