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Systems for Production of Calves from Hanwoo (Korean Native Cattle) IVM/IVF/IVC Blastocyst

Ⅰ. Hanwoo IVM/IVF/IVC Blastocyst Cryopreserved by Vitrification

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체외생산된 한우 배반포기배로부터 송아지 생산을 위한 체계

Ⅰ. 체외생산된 한우 배반포기배의 초자화 동결보존

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요 약

본 실험은 체외 생산된 한우 배반포기배의 생존능이 초자화동결 융해 후에도 유지될 수 있는지를 조사하고자 실시하였다. 한우 배반포기배는 2단계로 초자화동결되었는데, EG20에 3분간 평형 그리고 EFS40에 노출한 후 LN_2에 초자화되기까지 30~45초간 처리하였다. 체외에서의 생존능은 융해 후 24시간째의 re-expanded 와 48시간째의 hatched 발달율로 조사하였다. 그 결과는 다음과 같다. 12번의 반복실험으로부터 체외 수정 후 체외 배양 7일째에 52.5%의 배반포기배가 생산되었다. 배의 생존에 동결액이 미치는 영향을 조사하였던 바, 대조군 (100.0, 87.0%) 과 비교하였을 때 동결액 노출군 (100.0, 73.8%) 에서 유의한 독성은 확인되지 않았다. 그러나, 배반포기배가 초자화동결되었을 때, 높은 생존능 (86.2, 55.4%) 은 확인되었지만 그 결과는 노출군과 대조군에 비교하여 볼 때 유의하게 낮은 것이었다 ($p<0.05$). 배 발달단계와 배양날짜가 초자화동결된 난자의 생존에 미치는 영향을 조사하였던 바, 배양날짜와 상관없이 빠르게 발달한 난자군에서 유의하게 높은 생존능이 있음을 알 수 있었다 ($p<0.05$). 또한, 같은 발달단계라 하더라도, 배양 7일째 배의 (re-expanded: 75.0~87.5%, hatched: 21.4~66.7%) 생존능이 배양 8일째 배의 (re-expanded: 58.6~78.3%, hatched: 10.3~52.2%) 생존능보다 높다는 것을 확인하였다. 따라서, 이러한 결과에서 볼 때 체외에서 생산된 한우 배반포기배는 EFS40 동결액을 사용하는 간편 2단계 초자화방법으로 성공적으로 동결되어질 수 있으며 특히 배양 7일과 8일째의 expanded 배반포기배와 early hatching 배반포기배에서 더욱 효과적으로 사용될 수 있음을 확인하였다.

(Key words : Hanwoo IVM /IVF /IVC blastocyst, Vitrification, EFS40, *In vitro* survival rate)

Ⅰ. INTRODUCTION

At present, the production of *in vitro* derived bovine embryos has become routine and thus successful cryopreservation method is still more

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required for efficient utilization of these embryos (Leibo and Loskutoff, 1993). However, it has known that *in vitro* produced embryos were much more sensitive to freezing than *in vivo* derived counterparts. To outrace the chilling injury, many researchers suggested that rapid cooling may be beneficial than slow cooling for the survival of cattle embryos (Mamoudzadeh et al., 1994; Pollard and Leibo, 1994). Since Rall and Fahy (1985) introduced successful vitrification of mouse 8-cell, vitrification has been widely used and is now regarded as a potential alternative to traditional slow-rate freezing. In bovine, the first successful vitrification of *in vitro* derived embryos has demonstrated by Kuwamaya et al. (1992), with the cryoprotectants of including a mixture of glycerol and 1,2-propanediol. Also, Tachikawa et al. (1993) reported that a high survival of vitrified-thawed bovine blastocysts can be obtained by using EFS40. EFS which consisted of ethylene glycol, ficoll and sucrose, permeates the cell rapidly and has low toxicity, was first described by Kasai et al. (1990) at the mouse morula stage, has also been used rabbit embryos (Kasai et al, 1992), bovine morulae produced *in vivo* (Mamoudzadeh et al., 1993) and mouse expanded blastocysts (Zhu et al., 1993). In addition, in our previous study (Kim et al., 1996a), we confirmed that the efficiency of EFS40 on the survival of vitrified mouse IVF/IVC blastocysts. On the basis of these reports, this study was carried out to examine whether the *in vitro* produced Hanwoo blastocysts can be successfully cryopreserved by the simple two-step vitrification method using EFS40 freezing solution.

Ⅱ. MATERIALS AND METHODS

1. Production of Hanwoo IVM/IVF/IVC blastocysts

Hanwoo blastocysts were produced *in vitro* by the same procedure as outlined by Park et al. (1995). Briefly, Hanwoo 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 (1μg/ml), estradiol-17β (1μg/ml), and gentamycin (25 μg/ml) at 39℃, 5% CO_2 incubator. After incubation for 22~24 h in IVM medium, the COCs were inseminated using highly motile sperm recovered from frozen-thawed Hanwoo bull semen separated on a discontinuous percoll column. Fertilization was assessed as cleavage rate (≥2-cell) after 44±2 h co-incubation with the sperm. For *in vitro* culture, cleaved embryos were cultured in m-CR1 medium supplemented with fatty acid-free BSA (3mg/ml) and then transferred in 10% FBS added m-CR1 medium at day 4 after IVF. For the study, blastocysts produced *in vitro* at day 7 and day 8 after IVF were classified to early, expanded and hatching stage according to their developmental morphology and they were divided into control, exposure and vitrified group.

2. Vitrification procedure

The vitrification solution, EFS40, was prepared the same as described by Kasai et al. (1990). It consisted of 40% v/v ethylene glycol (EG, Sigma), 18% w/v ficoll (Ficoll70, Average MW: 70,000, Sigma), 10.26% w/v sucrose (Sigma) and 10% FBS supplemented in mDPBS. Also, as an equilibration solution, mDPBS (EG20) containing 20% EG and 10% FBS was prepared.

Hanwoo blastocysts (Fig. 1A) were vitrified using two-step freezing method described by Mamoudezadeh et al. (1995) at 25℃ room tem-

perature. In the first step, embryos were equilibrated in EG20 for 3 min. And then in the second step, embryos were exposed in EFS40 (Fig. 1B), loaded in a 0.25 ml French straw and vitrified in liquid nitrogen (LN_2) within 30 to 45 sec.

The vitrification solution was loaded in 0.25 ml French straw as the same procedure described by Kim et al. (1996b). Briefly, a 4 cm length was filled with 0.5 mol sucrose (prepared in mDPBS containing 10% FBS) followed by a 1.5 cm air bubble, 0.5 cm EFS40, 0.5 cm air bubble, 0.5 cm EFS40, 0.5 cm air bubble, 2 cm EFS40 and 1.5 cm air bubble. The remaining part of the straw was filled with 0.5 mol sucrose. Straw was sealed with powder and heat after embryo loading. Average loading embryo numbers in each straw were about five to ten. For vitrification, the first part of the straw filled with sucrose (4 cm) was slowly immersed into LN_2; the remaining part of the straw was then plunged in.

3. Thawing procedure

After a few hours or days of storage in LN_2, the embryos were warmed rapidly in water bath at 25℃. The contents of each straw were emptied into 0.8 ml of 0.3 mol sucrose (prepared in mDPBS containing 10% FBS), and then recovered embryos were transferred into a new 0.8 ml of 0.3 mol sucrose for 5 min. And then embryos were diluted in a 0.8 ml of mDPBS (containing 10% FBS). After 5 min., embryos were co-cultured in cumulus monolayer cell drop added m-CR1 medium supplemented with 10% FBS (Fig. 1C).

4. Assessment of embryo survival

The post-warming survival of embryos was observed every 24 h under microscope and assessed as the observed number of embryos which re-expanded and hatched during 24 h and 48 h (Fig. 1D), respectively.

5. Experimental design

To examine the toxicity of vitrification solution, Hanwoo blastocysts in exposed group were treated with the same procedures as in vitrified group except plunged into LN_2. Also, to compare the survival after thawing according to embryo developmental stage, day 7 or day 8 blastocysts were vitrified.

6. Statistical analysis

The significance of difference among treatment group in each experiment was compared with Chi-square test ($p<0.05$).

Ⅲ. RESULTS

This study was to investigate whether the viability of Hanwoo blastocysts can be maintained after vitrification and thawing. The rate of *in vitro* development of Hanwoo embryos cultured in m-CR1 medium after IVF was as follows; as shown in Table 1, in a total of 12 replicates, the average cleavage rate was 87.2% and blastocyst rate at day 7 after IVF was 52.5%. Also, development rates of classified blastocysts to early, expanded and hatching stage at day 7 after IVF were 27.4, 17.9 and 7.2%, respectively. In all of freezing study, *in vitro* survival was assessed as the re-expanded and hatched rates at 24 h and 48 h after thawing, respectively. When the *in vitro* survival of Hanwoo blastocysts according to the exposure of cryoprotectants (in EG20 for 3 min and EFS40 for 45 sec at 25℃) was examined, significant toxicity was not showed in the results of exposure group (100.0, 73.8%) compared to that of control group (100.0, 87.0%). However, when those embryos were vitrified, high survival (86.2, 55.4%) was obtained although it was significantly lower than those of exposure

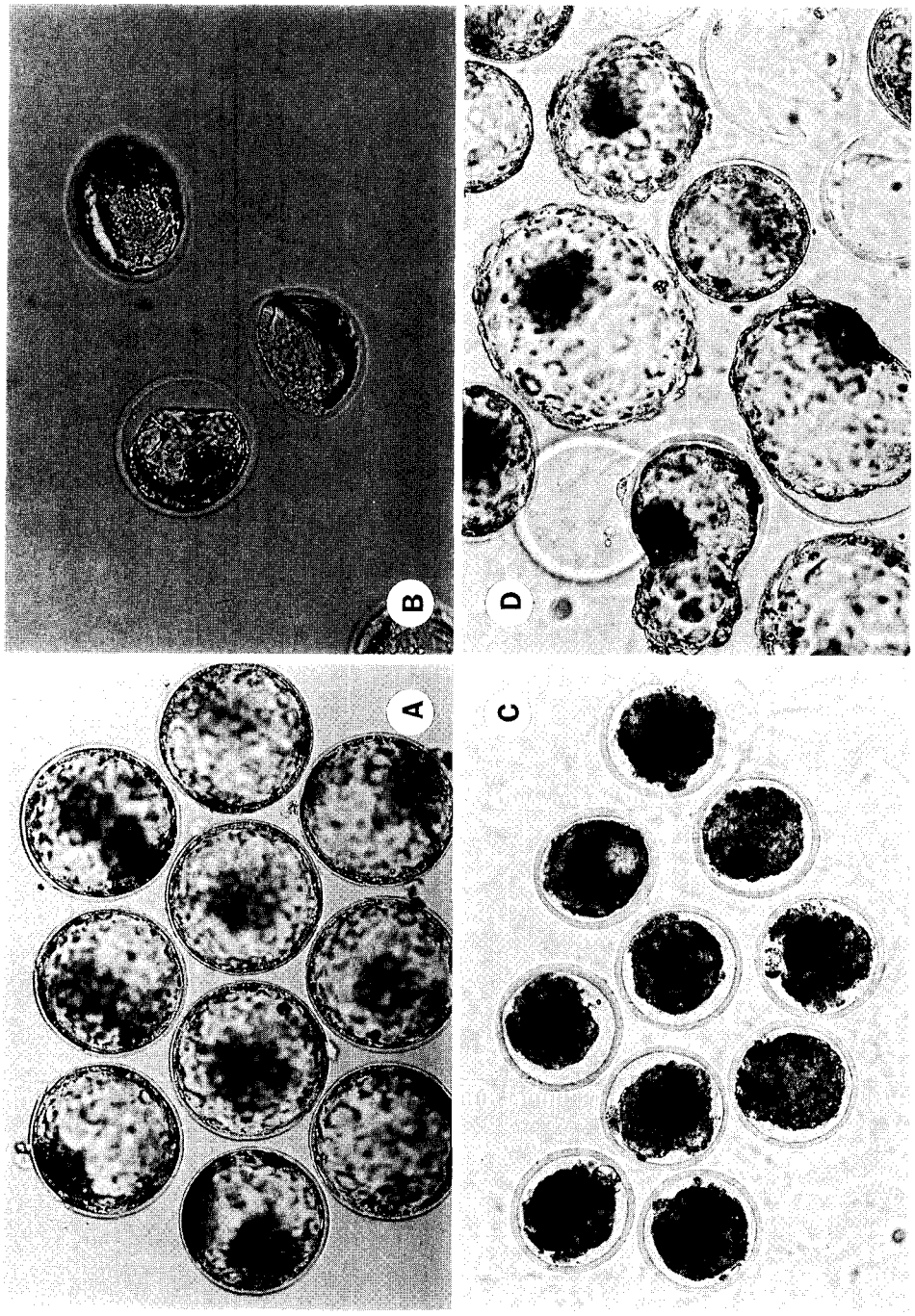

Fig. 1. ***In vitro*** **produced Hanwoo blastocysts (A) were equilibrated in EG20 for 3 min and then exposed in EFS40 for 30~45 sec (B) before being plunged into LN_2. After thawing, embryos were diluted their cryoprotectants and then cultured in cumulus cell monolayered drop (C). Thawed blastocysts were** ***in vitro*** **hatched at 48 h after culture (D). ×150**

Table 1. Development of Hanwoo follicular oocytes at day 7 after IVF (r=12)

No. of oocytes	≥2-cell(%)	No.(%) of developed to			
		≥Bla.*	ErB	EdB	HgB
2,350	2,049 (87.2)	1,075 (52.5)	561 (27.4)	366 (17.9)	148 (7.2)

*Bla.; Blastocyst, ErB; Early blastocyst, EdB; Expanded blastocyst, HgB; Hatching blastocyst

Table 2. *In vitro* survival of exposed or vitrified Hanwoo blastocysts in EFS40

Treatment	No. of embryos*	No.(%) of recovered	No. (%) of survived		
			24 h later	48 h later	
			≥EdB or** Re-edB	≥HgB	HdB
Control	54	–	54 (100.0)[a]	54 (100.0)[a]	47 (87.0)[a]
Exposed	65	65 (100.0)	65 (100.0)[a]	55 (84.6)[b]	48 (73.8)[a]
Vitrified	65	65 (100.0)	56 (86.2)[b]	49 (75.4)[b]	36 (55.4)[b]

* Day 7 expanded blastocysts were used for comparison

** EdB; Expanded blastocyst, Re-edB; Re-expanded blastocyst, HgB; Hatching blastocyst, HdB; Hatched blastocyst

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

Table 3. *In vitro* survival of vitrified day 7 Hanwoo blastocysts according to embryo development stage

Embryo stage	No. of embryos	No.(%) of recovered	No.(%) of survived		
			24 h later	48 h later	
			≥Re-edB	≥HgB	HdB
Early B.	56	56 (100.0)	42 (75.0)	30 (53.6)[b]	12 (21.4)[b]
Expanded B.	41	39 (95.1)	34 (87.2)	29 (74.4)[a]	21 (53.8)[a]
Hatching B.*	48	48 (100.0)	42 (87.5)	38 (79.2)[a]	32 (66.7)[a]

*Hatching B: Early hatching blastocyst which was shrunken and then re-expanding state

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

Table 4. *In vitro* survival of vitrified day 8 Hanwoo blastocysts according to embryo development stage

Embryo stage	No. of embryos	No.(%) of recovered	No.(%) of survived		
			24 h later	48 h later	
			≥Re-edB	≥HgB	HdB
Early B.	60	58 (96.7)	34 (58.6)[b]	14 (24.1)[b]	6 (10.3)[b]
Expanded B.	56	56 (100.0)	42 (75.0)[a,b]	32 (57.1)[a]	20 (35.7)[a]
Hatching B.*	46	46 (100.0)	36 (78.3)[a]	34 (73.9)[a]	24 (52.2)[a]

*Hatching B: Early hatching blastocyst which was shrunken and then re-expanding state

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

and control group ($p<0.05$). In addition, when the *in vitro* survival rates were examined according to the embryonic developmental stage, at day 7 blastocysts, re-expanded state at 24 h after thawing was not significantly different in all embryo development stages (early: 75.0%, expanded: 87.2% and hatching: 87.5%). But, at 48 h after thawing, more advanced embryo stage (expanded: 53.8% and hatching: 66.7%) indicated significantly higher hatched rates than delayed developed embryos (early: 21.4%) ($p<0.05$). However, this phenomenon was more clearly presented at day 8 blastocysts, all observation time (at 24 h and 48 h after thawing) indicated that *in vitro* survival of early blastocysts (58.6, 10.3%) was significantly lower than those of expanded (75.0, 35.7%) and hatched blastocysts (78.3, 52.2%). These results demonstrated that simple two-step vitrification using EFS40 is the more efficient cryopreservation method to expanded and hatching blastocyst regardless of culture duration (at day 7 or day 8 after IVF).

Ⅳ. DISCUSSION

This study presents that *in vitro* produced Hanwoo blastocysts can be successfully cryopreserved by simple two-step vitrification method using EFS40 freezing solution. Vitrification of embryos is a useful cryopreservation method since it does not require costly freezing equipment and technical skill and it provides time savings. Vitrification is defined as "the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during cooling" (Fahy et al., 1984). Thus, vitrification involves the addition of higher concentrations of cryoprotectant and a very rapid freezing rate, which prevents the formation of ice crystals that is a major cause of cell death (Fahy et al., 1984). Recent reports have indicated that vitrification of *in vitro* produced embryos is at least as efficient as conventional freezing (Tachikawa et al., 1993; Mamoudzadeh et al., 1994, 1995). However, the survival rates of frozen-thawed IVF bovine embryos are affected by the quality or age of the embryos, cryoprotectant used, the cooling rates during freezing, and the method of equilibration or removal of the cryoprotectant (Voelkel and Hu, 1992).

The first successful vitrification solution for mouse embryos (VS1; Rall and Fahy, 1985) was based on DMSO supplemented with acetamide, propylene glycol and polyethylene glycol as cryoprotectants. Thereafter, solutions containing glycerol and propylene glycol were reported to be successful in vitrifying not only mouse embryos (Scheffen et al., 1986), but also bovine embryos (Massip et al., 1986, 1987). In recent, ethylene glycol has gained attention as an important cryoprotectant for embryo cryopreservation (Kasai et al., 1990; Tachikawa et al., 1993). Its beneficial effect may be due to the high permeability of this cryoprotectant. Thus, during vitrification and dilution, the cryoprotectant is easily removed from the embryos, reducing the chance of embryo toxicity. Currently, EFS is widely used as a stable vitrification solution irrespective of species.

To yield success of pregnancy and baby-born from *in vitro* derived cryopreserved embryos, improved *in vitro* culture techniques may be more required than simply changes in cryopreservation methods (Leibo and Loskutoff, 1993). It means that it is important to establish the appropriate culture condition for production of high quality embryos to obtain a successful cryopreservation. In our culture condition, the development rate of day 7 Hanwoo blastocysts *in vitro* cultured in simple defined m-CR1 medium was 52.5%. This development rate was very high compared to that of others (Massip et

al., 1993: 19%; Mamoudzadeh et al., 1995: 25%). Moreover, in present study, final observation (embryo hatched from the zona) for assessment of embryo survival after thawing was done 24 h faster than any others (almost observed till 72 h). Also, when day 7 expanded blastocysts were vitrified in EFS40, our *in vitro* survival rates (re-expanded: 86.2%, hatched: 55.4%) were not bad compared to the results of Mamoudzadeh group (1995) (re-expanded: 89.0%, hatched: 68.8%) observed till 72 h after thawing. On the other hand, when the *in vitro* survival according to developmental stage and culture day were examined, it showed that more advanced embryo stage exhibited a significantly higher survival rate than delayed developed embryos ($p<0.05$). In addition, even in the same development stage, the *in vitro* survival of day 7 embryos (re-expanded: 75.0~87.5%, hatched: 21.4~66.7%) was higher than that of day 8 embryos (re-expanded: 58.6~78.3%, hatched: 10.3~52.2%). These results are consistent with other studies (Mamoudzadeh et al., 1994; Pollard and Leibo, 1994) demonstrating the developmental stage-dependent survival rate of *in vitro* produced bovine embryos after cryopreservation. Especially, unlike other groups, this study indicated that the survival of cryopreserved early hatching embryos, which has re-expanding morphology from shrunken state caused by osmotic difference between blastocoel fluid and culture medium from the zona rupture, was higher than that of any other stage embryos examined.

Therefore, these results suggested that *in vitro* produced Hanwoo blastocysts can be successfully cryopreserved by simple two-step vitrification method using EFS40 freezing solution, particularly at the expanded and early hatching blastocyst stage regardless of embryo culture duration (day 7 or day 8 after IVF).

V. SUMMARY

This study was to investigate whether the viability of Hanwoo IVM/IVF/IVC blastocysts was maintained after vitrification and thawing. *In vitro* produced Hanwoo blastocysts were vitrified by two-step method; equilibrated in EG20 for 3 min, and then exposed in EFS40 [40% ethylene glycol (EG), 18% ficoll and 10.26% sucrose in mDPBS containing 10% FBS]and vitrified in LN_2 for 30 - 45 sec. After thawing, *in vitro* survival was assessed as the re-expanded and hatched rates at 24 h and 48 h, respectively. The results obtained in these experiments were summarized as follows; From the 12 replicates, 52.5% of Hanwoo blastocysts were produced *in vitro* at day 7 after IVF. When the effects of freezing solution to the embryo survival were examined, there is no significant toxicity in exposure (100.0, 73.8%) compared to that of control group (100.0, 87.0%). However, when embryos were vitrified, high survival (86.2, 55.4%) was obtained although it was significantly lower than those of exposure and control group ($p<0.05$). When the *in vitro* survival of vitrified embryos according to developmental stage and culture day were examined, it showed that more advanced embryo stage exhibited a significantly higher survival rate irrespective of culture day ($p<0.05$). Also, even in the same development stage, the *in vitro* survival of day 7 embryos (re-expanded: 75.0~87.5%, hatched: 21.4~66.7%) was higher than those of day 8 embryos (re-expanded: 58.6~78.3%, hatched: 10.3~52.2%). Therefore, these results suggested that *in vitro* produced Hanwoo blastocysts can be successfully cryopreserved by simple two-step vitrification method using EFS40 freezing solution, particularly at the expanded and early hatching blastocyst stage regardless of embryo culture

duration (day 7 or day 8 after IVF).

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