大韓不妊學會雜誌：第24卷 第3號 1997
Kor. J. Fertil. Steril., Vol. 24, No. 3, 1997, 12

In Vitro/In Vivo Development after Thawing of Vitrified Mouse Blastocysts by Culture Condition and Embryo Transfer Method

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초자화 동결된 생쥐 배반포기배의 융해 후 배양조건과 수정란 이식방법에 따른 체외/체내발달

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본 실험은 초자화 동결된 생쥐 배반포기배의 융해 후 배양조건 및 이식방법이 난자의 생존에 미치는 효과를 조사하고자 실시하였다. 체외수정후, M16배양액에서 4일동안 배양하여 얻어진 생쥐 배반포기배는 EFS40 (40% ethylene glycol, 18% Ficoll, 0.5 M sucrose가 함유된 PBS)으로 초자화동결하였다. 실험 I에서는 융해 후 배양조건에 따른 난자들의 체외/체내 생존율을 조사하였다. 융해된 난자가 M16과 4 mg/ml 소혈청알부민과 20 가지 아미노산이 함유된 m-CR1 (2% BME 아미노산 용액, 1% MEM 아미노산 용액) 및 단층배양이 유도된 난구세포 (10% FBS가 함유된 m-CR1배양액)에서 각각 배양되었을때, 융해 후 24시간째 체외 생존율은 배양조건에 따라 차이가 없었다 (75.6, 83.1, 82.4%). 그러나 체내 발달율에 있어서 임신 15일째 생존 산자율은 39.0, 49.0, 38.1%로서 유사한 성적을 나타냈으나, 전체 착상율에 있어서는 m-CR1 (80.4%)에 배양되었을 때, M16 (51.2%), 난구세포와 공배양시 (57.1%) 보다 유의하게 높은 생존율을 보였다 ($p<0.05$). 실험 II에서는 수정란 이식 방법에 따른 체내발달율을 조사하였다. 배반포기배를 융해 후 체외배양없이 곧바로 가임신 2, 3일째 대리모에 이식을 실시하였을때, 가임신 2일째 대리모에서는 임신징후를 얻지 못하였고, 가임신 3일째 대리모에서는 50.0%의 착상율과 15.4%의 정상산자율을 얻었다. 그러나, 이러한 결과는 융해 후, 16시간 배양하여 가임신 3일째 대리모에 이식 (73.5, 57.1%)하는 경우보다 유의하게 낮은 결과였다 ($p<0.05$). 실험 III에서는 초자화 동결된 배반포기배의 융해 후 배양시 발달이 늦어진 수정란의 이용효율을 극대화시키기 위해 융해한 4일째 초기, 5일째 초기, 5일째 팽창 배반포기배의 체외/체내 생존율을 조사하였다. 가장 높은 체외 생존율은 5일째 팽창 배반포기배 (78.3%)에서 얻었으나, 체내 발달율 (산자율, 착상율)에 있어서는 4일째 초기 배반포기배 (33.3, 66.7%)의 경우가, 5일째 팽창 배반포기배 (29.0, 38.7%)의 경우보다 높았다 ($p<0.05$). 따라서 본 연구의 결과는 배양조건과 수정란 이식방법에 따라 초자화 동결된 배아의 체외/체내 발달율을 높일 수 있으며, 발달이 늦은 배반포기배의 체내 발달율은 체외 배양시간이 길어질수록 낮아짐으로, 5일째 팽창 배반포기배보다 4일째 초기 배반포기배를 동결하는 것이 더 유용하다는 것을 알 수 있었다.

INTRODUCTION

Vitrification of embryos at various developmental stages was successfully achieved in mouse and bovine embryos (Tachikawa *et al.*, 1991; Zhu *et al.*, 1993). Many investigators have examined *in vitro* and *in vivo* survival of vitrified-thawed embryos by various cryoprotectants (dimethyl sulfoxide, glycerol, propylene glycol etc.), vitrification procedures (concentration of cryoprotectants, exposure time and temperature etc.) (Kasai *et al.*, 1990; Ali *et al.*, 1993; Zhu *et al.*, 1993). Successful freezing of mouse blastocysts which were produced by *in vitro* fertilization and *in vitro* culture was obtained by using EFS40 vitrification solution (40% ethlyene glycol, 18% Ficoll and 0.5 M sucrose in m-DPBS) (Kim *et al.*, 1996). Also, 25~26.4% of *in vivo* development potential to live fetus have been reported (Kim *et al.*, 1997). Vergos *et al.* (1991) reported that only embryos of excellent quality can develop to term. This quality was an important factor to determine the developmental capacity of blastocysts after transfer or their ability to withstand freezing. In other words, the successful embryo survival was dependent on the efficient culture system after thawing (Massip *et al.*, 1993). Also, Vicente *et al.* (1996) stressed that a simpler method for the transfer of cryopreserved embryos is essential. Seidel *et al.* (1992) reported that direct transfer of embryos to recipient could be reduce the time required and improve the process in frozen embryos.

Therefore, this study was to test whether *in vitro/in vivo* survival of vitrified mouse blastocysts was influenced by culture conditions and embryo transfer (ET) method. In addition, it has been tested that day 4 early, day 5 early and day 5 expanding blastocysts were vitrified by EFS40 and compared their in *vitro/in vivo* survival rates to enhance usability of delayed blastocysts.

MATERIALS AND METHODS

1. Production of blastocysts *in vitro*

Production of mouse blastocysts was performed as a method described Kim *et al.* (1996). Briefly, hybrid F1 female mice (C57BL/6xCBA/N) were superovulated by intraperitoneal injection of 7.5 I.U. pregnant mare serum gonadotrophin, followed by 7.5 I.U. human chorionic gonadotrophin (hCG) 50 hr later. At approximately 13.5 hr post-hCG injection, the cumulus-oocyte complexes were inseminated with epididymal sperm of hybrid F1 male mice ($1x10^6$/ml). After *in vitro* fertilization, embryos were cultured in M16 medium at 37℃, 5% CO_2 for 4~5 days.

2. Vitrification studies

In vitro cultured blastocysts were vitrified by a method of Kim *et al.* (1997). As a vitrification solution, EFS 40 [containing 40% (v/v) ethylene glycol, 18% (w/v) Ficoll 70 (average molecular weight 70,000) plus 0.5 M sucrose in modified Dulbecco's phosphate-buffered saline (mDPBS)] was used. Embryos were equilibrated with D-PBS medium containing 20% ethlyene glycol for 5 min. And these embryos were transferred to EFS40 in the straw for 30 sec at room temperature (25℃). The straw was then slowly immersed into liquid nitrogen.

For embryo recovery, straws were warmed rapidly in water bath at 25℃. The contents of each straw were expelled into dish containing 0.8 ml of D-PBS containing 0.5 M sucrose (S-DPBS) and then put into fresh 0.5 M S-DPBS for 5 min. The embryos were transferred to culture medium.

3. Experimental design

Experiment I. *In vitro/in vivo* survival of vitrified day 4 mouse blastocysts after thawing by culture condition

To examine *in vitro* viability of vitrified blas-

tocysts, recovered blastocysts in fresh D-PBS were cultured in M16 medium, m-CR1 medium supplemented with amino acids (2% basal medium eagle's (BME) amino acids and 1% minimum essential medium (MEM) non-essential amino acids solution) and 4 mg/ml BSA, and cumulus monolayer cell cultured in m-CR1 (10% FBS) medium at 37℃, 5% CO_2 incubator. Their survival rates were assessed by re-expansion of the blastocoel (A) within 16~18 hr and (B) within 24 hr of culture. And survived blastocysts within 16~18 hr after thawing were transferred to one uterine horn of recipient ICR mice on day 3 of pseudopregnancy (6-8 embryos/uterine horn). To examine *in vivo* survival rates, the recipients were killed on the day 15 of gestation, and the number of normal fetuses and resorbing fetuses, as well as the implantation sites were recorded.

Experiment II. *In vivo* development of vitrified blastocysts according to ET method

This experiment was performed to compare *in vivo* survival rates of vitrified embryos between direct ET without culture time and ET after culture for 16 hr post thawing. In case of direct ET, after thawing within 1 hr, blastocysts were transferred to recipients at day 2 or 3 of pseudopregnancy. Live fetus and implantation rates were recorded as described above.

Experiment III. *In vitro/in vivo* survival after thawing of vitrified delayed-blastocysts

Delayed blastocysts (day 4 early, day 5 early, day 5 blastocysts) were vitrified as described above. And their *in vitro/in vivo* survival rates were compared.

4. Statistics

The survival rate in each treatment was compared using chi-square analysis.

RESULTS

Experiment 1. *In vitro/in vivo* survival of vitrified blastocysts after thawing by culture condition

To improve the embryo quality after thawing, blastocysts were cultured in M16 medium (used in previous study, Kim *et al.*, 1997), m-CR1 medium supplemented 4% BSA and cumulus monolayer cell in m-CR1 medium (contained 10% FBS). *In vitro* survival of embryos is summarized in Table 1. At 24 hr after thawing, the survival rates were in M16 (75.6%), m-CR1 (83.1%) and co-cultured medium (82.4%),

Table 1. *In vitro* survival rates of vitrified day 4 blastocysts according to culture condition after thawing

Treatment	No. of vitrified day 4 blastocysts	*Survival rates (%)	
		A	B
M16	86	60 (70.0)	65 (75.6)
m-CR1	201	159 (79.1)	167 (83.1)
Cumulus co-culture	199	148 (74.4)	164 (82.4)

A: after 16~18 hr, B: after 24 hr
*Not significantly different.

Table 2. Development *in vivo* of vitrified blastocysts according to culture condition after thawing

Treatment	*No. of pregnant recipient	**No. of blastocyst transferred		No. of day 15 of gestation (%)		
		Total	PR	Resorption sites	Live fetuses	Total implantation
M16	6/8	53	41	5	16 (39.0)	21 (51.2)[a]
m-CR1	8/8	51	51	16	25 (49.0)	41 (80.4)[b]
Cumulus co-culture	7/8	48	42	8	16 (38.1)	24 (57.1)[a]

*: Day 3 pseudopregnant recipient
**: Day 4 cultured embryos
PR: Pregnant recipient
[a,b] Different superscripts within column were significantly different ($p<0.05$).

Table 3. *In vivo* survival rates of vitrified-thawed blastocysts according to embryos transfer method

Treatment	Day of pseudo-pregnancy	No. of pregnant recipient	No. of blastocyst transferred		No. of day 15 of gestation (%)		
			Total	PR	Resorption sites	Live fetuses	Total implantation
Direct	2	0/8	56	–	–	–	–
	3	4/8	51	26	8	4 (15.4)[a]	12 (50.0)[a]
After culture	3	7/8	55	49	8	28 (57.1)[b]	36 (73.5)[b]

PR: Pregnant recipient
[a,b] Different superscripts within column were significantly different ($p<0.05$).

Table 4. Survival rate of vitrified-thawed day 4, day 5 early and day 5 expanding blastocysts (Bl)

Treatment	No. of vitrified blastocysts	No. of recovered embryos	No. of survival embryos (%)
Day 4 early Bl	108	106	67 (63.2)[a]
Day 5 early Bl	84	81	38 (47.0)[b]
Day 5 > Bl	108	105	83 (78.3)[a]

[a,b] Different superscripts within column were significantly different ($p<0.05$).

Table 5. Survival *in vivo* of day 4 early and day 5 expanding blastocysts (Bl)

Treatment	No. of pregnant recipient	No. of blastocyst transferred		No. of day 15 of gestation (%)		
		Total	PR	Resorption sites	Live fetuses	Total implantation
D4 early Bl	8/8	54	48	12	16 (33.3)	32 (66.7)[a]
D5 > Bl	5/9	57	31	3	9 (29.0)	12 (38.7)[b]

PR: Pregnant recipient
[a,b] Different superscripts within column were significantly different ($p<0.05$).

and there were not significantly different among groups. *In vivo* survival rates (Table 2), the rates of live fetuses on day 15 gestation were 39.0 49.0 and 38.1% in M16, m-CR1 and co-culture condition, respectively. Also, there were no differences among groups. However, *in vivo* total implantation rates were obtained significantly higher in m-CR1 medium (80.4%) than in M16 (51.2%) and co-culture medium (57.1%) ($p<0.05$).

Experiment 2. *In vivo* development of vitrified blastocysts according to ET method

To enhance live fetus rates, *in vivo* developmental potential was examined by ET methods. As shown in Table 3, no recipient became pregnant when transferred to day 2 pseudopregnant recipients soon after thawing. And 50% of recipients which directly transferred on day 3 pseudopregnancy became pregnant and 15.4% of the pregnant recipients developed to live fetuses. When thawed embryos cultured for 16～18 hr after thawing were transferred on day 3 of pseudopregnant recipients, the rates of pregnancy and live fetus were 87.5 and 57.1%, respectively.

Experiment 3. *In vitro/in vivo* survival after thawing of vitrified delayed-blastocysts

To elevate the usability of delayed embryos, the survival rates *in vitro/in vivo* of day 4 early, day 5 early and day 5 expanding blas-

tocysts were examined. As indicated in Table 4, *in vitro* survival rates of day 4 early, day 5 early and day 5 expanding blastocysts after thawing were 63.2, 47.0 and 78.3%, respectively. However, *in vivo* survival rates (live fetus, total implantation) were higher in day 4 early blastocysts (33.3, 66.7%) than in day 5 expanding blastocysts (29.0, 38.7%), although the highest *in vitro* survival rates were obtained in the day 5 expanding blastocysts (78.3%).

DISCUSSION

In previous study, we have reported that mouse blastocysts which were produced by *in vitro* fertilization could be vitrified using EFS-40 (Kim *et al.*, 1997).

In experiment I, *in vitro*/*in vivo* survival has been tested after thawing of day 4 expanding blastocysts (except early blastocysts) cultured in different culture conditions. Three culture conditions were considered as follows: M16, m-CR1 medium and cumulus monolayer cell co-culture. M16 medium has been used as basic medium of mouse embryos (Whittingham *et al.*, 1971). m-CR1 medium consisted of NaCl, KCl, $NaHCO_3$, lactate, pyruvate and 20 amino acids (Park *et al.*, 1995). In all of conditions, *in vitro* survival rates were excellent (75~83%) with no difference among conditions (Table 1).

However, *in vivo* survival after thawing was influenced by culture conditions (Table 2). The highest *in vivo* survival rates were obtained in m-CR1 culture condition. Rosenkrans *et al.* (1989) demonstrated that CR1 medium was as effective as co-culture in supporting preimplantation bovine embryo development. Uhm *et al.* (1996) reported that amino acids presenting high levels in oviduct and uterine fluids can stimulate the development of pig hatching blastocysts. These results suggest that amino acids might be support to improve of embryo quality, since implantation rates were increased in m-CR1 medium. Massip *et al.* (1993) reported that limited re-expansion of blastocysts in control medium (M199) was increased in conditioned medium. But hatching were only induced in epithelial cell co-culture system. However, in present study, low survival rates in the co-culture condition were due to the quality of cumulus monolayer cell which secretes benefitial proteins or harmful oxygen to embryos.

Optimal ET methods were necessary to achieve better cryopreservation of embryos. Landa (1982) reported that frozen-thawed embryos required about 48 hr for the resumption of mouse developmental potential. Seidel *et al.* (1992) demonstrated that frozen bovine embryos can survive without any of the steps which remove cryoprotectant before transfer. Therefore, in experiment II, thawed embryos were transferred to recipients (day 2, 3 pseudopregnancy) without a period of culture *in vitro*. We expected that transferred embryos without culture could be recovered developmental potential *in vivo* tract. However, in this study, no pregnant symptom was obtained at day 2 pseudopregnant recipients which directly transferred soon after thawing. The rates of live fetuses and total implantation on recipients which directly transferred at day 3 pseudopregnancy were 15.4 and 50.0%, respectively. High survival of vitrified embryos was obtained when thawed embryos cultured for 16 hr *in vitro* were transferred to recipients on day 3 of pseudopregnancy. This result suggested that vitrified-thawed embryos were required *in vitro* culture time for selection of good quality embryos before transfer. Therefore, 16 hr cultured embryos after thawing were transferred to recipient in following experiments.

This vitrification method was applied to the cryopreservation of early blastocysts and day 5 blastocysts. *In vitro* survival of embryos was similar between day 5 blastocysts and day 4 early blastocysts except day 5 early blastocysts which delayed after thawing, but *in vivo* de-

velopment rates were significantly higher in day 4 early blastocyst than in day 5 blastocysts (Table 4, 5). At 16 hr post thawing, most day 5 blastocysts reached to hatching and hatched blastocysts, but day 4 early blastocysts developed to expanding blastocysts. As we reported earlier, hatching and hatched blastocysts which have poor quality decreased *in vivo* survival rates (Kim *et al.*, 1997). In addition, none of the day 5 early blastocysts became pregnant (Data not shown).

In conclusion, it was expected that the present vitrification method using EFS40 will be suitable for cryopreservation of day 4 expanding blastocysts which have large blastocoels and also, day 4 early blastocysts could be more successfully vitrified than day 5 expanding blastocysts which further cultured for 1 day *in vitro*.

SUMMARY

This study was to test whether *in vitro/in vivo* survival of vitrified mouse blastocysts was influenced by culture conditions and ET method. Mouse blastocysts were obtained from *in vitro* fertilization and cultured for 4 days in M16 medium, and they were vitrified in EFS40 which contained 40% ethlyene glycol, 18% Ficoll and 0.5 mol sucrose in PBS. In experiment I, *in vitro* and *in vivo* survival rate of these embryos were evaluated in different culture condition after thawing. When thawed embryos were cultured in M16 medium as a control, m-CR1 medium contained 20 amino acids (2% BME amino acis and 1% MEM non-essential amino acids solution) and 4 mg/ml BSA and cumulus monolayer cell co-cultured condition in mCR1 medium (10% FBS), their *in vitro* survival at 24 hr after thawing was not affected by culture condition (75.6, 83.1, 82.4%). However, *in vivo* survival rates of implantation in m-CR1 medium (80.4%) were significantly higher than those of M16 medium (51.2%), co-culture (57.1%) condition, although there was no difference in live fetuses rates on day 15 gestation (39.0, 49.0, 38.1%). In experiment II, the *in vivo* development potential of embryos by ET methods was examined. When blastocysts were transferred to the day 2, 3 pseudopregnant recipient without culture soon after thawing, no pregnant recipient was obtained on the day 2 pseudopregnancy, and 50% of pregnancy rates and 15.4% of live fetus rates were obtained on the day 3 pseudopregnant recipients. These results were significantly lower than those of transferred group (day 3 pseudopregnant recipients) after culture for 16 hr post thawing (73.5, 57.1%) ($p<0.05$). In experiment III, to elevate usability of delayed embryos *in vitro/in vivo* survival of vitrified embryos (day 4 early, day 5 early and expanding blastocyst) were examined. *In vivo* survival rates (live fetus, total implantation) were higher in day 4 early blastocysts (33.3, 66.7%) than in day 5 expanding blastocysts (29.0, 38.7%), although the highest *in vitro* survival rates were obtained in the day 5 expanding blastocysts (78.3%). Therefore, these results suggest that the *in vitro/in vivo* survival rates of vitrified embryos could be improve by the culture condition and ET method and that the *in vivo* development rates of delayed embryos were decreased with longer culture duration *in vitro*. It means that more effective cryopreservation was obtained in day 4 early blastocysts than in day 5 expanding blastocysts.

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