韓國家畜繁殖學會誌 25(4) : 371~379 (2001)
Korean J. Animal Reprod.

Development of *In Vitro* Produced Bovine Embryos after Vitrification with Various Containers

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ABSTRACT

The objective of this study was to establish an effective cryopreservation method of *in vitro*-produced bovine embryos. For the vitrification, *in vitro*-produced embryos at 8-cell, morula and blastocyst stages were exposed to freezing solution containing 5.5 M EG (EG 5.5) for 20 sec, loaded on each containers such as EM grid, OPS and Cryo-loop, and then immediately plunged into liquid nitrogen at −196℃. Thawed embryos were serially diluted in 0.5, 0.25 and 0.125 M sucrose in m-DPBS, each for 1 min, and cultured in CRIaa medium supplemented with 10% FBS. Significant differences in the rates of re-expanded and hatched embryos were not observed among these embryo containers. The total cell number of expanded blastocyst cultured *in vitro* after vitrification was examined by Hoechst staining. There were no differences between non-vitrified (180.0 ± 5.4) and vitrified groups (178.0 ± 7.5). In addition, when the cellular injuries after vitrification were compared by double staining. There were no significant difference in the ratio of live and dead cells between non-vitrified group (176 : 4) and vitrified group (172 : 6). Therefore, these results suggest that bovine embryos can be cryopreserved easily, effectively and successfully by vitrification using various containers, such as EM grid, OPS or Cryo-loop in the presence of EG 5.5 freezing solution.
(Key words: Bovine embryos, EG 5.5, EM grid, OPS, Cryo-loop)

Ⅰ. INTRODUCTION

Currently, the various freezing methods have been developed to improve the efficiency of cryopreservation of oocytes or embryos. Especially, vitrification among them is useful freezing method regarded as a good alternative to transitional slow-freezing method. The transitional slow freezing method requires more accurate operation, expensive freezer and time-consuming process. Also, the survival rates are generally low and depend on the quality and developmental stage of embryos (Pollard and Leibo, 1994). The vitrification method was developed to overcome these disadvantages in cryopreservation of mammalian embryos (Rall and Fahy, 1985; Massip et al., 1986). Vitrification provides way to resolve most of these problems; it is easy to handle in simple container and takes only a few seconds for cooling embryos. In addition, the survival rates of embryos derived from *in vitro* and *in vivo* are reasonable (Agca et al., 1994; Mah-

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moudzadeh et al., 1994; Wurth et al., 1994). Nakagata (1989) reported a vitrification procedure, which required only very short exposure to the cryoprotectant mixture. In addition, Shaw et al. (1992) had found the optimal conditions to obtain higher survival rates of vitrified oocytes by very short exposure. The very short exposure times to cryoprotectant would minimize any potential problem of the toxicity of the cryoprotectant. Furthermore, when embryos are cryopreserved by vitrification, ice crystal formation that is one of the major causes of cell injuries is prevented by the use of high concentrations of cryoprotectants and/or high cooling and warming rates. If exposure is too brief, however, permeation of the cryoprotectant will be inadequate, and intracellular ice may form even in the absence of extracellular ice.

Otoi et al. (1998) reported that vitrified bovine embryos were very sensitive to cooling with ethylene glycol-based cryoprotectants in conventional straws. They obtained results better than those observed with the slow freezing method. However, these straws limit the maximum cooling and warming rate to less than 2,000℃/min. To obtain a more rapid cooling and warming rate, Martino et al. (1996 a, b), Vajta et al. (1998, 1999), Lane et al. (1999) and Lane and Gardner (2001) have developed the various methods by using EM grids, OPS and Cryo-loop to hold bovine embryos with a very small amount of solution ($< 1 \sim 2\ \mu l$) for vitrification. These methods achieved a faster cooling and warming rate more than 20,000℃/min. These techniques using the different containers have proved to be an efficient method for vitrifying both bovine oocytes and embryos. However, *in vitro*-produced bovine embryos were more sensitive to cold shock than *in vivo*-derived embryos (Leibo and Loskutoff, 1993), especially at stages earlier than the blastocyst. It has been suggested that sensitivity of embryos to cold shock depends on both their developmental stage and culture conditions, as well as on the presence of numerous lipid droplets in the *in vitro*-produced embryos prior to the blastocysts stage (Plante and King, 1994). Therefore, this study was performed to optimize the vitrification method of *in vitro*- produced bovine embryos with various containers.

Ⅱ. MATERIALS AND METHODS

1. *In Vitro* Production of Bovine Embryos

1) *In Vitro* Maturation

Bovine ovaries were collected at a slaughterhouse and transported to the laboratory at 35 to 39 ℃ in physiological saline. Follicles with a diameter of 2 to 8 mm were aspirated using 18- gauge needles. Selection of cumulus oocyte complexes (COCs) were based on their morphology and washed in TALP-Hepes containing 1 mg/ml bovine serum albumin (BSA: Fraction Ⅴ). The COCs were then matured in TCM199 (Earle's salts; Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) for 22-24 h at 39℃ in 5% CO_2 humidified air.

2) *In vitro* Fertilization

Matured oocytes were partially separated from cumulus cells by pipetting and transferred in each drop containing fertilization-TALP supplemented with a PHE mixture (2 mM Penicillamine, 1 mM Hypotaurine and 0.1 mM Epinephrine) and 2 μg /ml heparin. Frozen semen were thawed in a 37 ℃ water bath for 15 seconds, and were loaded on top of a discontinuous percoll gradient consisting of 2 ml of 45% and 2 ml of 90% percoll dissolved in sperm-TALP containing 6 mg/ml BSA (fatty acid free) and centrifuged at 2,000 rpm for 15min. The pellet was resuspended in sperm-TALP. Finally,

sperm number and motility were recorded. Prepared oocytes were incubated with capacitated spermatozoa at a concentration of 2 × 10^6 spermatozoa/ml.

3) *In Vitro* Culture

At 18 h after sperm-oocyte incubation, the cumulus cells were removed by pipetting and the embryos were cultured in CR I aa supplemented with 3 mg/ml BSA for 48 h. Then the embryos were transferred to CR I aa supplemented with 10% FBS. On day 3, 6 or 8 of *in vitro* culture (Day 0 was the day of fertilization), visually normal 8 cells, morulae and blastocysts were selected and frozen.

2. Vitrification and Thawing

The vitrification procedure was based on the method originally designed for *in vitro*-produced embryos by Martino et al. (1996a, and/or 1996b), Vajta et al. (1998) and Lane et al. (1999) with significant modifications (Hong et al., 1999; Chung et al., 2000). In brief, each stage embryos were placed in a cryoprotectant consisting of Dulbecco's phosphate-buffered saline (Gibco BRL, Grand Island, NY) supplemented with 5.5 M ethylene glycol, 1.0 M sucrose and 10% fetal bovine serum (FBS) for 20 seconds. They were then loaded on each vitrification containers (EM grid, OPS and Cryo-loop), then immediately plunged into liquid nitrogen at −196℃. Each vitrification containers stored in liquid nitrogen were directly transferred into 0.5 M sucrose solution as soon as possible. And then, the each vitrification containers were exposed serially in 0.25 M and 0.125 M sucrose solution with 1 min interval at 37 ℃. Thawed embryos were washed in culture medium and cultured in CR I aa supplemented with 10% FBS for 24 h at 39℃ in 5% CO_2 in humidified air, and then the re-expansion rate was evaluated under stereomicroscope.

1) Effect of Cryoprotectant Addition Procedure on the Developmental Competence of Embryo

The effect of stepwise addition of cryoprotectant before exposure to vitrification solution on the developmental competence of embryos vitrified in EG 5.5 solution (supplemented with 1.0 M sucrose and 10% FBS) was tested. In the one-step procedure, embryos were directly exposed to EG 5.5 solution. In the two-step procedure, embryos were equilibrated to EG 1.5 for 3 min before being exposed to EG 5.5 solution.

2) Effect of Container on the Development of Vitrified-thawed Embryos

Eight cell embryos on Day 3, compact morulae on Day 6, expanded blastocysts on Day 8 after insemination were selected for vitrification. All embryos were belong to Grade 1 according to the International Embryo Transfer Society standards. In this study, three types of vitrification containers, EM grid, OPS and Cryo-loop were used to cryopreservation of the bovine embryos. Electron microscope grid (EM grid; Gilder Co., West Chester, PA) consisted of a 400 mesh cooper in a 3.05 mm in diameter and 0.037 mm thick. Embryos were mounted on an EM grid using a fine glass pipette, and surplus cryoprotectant was removed using sterilized tissue. The open pulled straws (OPS) were made using French mini straws (250 μl, IMV, L'Aigle, France) as described by Vajta et al. (1998). Briefly, the French mini-straws were heat-softened over a hot plate and pulled manually until the inner diameter and the wall thickness of the central part decreased from 1.7 mm to approximately 0.8 mm. The straws were cooled in air then cut at the narrowest point with razor blade. Loading embryos into the tip of the OPS was done by means of the capillary effect by simply touching a microdrop (1~2 μl) of cryoprotectant solution

containing embryos. The cryo-loop consisted of a 20 μm nylon filament in a 0.7~1.0 mm diameter loop mounted on a stainless steel tube fixed to the inside of a 1.8 ml cryovial cap (Hampton Research, Laguna Niguel, CA, USA). Embryos were transferred to the nylon loop that had previously been dipped into cryoprotectant solution to create a thin film. The cryo-loops containing the embryos were then plunged into the cryovial, which is submerged and filled with liquid nitrogen. Then, vitrification containers containing the embryos were immediately plunged into liquid nitrogen at −196℃.

3) Counting of Total Cell and Live Cell Numbers of Expanded Blastocysts

For the count of total cells, the blastocysts developed *in vitro* from vitrified-thawed bovine 8-cells, morulae and blastocysts were fixed with 2% formaldehyde, 0.1% PVP (polyvinyl pyrrolidone) and 0.25% glutaraldehyde in DPBS for 2~3 min prior to 2.5 μg/ml bisbenzimide (No. 33342, Sigma) treatment. Fixed blastocysts were placed on a slide glass and covered with cover glass and then pressed down the edges until formation of sample for the good observation, and then on appropriate bisbenzimide solution dropped beside the cover glass, filled the square under cover glass with the solution and sealed the edges with fingernail polish. Observation was carried out under ultraviolet filter incorporated fluorescent microscope on 1 day after making sample.

4) Double Staining for the Counting Live and Dead Cells in Expanded Blastocysts

A double staining method was used to assess cell membrane alterations. One dye was 1) propidium iodide (PI; Sigma), marking a nucleic acid excluded from intact cells. It can only enter cells with altered membrane integrity and showing red color following UV excitation. The other was bisbenzimide (Hoechst 33342; Sigma), which enters all cells and binds both specifically and quantitatively to DNA showing blue color following UV excitation. In cells with membrane alteration, the bisbenzimide fluorescence is quenched by the PI, which absorbs the energy and emits red fluorescence. The hatched blastocysts were rinsed in fresh culture medium and incubated in DPBS containing propidium iodine (PI) and bisbenzimide (10 μg/ml, respectively) for 30 min in an incubator at 39℃ and 5% CO_2 in air. The embryos were washed in DPBS supplemented with 3 mg/ml BSA and pressed on to a slide glass and then examined under a fluorescence microscope. This resulted in live cell fluorescing blue (bisbenzimide-positive) and dead cell fluorescing red (PI-positive).

3. Statistical Analysis

The significant difference among treatment groups in each experiment was estimated by ANOVA (Abacus, Berkeley, CA) and t-test.

Ⅲ. RESULTS

1. Effect of Cryoprotectant Addition Procedure on the Developmental Competence of Embryo

As shown in Table 1, re-expended rate of blastocysts vitrified by the one-step addition procedure of cryoprotectant was 77.5%. This value was higher than that of two-step addition procedure, 64.0%. Similarly, the hatched rate of blastocysts, 70.8% after vitrification by one-step procedure was significantly higher than that, 54.0% of blastocyst vitrified by two-step procedure.

2. Effect of Container on the Development of Vitrified-thawed Embryos

As shown in Table 2, embryos at pre-morula stage showed lower resistance to cryopreservation than that of later-stage embryos, irrespective of vit-

Table 1. Developmental competence of bovine blastocysts vitrified-thawed in EG 5.5 by the one-step and two-step procedure of cryoprotectant addition

Procedure	Treatment	No. of blastocysts examined	No. (%) of blastocysts	
			Re-expanded	Hatched
One-step	EG 5.5*	120	93 (77.5)[a]	85 (70.8)[a']
Two-step	EG 1.5**/ EG 5.5	100	64 (64.0)[b]	54 (54.0)[b']

Values within columns with superscripts are significantly different between a-b and a'-b' ($p<0.05$).

* EG 5.5 : 5.5 M ethylene glycol, 1.0 M sucrose and 10% FBS in m-DPBS medium

** EG 1.5 : 1.5 M ethylene glycol and 10% FBS in m-DPBS medium

Table 2. Effect of embryo stage and container on the development of vitrified-thawed embryos

Embryo stage	Container type	No. of embryos examined	No. (%) of embryos	
			Re-expanded	Hatched
8-cell	EM grid	178	91 (51.1)[a]	62 (34.8)[a']
	OPS	190	97 (51.1)[a]	69 (36.3)[a']
	Cryo-loop	125	57 (45.6)[a]	40 (32.0)[a']
	Total (Mean)	493	245 (49.7)	171 (34.7)
Morula	EM grid	142	94 (66.2)[b]	73 (51.4)[b']
	OPS	117	72 (61.5)[b]	57 (48.7)[b']
	Cryo-loop	94	59 (62.8)[b]	47 (50.0)[b']
	Total (Mean)	353	225 (63.7)	177 (50.1)
Blastocyst	EM grid	146	114 (78.1)[c]	103 (70.5)[c']
	OPS	150	121 (80.7)[c]	106 (70.7)[c']
	Cryo-loop	105	78 (74.3)[c]	71 (67.6)[c']
	Total (Mean)	401	313 (78.1)	280 (69.8)

No significant difference was observed among a,b,c and a',b',c', respectively.

Values within columns with superscripts are significantly different among a-b-c and a'-b'-c' ($p<0.05$).

rification container. Namely, mean rates of 8-cell embryo, morula and blastocyst re-expanded and hatched after vitrification and thawing were 49.7 and 34.7%, 63.7 and 50.1% and 78.1 and 69.8%, respectively. However, no significant differences in the rate of re-expanded or hatched embryos were observed among embryo containers in the same stage embryos. And all the embryos in each container were observed to be morphologically normal (Fig. 1).

3. Counting of Total Cell and Ratio of Live and Dead Cells of Expanded Blastocysts

In this experiment, the effect of vitrification on the cell number and the ratio of live and dead cells of expanded blastocysts were investigated. As shown in Table 3, the total cell number of expanded blastocyst after vitrification and thawing were

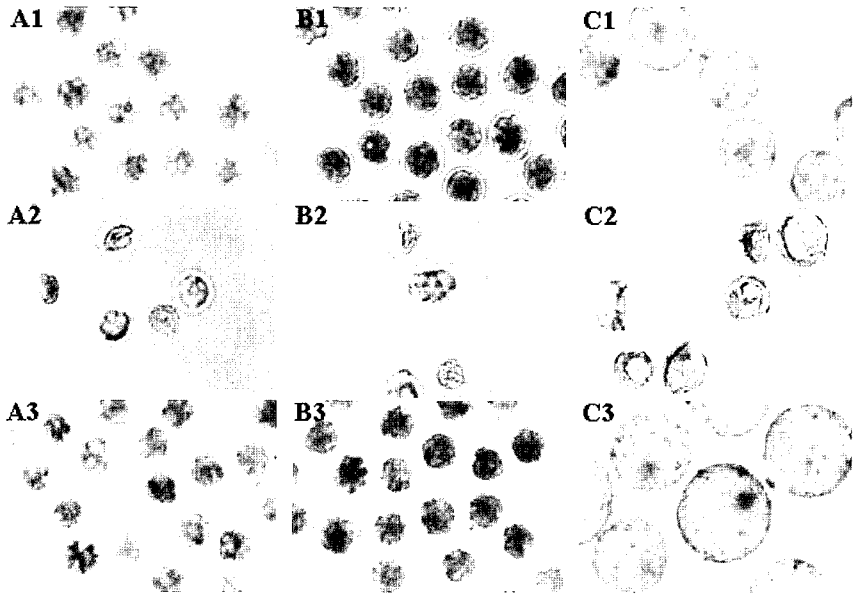

Fig. 1. Developmental morphology of vitrified and thawed bovine embryos. (A1); 8-cells before vitrification. (A2); 8-cells exposed to EG 5.5. (A3); 8-cells survived after thawing, (B1); Morulae before vitrification. (B2); Morulae exposed to EG 5.5. (B3); Morulae survived after thawing, (C1); Blastocysts before vitrification. (C2); Blastocysts exposed to EG 5.5. (C3); Blastocysts survived after thawing. (×100).

Table 3. Live and dead cell numbers to total cell of expanded blastocysts developed from bovine 8-cell embryos, morulae and blastocysts after vitrification and thawing

Treatment	No. of blastocysts examined	Mean No. of cell ± SD[a]	Live : Dead cell ratio[a]
Control (Fresh cultured)	12	180.0 ± 5.4	176 : 4
Vitrified group	24	178.0 ± 7.5	172 : 6

[a] No significant difference was observed ($p>0.05$).

about 178.0 ± 7.5 per embryo. This value was not significantly different from 180.0 ± 5.4 of nonvitrified control embryos. Also, the ratio of live and dead cell, to total cell in control and vitrified group were 176 : 4 and 172 : 6, respectively.

Ⅳ. DISCUSSION

The exposure time of embryos to the cryoprotectant must be shortened to prevent the toxic effects of vitrification solutions (Shaw et al., 1992; Nakagata. 1989). If exposure is too brief, however, permeation of the cryoprotectant will be inadequate, and intracellular ice may form even in the absence of extracellular ice. Thus, the embryos are required adequate exposure to cryoprotectant to prevent ice formation. In this experiment, the significantly higher survival and developmental competence of bovine embryos vitrified in EG 5.5 were observed in one-step procedure of adding cryoprotectant than that in stepwise procedures (Table 1).

Embryos held in EM grid, OPS and Cryo-loop

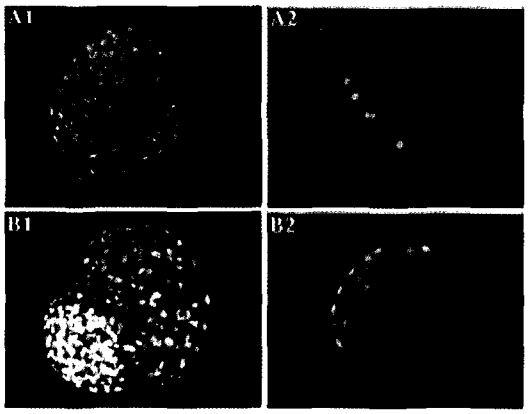

Fig. 2. Morphology of bovine embryo by staining (A1); Hoechst staining of non-vitrified embryo. (A2); Double staining of non-vitrified embryo, (B1); Hoechst staining of vitrified embryo. (B2); Double staining of vitrified embryo. (×200).

with a very small volume of vitrification solution achieve a faster cooling and warming rate (a theoretical rate of about 20.000~180.000℃/min) than those in conventional straws (2.000℃/min) (Rall and Fahy, 1995; Vajta et al., 1998) and they result in greater percentage of blastocyst formation than that of conventional straws (Park et al., 1999). Furthermore, the technique using the EM grid, OPS and Cryo-loop are easier and simpler than that using the conventional straws. On the other hand, one disadvantage of those all rapid cooling methods (EM grids, OPS and Cryo-loops) is the direct contact of the embryo suspension with liquid nitrogen contaminated with virus and other microorganism. Recently, Vajta (1998) reported that contamination could be prevented by cooling OPS straws in filtered liquid nitrogen.

An experimental and practical use of embryos is limited because bovine embryos at pre-morula stage, regardless of their origins are known to be sensitive to cryopreservation in comparison with later-stage embryos. This is caused by several reasons; Above all, however, the inherent quality of oocytes have influence on *in vitro* survival of embryos vitrified as well as *in vitro* maturation, fertilization and development. Thus, in this study embryos evaluated to be Grade 1 according to the International Embryo Transfer Society standards were used. The morphology of the blastocysts selected by such standard and vitrified on EM grid, in OPS and on Cryo-loop were normal and indistinguishable from that of the control (Fig. 1). And also, no significant differences in the rates of re-expanded or hatched embryos were founded among embryo containers (Table 2).

Finally, there were no differences in total cell numbers and the rate of live cell to total cells between blastocysts developed before and after vitrification (Table 3 and Fig. 2).

Taken together, it could be concluded that the vitrification using the EM grid, OPS and Cryo-loop with EG 5.5 offers great potential to 8-cell embryo, morula and blastocyst stage embryos for cryopreservation without cellular damage.

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요 약

다양한 Container로 유리화 동결된 체외생산 소 수정란의 발달에 관한 연구

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본 연구는 체외에서 생산된 소 수정란의 동결을 위한 최적의 조건을 규명할 목적으로 실시하였다. 동결을 위하여 체외에서 생산된 8 세포기, 상실배기 및 배반포기 단계의 수정란을 공시하여 EG 5.5 동결용액에 20초 동안 노출시키고, 각 용기에 장착한 후, 즉시 −196℃ 액체질소에 침지하는 유리화동결법을 채택하였다. 그 후 0.5 M, 0.25 M 및 0.125 M sucrose 용액에서 각 1분간씩, 연속으로 융해한 다음, 10 % FBS가 첨가된 CR I aa 배양액으로 옮겨 배양하였다. 그 결과 수정란의 재팽창률과 완전부화율은 EM grid, OPS 및 Cryo-loop 등과 같은 동결용기에 의해 큰 차이를 보이지 않았다. 또 Hoechst 염색에 의해 조사한 동결융해 후 체외에서 발달된 완전팽창 배반포의 총세포수에 있어서도, 대조군 (180.0 ± 5.4)과 동결군 (178.0 ± 7.5) 사이에 차이가 없었고, 동결융해 후 세포의 손상을 이중염색법으로 조사한 생존세포와 사멸세포의 비율도 대조군 (176 : 4)과 동결군 (172 : 6) 사이에 유의차가 인정되지 않았다. 이러한 결과로 보아 소 수정란은 EG 5.5 동결용액과 EM grid, OPS 또는 Cryo-loop과 같은 동결용기에 의해 성공적으로 동결보존할 수 있는 것으로 판단된다.

(접수일자 : 2001. 10. 8. / 채택일자 : 2001. 11. 15.)