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Cryopreservation of Bovine IVM/IVF/IVC Hatched Blastocysts

Lee, K. S., E. Y. Kim, B. K. Yi, H. K. Nam, S. H. Yoon*, S. P. Park and J. H. Lim*
Maria Infertility Medical Institute

체외생산된 소 완전탈출 배반포기배의 동결보존

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

요 약

본 실험은 체외 생산된 소 완전탈출 배반포기배의 생존능이 초자화동결 융해 후에도 유지될 수 있는지를 조사하고자 실시하였다. 완전탈출 배반포기배는 체외 수정 후 체외배양 9일과 10일에 얻었으며, 직경을 기준으로 small(S-HBs; ϕ ≤300 μm)과 large(L-HBs; ϕ>300 μm)로 구분하였다. 동결액은 35% ethylene glycol(EG), 18% ficoll, 0.3 M sucrose와 10% FBS가 첨가된 mDPBS로 만들어진 EFS35를 사용했다. 완전탈출 배반포기배는 2단계로 초자화동결되었는데, 10% EG에 5분간 평형 그리고 EFS35에 노출한 후 LN_2에 초자화되기까지 30~45초간 처리하였다. 체외에서의 생존능은 융해 후 2, 16 시간째의 재 팽창으로 조사하였다. 그 결과를 요약하면 다음과 같다. 1) 체외 수정 후 8일째에 얻어진 배반포기배(40.8%)를 24~48 시간 추가배양했던 바, 체외 수정 후 9일째와 10일째의 완전탈출 배반포기배의 발달율은 20.5%와 6.7%였다. 또한, 완전탈출 배반포기배의 총 세포수를 조사하였던 바, 배양 9일째의 완전탈출 배반포기배의 총세포수(232.7±16)가 배양 10일째의 완전탈출 배반포기배(157.5±9.3) 보다 많게 나타났다. 2) 체외수정 후 체외 배양 9일째 생산된 L-HBs의 생존에 동결액이 미치는 영향을 조사하였던 바, 동결군 (75.5%)이 대조군 (100%)과 노출군 (100%)에 비해 낮은 생존능을 보였다. 3) 완전탈출 배반포기배를 직경(L-HBs, S-HBs)과 배양일로 구분하여 초자화동결된 난자의 생존에 미치는 영향을 조사하였던 바, 9일에 얻어진 완전탈출 배반포기배(75.5%, 63.6%)는, 10일에 얻어진 완전탈출 배반포기배(64.3%, 60.7%) 보다 약간 높은 생존능이 있음을 알 수 있었다. 4) 융해 후 체외배양 조건이 난자의 생존능에 미치는 영향을 조사하였던 바, 동결 융해된 완전탈출 배반포기배의 생존능은 공배양(43.2%, 41.9%) 보다 mCR1aa (10% FBS) 배양액(79.3%, 62.5%)에서 유의하게 높게 나타나는 것을 확인하였다 ($p<0.05$). 따라서, 이러한 결과에서 볼 때 소 완전탈출 배반포기배는 EFS35 동결액을 사용하여 성공적으로 초자화동결 후 보존될 수 있음을 확인하였다.

(Key words: Hatched blastocyst, Vitrification, EFS35, *In vitro* survival)

I. INTRODUCTION

Recently, the production of *in vitro* derived bovine embryo has become routine, and attention has been focused on vitrification as a rapid and efficient method of cryopreservation. The first successful vitrification of mammalian embryos was achieved in eight-cell mouse embryos by Rall and Fahy(1985). The vitrification sol-

*마리아 불임클리닉 (Maria Infertility Clinic, Seoul)

ution they used was composed of dimethyl sulfoxide(DMSO), acetamide, propylene glycol and polyethylene glycol as cryoprotectants. Kasai et al.(1990) reported that mouse morulae have been vitrified using vitrification solution based on ethylene glycol without appreciable loss of viability. This method which permeates the cell rapidly has low toxicity. Zhu et al.(1993) reported that a high survival of expanded mouse blastocysts can be obtained using EFS40. Also, Tachikawa et al.(1993) reported that successful vitrification of *in vitro* derived bovine blastocysts has obtained using EFS40. As mentioned in above, zona-intact mammalian embryos can be successfully vitrified, while only a few investigation was carried out on the cryopreservation of hatched blastocysts(HBs). Shaw et al.(1995) reported that high survival of mouse HBs can be obtained by slow freezing. However, when mouse HBs were frozen by a rapid method, the survival rates were lower than those of embryos at earlier stages of development(Shaw et al., 1991). But, Zhu et al.(1996) reported that mouse HBs can be successfully cryopreserved by vitrification. Also, in our previous study (Kim et al., 1997; Yi et al., 1998), we confirmed that *in vitro* produced mouse HBs can be successfully vitrified by using EFS35, obtained good pregnancy rates, implantation rates and live fetus formation. Furthermore, if bovine HBs can be cryopreserved by simple method, it will be possible to make wide use of embryonic resources. Therefore, this study was carried out to examine whether the bovine HBs can be successfully cryopreserved by vitrification method using EFS35.

Ⅱ. MATERIALS AND METHODS

1. Production of bovine hatched blastocysts (HBs)

Bovine cumulus oocyte complexes(COCs) were collected from visible follicles (2~6 mm) of ovaries, washed with TALP-HEPES and cultured in maturation medium composed of TCM-199(Gibco) + 10%(v/v) fetal bovine serum(FBS) supplemented with sodium pyruvate (0.2 mM), follicle stimulating hormone(1 μg/ml), estradiol-17β(1 μg/ml), and gentamycin(25 μg/ml) at 39℃, 5% CO_2 incubator. After incubation for 22~24 h in *in vitro* maturation(IVM) medium, the COCs were inseminated using highly motile sperm recovered from frozen-thawed semen separated on a discontinuous percoll column. Fertilization was assessed as cleavage rate($\geq$2-cell) after 44±2 h co-incubation with the sperm. For *in vitro* culture, cleaved embryos were cultured in mCR1aa medium (Kim et al., 1997) supplemented with fatty acid-free BSA(3 mg/ml) and then transferred in 10% FBS added mCR1aa medium at day 4 after IVF. For the study, HBs produced *in vitro* at Day 9 and Day 10 after IVF were classified into small(S-HBs; $\phi \leq 300$ μm) and large(L-HBs; $\phi > 300$ μm) on the basis of embryo diameter using eyepiece micrometer(Fig. 1. A).

2. Vitrification procedure

Vitrification solutions were designated EFS35, it consisted of 35%(v/v) ethylene glycol(EG, Sigma), 18%(w/v) ficoll(Ficoll 70, Average MW: 70,000, Sigma), 0.5 M sucrose(Sigma) and 10%(v/v) FBS supplemented in mDPBS. Also, as an equilibration solution, mDPBS(EG10) containing 10%(v/v)EG and 10%(v/v)FBS was prepared.

Bovine HBs were vitrified using two-step freezing method. In the first step, embryos were equilibrated in EG10 for 5 minutes. (Fig. 1. B), in the second step, embryos were exposed in EFS35 at room temperature, loaded in a 0.25 ml French straw and vitrified in liquid nitrogen

(LN_2) within 30 to 45 sec. And then the straw was slowly immersed into LN_2.

3. Thawing procedure

Embryos were warmed rapidly in water bath at 25℃. The contents of each straw were expelled into dish containing 0.8 ml of 0.5 M sucrose(prepared in mDPBS containing 10% FBS), and then recovered embryos were transferred into fresh 0.8 ml of 0.5 M sucrose for 5 minutes. And embryos were transferred into 0.8 ml of mDPBS(containing 10% FBS) for 5 minutes and then embryos were transferred to mCR1aa(containing 10% FBS) medium for 1 minute. The embryos were cultured in mCR1aa medium only or co-cultured in cumulus monolayered cell drop.

4. Assessment of embryo survival

The post-warming survival of frozen embryos was observed every 2 h and 16 h under microscope(Fig. 1. C), respectively.

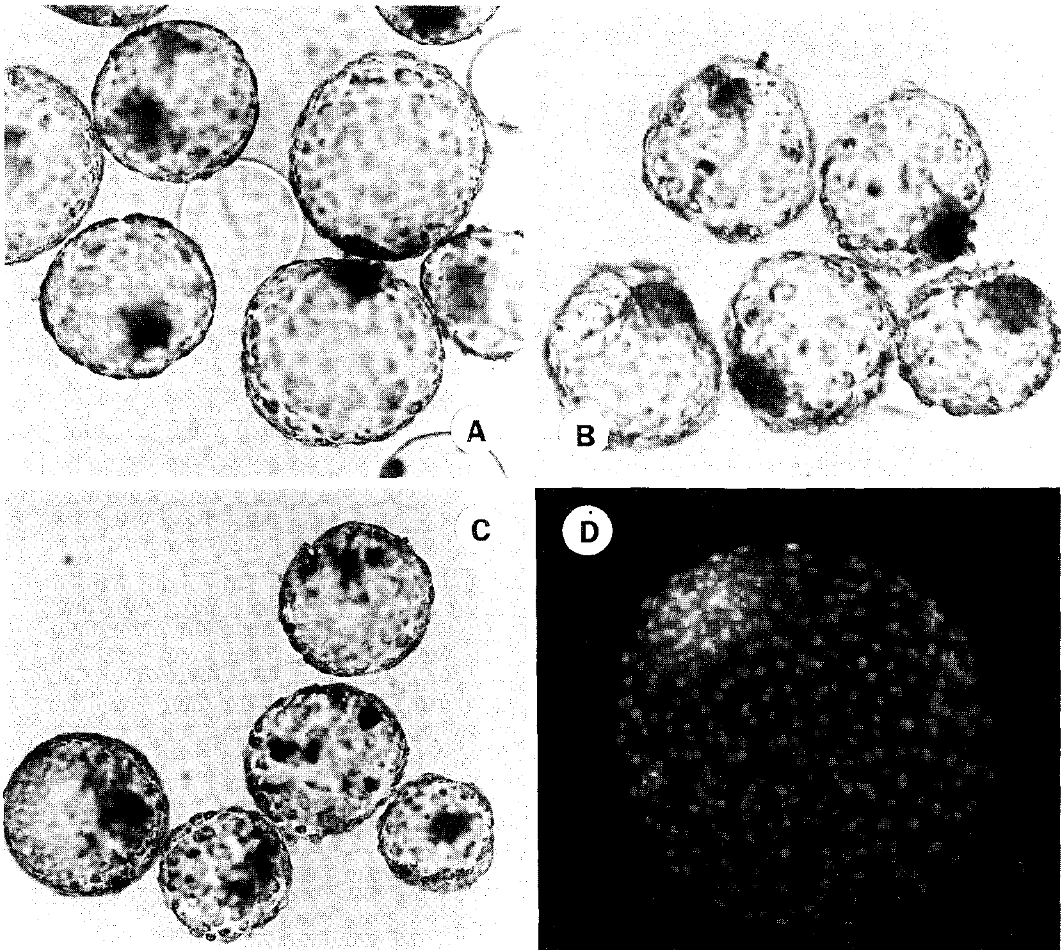

Fig. 1. Bovine hatched blastocysts on Day 9. (A) before vitrification(×150), (B) exposed to EFS35(×150), (C) survived embryos at 16 h after thawing(×100), (D) neuclei stained with bisbenzimide(total cell number) (×300).

5. Total cell number count

The HBs were fixed with 2% formalin solution for 2~3 minutes and stained with bisbenzimide solution(No. 33342, 2.5 μg/ml, Sigma). Observation was carried out under ultraviolet filter incorporated fluorescent microscope(Fig. 1. D).

6. Experimental design

To compare the embryo quality according to culture duration, total cell number of HBs recovered at Day 9 and Day 10 after IVF was examined. And, to examine the survival of Day 9 HBs by exposure to the cryoprotectant, bovine HBs in exposed group were treated with the same procedures as in vitrified group except being plunged into LN_2. Also, to compare the survival after thawing according to embryo age and embryo size, the HBs produced on Day 9 and Day 10 were vitrified, the *in vitro* survival of Day 9 HBs according to size was evaluated under different culture condition after thawing.

To determine the effect of culture condition to the embryo survival after thawing, frozen-thawed Day 9 HBs were cultured in medium only or culture environment.

7. Statistical analysis

The significance of difference in number of cells according to development age was compared with t-test($p<0.01$). Difference among treatment group in each experiment was compared with Chi-square test($p<0.05$).

Ⅲ. RESULTS

This study was to test whether bovine hatched blastocysts(HBs) can be successfully cryopreserved by vitrification method using EFS35. The rates of *in vitro* development of bovine embryos after IVF were as follows; as shown in Table 1, the cleavage rate was 80.5% and blastocysts rate at Day 8 after IVF was 40.8%. Also, when the blastocysts recoverd at Day 8 after IVF were further cultured for 24 h(Day 9 after IVF) and 48 h(Day 10 after IVF), HBs rates developed to small(S-HBs; $\phi \leq 300$ μm) and large(L-HBs; $\phi > 300$ μm) were 4.6%, 15.9% on Day 9(after IVF) and were 2.5% and 4.1% on Day 10(after IVF), respectively. And, the total cell number of HBs on day 9 (after IVF) was significantly higher(232.7±16) than that(157.5±9.3) on Day 10. When the survival of Day 9 HBs according to the exposure of cryoprotectant (in EG10 for 5 minutes and EFS35 for 30~45 sec. at 25℃) was examined, as indicated in Table 2, there was not detrimental effect in the exposed group. However, when those HBs were vitrified, high survival (75.5%) was obtained, although it was significantly lower than that

Table 1. *In vitro* development of bovine IVM/IVF/IVC embryos

No. of oocytes	No. of cleaved (%)	No. of blastocysts (Day 8)(%)	No. (%) of hatched blastocyst							
			Day 9				Day 10			
			Total	S*	L*	Cell No.**	Total	S*	L*	Cell No.**
539	434 (80.5)	177 (40.8)	89 (20.5)	20 (4.6)	69 (15.9)	232.7 ±16.0[a]	29 (6.6)	11 (2.5)	18 (4.1)	157.5 ±9.3[b]

* S; Small($\phi < 300\mu m$), L; Large($\phi \geq 300\mu m$)

** Values are means ± standard errors

[a, b] $p<0.01$

Table 2. *In vitro* survival of Day 9 hatched blastocysts(HBs) exposed or vitrified in EFS 35

Treatment	HBs size	No. (%) of embryos			
		Vitified	Recovered	Survived	
				2 h	16 h
Control	L	–	30 (100.0)	30 (100.0)[a]	30 (100.0)[a]
Exposed	L	34	34 (100.0)	34 (100.0)[a]	34 (100.0)[a]
Vitrified	L	35	33 (94.2)	27 (81.8)[b]	25 (75.7)[b]

* L; Large($\phi \geq 300\mu m$)

[a, b] Means in the same column without common superscripts are significantly different($p<0.05$)

(100%) of exposed and control groups($p<0.05$). In addition, when the *in vitro* survival rates of vitrified HBs according to embryo development stage were examinedand, the embryo size (L-HBs, S-HBs) and embryo age (Day 9 and 10) were larger and shorter, respectively, the survival rates of HBs were slightly higher(Day 9: 75.7%, 63.6%; Day 10: 65.5%, 60.7%). Also, when *in vitro* survival of Day 9(after IVF) HBs according to size was evaluated under the different culture condition after thawing(Table 4), the results of L-HBs in culture medium only(79.3%) were significantly higher than those in co-culture(43.2%) group($p<0.05$).

Ⅳ. DISCUSSION

Study on the characteristics of supernumerary blastocysts in different development stage was to wide the usability of the HBs embryos. In the viewpoint, we examined whether the IVM/IVF/IVC bovine HBs can be successfully cryopreserved by simple two-step vitrification method using EFS35 freezing solution.

Recently, EG is widely used for vitrification of various mammalian embryos (Kasai et al., 1990, 1996). The method using vitrification solution based on EG, which permeates the cell rapidly and has low toxicity, was introduced by Kasai et al.(1990) at mouse morula stage. And, high survival of frozen-thawed mouse expanded blastocysts was reported by Zhu et al.(1993). Also, *in vitro* derived bovine blastocysts can be successfully vitrified by the method using EFS40 (Mamoudzadeh et al., 1994). On the other hand, Zhu et al.(1996) reported that *in vitro* derived mouse HBs was obtained success of pregnancy by the method using EFS40. Also, in previous study, we reported that *in vitro* derived mouse HBs can be obtained good pregancy rates, and implantation with live fetuses formation by using EFS35.

The HBs have limitation than zona-intact embryos, because they are more sensitive to culture condition and poor tolerant to external stress(Yu, 1994). Thus, improved *in vitro* culture techniques were more required. Also, it is important to estabish the appropriate culture condition for production of high quality HBs to obtain a successful cryopreservation. In our culture condition, the development rate of Day 8 blastocysts cultured *in vitro* in simple defined mCR1aa medium was 40.8%(Table 1). When the blastocysts were further cultured for 24 h(Day 9 after IVF) and 48 h(Day 10 after IVF), the rates of HBs were 20.5% and 6.7%, respectively. However, the total cell number of HBs was significantly decreased in culture of delayed HBs at day 10 after IVF($p<0.01$). It was known that blastocysts that developed early have more cells and fewer chromosomal anomalies than those developed later(Goto et al., 1992). When the

survival rates of Day 9 HBs according to the exposure of cryoprotectant(in EG10 for 5 minutes and EFS35 for 30~45 sec. at 25℃) was examined(Table 2), significant toxicity was not showed in the results of exposed group(100%) when compared to that of control group(100%). Also, when those of HBs were vitrified, high survival(75.5%) was obtained, although it was significantly lower than those of exposed and control group($p<0.05$). For the efficient cryopreservation of *in vitro* derived embryos, it will also be important to produce embryos of high quality(Iwasaki et al., 1990). Voelkel et al.(1992) reported that the survival rates of frozen-thawed bovine embryos were affected by the quality or age of the embryos. Although the survival rates of Day 9 and Day 10 HBs were not significant, the survival rate of on day 9 was higher than that of Day 10(Table 3). The viability of delayed embryo at day 10 after IVF was low, it may be due to increased stress of cryoprotectant and cryopreservation. The early HBs (Day 9 after IVF) have more resistant to cryopreservation than later HBs at Day 10 after IVF (Zhu et al., 1996). On the other hand, *in vitro* survival after thawing on day 9 was influenced by two culture condition(Table 4). The two culture conditions were considered as follows; mCR1aa medium and cummulus monolayer cell co-culture group. The *in vitro* survival rate in mCR1aa culture condition was higher than that in co-culture condition. Rosenkrans et al.(1989) demonstrated that CR1 medium was as effective as co-culture in supporting preimplantation bovine embryo development. However, low survival rates in the co-culture condition may be due to the quality of cumulus monolayer cell which secretes beneficial proteins or harmful oxygen to

Table 3. *In vitro* survival of vitrified - thawed Day 9 or 10 HBs according to embryo age and embryo size

Embryo age	HBs size*	No. (%) of embryos		
		Vitrified	Recovered	Survived**
Day 9	S	33	33 (100.0)	21 (63.6)
	L	34	33 (97.0)	25 (75.7)
Day 10	S	28	28 (100.0)	17 (60.7)
	L	30	29 (96.6)	19 (65.5)

* S; Small($\phi<300\mu m$), L; Large($\phi\geq300\mu m$)

** No significant difference.

Table 4. Effect of culture condition after thawing on survival of Day 9 HBs

Culture condition	HBs size*	No. (%) embryos		
		Vitrified	Recovered	Survived
mCR1aa	S	32	32 (100.0)	20 (62.5)[ab]
	L	30	29 (96.6)	23 (79.3)[a]
Co-culture	S	31	31 (100.0)	13 (41.9)[b]
	L	31	30 (96.7)	12 (43.2)[b]

* S; Small($\phi<300\mu m$), L; Large($\phi\geq300\mu m$)

[a, b] Means in the same column without common superscripts are significantly different ($p<0.05$).

embryos.

In conculsion, the simple vitrification method may increase the use of cryopreserved embryos, because the vitrification method greatly simplifies the cooling process and eliminates the use of elaborate equipment to control the cooling rate.

These results suggested that *in vitro* produced bovine HBs can be successfully cryopreserved by simple two-step vitrification method using EFS35 freezing solution.

V. SUMMARY

This study was to test whether the viability of bovine hatched blastocysts (HBs) can be maintained after vitrification and thawing. The HBs were produced *in vitro* at Day 9 and Day 10 after IVF, and they were classified to small (S-HBs; $\phi \leq 300$ μm) and large(L-HBs; $\phi > 300$ μm) on the basis of embryo diameter using eyepiece micrometer. As freezing solution, we used EFS35 which consisted of 35% ethylene glycol (EG), 18% ficoll, 0.3 M sucrose and 10% FBS added in mDPBS. Vitrification was taken by two-step method, the HBs were equilibrated in 10% EG for 5 minutes and then shortly exposed in EFS35 and plunged into LN_2 for 30~45 sec. After thawing, the survival rates were assessed by the re-expansion of the blastocoel during 2 h and 16 h of culture. The results obtained in these experiments were summarized as follows; 1) When the blastocysts(40.8%) recovered at Day 8 after IVF were further cultured for 24 h(Day 9 after IVF) and 48 h(Day 10 after IVF), the rates of HBs were 20.5% and 6.7%, respectively. Also, the total cell number of HBs on Day 9 was significantly higher than that of HBs on Day 10 ($p<0.01$). 2) When the effects of freezing solution to the survival of Day 9 L-HBs were examined, the rate of vitrified group(75.7%) was significantly lower than 100% of control and exposed group($p<0.05$). 3) When the survival rates of vitrified HBs according to size and developmental age were examined, the data of L-HBs (75.5%) and S-HBs(63.6%) on Day 9 were slightly higher than those of L-HBs(64.3%) and S-HBs(60.7%) on Day 10. 4) Also, when the *in vitro* survival of Day 9 HBs was evaluated under different culture condition after thawing, the result in culture medium only (79.3%) was significantly higher than 43.2% in co-culture group ($p<0.05$). These results demonstrated that bovine HBs can be successfully cryopreserved by two-step vitrification method using EFS35.

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