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Optimization of One-Step Dilution Method of Vitrified Bovine IVM/IVF/IVC Blastocysts

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초자화 동결된 체외생산 소 배반포기배의 1단계 융해 방법의 적정화

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ABSTRACT

This study was to establish an effective dilution technique in a vitrification of bovine blastocysts for the field trial. For vitrification, blastocysts were exposed in glycerol (G) and ethylene glycol (EG) mixture in m-DPBS supplemented with 10% FBS. Blastocysts were first exposed to 10% (v/v) G for 5 min, and subsequently were transferred to 10% G plus 20% EG (v/v) for 5 min. Finally, embryos were transferred to 25% G plus 25% EG (v/v) for 30 sec and were placed in nitrogen vapor for 3 min, and then were plunged into LN_2. At thawing, the straw containing blastocysts was placed in air for 10 sec, and then plunged into a water bath at 25℃ until all ice had disappeared. They were placed in 25℃ and 36℃ water according to treatment group for different time. Also, *in vitro* survival was assessed by the re-expansion and hatched rates at 24 h and 48 h postwarming, respectively. The results obtained in these experiments were summarized as follows; 1) In the survival rates of vitrified bovine blastocysts according to different dilution time at thawing, the data of 1 min group (86.6, 56.6%) were higher than those of other treatment groups (2 min; 93.5, 35.4%, 2.5 min; 76.9, 30.7%, 3 min; 88.8, 36.1% and 3.5 min; 83.7, 8.1%). 2) When the *in vitro* survival of vitrified groups according to different developmental stage was examined at 48 h after thawing using 1 min dilution method, the hatching rates of fast developed embryos (expanded blastocyst: 81.3%; early hatching blastocyst: 86.2%) were higher than that of delayed developed one (early blastocyst: 46.6%). 3) In addition, when the *in vitro* survival of vitrified groups according to different embryo age was compared, the hatched rates at 48 h after thawing of Day 7 (66.6%) and Day 8 embryos (60.0%) were significantly higher than that of Day 9 embryos (22.7%) ($P<0.05$). These results demonstrate that vitrified bovine IVM/IVF/IVC blastocysts can be successfully survived *in vitro* using one-step dilution (1 min) method.

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(Key words: Bovine blastocyst, Vitrification, One-step dilution, *In vitro* survival)

I. INTRODUCTION

Embryo transfer has become a routine procedure in cattle breeding programs, and cryopreservation of embryos plays an important role in such programs. Many researchers reported a one-step thawing method, in which bovine embryos were slowly frozen in glycerol (G) or ethylene glycol (EG) and the frozen embryos were thawed and diluted in straw sucrose solution before being transferred into the recipients (Leibo, 1984; Massip et al., 1984; Voekel and Hu, 1992; Dochi et al., 1995; 1998). In recent years, vitrification has become to be widely used, because of the cryopreservation of cells without ice crystal formation, the possibility of chilling injuries is lower due to the high cooling rates (Massip et al., 1995). Furthermore, this method would be practical and acceptable procedures for using in field application. This study was to develop a easier and more effective dilution technique of vitrified bovine blastocysts for the field trial.

II. MATERIALS AND METHODS

1. Production of Bovine Blastocysts

Bovine cumulus oocyte complexes (COCs) were collected from visible follicles (2~6 mm) of ovaries, washed with TALP-HEPES medium and cultured in maturation medium consisting of TCM-199 (Gibco) suppleme-nted with 10% (v/v) fetal bovine serum (FBS) 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 (Park et al., 1995). 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 (≥2-cell) after 44±2 h co-incubation with the sperm. For *in vitro* culture, cleaved embryos were cultured in mCR1aa medium 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, Day 8 blastocysts produced *in vitro* after IVF were mainly used and they were classified into early, expanded and early hatching stages according to their developmental morphology. Also, expanded blastocysts produced *in vitro* at Days 7, 8 and 9 after IVF were applied to comparison of developmental capacity after freezing and one-step dilution (Fig. 1. A).

2. Vitrification Procedure

Vitrification solutions were prepared in modified Dulbecco's phosphate buffered saline (m-DPBS) supplemented with 10% FBS. Vitrification solutions were mixtures of G and EG. Selected blastocysts for the experiments were first exposed to 10% (v/v) G for 5 min, then were transferred to 10% G plus 20% EG (v/v) for 5 min and finally were transferred to 25% G plus 25% EG (v/v) for 30 sec (Fig. 1. B). And, then they were loaded in a 0.25 ml French straw. Briefly, a 7.5 cm length was filled with 0.5 M sucrose solution (m-DPBS containing 10% FBS) followed by a 0.5 cm air bubble, 1.0 cm G 25% plus EG 25% and 0.5 cm air bubble. And, the remaining part of the straw was filled with 0.5 M sucrose. Before being plunged into LN_2, straw was exposed by the cold nitrogen vapor for 3 min.

3. Thawing Procedure

Thawing was modified from a method de-

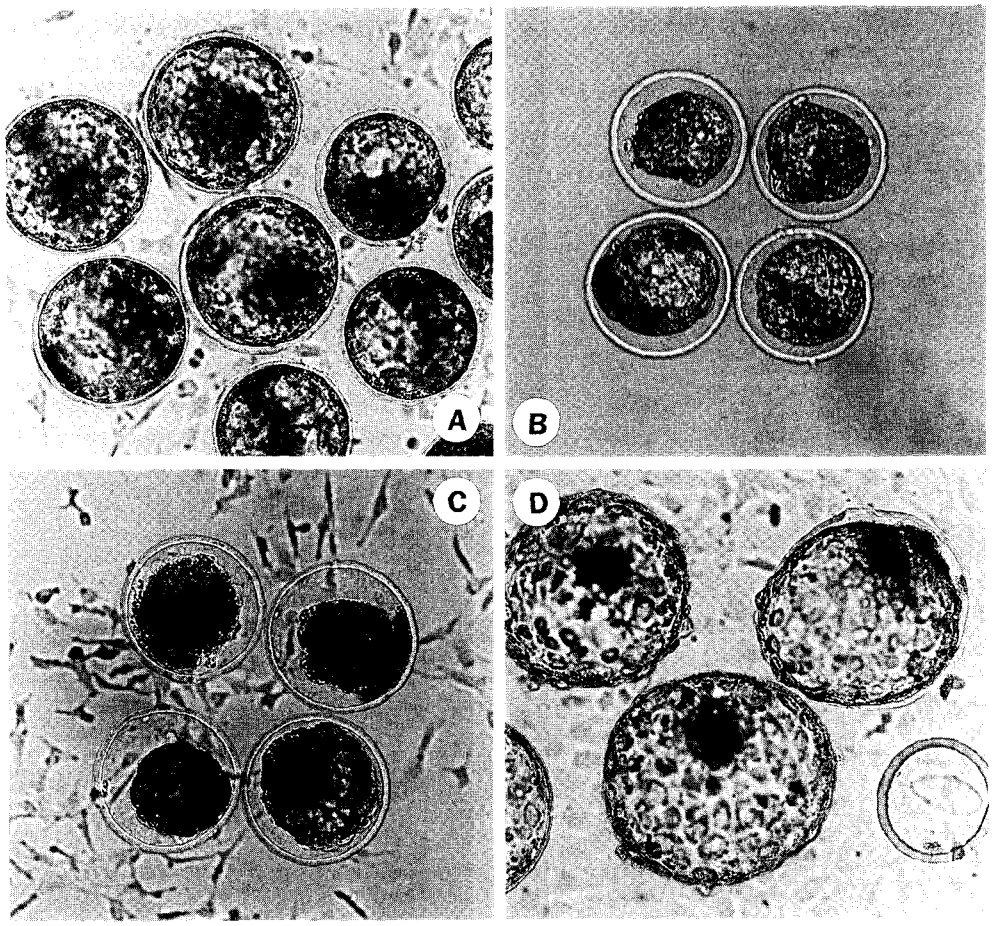

Fig. 1. Bovine expanded blastocysts on Day 7. (A) before vitrification (×300), (B) exposed to cryoprotectant (×300), (C) after thawing (×300), (D) survived embryos at 48 h after thawing (×150).

scribed by van Wagtendonk-de Leeuw et al. (1997). Vitrified straws were classified into five groups (1, 2, 2.5, 3 and 3.5 min) according to dilution time at thawing. Vitrified straws were thawed in air of 25℃ for 10 sec and then water of 25℃ until all ice had disappeared. One-step dilution of thawed straws was done as follows. Straws were held at the plug end and yield single column of air and liquid. Ⅰ) In the 1 min method, straws were placed vertically (plug-end down) 25℃ water for 0.5 min and then in 25℃ water for about 0.5 min (plug-end up). Ⅱ) In the 2 min method, in 36℃ water for 1 min (plug-end down) and then in 25℃ water for 1 min (plug-end up). Ⅲ) In the 2.5 min method, in 36℃ water for 1.5 min (plug-end down) and then in 25℃ water for 1 min (plug-end up). Ⅳ) In the 3 min method, in 36℃ water for 1.5 min (plug-end down) and then in 25℃ water for about 1.5 min (plug-end up). Ⅴ) In the 3.5 min method, in 36℃ water for 1.5 min (plug-end down) and then in 25℃ water for about 2 min

(plug-end up).

Each one-step diluted embryos were transferred to mCR1aa (containing 10% FBS) medium for 1 min. The embryos were co-cultured in cumulus monolayered cell droplets added mCR1aa medium supplemented with 10% FBS (Fig. 1. C).

4. Assessment of Embryo Survival

The post-thawing survival of embryos was observed every 24 h and 48 h under a stereomicroscope (Fig. 1. D), respectively.

5. Experimental Designs

To establish an effective method for better embryo survival after one-step dilution, dilution times at thawing were varied. And, the survival of vitrified groups according to the different development stage at Day 8 bovine blastocysts was examined after one-step dilution by using 1 min.

Also, to compare the survival of vitrified groups according to embryo age, Days 7, 8 or 9 bovine expanded blastocysts were one-step diluted by using 1 min.

6. Statistical Analysis

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

Ⅲ. RESULTS

When the *in vitro* survival of vitrified bovine blastocysts according to different one-step dilution time was examined at 24 h after thawing, although the re-expanded rates of 2.5 min group (76.9%) was lower than that of control group (100.0%) ($P<0.05$), there were no significantly differences among the treatment groups. Especially, at 48 h after thawing, the survival rates of 1 min group (≥hatching: 73.3, hatched: 56.6%) were higher than those of the other treatment groups (2 min: 61.3, 35.4%; 2.5 min: 57.6, 30.7%; 3 min: 55.5, 36.1% and 3.5 min: 56.7, 8.1%). Also, the hatched rate of 3.5 min group was significantly lower than that of the other treatment groups and control group ($P<0.05$). However, at 48 h after thawing, there were significant differences between control group and treatment groups ($P<0.05$). *In vitro* survival of vitrified groups according to differ-

Table 1. *In vitro* survival of vitrified bovine blastocysts according to one-step dilution time at thawing (r=8)

Thawing time (min)		No. of* embryo	No. (%) of recovery	No. (%) of survived		
				24 h later	48 h later	
36℃	25℃			≥Re-edB**	≥HgB**	HdB**
		30	–	30 (100.0)[a]	30 (100.0)[a]	26 (86.6)[a]
	1	30	30 (100.0)	26 (86.6)[a,b]	22 (73.3)[b]	17 (56.6)[b]
1	1	31	31 (100.0)	29 (93.5)[a,b]	19 (61.3)[b]	11 (35.4)[b]
1	1.5	29	26 (89.6)	20 (76.9)[b]	15 (58.8)[b]	8 (30.7)[b]
1.5	1.5	37	36 (97.3)	32 (88.8)[a,b]	20 (55.5)[b]	13 (36.1)[b]
1.5	2	38	37 (97.4)	31 (83.7)[a,b]	21 (56.7)[b]	3 (8.1)[c]

* Expanded blastocysts (Day 8)

** Re-edB; Re-expanded blastocysts, HgB; Hatching blastocysts, HdB; Hatched blastocysts

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

Table 2. *In vitro* survival of bovine blastocysts after vitrification and one-step dilution according to embryo development stage (r=8)

Embryo stage	No. of embryo	No. (%) of recovery	No. (%) of survived		
			24 h later	48 h later	
			≥Re-edB*	≥HgB*	HdB*
Early blastocyst	32	30 (93.8)	25 (83.3)	14 (46.6)[a]	11 (36.6)
Expanded blastocyst	32	32 (100.0)	30 (93.8)	26 (81.3)[b]	18 (56.3)
Early Hatching blastocysts	30	29 (100.0)	25 (86.2)	25 (86.2)[b]	17 (58.6)

* Re-edB; Re-expanded blastocysts, HgB; Hatching blastocysts, HdB; Hatched blastocysts

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

ent development stage (classified Day 8 bovine blastocysts) was examined after one-step dilution by using 1 min (Table 2). The re-expansion rates of early, expanded and early hatching blastocysts rates were 83.3, 93.8 and 86.2%, respectively, and there were not significant different among the groups. At 48 h after thawing, the hatching rate of early blastocysts (46.6%) were lower than that of expanded and early hatching blastocysts (81.3 and 86.2%) ($P<0.05$), while the hatched rates were not different among the groups. When the *in vitro* survival of vitrified groups according to different embryo age (classified Days 7, 8 or 9 bovine expanded blastocysts) was examined (Table 3). The re-expansion rates of Days 7, 8 and 9 blastocysts rates at 24 h after thawing were 88.8, 88.6 and 81.8%, respectively. However, the hatching and hatched rates of Day 9 group (50.0, 22.7%) at 48 h after thawing were significantly lower than those of Day 7 and Day 8 groups (81.5, 66.6% and 82.8, 60.0%) ($P<0.05$).

Ⅳ. DISCUSSION

These results demonstrate that the one-step dilution method greatly simplifies the embryo transfer process. The application of vitrification and one-step dilution to on-farm transfer reduces the equipments and embryological skills

Table 3. *In vitro* survival of bovine blastocysts after vitrification and one-step dilution according to embryo development age (r=7)

Embryo age (Day)	No. of* embryo	No. (%) of recovery	No. (%) of survived		
			24 h later	48 h later	
			≥Re-edB**	≥HgB**	HdB**
7	28	27 (96.4)	24 (88.8)	22 (81.5)[a]	18 (66.6)[a]
8	30	30 (100.0)	25 (88.6)	23 (82.8)[a]	18 (60.0)[a]
9	23	22 (95.6)	18 (81.8)	11 (50.0)[b]	5 (22.7)[b]

* Expanded blastocysts

** Re-edB; Re-expanded blastocysts, HgB; Hatching blastocysts, HdB; Hatched blastocysts

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

required for dilution and transfer and yields a considerable saving of time per transfer. In our study, the vitrificatin solution was G and EG mixture. In the results, when the *in vitro* survival of vitrified bovine blastocysts according to different one-step dilution time was examined at 48 h after thawing, the *in vitro* survival rates of 1 min group were higher than those of the other treatment groups ($P<0.05$).

Thus, in this study, 1 min dilution method was selected. Recently, van Wagtendonk-de et al. (1997) reported that the high pregancy rate obtained in field trial by vitrified and 7 min diluted method of bovine embryos produced *in vitro*. In present study, *in vitro* survival rates of 1 min dilution method and 7 min dilution method were similar (data not shown).

Especially, as shown in Table 2, fast developed embryo groups (expanded blastocysts and early hatching blastocysts) showed the better resistance to cryopreservation than delayed developed one (early blastocysts). Our previous results were shown that the highest survival rates among the developmental stage were obtained in expanded and early hatching blastocysts (Park et al., 1998). Also, Mahmoudzadeh et al. (1995) and Hasler et al. (1997) reported that the stage of embryonic development has influence on frozen-thawed survival rate. It is caused that early blastocysts stage of *in vitro* produced embryos has been shown to be more sensitive to chilling injury than expanded blastocysts (Pollard and Leibo, 1994). Also, when embryo age was younger, *in vitro* survival was significantly higher. It is known that blastocysts developed earlier have more cells and fewer chromosomal anomalies than those developed later (Zhu et al., 1996). Taken altogether, these results suggested that bovine IVM/IVF/IVC blastocysts cryopreserved using vitrification can be successfully survived *in vitro* by one-step dilution (1 min) method.

V. 요 약

본 실험은 초자화동결된 소 배반포기배를 실험현장에서 효율적으로 융해할 수 있는 기술을 찾고자 실시하였다. 초자화동결은 glycerol (G)과 ethylene glycol (EG) 그리고 10% FBS가 들어있는 m-DPBS를 이용하였으며, 배반포기배는 3단계로 초자화동결되었는데, 10% G에 5분간 평형, 10% G와 20% EG에 5분간 평형, 그리고 25% G와 25% EG에 30초간 노출하였다. 질소 증기를 3분간 쐬고 액화질소에 침지하였다. 융해는 straw를 공기 중에서 10초간 노출시키고, 25℃ 물에서 빙정이 없어질 때까지 녹인 후 25℃와 36℃에 각각 시간차에 따라 처리군을 나누었다. 초자화동결된 배반포기배를 융해시 시간차에 따라 체외생존능은 융해 24시간과 48시간 후 재팽창과 완전탈출 배반포기배로 평가하였다. 그 결과를 요약하면 다음과 같다.

1) 초자화 동결된 배반포기를 융해시 시간차에 따라 체외생존능을 보았을 때, 1분으로 융해한 군이 (86.6, 56.6%) 다른 처리군들보다 (2분: 93.5, 35.4%; 2.5분: 76.9, 30.7%; 3분: 88.8, 36.1%; 3.5분: 83.7, 8.1%) 체외생존능이 높게 나타났다.
2) 1분 융해방법으로 배반포기배의 발달단계에 따라 생존능을 조사하였을 때, 융해 48시간 후 빠르게 발달된 배반포기배의 부화율 (팽윤: 93.8, 56.3%; 부화초기: 86.2, 58.6%)은 느리게 발달하는 난자군의 부화율 (초기: 83.3, 36.6%) 보다 높은 체외생존능을 나타내었다.
3) 또한, 1분 융해방법으로 배반포기배가 생산된 나이에 따라 체외생존능을 조사하였을 때, 융해 48시간 후, 7일 (66.6%)과 8일 (60.0%)에 생산된 배반포기배가 9일 (22.7%)에 생산된 완전탈출 배반포기배율 보다 유의하게 높은 체외생존율을 나타내었다 ($P<0.05$). 그러므로 초자화동결된 배반포기배를 1분 융해방법으로 융해하였을 때 빠르고 효율적으로 체외생존능을 얻을 수 있음을 알 수 있었다.

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