Post-Thaw Cryosurvival of Bovine Embryos Produced *In Vitro* and *In Vivo* after Controlled Freezing

Sang-Rae Cho, Sun-Ho Choi, ChangYong Choe, PoongYeon Lee, Jun-Kyu Son, Jae-Bum Kim, Sung-Jae Kim, Hyun-Jong Kim, Seung-Oh Shin and Dong-Soo Son*

Animal Genetic Resources Station, National Institute of Animal Science, RDA, Namwon 590-832, Korea

ABSTRACT

To enhance the embryo preservation technology and better application of embryo transfer technique to the field (dairy science or animal reproduction. etc.), we examined the viabilities of bovine embryos produced *in vitro* and *in vivo* after cryopreservation according to their developmental stage and thawing temperature. Bovine embryos from *in vivo*/*vitro* fertilization (Hanwoo) were examined at day 7, 8, and 9. Survival rates and total cell numbers of *in vivo* fertilized embryos were as follows: morulae 68.8% and 67 ± 6.0; blastocysts 80.5% and 120 ± 10; expanded blastocysts 77.4% and 138 ± 9.7, respectively. Rates of embryo development for blastocysts and expanded blastocysts after thawing were significantly higher than that of morula stage embryos ($p<0.05$). While survival rates of *in vitro* fertilized embryos according to developmental stage showed no significant difference among groups (morula 67.9%; blastocyst 74.3%; and expanded blastocyst 79.4%), total cell numbers were significantly lower than those of other groups (morula 64 ± 5.9; blastocyst 116 ± 8.7; and expanded blastocyst 135 ± 9.1) For the viability according to thawing temperature, survival rate was higher in 37℃.

(Key words : bovine, embryo, cryopreservation, *in vivo*, *in vitro*, thawing)

INTRODUCTION

Bovine embryos derived from *in vitro* produced (IVP) and *in vivo* have been used for animal production and other biotechnological purpose (Rall, 1992). In view of the fact that the import and export of cattle embryos must be based on frozen rather then fresh ones, the international trade in embryos is very much dependent on effective freeze-thaw procedures. Cryopreservation of bovine embryos has been widely used commercially (Hasler, 2003; Stringfellow and seidel 1998), since the frist successful cryopreservation of mammalian embryos (Whitingham *et al*., 1972). Recently, advances in reproductive technologies, such as *in vitro* and *in vivo* embryos production, have stimulated research for efficient cryopreservation techniques for embryo transfer. Determination in the field of cryobiology are still dependent primarily on visual or morphological examination and on pregnancy rates after the transfer of cryopreserved embryos. However, the selection of available embryos for cryopreservation is still dependent on tolerate freeze-thawing or appropriate freezing agents. Conventional Slow freezing of IVP bovine embryos reduced post-thaw survival rates compared with their *in vivo* counterparts, mostly due to their susceptibility to ice crystal formation (Kasai *et al.*, 2002; Ishimori *et al.*, 1992). Utilization of IVP bovine embryos is currently also hampered by several substantial concerns. Pollard and Leibo (1993) raised doubts as to whether IVP bovine embryos can survive freezing as well as *in vivo* derived embryos. The thawing procedure is known to be important in determining embryo survival (Fashing and Garcia, 1992; Miyamoto and Ishibashi, 1983). To achieve optimal success after cryopreservation research into the physical and chemical changes that an embryo must endure if it to survive and develop normally are needed (Leibo, 1989; King *et al*., 1985). Among cryoprotectants, ethylene glycol (EG) is one of the most widely used agents for conventional freezing or vitrification (Kojima *et al*., 1984; Voelkel *et al*., 1992). However, results are always inconsistent. To enhance the embryo preservation technology and better application of embryo transfer technique to the field. In the present study, we examined survival rates after cryopreservation of bovine *in vitro*/*in vivo* fertilized embryo according to developmental stage and thawing temperature in this study.

MATERIALS AND METHODS

All Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

* Correspondence : E-mail : sonds@korea.kr

1. *In Vitro* Maturation

Bovine ovaries were collected at a local abbatoir and transported to the laboratory (within 3 h) in sterile 0.9% NaCl solution at 28℃. Cumulus-oocyte complexes(COCs) were recovered by aspiration of 2～8 mm follicles using 18-gauge needle attached to a 10 ml syringe. The COCs were washed three times and then cultured in TCM-199 supplemented with 50 μg/ml gentamycin, 5.5 mM calcium lactate, 2.3 mM Na pyruvate, 36 mM $NaHCO_3$, 5 mM Hepes and 10% Fetal Calf Serum (FBS). Maturation was performed in four-well plates (Nunc® Denmark) in groups of 50 COCs in 500 μl of a maturation media for 22～24 h at 38.5℃ under 5% CO_2, 5% O_2 and 90% N_2 in air with maximum humidity.

2. *In Vitro* Fertilization

Frozen semen from a single bull were thawed in water bath (35～37℃), washed with Brackett and Oliphant's medium (BO medium; 1) supplemented with 5 mM-Caffeine (Caff-BO) by centrifugation at 1,500 g for 5 min. The sperm pellet was resuspended in Caff-BO supplemented with 1% bovine albumin serum (BSA, Sigma) and 20 μg/ml heparine for a sperm concentration of 2×10^5 /ml. Oocytes matured *in vitro* were transferred sperm into the BO medium (15 to 20 oocytes) for insemination. After incubation for 6 h oocytes were washed 2 to 3 times and transferred to the culture medium (CR1aa) supplement 0.5% BSA and 50 μg/ml gentamycin for development using the cumulus cell co-culture system.

3. *In Vitro* Culture

After 6 h in IVF, oocytes were removed cumulus cells and sperm attached by vortexing and transferred each 15 oocytes into 50 μl of drop of CR1aa medium for 3 day and additionally cultured in CR1aa medium containing 30 mg BSA for 5 days at 38.5℃ under 5% CO_2, 5% O_2 and 90% N_2 in air with maximum humidity. Development of the embryos was observed every 24 h under a inverted microscope at × 100 magnification.

4. Production of *In Vivo* Embryo

Korean native cows (Hanwoo: *Bos taurus coreanae*), at random stages of the estrous cycle, received a CIDR containing 1.9 g progesterone (EAZI-BREED™ CIDR®; InterAg, Hamilton, New Zealand), 1 mg estradiol benzoate (SY Esrone; Samyang, Seoul, Korea) and 50 mg progesterone (SY Ovaron; Samyang). Gonadotropin treatment began 4 day after CIDR implantation, that were superovulated with 28 mg of porcine follicle stimulating hormone (FSH) (Antrin-R10®; Kawasaki Mitaka Pharmaceutical, Tokyo, Japan) in twice daily im injections, with a gradual decrease over 4days (5,5,4,4,3,3,2 and 2 mg),. Along with the 5th and 6th injections of FSH, 25 mg and 15 mg $PGF_2\alpha$ were administered, respectively. The cows received 200 μg gonadorelin (GnRH) (Fertagyl®; Intervet, Boxmeer, Netherlands) 12 h after the final (8th) injection of FSH. The cows were artificially inseminated 24 and 36 h after the final injection of FSH using commercial semen from a Korean native bull. The embryos were recovered 7 days after the frist insemination by embryo collection medium (Agtech, U.S.A) (Son *et al*., 2007).

5. Freezing and Thawing of Embryos

Selected embryos for morphological normality and pipette them either a directly into 1.8 M EG solution for 10～15 min at room temperature. Groups of 1～2 embryos were frozen in 0.25 ml plastic straws (IMV International, Minneapolis, MN) containing a cryoprotectant solution composed of 1.8 M EG in Dulbecco's PBS (Gibco BRL)＋4 g BSA. After 10 min at room temperature, place the straws into a freezing machine set at −7℃. After 3 min, seed each straw by grasping the straw with a forceps dipped in liquid nitrogen at the air bubble; make sure that the seed forms by checking for ice about a minute later. After 10 min, start the freezing machine with a cooling rate of 0.5℃/min until straws reach −35℃. No later than 3 min after straws reach −35℃, plunge straws into liquid nitrogen. After cryopreservation, thaw the straws in air for 10 seconds, and then place in a 35 and 37℃ water bath until all ice melted (about 20 sec). and then cultured in CR1aa medium containing 0.5% (v/v) BSA (Bovine Serum Albumin).

6. Post Thaw Embryos Evaluation

Following thawing and rehydration, all embryos were placed in CR1aa culture medium. Embryos were evaluated at 12, 24 and 48 h post- thawing. Embryos that survived were recorded as either blastocysts that had re-expanded or hatching at 24 or had hatched at 48 h. To determine cell number, after thawing, half of the embryos in each treatment were removed on Day 7 and 8 (Morulae, blastocysts), and the remainder were left to continue their development until Day 9 (hatching and hatched blastocysts). morulae, blastocysts and expanded blastocysts were mounted on slide, stained with Hoechst 33342 (10 μg/ml) and covered with a cover slip. The total number of cells in blastocysts was determined by counting the number of nuclei under epifluorescent microscopy.

7. Statistical Analysis

Difference were analyzed among treatments was evaluated using the General Linear Modeal (GLM) procedure in the Statistical Analysis System (SAS) pakage (version 6,12). A probability of $p<0.05$ was to be statistically significant.

RESULT AND DISCUSSION

1. Thawing of *In Vivo* Fertilized Embryo

The objective of the present study was to identify a practical and useful freezing and rehydration method for successful embryo development and hatching under field conditions. Table 1 shows outcomes of conventional freezing (slow freezing) method according to developmental stage of *in vivo* fertilized embryo. For Table 1, a total of 99 embryos were used (n=7). At morula stage (a total of 32 frozen embryo), survival rate after thawing was 68.8% (22/32). Survival rates for blastocysts and expanded blastocysts were 80.5% (29/36) and 77.4% (24/31) respectively. Rates of embryo development for blastocysts and expanded blastocysts after thawing were significantly higher than that of morula stage embryos. However, no difference was noted between blastocysts and expanded blastocysts after thawing. While similar result was noted for total cell number and survival rates, for total cell number of expanded blastocysts (1388 ± 9.7) was higher than that of blastocyst (120 ± 10). Nagashima *et al*. (1999) compared survival rates of porcine embryo according to developmental stage to address the low survival rate due to the accumulation cytoplasmic lipid. When cytoplasmic lipid of morula embryo was removed and processed with freezing and thawing, blastocyst rate was 92.6% (25/27) and 63% (17/27) of surviving embryo developed to hatching. This result suggests that accumulation of cytoplasmic lipid during production of IVF embryo highly affects survival rate of the embryo after thawing. In general, for IVF production and cryopreservation, serum is not added to culture medium. The beneficial effects of FBS on advanced embryo development may be due to not only phophate but also other factor such as growth factors in cultured in FBS (Yonis *et al*., 1989). When cultured in media with FBS, significantly higher proportion of bovine (Pinyopummintr and Bavister, 1991), mouse (Miyamoto and Ishibashi, 1983), pig (Dobinsky *et al*., 1996) and rat morulae developed with morulae cultured in FBS-free media. However, The precise factors in FBS responsible for the biphasic effect on the development of embryos is not known. it is suggested that one of the main factors may be phophate, because FBS contains comparatively higher concentration of inorganic phosphorous (Lindle and Bauer, 1989).

Table 1. Comparison of survival rates and total cell number following to conventional freezing *in vivo* embryos

Embryo stage	Used-embryos	Evaluation to embryos (%)	
		Survival (re-expansion)	Total cell number (Mean ± SEM)
Morula	32	22 (68.8)[a]	67 ± 6.0[a]
Blastocyst (BL)	36	29 (80.5)[b]	120 ± 10.0[b]
Expansion BL	31	24 (77.4)[b]	138 ± 9.7[b]

[a,b] Percentage with different superscripts within columns indicate significant different ($p<0.05$). repetition: 7 (n = 7).

2. Thawing of *In Vitro* Fertilized Embryo

Table 2 shows result of slow freezing method according to developmental stage of *in vitro* fertilized embryo. For Table 2, a total of 189 embryos were used (n=8). At morula stage (a total of 56 frozen embryo), survival rate after thawing was 67.9% (38/56) which is similar to *in vivo* fertilzed embryo. Similar result was noted for total cell number and survival rates, for total cell number of morula being lower than that of blastocysts. For IVF embryos, survival rates for blastocysts and expanded blastocysts were 74.3% and 79.4% respectively. For total cell number, blastocyst was 117 and expanded blastocyst was 136. All together, the result was similar to that of *in vivo* fertilized embryo. While the cause for this phenomenon is not clear, embryo culture condition might be a factor. Dehydration and rehydration of embryo due to the different culture condition might affect calcium channel and cytoplasmic ice crystal formation. Viability of embryo after thawing is largely

Table 2. Comparison of survival rates and total cell number following to conventional freezing *in vitro* embryos

Embryo stage	Used-embryos	Evaluation to semen (%)	
		Survival (re-expansion)	Total cell number (Mean ± SEM)
Morula	56	38 (67.9)	64 ± 5.9[a]
Blastocyst(BL)	70	52 (74.3)	116 ± 8.7[b]
Expansion BL	63	50 (79.4)	135 ± 9.1[b]

Repetition: 8 (n = 8).

affected according to the developmental stage. Although we can not estimate pregnancy rate after embryo transfer using thawed morula embryo, pregnancy rate for fresh morulae embryo was higher than that of later stage embryos (data is not shown). The survival rates observed with embryo stage in Experiment 1 and 2. IVP morulae have resulted in poor survival rates in other studies (Hasler, 2003; Holm and Callesen, 1998). The development competence and visual selection of *in vitro* produced morulae may be important. in this study, only about 68% of selected morulae develop into blastocysts. This shows that morphological selection of normal morulae is extremely difficult. Along with increased degeneration due to poor selection methods, morulae appear to be more sensitive to cryoprotectant exposure, possibly needing shorter exposure time. Cell death may possibly be attributed to exposure of morulae to toxic levels of cryoprotectant before cryopreservation. Cryotolerance of *in vitro*-produced embryos is generally lower than those obtained *in vivo*, and it can be affected by the embryo culture system. Saha *et al*. (1994) compared survival rates of IVF embryos according to stage of embryo and reported the most appropriate stage as Day 7 blastocysts (84%) that is higher than Day 8 and 9 blastocysts (71% and 46%). Average of total cell number for Day 7 embryo was 124, while that of Day 8 and 9 were 119 and 109 respectively. The reason for low viability and total cell number of Day 9 is possibly because younger cells are more stable under osmotic stress and recover easily than older cells.

3. Viability of Embryos for Different Thawing Temperatures

Results for experiment 3 are presented in Table 3. There was no significant difference among embryo development stages in re-expansion (12～24 h). As shown Table 3, Embryos exposed for 20 sec in air and then warmed at 35℃ and 37℃ water bath maintaining 15℃ angle for the straw. Although there was no significant difference between two treatments, 37℃ treatment showed relatively higher survival rate. Longer exposure times were favorable for blastocysts and detrimental to morulae, which may correspond with the larger amount of water content in the blastocoele cavity. Furthermore, *in vitro*-produced morulae survival rate was lower compared to blastocysts (Campos-Chillon, *et al*., 2006). In this experiment results, temperature is a very important factor which might increase the cryoprotectant permeability through the embryo membrane to a toxic level, ultimately causing damage to embryonic cells. Saha *et al*. (1996) compared viability of vitrified IVF embryo for thawing temperatures (5℃ and 20℃) and reported significantly higher developmental rate for embryo kept at 5℃. Survival rate of 100% for embryo kept at 5℃ was reported while that of 20% for embryo kept at 20℃. The authors suspected that increased temperature might increased the cryoprotectant permeability through the embryo membrane to a toxic level, eventually damaging the embryos. Campos-Chillon *et al*. (2006) compared thawing temperature between 20℃ and 37℃ for 5 minutes exposure and reported survival rate as 84% and 96% respectively. These results show similarity with present study. The mechanism of this benefit was reported to be unknown. Holding the embryos after in-straw dilution at the higher temperature likely speed up removal of cryoprotectant from cell; a less likely possibility is prevention of ice crystal formation during warming to 37℃ (Macfarlane DR, 1986). Holding embryos in air or water did not significantly change the survival blastocysts or morulae. For thawing temperature for the embryo, it seems the higher the concentration of cryoprotectant, the higher thawing temperature.

Table 3. Effect of post-warming conditions on mean responses of *in vitro* cryopreservation bovine embryos in Korean native cows

Temperature	Used-embryos	Survival	Degeneration
35℃	48	34 (70.8)	14 (29.2)
37℃	56	47 (85.7)	9 (16.1)

The objective of the present study was to transfer, after freeze and thaw, *in vivo*/*in vitro* fertilized embryos according to developmental stage. Blastocysts showed highest survival rate for *in vivo* fertilized embryos, while extended blastocysts was highest for *in vitro* fertilized embryos. For the viability according to thawing temperature, 37℃ treatment showed higher survival rate than 35℃.

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