韓國家畜繁殖學會誌 25(2) : 191~198 (2001)
Korean J. Animal Reprod.

Effects of Embryo Sources and Culture Systems on the Membrane Permeability and Viability of Bovine Blastocysts Cryopreserved by GMP Vitrification

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소 수정란의 생산체계가 세포막 투과력 및 GMP Vitrification 동결융해 후 생존성에 미치는 영향

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ABSTRACT

The purpose of this study was to investigate the effects of embryo sources such as *in vivo* vs. *in vitro* produced blastocyst, and culture systems on the membrane permeability and viability of bovine blastocyst following GMP vitrification. To produce *in vivo* embryos, six cows were superovulated by administration of follicle stimulation hormone (FSH) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). *in vitro* embryos were produced by two different culture systems, oviduct co-culture (OCS) and defined culture system (HECM-6; DCS). Ovaries were picked up at a local slaughterhouse and transported to laboratory in 30℃ saline within 2 h. Ovaries were washed with same saline three times and then placed in saline on warm plate adjusted at 30℃ during aspiration. The blastocysts produced were assigned for membrane permeability and viability following GMP vitrification. The membrane permeability of blastocysts was checked in 0.5 M sucrose solution on warm plate at 35℃ for 0, 2, 5 and 7 min, respectively. Then the diameters (width and length) of embryo cytoplasms were measured by a eyepiece meter, and they were converted to their volume by $4/3 \cdot \pi r^3$. The blastocysts were cryopreserved by GMP vitrification method, where they were sequentially placed into vitrification solution before being loaded into GMP vessels and immersed into LN_2 within 20 to 25 sec. Post-thaw blastocysts were serially washed in 0.25 and 0.15 M sucrose in HM and TCM-199 for 5 min each, and then cultured in TCM 199 supplemented with 10% FCS for 24 or 48 h.

The volume change of *in vivo* blastocyst at 0, 2, 5 and 7 min (100, 37.1, 34.3 and 31.6%) was significantly more shrunk than those of *in vitro* blastocysts derived from OCS (100, 59.8, 48.9, 47.9%) and DCS (100, 57.2, 47.3 and 46.9%) ($P<0.05$). The viability of post-thaw blastocyst derived from *in vivo* (93.6%) was also significantly different from those in OCS and DCS (81.9 and 83.6%; $P<0.05$). In the present culture system, the morphology of embryos produced *in vitro* was similar to that of *in*

「This research supported by Sunchon National University Non-directed Research Fund, 2000.」

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vivo embryos, but the quality in membrane permeability and post-thaw viability showed a big difference from their sources as *in vivo* or *in vitro* derived from OCS and DCS.

The results indicated that the quality of *in vivo* embryos in membrane permeability and post-thaw viability was better than those of *in vitro* embryos derived from OCS or DCS.

(Key words: Embryo source, Culture system, Membrane permeability, Viability, Bovine)

Ⅰ. INTRODUCTION

The quality of bovine embryos produced *in vitro* is difficult to assess because they differ from their *in vivo* counterparts in many respects (Greve et al., 1993; Massip et al., 1995; Plante and King, 1994), particularly in their cryobiological characteristics (Leibo and Loskutoff, 1993; Pollard and Leibo, 1994). Moreover, their developmental capacity after freezing is low. Several studies have shown that post-thaw survival is strongly influenced by the age, developmental stage, and quality of embryos developed *in vitro* (Carvalho et al., 1995; Han et al., 1994; Massip et al., 1995). Permeability to cryoprotectants depends on several factors, including the type and concentration of the cryoprotectant, the temperature, and the duration of exposure (Jackowski et al., 1980; Kasai et al., 1981; Mahmoudzadeh et al., 1994). The addition and removal of the cryoprotectants must be progressive to reduce osmotic shocks generated by rapid water movements across the membranes (Kuwayama et al., 1994; Rall, 1987).

The efficiency of vitrifying embryos has been markedly improved by increasing the speed of cooling and warming. Various techniques have been established for this purpose: direct immersion into LN_2 (Landa and Tepla, 1990; Riha et al., 1991), using an electron microscope grid to provide support (Martino et al., 1996), the OPS (Hurtt et al., 1999; Lewis et al., 1999; Vajta et al., 1997, 1998b), cryo-loop method (Lane et al., 1999), and GMP straw (Kong et al., 2000). Vajta et al. (1998a) demonstrated that with the OPS method the cooling and warming rates can be increased (over 20,000℃ /min) and the toxic and osmotic damage can be decreased. Kong et al. (2000) reported that the GMP straw can be decreased the volume of straw loaded by smaller diameter of GMP straw and then increased the cooling and warming speed of straw.

To get high quality of IVP embryos, we have investigated the effects of different culture system and *in vivo* embryos for membrane permeability and viability following GMP vitrification.

Ⅱ. MATERIALS AND METHODS

1. Reagents and Culture Media

Inorganic salts were analytical grade from Mallinckrodt, Paris, KY. Fetal bovine serum (FBS) and packaged media were from HyClone Laboratories, Logan, UT. All other reagents were purchased from Sigma, St. Louis, MO, unless otherwise noted. "Embryo culture tested" grade was used whenever possible.

2. *In Vitro* Embryo Production

Bovine ovaries were obtained from a local abattoir. Cumulus-oocyte complexes were aspirated from antral follicles of 2 to 8 mm diameter with an 18 gauge hypodermic needle, selected for an envelope of compact and complete cumulus cells, and then washed three times in HEPES-buffered Tyrode's medium (Fissore et al., 1992) supplemented with 3 mg/mL BSA, 0.2 mM pyruvate, 100 IU /mL penicillin, 100 (g/mL streptomycin (TL-HEPES). Oocytes were transferred into 500 μL TCM 199

maturation medium containing 10% FBS, 0.5 μg/mL bovine FSH, 5 μg/mL bovine LH, 100 IU/mL penicillin, 100 μg /mL streptomycin (M199) in 4-well culture dishes and cultured at 39℃ in a humidified atmosphere of 5% CO_2 and air for 24 h. Fertilization was initiated 22 h after the onset of maturation and was counted as Day 0. Spermatozoa were prepared for IVF as previously described (Parrish et al., 1988). Cryopreserved semen was thawed in 37℃ water and transferred into 10 mL PBS for washing by centrifugation, and then capacitated with 400 μL of 50 ng/mL heparin solution at 39℃ for 15 min. The capacitated sperm were diluted with TL-FERT medium to approximately $1\sim2\times10^6$ sperms/mL in drops containing the oocytes.

Embryos were cultured in OCS or DCS containing amino acids and 4 mg/mL BSA, 3 mg/mL PVA, 150 μg/mL sodium citrate and/or 500 μg/mL myo-inositol for 72 h after insemination. After 3 days in culture the cleaved embryos were counted. The embryos cleaved over 2-cell were cultured in TCM199 supplemented with 10% FCS on in 50 μL volume 30 mm dish and approximately 30 embryos with a paraffin oil overlay. Embryos that reached the expanded blastocyst stage at Day 7 and 8 after insemination (initiation of insemination on Day 0) were recovered and randomly assigned to either membrane permeability check or GMP vitrification.

3. *In Vivo* Embryo Production

Korean native cows in a Sunchon local farm were superovulated by i.m. administration of 20~28 mg FSH (Ovagen, Immuno-Chemical Products Ltd, New Zealand) given in a series of decreasing doses over a 3 days period beginning Day 9 of the estrous cycles. Estrous was induced by the administration of a single i.m. injection (24 mg) of $PGF_{2\alpha}$ (Lutalyse; Upjohn, Belgium) at intervals of 8 h on the third day after the initial injection of FSH. Donors were inseminated with frozen-thawed semen from a Korean native bull at 12 and 24 h after the detection of standing estrous. Embryos were recovered non-surgically from 6 donors using a balloon catheter on Day 7 after the first detection of estrous and first insemination (Day 0).

4. Volume Check of Embryos

Embryos were exposed in 100 μL of 0.5 M sucrose solution on the warm plate adjusted at 35℃ with inverted microscope to check the diameter of cytoplasm by the ocular micrometer for 0, 2, 5 and 7 min. The volume of cytoplasm was calculated by $4/3 \cdot \pi r^3$ (Takahashi and Kanagawa, 1990).

5. Making of GMP Straw

GMP straws were made with capillary glass pipette (outer/inner diameter: 1.0/0.8 mm; Drummond Sci Co., USA) as described by Kong et al. (2000). Briefly, the capillary glass pipettes were pulled with pipette puller (Narishige, Japan) until the outer diameter of central part decreased from 1.0 mm to approximately 0.3 mm. The GMP vessels were cooled in air then broken at the narrowest point by scribing with a diamond tip. All straws were sterilized by flushing with 70% ethanol and dried in a clean bench.

6. Vitrification Procedure

The vitrification solution (VS) consisted of VS1: 10% ethylene glycol (EG), 10% DMSO in holding medium (HM, TCM199 supplemented with 10% FCS) and VS2: 16.5% EG, 16.5% DMSO in HM (EDS). The blastocysts collected were vitrified using EDS as reported previously (Kong et al., 2000). In brief, the embryos were first incubated in VS1 for 1 min, and then transferred within approximately 1 to 2 μL of VS1 solution into a 20 μL droplet of VS2. Embryos were mixed quickly by pipetting then another drop containing approxi-

mately 1 to 2 μL of VS2 solution and embryos was made using a 10 μL automatic pipette. Loading and cooling were performed as described by Vajta et al. (1998a). The time interval between the contact of the embryos with the concentrated cryoprotectant solution and cooling did not exceed 25 sec. In the GMP vitrification, the capillary reaction was controlled with fingers carefully because the GMP was sensitive to capillary phenomenon. The loaded OPS or GMP straws were laying them down into LN_2 at first horizontally and then immersed in the LN_2 vertically. Warming was performed by immersing the straw end with embryos into 1.0 mL of 0.25 M sucrose in HM prepared in a 4-well dish. After 1 min, the embryos were transferred into 1.0 mL of 0.15 M sucrose in HM for another 5 min, and then washed with HM twice for 5 min each time. The warming temperature of the media was held at approximately 35℃. Survival of cryopreserved embryos was determined by development to re-expanding or hatching blastocysts during in vitro culture for 24 or 48 h, respectively. The embryos were cultured in TCM199 supplemented with 10% FCS in humidified atmosphere of 5% CO_2 at 39℃.

7. Statistical Analysis

Data were analyzed by a General Linear Model technique (SAS, 1990). Statistical significance was established at the $P<0.05$ levels.

Ⅲ. RESULTS

1. Production of *in vivo* or *in vitro* Bovine Blastocysts

To obtain *in vivo* bovine blastocysts, 6 cows were superovulated by i.m. administration of FSF given in a series of decreasing doses and the embryos were collected by non-surgical flushing. As shown in Table 1, total 56 blastocysts were collected from 6 cows (9.3 ± 2.9 blastocysts per cow). The donor cows were selected by ovary and uterus palpation. To obtain *in vitro* bovine blastocysts, two different culture systems were applied in this study, such as OCS or DCS. The cleavage and blastocyst development rates were not different between OCS (78.5; 38.8%) and DCS (80.1; 40.8 %), respectively.

2. Change of Membrane Permeability

To analysis of embryo membrane permeability, the embryo volume was checked during exposing to a 0.5 M sucrose solution on the warm plate of a microscope and checked at 0, 2, 5 and 7 min. The volumes of *in vivo* embryos (100, 37.1, 34.3 and 31.6%) at 0, 2, 5 and 7 min after exposing to a 0.5 M sucrose solution was significantly shrunk more rapidly than those in OCS (100, 59.8, 48.9 and 47.9%) and DCS (100, 57.2, 47.3 and 46.9%) as shown in Table 2 and Fig. 1 ($P<0.05$). Even though the volume change of *in vivo* embryos was decreased to 31.6% at 7 min after the exposure, there was not significantly different between OCS and DCS. The results indicated that the embryo source was important for membrane permeability for *in vivo* or *in vitro* produced embryos, but the culture system such as OCS or DCS was not so important for membrane permeability.

In the permeating cryoprotectant, embryos shrunk by losing water for two reasons: 1) hyperosmolarity of the cryoprotectant solution and 2) the higher permeability of the embryo to water than to protectants. Indeed, the permeability of cell membranes to water is 2,000 to 3,000 times greater than that to most permeating cryoprotectants (Jackowski et al., 1980; Leibo, 1980). The shrinkage was stopped when the efflux of water was balanced by the influx of cryoprotectants. The presence of lipid droplets, for example, is more apparent with *in vitro* embryos, giving them their 'sunburnt' appearance (Tsuzuki et al., 1992; Greve et al., 1993).

Table 1. Production of embryos derived from *in vivo* or *in vitro* culture system

Embryo sources	No. of embryos collected per cow (Mean ± S.E)/total	No. of embryo cleaved/inseminated (%)	No. of blastocysts developed at Day 8 /cleaved (%)
In vivo	9.3 ± 2.94/56		
OCS		165/210 (78.5)	64 (38.8)
DCS		174/217 (80.1)	71 (40.8)

* Six cows were superovulated by FSH treatment.

Table 2. Volume change of bovine blastocyst derived from *in vivo*, OCS and DCS

Embryo source	No. of embryos examined	Volume change of blastocyst according to exposed time Mean volume/Volume ratio (%)			
		0	2	5	7
In vivo	5	2,143/100	795/37.1	735/34.3	677/31.6
OCS	10	2,690/100	1,608/59.8	1,318/48.9	1,291/47.9
DCS	11	2,709/100	1,549/57.2	1,281/47.3	1,270/46.9

* Mean volume is calculated $4/3 \cdot \pi r^3$ following checking the width x length of cytoplasm

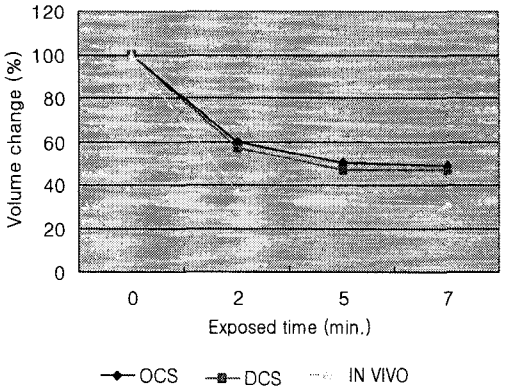

Fig. 1. Volume change of blastocyst in 0.5 M sucrose solution on 35°C warm plate for 0, 2, 5 and 7 min. Number of blastocyst for volume checking in OCS, DCS and *in vivo* used 10, 11 and 5 embryos, respectively.

Niemann (1991) noted information suggesting that the high lipid content of the pig embryo might lead to uneven intracellular ice formation and that it was the cause of the sensitivity to low temperatures; it might be that similar events occurred with some of the IVMFC embryos. There were other differences between IVMFC embryos produced by some other methods and *in vivo* embryos. It took the enzyme pronase twice as long to digest the ZP of the *in vivo* embryos than those of the *in vitro* embryos (Pollard and Leibo, 1993). Such differences presumably are likely to influence the permeability of the zona to water and cryoprotectants.

3. Viability of Post-thaw Blastocysts

The blastocysts produced *in vivo*, OCS or DCS culture system were cryopreserved by GMP vitrification to analyze of viability of post-thaw blastocysts. Even the recovery rate was also high in all of groups (100, 98.3 and 96.4%), the viability rate of *in vivo* blastocysts (93.6%) was significantly higher than those of OCS or DCS (81.9 and 83.6%) as shown in Table 3 ($P<0.05$). The results indicated that embryo source derived from *in vivo* or *in vitro* contributed to post-thaw viability, but culture system

Table 3. Effect of embryo sources on the viability of post-thaw blastocysts following GMP vitrification

Embryo source	No. of blastocyst frozenl	No. of blastocyst recovered (%)	No. of blastocyst survived (%)
In vivo	31	31(100)	29(93.6) [a]
OCC	62	61(98.3)	50(81.9) [b]
DCS	57	55(96.4)	46(83.6) [b]

* OCC and DCS denoted oviduct co-culture and defined culture system.
* Values with different superscript were significantly different (P<0.05).

just as OCS or DCS did not affect on viability of bovine blastocysts following GMP vitrification method. As shown in Fig. 1 and Table 3, the membrane permeability and viability of post-thaw blastocyst were variable between *in vivo* and in vitro embryos, but not variable between OCS and DCS.

It were generally accepted that *in vitro* produced cattle embryos were much more sensitive to freezing than those produced *in vivo*. Pollard and Leibo (1993) brought the attention to marked differences between *in vitro* and *in vivo* produced cattle embryos in their sensitivity to low temperatures. Similar differences were reported in the freezing of *in vitro* and *in vivo* sheep embryos (Tervit et al., 1994). Leibo and Loskutoff (1993) also found much evidence of adverse effects on *in vitro* embryos, when using those produced in the bovine oviductal epithelial cell co-culture system with B2 medium.

The GMP vitrification method was established to increase the speed of freezing and warming, heat conductivity, post-thaw survival rates, and then decrease the loading volume and embryo damage by reducing of straw size and loading column (Kong et al., 2000). We reported that GMP method attained almost 100% post-thaw survival rates in mouse blastocyst was a potentially valuable technique applicable to other species (Kong et al., 2000).

The results indicated that the embryo source was important in membrane permeability and viability of post-thaw bovine blastocysts, regardless of the embryos derived from OCS and DCS.

Ⅳ. 요 약

본 연구는 체내, 체외수정란 및 배양체계가 세포막투과력 및 GMP vitrification 후 생존성에 미치는 영향을 조사하고자 실시하였다. 체내수정란은 6마리 한우를 FSH와 $PGF_{2\alpha}$에 의한 과배란처리하여 생산하였다. 체외수정란은 난관상피세포 공배양 (OCS) 및 HECM-6 (DCS) 방법으로 생산하였다. 생산된 배반포기 배는 세포력투과력과 GMP vitrification 후 생존성의 조사를 위하여 사용되었다. 세포력투과력은 35℃ 가온판과 0.5 M sucrose 용액에서 0, 2, 5 및 7분간의 노출시간에 세포질의 "가로 × 세로"의 직경을 조사하였다. 세포질의 용적은 조사한 직경을 $4/3 \cdot \pi r^3$ 공식으로 계산하였다. 배반포의 동결보존은 GMP vitrification 방법으로 실시하였으며, 융해 후 0.25와 0.15 M sucrose 용액 및 TCM199에 각각 5분간 세척한 후 TCM 199에 24 또는 48시간동안 배양하였다. 체내수정란의 0, 2, 5 및 7분 때의 용적변화(100, 37.1, 34.3 및 31.6%)는 OCS(100, 59.8, 48.9 및 47.9%)와 DCS(100, 57.2, 47.3 및 46.9%) 보다 유의적으로 높게 수축되었다(P<0.05). 또한 체내수정란(93.6 %)의 동결융해 후 생존성은 OCS 및 DCS (81.9 및 83.6%) 보다 유의적으로 높았다(P<0.05). 현 배양체계에서 체외수정란의 형태는 체내수정란과 유사하였지만, 세포막투과력 및 융해 후 생존성 등의 질적인 면에서는 큰 차이를 보였다. 결론적으로 세

포력 투과력 및 동결융해 후 생존성 등의 질적인 면에서 체내수정란은 OCS 또는 DCS 배양체계에서 생산된 체외수정란보다 우수하였다.

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(접수일자 : 2001. 3. 19. / 채택일자 : 2001. 4. 8.)