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

Ⅱ. Simple, Efficient and Successful Vitrification of Hanwoo Blastocyst

Kim, E. Y.[1,*], D. I. Kim[2], N. H. Park[2], Y. S. Weon[2], H. K. Nam[1], K. S. Lee[1], S. Y. Park[1], S. H. Yoon[3], S. P. Park[1], K. S. Chung[4] and J. H. Lim[3]

Maria Infertility Medical Institute, Seoul

ABSTRACT

The objective of this study was to optimize the freezing/thawing method of *in vitro* produced Hanwoo blastocysts. Day 7 blastocysts after IVF were vitrified using EFS40 (40% ethylene glycol, 18% ficoll, 0.3 M sucrose and 10% FBS added m-DPBS) as a freezing solution and electron microscope (EM) grid (V-G) or straw (V-S) as an embryo container. In both method, freezing/thawing were treated by 2-step, treatment time was required in V-G method and V-S method, for 2 min/3 min and 3.5 min/10 min, respectively. Embryo survival was assessed as re-expanded and hatched rates at 24 h and 48 h after warming, respectively. The results obtained in these experiments were summarized as follows: when the effect of exposure in vitrification solution and chilling injury from freezing procedure on *in vitro* produced expanded blastocysts were examined, at 24 h after warming, embryo survival in exposure group (100.0%) was not different compared to that in control group (100.0%), although those results were significantly different with two vitrified groups (V-G: 87.8, V-S: 77.8%) ($P<0.001$). However, at 48 h after warming, hatched rates of V-G group (67.8%) were significanlty higher than those of V-S group (53.3%) ($P<0.05$). In addition, this hatched rate in V-G group was not different with that in exposure group (73.3%). When the effects of embryo developmental stage (early, expanded and early hatching blastocysts) and embryo container (EM grid and straw) to the *in vitro* survival of vitrified-warmed day 7 Hanwoo blastocysts were simultaneously examined, fast developed embryos were indicated the better resistance to freezing than delayed developed one, irrespective of embryo containers (early; 57.1 & 24.4%, expanded; 84.7 & 60.6%, early hatching; 91.7 & 80.0%) ($P<0.001$). Especially, in expanded and early hatching blastocysts, embryo survival of V-G group (67.8, 95.0%) was significantly higher than those of V-S group (53.0, 65.0%) at 48 h post warming, respectively ($P<0.05$, $P<0.001$). Therefore, this study indicates that Hanwoo blastocysts can be cryopreserved more simple, efficient and successful by vitrification method using EM grid.

(Key words: Hanwoo IVM/IVF/IVC blastocyst, Vitrification, EFS40, EM grid, Straw)

[1,*] Maria Infertility Medical Institute, 103-11 Sinseol-dong, Dongdaemun-Gu, Seoul 130-110, Tel: (02) 924-8757, FAX: (02) 924-8759, E-mail: MIMI60@unitel.co.kr

[2] Hanwoo Improvement Center, NLCF, [3] Maria Infertility Clinic, Seoul

[4] College of Animal Husbandry, Konkuk University

I. INTRODUCTION

The production of *in vitro* derived bovine embryos has become routine procedure and thus successful cryopreservation method is still more required for efficient utilization of these embryos (Leibo and Loskutoff, 1993). In the past decade, various new methods for embryo cryopreservation have been developed. Among these methods, vitrification has been widely used and is now regarded as a potential alternative to conservative slow freezing. In addition, *in vitro* produced bovine embryo cryopreservation, to outrace the chilling injury from the freezing procedures, it suggested that sufficient rapid cooling may be beneficial than slow cooling (Pollard and Leibo, 1994). Vitrificaton has potential advantages over conventional freezing in that it takes only a few seconds for cooling embryos, and there is no extracellular crystallization, which is one of the major causes of cell injury (Rall and Fahy, 1985). Also, by acceleration of the speed of temperature changes, it offers lower osmotic and toxic effects and less severe chilling injury as a result of the rapid passage though the "dangerous" temperature zone (Vajta et al., 1998). However, most vitrification methods use the standard French mini-straws for holding the embryos during cooling, storage and warming. Recently, to overcome the disadvantages of straw which has low cooling and warming rates, a few study has being introduced. Among them, Martino et al. (1996) reported that ultra-rapid freezing method using electron microscope (EM) grid was efficient for the cryopreservation of chilling sensitive bovine oocyte. But, until now, freezing study using EM grid was limited only at oocyte stage (Martino et al., 1996; Kim et al., 1998a, b). On the other hand, successful results on the vitrification of *in vitro* derived bovine blastocyst were immediately demonstrated by many researchers (Kuwamaya et al., 1992; Tachikawa et al., 1993; Mamoudzadeh et al., 1995). In previous study (Park et al., 1998), we already reported that higher survival of Hanwoo IVM/IVF/IVC blastocysts can be obtained by simple 2-step vitrification method using straw and EFS40 freezing solution. On the basis of these results, in this study, to find the better cryopreservation method for *in vitro* produced Hanwoo blastocysts, we examined the *in vitro* survival rates of vitrified-warmed embryos from a new freezing method using EM grid, and also those results were compared with the survival rates of vitrification method using straw which was established before.

II. MATERIALS AND METHODS

Chemicals were obtained from Sigma Chemical Company (St. Louis, MO) and media from GIBCO (Grand Island, NY), unless otherwise stated. All of the manipulation procedures of oocytes, sperm, freezing and warming were conducted at 25℃ room temperature.

1. Production of Hanwoo IVM/IVF/IVC Blastocysts

The culture procedures employed in the production of preimplantation embryos from Hanwoo follicular oocytes were as outlined by Park et al. (1998). Briefly, Hanwoo ovaries were obtained from a slaughterhouse, and cumulus-oocyte complexes (COCs) were aspirated from visible follicles (2~6 mm in diameter). COCs were then washed with HEPES-buffered Tyrode's medium and cultured in maturation medium composed of TCM199 + 10% fetal bovine serum (FBS) supplemented with 0.2 mM sodium-pyruvate, 1 μg/ml follicle-stimulating hormone, 1 μg/ml estradiol-17β, and 25 μg/ml gentamycin

sulfate 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 CR1 (Rosenkrans et al., 1993) medium supplemented with 3 mg/ml fatty acid-free BSA and then transferred into 10% FBS added CR1 medium at day 4 after IVF. For the study, blastocysts produced *in vitro* at day 7 after IVF were classified into early, expanded and early hatching stage according to their developmental morphology (Kim et al., 1996) and they were divided into control, exposure and vitrified group.

2. Vitrification Procedures

In this study, two types of embryo containers (EM grid and straw) were used to cryopreserve the Hanwoo IVM/IVF/IVC blastocysts. We will refer to vitrification using EM grid and vitrification using straw as V-G and V-S, respectively. Detailed information about two different freezing method is indicated in Table 1.

In both vitrification methods, freezing was carried out by two-step. As a freezing solution, EFS40, was prepared equally to method described by Kasai et al. (1990), was consisted of 40% (v/v) ethylene glycol (EG) 18% (w/v) ficoll (Ficoll 70, Average MW: 70,000), 10.26% (w/v) sucrose and 10% FBS supplemented in m-DPBS, was used. To decrease the embryo damage according to abrupt exposure in high concentration of cryoprotective agents, as a pre-treatment solution, m-DPBS (EG20) containing 20% EG and 10% FBS was prepared.

1) Vitrification Using Grid (V-G)

Two-step freezing method was modified from that of Kim et al. (1998a,b). Shortly, in this method, 400 mesh copper EM grids (IGC 400; Pelco international, USA) were used as a physical support to maximize cooling rates when it was directly plunged into liquid nitrogen (LN_2). Before the exposure in vitrification solution, embryos (Fig. 1A) were partially equilibrated in EG20 for 1.5 min. And then embryos were exposed in EFS40 (Fig. 1B), loaded onto EM grid (Fig. 1C, D) and directly plunged in LN_2 within 30 sec. A mean number of oocytes loaded on one grid were eight to ten.

2) Vitrification Using Straw (V-S)

Freezing was done as described by Park et al. (1998). In the first step, embryos were fully equilibrated in EG20 for 3 min. And then in the second step, embryos were exposed in EFS40,

Table 1. Comparison of characteristics between vitrification method using two types of embryo container

Characteristic \ Type	EM grid	Straw
Material	Copper	Plastic
Freezing step	2-step (1.5 min./30 sec.)	2-step (3 min./30~45 sec.)
Prefreezing	–	Need
Warming	Directly in warming sol.	Air 5 sec/water 15 sec.
Dilution step	2-step (1.5 min./1.5 min.)	2-step (5 min./5 min.)

loaded in a 0.25 ml French mini straw (IMV, L'Aigle) and plunged in LN_2 within 30 to 45 sec. Plastic 0.25 ml straws were loaded as follows. Briefly, a 4 cm length was filled with 0.5 M sucrose solution (prepared in m-DPBS 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 M sucrose. Straw was sealed with powder and heat after embryo loading. To prevent cracking of the straw, the first part of the straw filled with 0.5 M sucrose (4 cm) was slowly immersed into LN_2; the remaining part of the straw was then plunged in. Average embryo numbers loaded in each straw were about thee to five.

3. Warming Procedures

1) In V-G

Cryoprotectants in vitrified-warmed embryos were removed by 2-step. After a few hours or days of storage in LN_2, embryos were warmed ultra-rapidly. EM grid stored in LN_2 was directly transferred into 0.3 M sucrose solution (prepared in m-DPBS containing 10% FBS) as soon as possible, was fast transferred into fresh 0.3 M sucrose and was diluted for 1.5 min. Recovered embryos were transferred in m-DPBS containing 10% FBS. After another 1.5 min, dilution finished embryos were washed with culture medium and co-cultured in cumulus cell mono-layered drop (10μl) added CR1 medium containing 10% FBS (Fig. 1E). Cumulus cell drop was prepared with cumulus cells recovered from *in vitro* matured Hanwoo oocytes before IVF treatment.

2) In V-S

Embryos were warmed rapidly by pacing straws in air for 5 sec. and then in 25℃ water bath for 10~15 sec. The contents of each straw were emptied into 0.8 ml of 0.3 M sucrose solution, and then recovered embryos were transferred into a new 0.8 ml of 0.3 M sucrose for 5 min. And then embryos were incubated in a 0.8 ml of m-DPBS containing 10% FBS for 5 min. Dilution finished embryos were co-cultured in the same culture condition as V-G for 48 h.

4. Assessment of Embryo Survival

The post-warming survival of embryos was observed every 24 h under microscope and judged as morphological survivors if they were expanded into blastocysts within the first 24 h of culture, and hatched out totally within the next 48 h (Fig. 1F).

5. Experimental Design

To examine embryo damage by toxicity of vitrification solution and to compare difference of chilling injury from the freezing procedure by embryo containers, IVM/IVF/IVC Hanwoo blastocysts were exposed in freezing solution or/and vitrified into LN_2, respectively. In exposure group, all treatment including before-freezing and warming procedures was done as the same method as freezing group. Also, to examine the embryo survival *in vitro* after warming according to embryo developmental stage, and to determine the effect of two kinds of sample container on the embryo survival, each classified blastocysts produced at day 7 were vitrified.

6. Statistical Analysis

The significance of difference among treatment group in each experiment was compared with Chi-square test using SAS Institute software package (SAS Institute Inc., 1985).

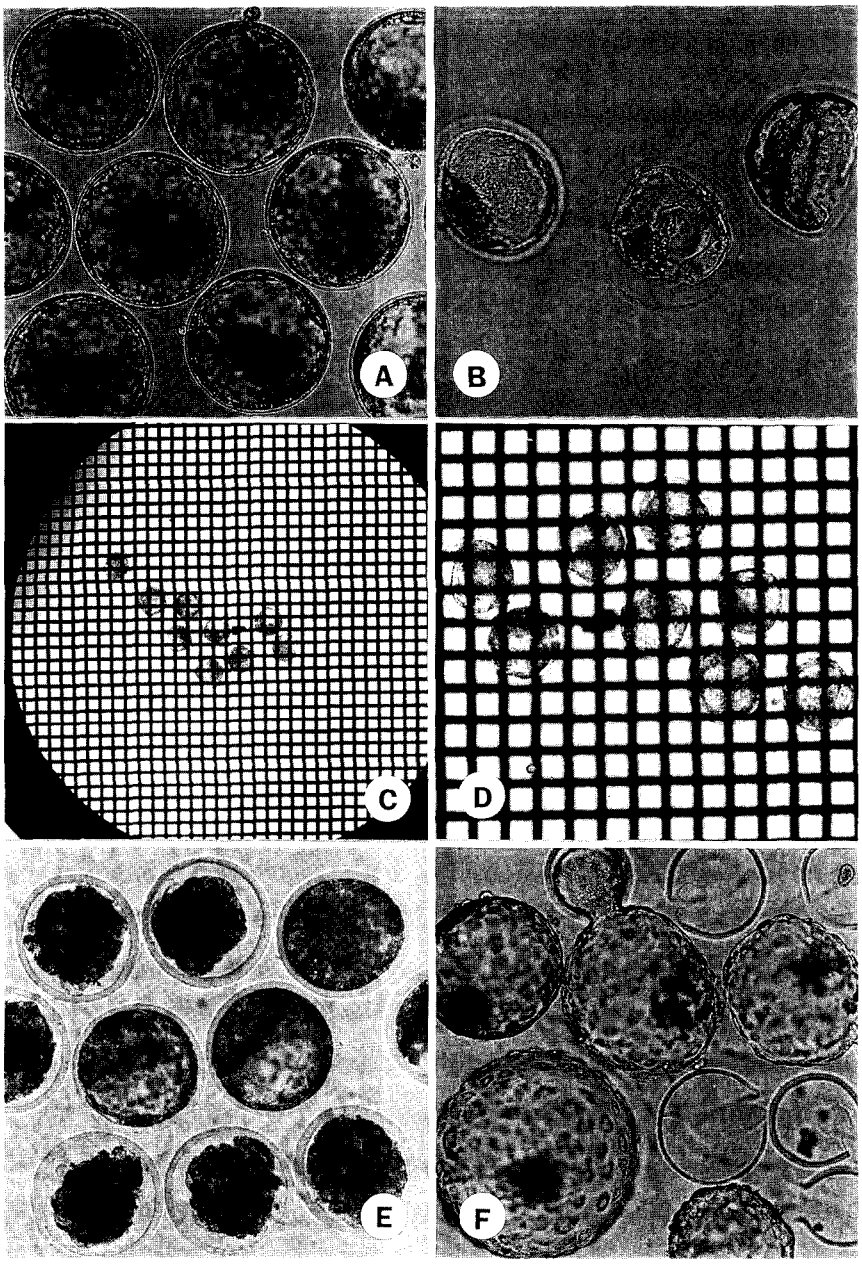

Fig. 1. A series course of freezing and warming of *in vitro* produced Hanwoo blastocysts using electron microscope (EM) grid (A-F). IVM/IVF/IVC Hanwoo blastocysts (A) were equilibrated in EG20 for 3 min, were exposed in EFS40 (B) and then were loaded onto EM grid immediately before being plunged into LN_2 (C, D) within 30 sec. After warming, embryos were diluted their cryoprotectants and then cultured in cumulus cell mono-layered drop (E). Thawed blastocysts were *in vitro* hatched 48 h after culture (F). (A-B: ×150, C: ×40, D: ×60, E: ×100, F: ×80).

Ⅲ. RESULTS

1. Hanwoo Embryo Production *in vitro*

In a total of 20 replicates, 3,318 Hanwoo oocytes were subjected to IVM and IVF procedures. On day 2, a total of 2,919 (88.0%) embryos were cleaved. The total number of embryos that developed to blastocysts at day 7 after IVF were 1,503 (45.3%). Also, when they were classified to early, expanded and beyond hatching blastocyst stage, 734 (22.1%), 582 (17.5%) and 187 (5.6%) were produced, respectively (Table. 2). For study, day 7 blastocysts showed morphologically good to excellent quality were selected from the each replicate, and thus 156 of early blastocysts, 534 of expanded blastocysts and 120 of early hatching blastocysts were utilized according to each experimental purpose.

2. *In vitro* Survival of Exposed or Vitrified Hanwoo IVM/IVF/IVC Blastocysts

The effect of exposure in vitrification solution and chilling injury from freezing procedure on *in vitro* produced Hanwoo blastocysts were examined. When the embryo survival *in vitro* was assessed to re-expanded and hatched rates at 24 h and 48 h after dilution, as shown in Table 3, viability of Hanwoo blastocysts was not significantly decreased in spite of exposure in freezing solution (100.0, 73.3%) compared to control group (100.0, 84.4%), respectively. However, their *in vitro* survival rates at 24 h were significantly higher than those of two vitrified groups (V-S: 77.8, V-G: 87.8%) ($P<0.001$). But, embryo hatched rates in V-G group (67.8%) at 48 h after warming were not significantly different with those in exposure group (73.3%), although

Table 2. Development of Hanwoo follicular oocytes at day 7 after IVF (r=20)

No. of oocytes	No.(%) of ≥2-cell	No.(%) of developed to			
		≥Bla.*	ErB	EdB	≥HgB
3,318	2,919 (88.0)	1,503 (45.3)	734 (22.1)	582 (17.5)	187 (5.6)

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

Table 3. *In vitro* survival of exposed or vitrified Hanwoo IVM/IVF/IVC blastocysts in EFS40

Treatment		No. of embryos examined*	No. of embryos recovered	No (%). of embryos survived after warming		
				24 h later	48 h later	
				≥EdB or Re-edB**	≥HgB	HdB
Control		90	–	90(100.0)^{a}	80(88.9)^{c}	76(84.4)^{c}
Exposed		90	90(100.0)	90(100.0)^{a}	78(86.7)^{c}	66(73.3)^{c,d}
Vitrified	Straw***	90	90(100.0)	70(77.8)^{b}	61(67.8)^{d}	48(53.3)^{e}
	Grid***	90	90(100.0)	79(87.8)^{b}	71(78.9)^{c,d}	61(67.8)^{d}

* Day 7 expanded blastocysts were used for comparison.

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

*** Types of embryo container used in freezing.

Means in the column without common superscripts are significantly different ($P<0.001$)$^{a-b}$, ($P<0.01$)$^{c-d}$, ($P<0.05$)$^{d-e}$.

there are significant difference with those in control group (84.4%) ($P<0.01$). Especially, between two vitrified groups, there are significant difference in hatched rates at 48 h after warming (V-S: 53.3%, V-G: 67.8%) ($P<0.05$).

3. *In vitro* Survival of Vitrified Hanwoo Blastocysts according to Developmental Stage and Embryo Container

The effects of embryo developmental stage (early, expanded and early hatching blastocysts) and embryo container (straw and EM grid) used at freezing to the *in vitro* survival of vitrified-warmed day 7 Hanwoo blastocysts were simultaneously examined. As shown in Table 4, fast developed embryos (expanded blastocyst and early hatching blastocyst stage) showed the better resistance to cryopreservation than delayed developed one (early blastocyst stage), irrespective of embryo containers (early; 57.1 & 24.4%, expanded; 84.7 & 60.6%, early hatching; 91.7 & 80.0%) ($P<0.001$). Especially, in this study, among the beyond hatching blastocysts, early hatching stage embryos which was shunken and then re-expanding state were used as a comparison group. However, survival rates of day 7 early hatching blastocysts were indicated higher than those of two other developmental stage embryos. In addition, when *in vitro* survival of vitrified early hatching blastocysts according to embryo container was compared, the results of V-G group (98.3, 95.0%) were significantly higher than those of V-S group (85.0, 65.0%) at 24 h and 48 h post warming, respectively ($P<0.05$, $P<0.001$).

Ⅳ. DISCUSSION

These data demonstrate that more simple and efficient cryopreservation of Hanwoo IVM/IVF/IVC blastocysts can be obtained by vitrification method using electron microscope grid (V-G). In previous study (Park et al., 1998), we already reported that *in vitro* produced Hanwoo blastocysts can be successfully cryopreserved by simple two-step vitrification method using straw (V-S) and EFS40 freezing solution. However, in this study, the faster developed em-

Table 4. *In vitro* survival of vitrified day 7 Hanwoo blastocysts according to embryo development stage and embryo container

Embryo stage	Container type	No. of embryos examined	No. of embryos recovered	No. (%) of embryos survived after warming		
				24 h later	48 h later	
				≥Re-edB	≥HgB	HdB
Early blastocyst	Straw	75	75(100.0)	42(56.0)^{a}	27(36.0)^{a}	17(22.7)^{a}
	Grid	81	81(100.0)	47(58.0)^{a}	24(29.6)^{a}	21(25.9)^{a}
		156	156(100.0)	89(57.1)*	51(32.7)*	38(24.4)*
Expanded blastocyst	Straw	87	83(94.8)	72(86.7)^{b,†}	59(71.1)^{b}	44(53.0)^{b,†}
	Grid	87	87(100.0)	72(82.8)^{b,†}	66(75.9)^{b}	59(67.8)$^{b,\mp}$
		174	170(98.5)	144(84.7)**	125(73.5)**	103(60.6)**
Early hatching blastocyst	Straw	60	60(100.0)	51(85.0)^{b,†}	48(80.0)^{b}	39(65.0)^{b}
	Grid	60	60(100.0)	59(98.3)$^{b,\mp}$	59(98.3)^{c}	57(95.0)^{c}
		120	120(100.0)	110(91.7)**	107(89.2)***	96(80.0)***

Means in the column without common superscripts are significantly different($P<0.001$)$^{a-c}$, *-***($P<0.05$)$^{\dagger-\mp}$.

bryos in V-G group were tend to significantly higher survival *in vitro* than those of V-S group after warming. This difference was indicated very high in early hatching blastocysts which has re-expanding morphology from shunken state caused by osmotic difference between blastocoele fluid and culture medium from the zona rupture. Almost of vitrified-early hatching embryos using EM grid were hatched from their zona at 48 h after warming. This result indicated that hatching stage embryos were also efficiently utilized for future use. Embryo cryopreservation using EM grid was originally designed for the vitrification of exceedingly chilling sensitive *Drosophila* embryos (Mazur et al., 1992; Steponkus et al., 1993). As described in Table 1, V-G method is more simple and time saving than V-S method. However, recent most attempt to improve the cryopreservation of bovine embryos have been directed the simplification of freezing/thawing procedures. Vitrification method using EM grid for the cryopreservation of bovine oocytes was firstly introduced by Martino et al. (1996). In their study, they demonstrated that *in vitro* survival of vitrified-warmed oocytes using EM grid was significantly higher than that of using straw. They denoted that these results were may be caused by the characteristic of grid which has about thee fold higher cooling rates than those obtained with straw. It has known that the increased rate of cooling and warming may considerably decrease the chilling injury of *in vitro* produced bovine embryos. In addition, in our previous study (Kim et al., 1998a, b), it proved that innate developmental capacity of bovine immature or mature oocytes vitrified and thawed by using EM grid can be maintained. Although cryopreservation is now a routine procedure, considerable differences of efficiency exist depending on stage, species and origin (*in vivo* or *in vitro* produced) (Fahning and Garcia, 1992). Generally, *in vitro* produced embryos were much more sensitive to freezing than *in vivo* derived counter parts (Leibo and Loskutoff, 1993). But, recently reported improved survival rates after cryopreservation of *in vitro* produced morulae and blastocysts are attributed more to the improved culture conditions than changes in the cryopreservation technology itself (Voelkel et al., 1992; Massip et al., 1995). In our culture system using CR1 medium, 45.3% blastocysts from oocytes were developed at day 7 after IVF. Also, for this study, 810 of day 7 healthy blastocysts were used. Especially, in expanded and early hatching blastocysts produced *in vitro* at day 7, using V-G method, 67.8, 95.0% of hatching out embryos were obtained at 48 h after warming, respectively. These embryo survival *in vitro* were not bad compared to that of Mamoudzadeh group (1995) (68.8%/expanded blastocysts) observed till 72 h after thawing. But, day 7 delayed developed blastocysts (early blastocysts) have been shown to be more sensitive to chilling injury than fast developed blastocysts (expanded blastocyst and early hatching blastocyst), irrespective of embryo containers. Their survival *in vitro* was significantly lower than those of other two groups. However, to compare the survival between two container types, re-expansion of embryos in V-G group was seem to carry out fast than that in V-S group (Data not shown). In addition, cryoprotective agents were very intimately related with embryo survival (Tachikawa et al., 1993). From the year 1990, EFS is widely used to cryopreserve the various stage embryos of several species, which permeates the cell rapidly and has low toxicity. Until now, in our lab, using EFS freezing solution, we tried to find the freezing method covering a wide range from immature oocyte to hatched blastocyst, thus appropriate concentration of freezing solution and treatment

method according to embryo stage were developed. By this report, in cryopreservation of IVM /IVF /IVC Hanwoo blastocysts, two-step vitrification method using EM grid and EFS40 freezing solution was very efficient, particularly at the day 7 expanded and early hatching blastocysts.

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요 약

체외생산된 한우 배반포기배로부터 송아지 생산을 위한 체계

Ⅱ. 한우 배반포기배를 간편하면서 효율적이고 성공적으로 유리화 동결하는 방법

김은영.[1,*], 김덕임[2], 박노형[2], 원유석[2], 남화경[1], 이금실[1], 박세영[1], 윤산현[3], 박세필[1], 정길생[4], 임진호[3]

[1,*]마리아 기초의학연구소, [2]한우개량부, [3]마리아 불임크리닉, [4]건국대학교 축산대학

본 실험은 체외 생산된 한우 배반포기배에 적합한 동결/융해 방법을 찾고자 실시하였다. 체외배양 7일째에 생산된 배반포기배는 동해제 EFS40(40% ethylene glycol, 18% ficoll, 0.3 M sucrose 그리고 10% FBS가 첨가된 m-DPBS)과 embryo container인 EM grid(V-G) 또는 straw(V-S)를 이용해서 초자화동결하였다. 동결과 융해는 두 방법 모두 2단계로 실시하였으며, 처리시간은 V-G 방법이 2분과 3분, V-S 방법이 3.5분과 10분 각각 소요되었다. 체외 생존능 평가는 융해 후 24시간째의 재팽창율과 48시간째의 부화율로 조사하였다. 본 실험에서 얻어진 결과는 다음과 같다. 팽창 배반포기배를 이용하여 동결액 노출과 동결과정의 냉해가 배의 생존에 미치는 영향을 조사하였던 바, 융해 후 24시간째, 동결액 노출군(100.0%)의 결과는 대조군(100.0%)과 차이가 없었으며, 두 동결군(V-G: 87.8%, V-S: 77.8%)의 생존율과 비교해 볼 때 유의하게 높았다($P<0.001$). 그러나, 융해 후 48시간째 각 처리군의 부화율을 조사하였던 바, V-G군(67.8%)은 V-S군(53.3%)보다 유의하게 높게 나타났으며($P<0.05$), 동결액 노출군(73.3%) 과도 유의한 차이를 나타내지 않았다. 또한, 배발달단계(초기, 팽창, 부화초기 배반포)와 동결에 사용된 embryo container(EM grid, straw)가 체외 생존율에 미치는 영향을 동시에 비교하였던 바, embryo container에 상관없이 빠르게 발달된 배반포기배가 느리게 발달하는 난자군보다 유의하게 높은 생존율을 나타내었다(초기: 57.1, 24.4%; 팽창: 84.7, 60.6%; 부화초기: 91.7, 80.0%)($P<0.001$). 특히, 팽창 배반포기배와 부화초기 배반포기배에서, 융해 후 48시간째, V-G군(67.8, 95.0%)의 부화율이 V-S군(53.0, 65.0%)보다 유의하게 높게 나타나 동결시 EM grid의 유용성을 확인할 수 있었다($P<0.05$, $P<0.001$). 따라서, 한우 배반포기배는 EM grid를 사용하는 초자화 동결방법으로 간편하면서도 효율적이고 성공적으로 동결보존할 수 있다는 것을 알았다.

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