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Production of Korean Native Cow from Mongolian Cow following Transfer of Vitrified Blastocyst

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Mongolian 수란우에 한우 동결수정란의 이식 후 산자 생산

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SUMMARY

The purpose of this study was to investigate the comparison of viability of bovine blastocysts following glass micropipette (GMP) vitrification and the possibility of production of Korean Native Cow ("Hanwoo," *Bos taurus coreanae*) following embryo transfer into Mongolia cows (*Bos taurus mongolian*). The embryos of Korean Native Cow were produced by IVMFC or superovulation methods in Korea, cryopreserved by GMP vitrification, and subsequently transported to Mongolia. The recipient cows were synchronized using a CIDR plus and prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$) treatment. To produce *in vivo* embryos, seven cows were superovulated using FSH and $PGF_{2\alpha}$ treatment. A total of 64 blastocysts (9.1±2.94 per cow) were collected. *In vitro* embryos were produced using a defined culture system which cleaved in 80.1% ova (174/217), and developed to blastocyst stage embryos of 40.8% (71/174). The post-thaw survival rate of *in vivo* blastocysts (93.7%; 45/48) was significantly higher than that of *in vitro* blastocysts (82.5%; 52/63, $P<0.05$). Embryo transfer was carried out using 8 Mongolian recipient cows and 2 post-thaw blastocysts per recipient. Five of 8 recipients were found pregnant at Day 60 but one abortion occurred by Day 240. Two offspring were produced from the Mongolian cows at 275 days after embryo transfer. These results indicated that a GMP vitrification method could be used as a cryopreservation technique for *in vivo* or *in vitro* bovine blastocysts and produced effectively a Korean Native Cow following embryo transfer into a Mongolian recipient cow.

(Key words : GMP vitrification; bovine; embryo transfer; Korean native cow)

INTRODUCTION

The development of a vitrification method for the cryopreservation of embryos was a major cryobiologic advance (Rall and Fahy, 1985) and the technique has application for *in vitro* produced (IVP) bovine embryos (Agca et al., 1994). Vitrification of embryos is a useful cryopreservation method because it does not require freezing equipment. The first successful report on the vitrification of mouse 8-cell embryos was done by Rall and

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Fahy (1985). In cattle, Massip et al. (1986) obtained the first calves from vitrified late morula and very early blastocysts. When subjected to cryopreservation, *in vitro* produced bovine embryos were more sensitive to injury resulting from direct toxic and osmotic effects as well as from ice crystal formation than *in vivo* derived counterparts. The background of this difference is only partially known. But structural changes as mean numbers of cells in the inner cell mass become lower (Machaty et al., 1998), more intracellular lipid droplets form and permeability of the zona increases (Polard and Leibo, 1994; Leibo et al., 1996; Massip et al., 1995a); these features are probably only some of the things reflecting inappropriate *in vitro* culture conditions. Vitrification, or cryopreservation of tissues and cells without ice crystal formation, seems promising to overcome the problem for embryos (Massip et al., 1995b), because the possibility of chilling injuries is lower due to the high cooling rates. Mahmoudzadeh et al. (1995) and Vajta et al. (1997) described an increase in survival rates following vitrification of bovine *in vitro* produced embryos compared to the traditional slow-rate freezing methods and recently Vajta et al. (1997) reported that a new vitrification technology, the open pulled straw (OPS) method, would allow satisfactory survival rates of all developmental stages of bovine embryo. The advantages of this method are 1) the thickness of the straw wall, which decreases the thermo-insulation effect; 2) the small inner diameter of the straw, which reduces the volume of the liquid column; 3) direct contact between the liquid nitrogen and embryo containing solution, which increases the speed of cooling; and 4) warming may be performed with direct contact between cryopotectant and diluent solution, which allows immediate rehydration and short contact with concentrated cryoprotectant additives, less than 30 sec over −180℃ (Vajta et al., 1996).

The importance of morphology and biochemistry of IVP bovine embryos with respect to cryopreservation has been intensively discussed (Massip et al., 1995a). Since transfer of poor quality cryopreserved-thawed embryos often contributes to inefficiency in bovine embryo transfer technology, post-thaw gross morphologic evaluation of vitrified IVP embryos with respect to their *in vivo* developmental competence is important. IVP embryos were reported to be much more sensitive to freezing and thawing injury than *in vivo* produced embryos (Leibo and Loskutoff, 1993).

This study was to compare the post-thaw viability of *in vivo* or *in vitro* blastocysts cryopreserved by GMP vitrification, and the possibility of production of Korean native cattle following embryo transfer into Mongolian cows.

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

Ovaries of Korean native cattle were obtained from a local abattoir. Cumulus-oocyte complexes were aspirated from 2 to 8 mm diameter antral follicles using an 18 G hypodermic needle. Oocytes enveloped with compact and complete cumulus cells were selected and 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 μg/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 (TCM 199) 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 23 h after onset of maturation and was designated Day 0. Spermatozoa were prepared for IVF as described by Parrish et al. (1988) with frozen semen thawed at 37℃ before transfer to 10 ml PBS for washing by centrifugation, and then capacitation with 400 μl of 10 ug/ml heparin solution for 15 min at 39 ℃. The capacitated sperm were diluted with TL-FERT medium to approximately 1 to 2×10^6 sperm/ml in drops containing the oocytes. Embryos were cultured in HECM-6 (McKiernan et al., 1995) 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. Embryos that had cleaved beyond the 2-cell stage were cultured in TCM 199 supplemented with 10% FCS in 50 μl droplets with approximately 30 embryos per droplet in a 30 mm dish with paraffin oil overlay. Embryos that reached the expanded blastocyst stage at Day 8 after insemination were recovered and used for vitrification.

3. *In Vivo* Embryo Production

Korean native cows (n=7) in Sunchon local farms were superovulated by i.m. administration of 20 to 28 mg FSH (Ovagen, Immuno-Chemical Products Ltd, New Zealand) given in a series of decreasing doses over a 3 day period beginning between Day 9 and 13 after initiation of the estrous cycle. Oestrous was induced by the administration of one 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 oestrous and embryos were recovered non-surgically from the 6 donors on Day 7 after the first detection of estrous and first insemination (Day 0).

4. Making of GMP Straw

The GMP vessels were constructed from a capillary glass pipette (outer/inner diameter (o.d./i.d.): 1.0/0.8 mm; Drummond Sci Co., USA) by the method of Vajta et al. (1998) with some minor modifications. The capillary glass pipettes were pulled with a pipette puller (Narishige, Japan) until the o.d. of central part decreased from 1.0 mm to approximately 0.3 mm. The GMP vessels were cooled in air and broken at the narrowest point after scribing with a diamond tip pen. The weights of OPS and GMP straw were approximately 0.070 vs 0.098 g, respectively, and the volume of the 10 mm long narrow column was 2.68 vs 0.14 mm^3 per straw, respectively. All straws were sterilized by flushing with 70% ethanol and air dried.

5. Vitrification Procedure

The vitrification solution consisted of vitrification solution 1 (VS1) {10% ethylene glycol (EG), 10% dimethyl sulfoxide (DMSO) in holding medium (D-PBS supplemented with 5% FCS: HM)} and vitrification solution 2 (VS2) {16.5% ethylene glycol (EG), 16.5% DMSO in HM; EDS}. The blastocysts collected were vitrified using EDS as reported previously (Vajta et al., 1998). In brief, the embryos were first incubated in VS1 for 1 min, and then transferred in approximately 1 to 2 μl VS1 solution into a 20 μl droplet of VS2. Embryos were mixed quickly by pipetting and another drops containing approximately 1 to 2 μl VS2 solution using a 10 μl automatic pipette. Loading and cooling were performed as described by Vajta et al. (1998). The time between the contact of the embryos with the concentrated cryoprotectant solution and cooling time did not exceed 25 sec. In GMP vitrification, the capillary reaction has to be controlled carefully because the GMP is very sensi-

tive to capillary action. The loaded OPS or GMP straws were placed into LN_2 first almost horizontal and then immersed them in the LN_2 vertically. Warming was performed by immersing the end of the straw containing the embryos in 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 temperature of the media used for warming was 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℃.

6. Assessment of Embryo Viability

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 TCM-199 supplemented with 10% FCS in a humidified atmosphere of 5% CO_2 at 39℃.

7. Embryo Transfer and Pregnancy Diagnosis

Prior to transfer, the embryos cryopreserved by GMP vitrification were thawed at the Mongolian farm, Mongolia as described by Vajta et al. (1998). Post-thaw blastocysts were loaded in the middle part of a 0.25 ml straw in TCM-199 + 10% FCS. The straw was mounted in a transfer gun (IMV, l'Aigle, France). Two post-thaw embryos were transferred immediately after thawing and loading into a straw to the uterine horn ipsilateral to the corpus luteum of Mongolian recipient cows (n=8). Synchronization of recipient cows had previously been performed using a CIDR plus and $PGF_{2\alpha}$ treatment. Pregnancy diagnosis was by rectal palpation on Days 60 and 240.

8. Statistics

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

RESULTS

Production of embryos derived from *in vivo* or *in vitro* culture systems are shown in Table 1. A total of 64 blastocysts were collected from seven cows (9.1 ± 2.94 per cow). HECM-6 was used to produce *in vitro* embryos and so cleaved embryos (80.1%; 174/217) and developed to blastocyst per cleaved embryos (40.8%; 71/174).

The survival rates of post-thaw blastocyst are shown in Table 2. The post-thaw survival rates of *in vivo* blastocysts (93.7%; 45/48) were significantly higher than that of *in vitro* blastocyst (82.5; 52/63, $P<0.05$), although the post-thaw recovery rate was not significantly different between *in vivo* and *in vitro* (100; 48/48 vs 94.0; 63/67) ($P<0.05$).

Two post-thawed blastocysts were transferred into Mongolian recipient cows, which were synchronized by $PGF_{2\alpha}$ treatment at CL stage. Two offspring were produced from Mongolian cows

Table 1. Production of *in vivo* and *in vitro* bovine embryos

Embryo source	No. of embryos collected per cows (Mean±S.E)	No. of embryo cleaved/inseminated (%)	No. (%) of blastocysts developed at Day 8/cleaved
In-vivo	9.1±2.94		
In-vitro		174/217 (80.1)	71 (40.8)

* Seven cows were superovulated by FSH treatment.

Table 2. Effect of embryo source on the viability of post-thaw blastocysts following GMP vitrification

Embryo source	No. of blastocysts frozen	No. (%) of blastocysts recovered	No. (%) of blastocysts survived
In vivo	48	48 (100)	45 (93.7)[a]
In vitro	67	63 (94.0)	52 (82.5)[b]

* Values with different superscript were significantly different ($P<0.05$).

Table 3. Production of Korean native cows following embryo transfer into Mongolia recipients

Items	Results
No. of recipients transferred	8
No. of recipients pregnancy at Day 60	5
No. of recipients pregnancy at Day 240	3
No. (%) of recipients delivered/recipient	2/8 (25.0)
No. of offspring	2

* Two embryos were transferred per recipient.

about 275 days after embryo transfer as shown in Table 3. The recipient cows were detected pregnancy diagnosis at Day 60 by rectal palpation and 5 out of 8 recipients were pregnant at this time. The pregnancy diagnosis was conducted one more time at Day 240 by rectal palpation and 3 out of 8 recipients were still on going, but two recipients died due to cold weather. Two recipients delivered offspring approximately 285 days after embryo transfer. Two offspring produced were still alive in Mongolia actual place.

DISCUSSION

This study was conducted to investigate the production of Korean native cow following embryo transfer into a Mongolian recipient cow in Mongolia. To produce Korean native cow from Mongolian recipient cow, Korean native cow embryos were produced either by *in vivo* or *in vitro* production system, and cryopreserved by GMP vitrification method and then transported them from Korea to Mongolia.

The GMP vitrification method has been 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). Kong et al. (2000) reported that the GMP method attained almost 100% post-thaw survival rates in mouse blastocyst and is a potentially valuable technique applicable to other species.

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

In the permeating cryoprotectant, embryos shrank 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 stopped when the efflux of water was balanced by the influx of cryoprotectants. The presence of lipid droplets, for example, is much more in evidence with *in vitro* embryos, giving them their 'sunburnt' appearance (Tsuzuki et al., 1992; Greve et al., 1993). Niemann (1991) notes information suggesting that the high lipid content of the pig embryo may lead to uneven intracellular ice formation and that this is the cause of the sensitivity to low temperatures; it may be that similar events occur with some of the IVMFC embryos that are produced. There can be other differences between IVMFC embryos produced by some methods and *in vivo* embryos. It may take the enzyme pronase twice as long to digest the ZP of the *in vivo* embryo than that of the *in vitro* embryo (Pollard and Leibo, 1993). Such differences presumably are likely to influence the permeability of the zona to water and cryoprotectants. Difference of membrane permeability derived from *in vivo* or *in vitro* could be much affecting in viability of post-thaw bovine blastocyst.

Two offspring of Korean native cows were produced after transferring vitrified *in vivo* or *in vitro* bovine blastocyst into Mongolian recipient cows in Mongolia. These results confirm that vitrified embryos can be succeeded or reported by Agca et al. (1998) and Vajta et al. (1998). The latter author reported that five of 14 recipient animals did not return to the subsequent estrous cycle, three of them were found pregnant at 28 and 92 days after transfer, three bull calves were born.

In conclusion, an *in vivo* or *in vitro* blastocysts vitrified by the GMP method were capable of establishing pregnancies and production of offspring. The GMP method is simple, rapid and inexpensive, and the handling and storage of vitrified embryos can be based on the existing tools and methods of cryopreservation.

요 약

본 연구는 체내, 체외 소 배반포기 배의 GMP vitrification 후 활력도의 비교와 한우 수정란을 몽골 소에 수정란이식 후 산자생산 가능성을 조사하고자 실시하였다. 한우 수정란은 체외수정란 또는 과배란처리에 의한 체내수정란을 생산하여 GMP vitrification 방법으로 동결 후 몽고로 수송하였다. 수란우는 CIDR과 $PGF_{2\alpha}$ 처리에 의하여 동기화를 유도하였다. 체내수정란생산을 위하여 7두를 과배란처리하였다. 총 64개의 배반포기를 회수하였다 (9.1±2.94 per cow). 체외수정란생산은 80.1% 분할율(174/217)과 40.8% 배반포기 발달율(71/174)을 얻었다. 체내수정란(93.7%; 45/48)의 동결융해 후 생존율은 체외수정란(82.5%; 52/63)보다 유의적으로 높았다($P<0.05$). 8두의 몽골 소에 2개의 수정란을 이식하여 5두가 이식 후 60일째 임신이 확인되었으나, 그 중 1두는 240째 유산을 확인하였다. 그 중 2두의 수란우에서 2두의 산자를 275일째 생산에 성공하였다. 이러한 결과는 GMP vitrification 방법은 체내, 체외수정란의 동결보존방법으로 이용될 수 있을 뿐만 아니라 동결융해란의 몽골 소에 이식 후 한우를 생산할 수 가능성을 확인하였다.

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