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In Vitro/In Vivo Development of Mouse Oocytes Vitrified by EFS

M.K. Kim, E.Y. Kim, B.K. Yi, S.H. Yoon[1], S.P. Park, K.S. Chung[2] and J.H. Lim[1]

Maria Infertility Medical Institute, [1]Maria OB/GYN, Seoul, [2]College of Animal Husbandry, Kon-Kuk University

EFS로 초자화 동결된 생쥐 미수정란의 체내/외 발달

마리아 기초의학 연구소, [1]마리아 산부인과, [2]건국대학교 축산대학

김묘경 · 김은영 · 이봉경 · 윤산현[1] · 박세필 · 정길생[2] · 임진호[1]

= 국문초록 =

본 연구는 생쥐 미수정란을 초자화 동결하였을 때, 체내/외 발달율에 미치는 영향을 연구하고자 실시하였다. 생쥐 미수정란은 30, 35, 40% ethylene glycol, 18% ficoll 과 0.5 M sucrose가 함유된 M2 배양액으로 구성된 EFS30, 35, 40을 이용하여 초자화 동결되었다. 노출 또는 초자화 동결-융해 후, 형태학적으로 정상적인 미수정란은 1-2×10^6/ml 농도의 정자로 체외수정되었고, 수정율과 체내/외 발달율 그리고 배반포의 세포수 (inner cell mass 와 tropectoderm cell)가 조사되었다. 본 실험에서 얻어진 결과는 다음과 같다. 35%의 ethylene glycol이 함유된 EFS35에서 EFS30과 EFS40보다 높은 분할율을 나타냈다. 초자화 동결-융해 후 체외수정된 미수정란의 2-세포기까지의 발달율 (51.1%)은 동결없이 초자화 동결액에 노출만된 군 (60.0%)과 대조군 (68.2%)에 비해 유의하게 감소하였다 ($p<0.05$). 그러나 이들 처리군에 있어서 난할된 난자로 부터의 배반포기배까지의 발달율에는 유의한 차가 없었다 (75.0, 73.3 과 80.0%). 또한 초자화 동결된 군의 배반포기배 세포수 (92.5±2.9)도 노출군 (98.5±5.3), 대조군 (100.9±4.8)과 유사하였다. 초자화 동결-융해하여 얻어진 배반포기배를 가임신 생쥐에 이식하였을 때 체내발달율인 산자발달율 (50.7%)과 착상율 (80.0%)도, 대조군 (58.2, 78.2%)과 유사하였다. 이러한 결과로 보아, 생쥐 미수정란은 ethylene glycol를 기본으로 한 EFS35라는 초자화 동결액을 이용하여 동결보존될 수 있음을 알 수 있었다.

INTRODUCTION

Many investigators have reported that mammalian embryos could be successfully cryopreserved (Whittingham *et al.*, 1972; Friedler *et al.*, 1988; Kim *et al.*, 1996a). However, developmental capacity of cryopreserved oocytes was still poor compared to that of embryonic stages, because the cryopreservation method of oocytes was not optimal resulting in overall reduced survival and fertilization rates (Glenister *et al.*, 1987). These failure may be due to zona hardening (Carroll *et al.*, 1990), spindle disruption (Aigner *et al.*, 1992) and chromosome abnormality (Bouquet *et al.*, 1992) by freezing. Earlier studies on oocytes cryopreservation have performed the slow or conventional cooling procedures using dimethylsulfoxide (DMSO) and 1,2-propanediol (Whittingham, 1972; Trounson and Kirby, 1989; Aigner *et al.*, 1992). Recently, ultra-rapid freezing and vitrification which were rapid and simple freezing method (Kola *et al.*, 1988; Nakagata, 1989; Kono *et al.*, 1991) have been introduced in the oocytes cryopreservation. Shaw *et al.* (1991) reported

high development rates of vitrified-thawed mouse oocytes after fertilization *in vitro*. Successful vitrification requires a high concentration of cryopreservation and optimal freezing procedure that minimize both osmotic damage and chemical toxicity.

Kim *et al.* (1996a) reported that mouse zygotes could be vitrified by EFS40 which based on 40% ethylene glycol which exposed to EFS 40 for 30 sec. However, unfertilized oocytes of mouse have lower permeability to cryoprotectant, thus sufficient time for the cryoprotectant to permeate into the cells is need (Rayos *et al.*, 1994).

Therefore, this study was conducted to select the optimal vitrification solution to maintain the survival of mouse oocytes. *In vitro/in vivo* development and cell number of cryopreserved mouse oocytes using selected vitrification solution were examined.

MATERIALS AND METHODS

1. Production of zygotes *in vitro*

Superovulated mouse oocytes were obtained from C57BL/CBA F1 hybrid female mice by 7.5 IU PMSG and 7.5 IU hCG 48 hr apart. Cumulus cells were removed by incubating in M2 medium containing 0.03% hyaluronidase. The denuded oocytes were washed three times and pooled in fresh M2 medium before vitrification or fertilization *in vitro*.

2. Experimental groups

Oocytes with normal morphology were distributed to three experimental groups. The first group was served as a control. The second group was put through the same procedure as vitrification except being plunged into liquid nitrogen (LN_2) (exposed). The third group was vitrified using the procedure described below.

3. Vitrification and thawing

Oocytes were frozen according to our modified protocol described by Kim *et al.* (1996a). All freezing solution were made with M2 containing 10% FBS, and manipulations were carried out at 25℃ room temperature. Vitrification solution (VS) was used EFS (ethylene glycol, 18% ficoll and 0.5 M sucrose in M2 medium). The cumulus-free mouse oocytes were first exposed to 10% ethylene glycol (EG) for 10 min. Then, oocytes were transferred individual EFS consisting of 30, 35 and 40% EG for 30 sec before being plunged into LN_2. Vitrified samples were warmed in a 25℃ water bath; then cryoprotectant was removed by two-step methods which oocytes were exposed to 0.5 M sucrose-M2 medium and M2 medium for 5 min at 25℃, respectively and then transferred to fresh M2 medium for 10 min at 37℃.

4. Assessment of *in vitro/in vivo* development

Morphologically normal eggs at 1 hr after thawing or exposure were underwent insemination *in vitro* with epididymal sperm ($1\text{-}2\times10^6$/ml) in M16 medium. After being incubated for 6 hr, some oocytes were stained by hoechst for the assessment of fertilization. Also, the remainders were cultured and scored the cleavage rates to the two-cells stage at 24 hr after insemination. Development to the blastocysts was assessed at day 4 after insemination. And some of these blastocysts were transferred to the uterus of day 3 pseudopregnant female ICR recipients. The recipients were killed on day 15 of pregnancy and the number of live and resorbing fetuses as well as the implantation sites were determined.

5. Inner cell mass (ICM) and trophectoderm (TE) cell count

Day 5 cultured embryos were stained as described by Kim *et al.* (1996b). The cell numbers of ICM and TE cell in vitrified-thawed embryos by differential labelling of the nuclei with immunosurgery and polynucleotide-specific fluorochromes were compared to that of exposed and control.

6. Data analysis

All the experiments were repeated at least three times. Data were analyzed by chi-square tests.

RESULTS

1. Effect of ethylene glycol concentration on development

To select the optimal vitrification solution of mouse oocytes, we examined the *in vitro* fertilization and blastocysts formation of oocytes vitrified to various EG levels (30, 35 and 40%). The survival rates at EFS30, 35 and 40 were 95.5, 94.3 and 20.8%, respectively (Table 1). The cleavage and blastocysts formation of EFS30 and 35 were significantly higher than those of EFS40 ($p<0.01$). No significant difference were between EFS30 and EFS35. However, higher fertilization and blastocysts formation rates were obtained in EFS35 than in EFS30. Therefore, in the following experiment, oocytes were vitrified in EFS35

2. Fertilization rates

The results of sperm penetration into the oocytes at 6 hr after insemination were summarized in Table 2. The pronuclear rates of vitrified oocytes were significantly lower than those of control oocytes ($p<0.05$). But there were not significant differences between control and exposed group. Moreover, polyspermy rates of vitrified group (28.9%) were significantly higher than those of control (5.2%) and exposed (9.3%) group.

Table 1. Survival of vitrified-thawed oocytes exposed to EFS30, 35, 40 vitrification solution

Treat.	No. of vitrified oocytes	No. of survived oocytes	No. of inseminated oocytes	No. of embryos (%)	
				2-cell	Day 4 blastocyst
EFS30	155	148 (95.5)^{a}	141	43 (30.5)^{ab}	30 (70.0)
EFS35	157	148 (94.3)^{a}	148	71 (48.0)^{a}	55 (77.5)
EFS40	125	26 (20.8)^{b}	24	3 (12.5)^{b}	2 (66.7)

^{a,b} Superscripts were significantly different within the same column ($p<0.01$)

Table 2. Fertilization of vitrified and exposed mouse oocytes in EFS35

Treatment	No. of examined oocytes	No. of oocytes fertilized			Mean no. of sperm/oocyte
		Total	2PN	Polyspermic	
Control	83	58 (69.9)^{a}	55 (66.3)^{c}	3 (5.2)^{c}	1.1
Exposed	85	54 (63.5)^{a,b}	49 (57.7)^{c}	5 (9.3)^{c}	1.2
Vitrified	83	45 (54.2)^{b}	28 (33.7)^{d}	13 (28.9)^{d}	1.6

^{a,b} Superscripts were significantly different within the same column ($p<0.05$)
^{c,d} Superscripts were significantly different within the same column ($p<0.01$)

Table 3. *In vitro* development of mouse oocytes vitrified in EFS35

Treat.	No. of examined oocytes	No. of survived oocytes (%)	No. of inseminated oocytes	No. of (%)	
				2-cell	Day 4 blastocyst
Control	110	110 (100)	110	75 (68.2)^{a}	60 (80.0)
Exposed	110	108 (98.2)	100	60 (60.0)^{a,b}	44 (73.3)
Vitrified	110	101 (91.8)	94	48 (51.1)^{b}	36 (75.0)

^{a,b} Superscripts were significantly different within the same column ($p<0.05$)

3. *In vitro* development

The cleavage rates of vitrified oocytes (51.1%) (Table 3) were significantly lower than those of control and exposed (68.2, 60.0%) ($p<0.05$). However, 75.0% of cleaved embryos in vitrified group developed to the blastocysts stages at day 4 after insemination. This data was similar to that of control and exposed (80.0, 73.3%).

4. Number of ICM and TE cell per blastocysts

To evaluate embryo qualities, day 5 blastocysts were fixed for counting their number of ICM and TE cell. The counts of total and ICM/TE cell number using differential labelling are summarized in Table 3. There were no significant differences in the number of total cell among fresh cultured blastocysts (100.9±4.8), exposed (98.5±5.3) and vitrified (92.5±2.9). Also, the ICM and TE cell number among groups was not different.

5. *In vivo* development

The development *in vivo* after embryo transfer of blastocysts derived from vitrified oocytes and of control blastocysts is shown in Table 5. The blastocysts derived from vitrified-thawed oocytes that developed to live fetuses (50.7%) on 15 day were similar to those from control (58.2%). Moreover, the implantation rate of vitrified (80.0%) was not significantly different from that of control (78.2%).

Table 4. Numbers of ICM and TE cell of day 5 blastocysts derived from vitrified oocytes

Treatment	No. of total cell (Mean±S.E.)	Cell number (Mean±S.E.)	
		ICM	TE
Control	100.9±4.8	19.1±1.7	81.8±3.7
Exposed	98.5±5.3	18.5±1.4	78.5±5.0
Vitrified	92.5±2.9	17.5±1.0	74.9±2.7

S.E.: standard error, ICM: inner cell mass
TE: trophectoderm cell

DISCUSSION

The EG as a cryoprotectant was first examined by Miyamoto and Ishibashi (1994) who found that embryos frozen in EG showed survival rates that were equal to or higher than embryos frozen in DMSO. In this study, more than 90% of the frozen oocytes were recovered and survived in EFS30 and 35 after thawing, but most of oocytes were lysed immediately in EFS40 (Table 1). When the straw vitrified in EFS30 was thawed, vitrified glass of the EFS 30 droplet becomes temporarily opaque during warming which is an indication of devitrification (ice formation). It causes thermal stress to oocytes. Forty percent EG cause lysis of oocytes. Prolonged exposure of oocytes to high EG concentration caused bleb formations on the embryos (Hotamisligil *et al.*, 1996). Rayos *et al.* (1994) reported that ethylene glycol in combination with either sucrose or trehalose can be used effectively in the quick freezing of unfertilized mouse oocytes.

A number of studies have suggested that cooling of unfertilized oocytes, even to room temperature, may impair their ability to be fertilized and developed normally (Glenister *et al.*, 1987; Wood *et al.*, 1992). In the study reported here, we obtained a high mean per-

Table 5. *In vivo* development of blastocysts derived from vitrified mouse oocytes

Treat.	No. of pregnant recipient (%)	No. of transferred blastocyst		No. of Day 15 of gestation (%)		
		Total	PR	Live fetuses	Resorption site	Total implantation
Control	10/13 (76.9)	73	55	32 (58.2)	11 (20.0)	43 (78.2)
Vitrified	10/12 (83.3)	87	75	38 (50.7)	22 (29.3)	60 (80.0)

PR: Pregnant recipient

centages of morphologically normal oocytes (over 90%). Although a high survival rate was obtained, we reported a mean percentages of two-cells that was lower than exposed and control. In addition, polyspermy was significantly increased in vitrified group. Carroll *et al.* (1990) reported an increased frequency of polyploidy in frozen-thawed oocytes compared with the non-frozen control. They explained that this was caused mainly by the retention of the second polar body. Also, an increased frequency of polyploidy may be due to polyspermy (Rayos *et al.*, 1994). However, the development rate of vitrified-thawed oocytes to the blastocysts stage after *in vitro* fertilization was not significantly different compared to that of exposed and control. It has been reported that cooling of oocytes, exposure to vitrification solution can have a zona-hardening and fertilization-decreasing effect (Johnson *et al.*, 1988; Wood *et al.*, 1992). Since exposure to the cryoprotectant did not decrease fertilization rates in our study, it is possible that the fertilization decreasing effect is related to variations in the freezing itself.

We also performed comparing ICM and TE cell number in control (19.1 ± 1.7, 81.8 ± 3.7), exposed (18.5 ± 1.4, 78.5 ± 5.0) and vitrified group (17.5 ± 1.0, 74.9 ± 2.7). There were no differences among groups. Live normal fetuses on day 15 obtained after transfer of blastocysts derived from frozen-thawed oocytes were similar to those of control. These results demonstrated that blastocysts derived from frozen-thawed oocytes had not lost their ability to enter mitotic division. Moreover, the implantation rates and live fetus rates to the blastocysts obtained from vitrified-thawed oocytes that were inseminated and cultured *in vitro* were similar to those of control (Table 5). This finding was inconsistent with reports by Van der Elst *et al.* (1993), who showed that ultrarapid freezing induced abnormal fetus.

In conclusion, mouse unfertilized oocytes were cryopreserved by in this proposed vitrification method which used ethylene glycol (EFS35). However, further study should be necessitated to increase fertilization rates and find more suitable method. Overall, the results presented here suggest that vitrification could be an acceptable method for cryopreservation of unfertilized ova.

SUMMARY

This study was carried out to investigate *in vitro/in vivo* development of vitrified mouse oocytes. Mouse oocytes were vitrified using EFS30, 35 and 40 (30, 35 and 40% ethylene glycol, 18% ficoll and 0.5 M sucrose in M2 medium). After being exposed or vitrified-thawed, oocytes of normal morphology were inseminated *in vitro* by $1\text{-}2 \times 10^6$/ml of epididymal sperm. The rates of fertilization, *in vitro/in vivo* development and cell number (inner cell mass and tropectoderm cell) of blastocysts in each treatment group were examined. The results obtained in these experiments were summarized as follows: The cleavage rates were obtained in EFS35 containing 35% ethylene glycol higher than in EFS30 and EFS40. The development rate of vitrified-thawed oocytes to two-cell stage after *in vitro* fertilization (51.1%) was significantly different compared to that of exposed to vitrification solution without cooling (60.0%) and control (68.2%) ($p<0.05$). However, there were no differences in the blastocyst formation from the cleaved embryos among groups (75.0, 73.3 and 80.0%). Also, the mean number of cells per blastocysts of vitrified group (92.5 ± 2.9) was similar to that of the exposed (98.5 ± 5.3) and control (100.9 ± 4.8). *In vivo* development of the blastocysts derived from vitrified-thawed oocytes resulted in fetal development (50.7%) and implantation rates (80.0%) which are very similar to those of control (58.2, 78.2%). These results suggest that mouse oocytes could be cryopreserved using vitrification solution (EFS35) based on ethylene glycol.

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