J. Emb. Trans. (2012) Vol. 27, No. 4, pp. 259~264
http://dx.doi.org/10.12750/JET.2012.27.4.259

Effects of Osmolality Step during Vitrification on Survival of Vitrified IVP Embryos in Korean Native Cattle (Hanwoo)

Han-Jun Yoo[1], Hye-Won Choi[1], Ki-Soo Cheong[3], Ji-Tae Kim[3], Chang-Woo Lee[4], Choon-Keun Park[2] and Joung-Jun Park[1,*]

[1]*Animal Reproduction & Biotechnology Center, Hoengseong 225-807, Korea*
[2]*College of Animal Life Sciences, Kangwon National University, Chuncheon 200-701, Korea*
[3]*South Branch of Gangwondo Veterinary Service Lab, Wonju 220-170, Korea*
[4]*Gangwon Provincial Livestock Research Center, Hoengseong 225-831, Korea*

ABSTRACT

Solution of glycerol, ethylene glycol, sucrose, dextrose (GESD) and cryotop methods were carried out to investigate the survivability on vitrification of embryos. Embryos cultured *in vitro* were vitrified by GESD of 10 or 8 step and cryotop methods of 6 step, from cryopreservation step to frozen-thawed and culture step. Survival rate and ICM, TE cells of embryos were investigated after frozen-thawed 24 h. As a results, cryotop method was significantly ($p<0.05$) higher (85.76 ± 5.3 vs. 66.71 ± 2.4, $44.80 \pm 2.1\%$) than GESD 10 or 8 step methods on survivability. Also, In ICM cell number, cryotop method was significantly ($p<0.05$) higher to 45.67 ± 4.7 cells than GESD 8 step method. TE cell number was significantly ($p<0.05$) highest to 111.00 ± 11.0 cells in cryotop method. On the other hand, survival rate, TE and total cell number were all the significantly ($p<0.05$) high, except ICM in GESD 10 step method between GESD 10 step method and GESD 8 step method. In conclusion cryotop method was to be most effective, but it is considered necessary to study vitrification method for step-by-step freezing and thawing process.

(Key words : vitrification, GESD, cryotop, osmolality, hanwoo)

INTRODUCTION

Cryopreserved embryos can be physically and biologically damaged easily. Possible factors can be formation of ice within the cells and its affect on abnormal activity of microstructures of cells (1993; Baka *et al*., 1995) as well as abnormal osmotic pressure, changes in temperature, endotoxin of cryoprotectants and pH change according to the exposed time to cryoprotectant. Researchers have published all different reports on survivability by these factors including freezing method (Damien *et al*., 1990; Hunter *et al*., 1991, 1995). There are two freezing methods for cryopreservation of embryos from cow, slow freezing method - transferring embryo directly after freezing and thawing- and vitrification method which is more comfortable and easily done. Slow-freezing method has several disadvantages; damage can be made within the cells due to a formation of ice, 2 to 3 h have to be consumed for freezing, large amount of liquid nitrogen consumption is necessary and constant care of the researcher is required. The method also requires an expensive cell freezer (Gardner *et al*., 2004). Vitrification method is developed to make up such shortcomings. Rall and Fahy (1985) used the embryo from rats and proved its first success. Since then, vitrified rat embryo successfully has been proven effective and economical according to production of newborn after thawing embryo transfer (Rall *et al*., 1987).

Vitrification method essentially requires the addition of high-concentrated cryoprotectant to keep embryos from a formation of ice (Fahy *et al*., 1984). Type of cryoprotectant and its solution greatly affect the survival rates; solution of ethylene glycol, ficoll, sucrose (EFS; Kasai *et al*., 1990) and solution of glycerol, ethylene glycol (GE; Yang *et al*., 1992) have been reported to be successful. In GE, vitrification of blastocysts has been reported to show high survivability (Yang *et al*., 1992; Saito *et al*., 1994). Saito *et al*. (1994) reported vitrification of *in vitro* blastocyst using GESD solution with the addition of sucrose and dextrose to the base of glycerol and ethylene glycol to show high survival rate.

Not only the components of cryoprotectant solution affect

† This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development(Project No. PJ907008)" Rural Development Administration, Republic of Korea.
* Correspondence : E-mail : jjparkcow@yahoo.co.kr

the survival rates of vitrified embryos (Ali and Shelton, 1993; Saito *et al*., 1994), but also equilibration method and dilution method after thawing also determines the survival rates of embryos (Kuwayama *et al*., 1994). Kuwayama *et al*. (1994) used solution of glycerol and 1,2-propanediol to vitrify *in vitro* blastocysts after equilibrating with 2 steps and 16 steps. And he reported the result that 16 step equilibration method showed much higher survival rate of embryos.

The following research was carried out, using bovine *in vitro* embryos, to investigate survivability difference between step-wise freezing-thawing vitrification by GESD and Cryotop method.

MATERIALS AND METHOD

1. Production of *In Vitro* Fertilized Embryos

COCs (Cumulus Ooctyes Complexes) from the ovaries of slaughtered cows were used in this experiment. Ovaries were kept in the saline solution under 37℃ and were transported to the laboratory right after being collected, 18-gauge needle 10 ml syringe was used to collect the COCs from the follicles below 10 mm. COCs were matured under IVM media -TCM-199 was added with FSH (0.5 ug/ml), LH (5 ug/ml), 5% FBS- for 22 h within the incubator with 5% CO_2 and the temperature of 38.5℃. In IVF, TL media was used with the sperm concentration of 1×10^6/ml and *in vitro* fertilization was induced for 18~20 h within the incubator with 5% CO_2, 38.5℃ (Table 1). Synthetic oviduct fluid-bovine embryo1(SOF-BEI) was used as the developmental culture media based on $CaCl_2 \cdot 2H_2O$ 1.17 mM, $MgCl_2 \cdot 6H_2O$ 0.49 mM, KH_2PO_4 1.19 mM, KCl 7.16 mM, NaCl 107.7 mM, Tri-Na-cirtate 0.50 mM, Myo-Inositol 2.77 mM, $NaHCO_3$ 25.07 mM, Na-lactate 5.3 mM, Gentamicin 25 ug/ml and for the culture of fertilized embryos, glutamine 1 mM, Na-pyruvate 0.4 mM, non-essential amino acids 10 ul/ml, essential amino acids 20 ul/ml were added (Fields *et al*., 2011). IVC was done with the microdrop of 30 ul, and the change of culture media was carried out with the 48 h interval. Developmental culture was carried out under the condition with 5% CO_2, 5% O_2 and 38.5℃.

2. Cryopreservation of *In Vitro* Embryos

Solution of glycerol, ethylene glycol, sucrose, dextrose (GESD) method (Saito *et al*., 1994) was practiced on blastocysts for vitrification. Base culture solution was made by adding 20% FBS (Gibco) to D-PBS (Dulbecco's PBS, Gibco) for vitrification. Vitrification solution 1, 2, and 3 (VS1, VS2 and VS3) were produced by adding glycerol, ethylene glycol, sucrose/glucose to the main culture solution. Sucrose solutions with different concentration level of 0.75, 0.5, 0.25 and 0.125 M were used as Thawing Solution (TS) (Table2). Cryotop Safety Kit – Vitrification/Thawing (Kitazato, Japan) was used for the experiment of vitrification. Embryos were equilibrated in VS1, VS2, and VS3 for 5 min, 5 min and 1 min for vitrification by GESD solution. Within the 1 min of equilibration of VS3, it was put in 0.25 ml straw. The tips of each end of straw was heated and sealed and they were immersed into the liquid nitrogen immediately for being cryopreserved. Cryopreservation by Cryotop method was done by equilibrating embryos for 15 min in equilibration solution (ES) from the kit. They were washed about 5 times with vitrification solution (VS) and loaded on Cryotop within 90 seconds. VS culture solution around embryos was removed as much as possible on Cryotop and embryos were immersed within liquid nitrogen to complete cryopreservation step.

Table 1. Composition of TL media for *in vitro* fertilization

Component	mM		
	TL-Hepes	TL-Fert.	TL-Sperm
NaCl	114.0	114.0	100.0
KCl	3.2	3.2	3.1
$NaHCO_3$	2.0	25.0	25.0
NaH_2PO_4	0.4	0.4	0.29
Na Lactate (60%)	10.0	10.0	26.0
$CaCl_2 \cdot 2H_2O$	2.0	2.0	2.107
$MgCl_2$	0.5	0.5	0.4
HEPES	10.0	–	10.0
Na pyruvate	0.2	0.2	1.05
Caffeine	–	–	10
Heparin	–	0.02 mg/ml	–
Gentamycin sol.	0.75 ul/ml	0.5 ul/ml	1.06 ul/ml
BSA	0.003 g/ml	0.006 g/ml	0.0063 g/ml

3. Freezing-Thawing

Cryopreserved 0.25 ml straw was exposed to the air for about 10 seconds and was thawed in the incubated water bath of 36℃ for 20 seconds for freezing-thawing step of embryos.

Table 2. Composition of vitrification and dilution solution in GESD solution Type

Typ of solution		DPBS	FBS (%)	Glycerol (%)	Ethylene glycol (%)	Sucrose (M)	Glucose (M)	Exposure time (min)
Vitr.[1)]	1	Basic	20	10		0.125	0.125	5
	2	Basic	20	10	10	0.25	0.25	5
	3	Basic	20	20	20	0.375	0.375	1
Thaw-ing[2)]	1	Basic	20	−	−	0.75	−	5
	2	Basic	20	−	−	0.5	−	5
	3	Basic	20	−	−	0.25	−	5
	4	Basic	20	−	−	0.125	−	5
Washing		Basic	20	−	−	−	−	-

[1)] Vitrification solution.
[2)] Thawing soltuion.

In GESD case, it was divided into two experiment groups and each group had five steps and 3 steps for thawing procedure. For the group 1, to have 5 steps of thawing procedure, it was divided into VS3 (1/2) + 0.5 M TS (1/2), 0.75 M TS, 0.5 M TS, 0.25 M TS and 0.125 M TS accordingly and equilibration time was given about 5 min. For the second group, it was divided into three solutions of 0.75 M TS, 0.5 M TS and 0.5 M TS and step-wise thawing procedure has been practiced. In the case of Cryotop, Cryotop in which embryos were loaded, was immediately moved from the liquid nitrogen to the TS dish, heated up to 36℃, and let remained for 1 min to start thawing. While being thawed, embryos were removed from Cryotop to TS. Equilibration and Thawing step was completed by equilibrating them in Diluent Solution (DS) from the Cryotop Safety Kit-Thawing for 3 min, and in Washing Solution (WS) for 5 min and 1 min each (Fig. 1) Embryos after completion of thawing, were cultured by developmental media under the condition of 5% CO_2, 5% O_2 and 38.5℃ to investigate survivability rate by re-expansion after freezing-thawing.

4. Measurement to Osmolality of Vitrification and Thawing Solution Used in GESD and Cryotop

Osmolality of vitrification and thawing solution were measured (Micro Osmometer 3300; Advaced. Instruments Inc, MA USA) for design the step wise graph all procedures from equilibration to developmental culture (Fig. 1). 20 ul of vitrification and thawing solutions of GESD 10 or 8 step and Cryotp methods were used in measurement. The equipment works on the principle of freezing point measurement with precision of 2 mOsm/kg.

5. Analysis the Inner Cell Mass and Trophectoderm of *In Vitro* Fertilized Embryos

Number of cells was investigated by using blastocysts 24h after vitrification-thawing. In order to count the number of cells of the embryos, Hoechst 33342 (B2661, Sigma, USA) and Propidium Iodide Sol .(PI, P4864, Sigma, USA) were used to do

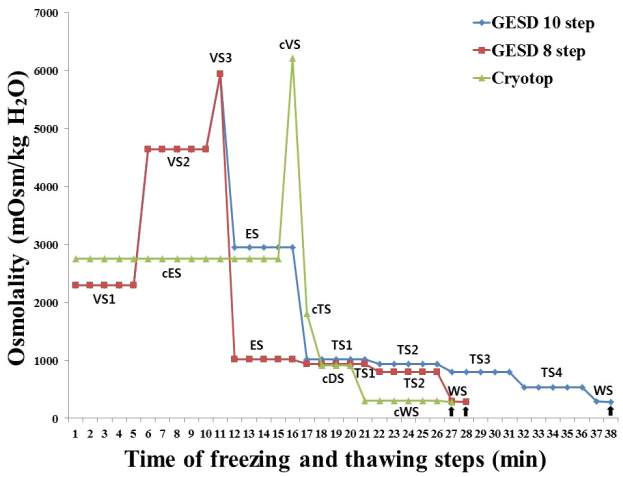

Fig. 1. Changes of Osmolality flow during vitrification and thawing steps in vitrification 1, 2 and Cryotop methods. ●: vitrification(GESD) 1 method using 10 step of osmolality, ■: vitrification(GESD) 2 method using 8 step of osmolality, ◆: cryotop method using 6 step of osmolality, VS: vitrification solution, ES: equilibration solution, TS: thawing solution, DS: diluent solution, WS: washing solution, ⬆: incubation of frozen-thawed incubation.

fluorescent staining and the method stated through the paper (Thouas *et al*., 2001) was used. 1 mg/ml of PVP was diluted into 0.1 M PBS and the solution was used as the basic culture medium and embryo washing solution. Fluid for fluorescent staining was a 10 mg/ml of stock solution made from Hoechst 33342 and diluted into 1,000 times and was used as H working solution. PI was diluted into 50 times and made into P working solution. Also, Triton X-100 (T8532, Sigma, USA) was diluted into 0.1% (v/v). For the inner cell mass (ICM) staining, the fertilized embryos were cultured in H solution for 30 min and the remaining H Solution was removed after washing the embryos with the PBS/PVP culture media. After washing it more than three times, TE cell staining was made sure to be done by letting it remain in 0.1% Triton X-100 solution for 1 min. Again, the fertilized embryos were washed more than three times. After staining the TE cells by culturing the embryos within the heated P solution of the temperature of 37℃, the fluorescent staining of fertilized embryos was completed with the last washing procedure. Stained embryos were transferred to the slide glasses for the observation of number of cells and the culture media was removed as much as possible on slide glasses. To observe the fluorescent stained cells, glycerol was added into the fertilized embryos and covered with the cover glass and fluorescent microscope was used to observe with the ×200 and ×400 magnifications.

6. Statistical Analysis

ANOVA analysis and Duncan's multiple range test using SAS 9.1 (SAS Institute INC., Cary, NC, USA) showed the significant difference ($p<0.05$) on the results on the survival rates of embryos 24 h after freezing and thawing and the results of the number of cells on each cultured group.

RESULTS

Table 3 shows survivability of frozen-thawed embryos following 24 h after vitrification by GESD and cyrotop methods. GESD group is divided into two groups which had gone 5 steps and the other with 3 steps of thawing. In the Table, 3 steps of freezing and 2 steps of washing and culture, were considered so in total, it is stated to be gone through 10 steps and 8 steps. Vitrification by cryotop method showed significantly higher survival rate compared to the vitrification by GESD method on 24 h after freezing-thawing. Considering the cell number counted, as well, vitrification by cryotop method showed highest ($p<0.05$) result (156.67 ± 15.0 vs. 119.5 ± 2.8 and 82.6 ± 6.9). In ICM, there was no significant difference between cryotop and GESD 10-step group. On the contrary, GESD 8 step group showed the lowest result ($p<0.05$). On the account of TE cell number, GESD 8 step group showed the lowest number of cells, 49.40 ± 6.1, while Cryotop group showed the highest ($p<0.05$) numbers of cells, 111.00 ± 11.0.

DISCUSSION

Vitrification method is useful and better than slow-freezing method in that the method can minimize the formation of ice on cells, no need of an expensive machine, less time consumption, comparably higher survival rates (Kuwayama *et al*., 1992; Saito *et al*., 1994; Kim *et al*., 1998) On the account of GE, Ishimori *et al*. (1992) vitrified rat blastocysts by using solution of 25% glycerol and 25% ethylene glycerol and reported high survival rate of 72%. Yang *et al*. (1992) reported high hatchability of 80.8% after vitrifying blastocyst using GE. Saito *et al*. (1994) as well reported the highest survival rate of 83.3 ~ 95.8% and hatchability of 69.2~85.9% when vitrification of blastocysts was done by GESD method using sucrose and dextrose added to GE. Even though the production of real fetus after transfer has not been proven, addition of sucrose and dextrose was proven to affect the survival of embryo positively. Kasai *et al*. (1990) got a high survival rate result after vitrifying morula embryos of rat by using vitrification solution com-

Table 3. Survivability of freezing-thawed embryos following freezing methods after 24 h of thawing

Tre.	No. of thawed embryos	Survivability (%)	No. of ICM	No. of TE	Total cell No.
GESD[1)] (8-step)	51	44.80 ± 2.1^{c}	33.20 ± 1.4^{b}	49.40 ± 6.1^{c}	82.6 ± 6.9^{c}
GESD (10-step)	54	66.71 ± 2.4^{b}	41.00 ± 1.7^{ab}	78.50 ± 1.6^{b}	119.5 ± 2.8^{b}
Cryotop	42	85.76 ± 5.3^{a}	45.67 ± 4.7^{a}	111.00 ± 11.0^{a}	156.67 ± 15.0^{a}

[1)] Solution of glycerol, ethylene glycol, sucrose, dextrose (Mean ± SEM, $p<0.05$).

posed of ethylene glycol, ficoll and sucrose. Adding sucrose solution prevents excessive permeatability of cryoprotectant (Szell and Shelton, 1987). It is reported that increasing the amount of protein within the cells helps vitrification within the cells (Rall, 1987).

Kuwayma *et al*. (1994) compared two different equilibration methods - one is two- step equilibration, each for 5 min and the other is 16 step equilibration method, each for 1 min. He vitrified *in vitro* embryos by using solution of glycerol and 1,2-propanediol. The result showed a great difference; in case of 2 step equilibration, survival rate was 0%. On the contrary, when embryos were equilibrated through 16 steps, survival rate was 83.3%. Equilibration method before vitrification has been proven to be affective also on the microstructures of the cells since cytoplasmic vacuolization was observed on the 2 step equilibration. In Ohboshi *et al*. (1997), a solution of ethylene glycol, polyethylene glycol and sucrose, one-level equilibration showed 13% of survival rate while two-level equilibration showed 72.7% survival rate; significant difference was observed. Appropriate levels of equilibration help to buffer the osmotic pressure from direct exposure to the high-concentrated freezing solution and such method helps to minimize the damage on cells during vitrification. Such method helps the vitrification be done more smoothly and stably.

As a result from this research, survival rates from embryos frozen-thawed by 10 step GESD method were significantly higher than the 8 step procedure. To find appropriate number of steps for freezing and thawing, additional experiments have to be done continuously. Cryotop method - known for high survivability on human fertility study, showed the highest survival rates and cell number (Lin *et al*., 2010). It seems that components such as ethylene glycol, DMSO and sucrose that were included in the kit and freezing method by cryotop which drew better effect.

Vitrification has not been widely adopted by ET practitioners for commercial use in cattle (Vajta, 2000). In general, vitfirication requires gradual cryoprotectant dilution in a laboratory setting because it uses a high concentration of cryoprotectants (Ishimori *et al*., 1993). Therefore, it is difficult to perform vitrification in the field not yet.

In vitro embryos can be frozen by vitrification method using the Cryotop or GESD. However, Cryotop method need to specially equipment such as cryotop instead of straw and optical microscopy for remove the attached embryos at cryotop in thawing step. Also, GESD 10 step method needs to simplify and consider osmolality, appropriate step-wise freezing and thawing procedure for direct transfer.

REFERENCES

Ali J and Shelton JN. 1993. Successful vitrification of day-6 sheep embryos. J. Reprod. Fertil. 99:65-70.

Baka SG, Toth TL, Veeck LL, Jones Jr HW, Muasher SJ and Lanzendorf SE. 1995. Evaluation of the spindle apparatus of *invitro* matured human oocytes following cryopreservation. Hum. Reprod. 10:1816-1820.

Damien M, Luciano AA and Peluso JJ. 1990. Propanediol alters intracellular pH and developmental potential of mouse zygotes independently of volume change. Hum. Reprod. 5: 212-216.

Fahy GM, MacFarlane DR, Angell CA and Meryman HT. 1984. Vitrification as an approach to cryopreservation. Cryobiology 21:407-426.

Fields SD, Hansen PJ and Ealy AD. 2011. Fibroblast growth factor requirements for *in vitro* development of bovine embryos. Theriogenology 75:1466-1475.

Gardner DK, Weissman A, Howles CM and Shoham Zeev. 2004. Textbook of Assisted Reproductive Techniques. 2nd ed, Taylor & Francis, United Kingdom, pp. 257-289.

Hunter JE, Bernard A, Fuller B, Amso N and Shaw RW. 1991. Fertilization and development of the human oocyte following exposure to cryoprotectants, low temperatures and cryopreservation: a comparison of two techniques. Hum. Reprod. 6:1460-1465.

Hunter JE, Fuller BJ, Bernard A, Jackson A and Shaw RW. 1995. Vitrification of human oocytes following minimal exposure to cryoprotectants; initial studies on fertilization and embryonic development. Hum. Reprod. 10:1184-1188.

Ishimori H, Saeki K, Inai M, Nagao Y, Itasaka J, Miki Y, Seike N and Kainuma H. 1993. Vitrification of bovine embryos in a mixture of ethylene glycol and dimethyl sulfoxide. Theriogenology 40:427-433.

Ishimori H, Takahashi Y and Kangawa H. 1992. Viability of vitrified mouse embryos using various cryoprotectant mixtures. Theriogenology 37:481-487.

Kasai M, Komi H, Takakamo A, Tsudera H, Sakurai T and Machida T. 1990. A simple method for mouse embryo cryopreservation in low toxicity vitrification solution without appreciable loss of viability. J. Reprod. Fert. 89:91-97.

Kim CI, Yoo JW, Park CK, Yang BK, Cheong HT. 1998. Effects

of equilibration and dilution methods on the survival of vitrified bovine IVF embryos. Korea J. Emb. Trans. 13: 313-321.

Kuwayama M, Fujikawa S and Nagai T. 1994. Ultrastructure of IVM-IVF bovine blastocysts vitrified after equilibration in glycerol 1,2-propanediol using 2-step and 16-step procedures. Cryobiology 31:415-422.

Kuwayama M, Hamano S and Nagai T. 1992. Vitrification of bovine blastocysts obtained by *in vitro* culture of oocytes matured and fertilized *in vitro*. J. Reprod. Fertil. 96:187-193.

Lin TK, Su JT, Lee FK, Lin YR and Lo HC. 2010. Cryotop vitrification as compared to conventional slow freezing for human embryos at the cleavage stage: survival and outcomes. Taiwan J. Obstet. Gynecol. 49:272-278.

Ohboshi S, Fujihara N, Yoshida T, Tomogane H. 1997. Usefulness of polyethylene glycol for cryopreservation by vitrification of *in vitro*-derived bovine blastocysts. Anim. Reprod. Sci. 48:27-36.

Rall WF and Fahy GM. 1985. Ice-free cyropreservation of mouse embryos at −196℃ by vitrification. Nature 313:573-575.

Rall WF, Wood MJ, Kirby C and Whittingham DG. 1987. Development of mouse embryos cryopreserved by vitrification. J. Reprod. Fert. 80:499-504.

Rall WF. 1987. Factors affecting the survival of vitrified mouse embryos. Cryobiology 24:387-402.

Saito N, Imai K and Tomizawa M. 1994. Effect of sugars-addition on the survival of vitrified bovine blastocysts produced *in vitro*. Theriogenology 41:1053-1060.

Széll AZ and Shelton Jn. 1987. Osmotic and cryoprotective effects of glycerol-sucrose solution on Day-3 mouse embryos. J. Reprod. Fertil. 80:309-316.

Thouas GA, Korfiatis NA, French AJ, Jones GM and Trounson AO. 2001. Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. Reprod. Biomed. Online 3:25-29.

Vajta G. 2000. Vitrification on the oocytes and embryos of domestic animals. Anim. Reprod. Sci. 60-61:357-364.

Yang NS, Lu KH, Gordon I and Polge C. 1992. Vitrification of blastocysts *in vitro*. Theriogenology 37:326.

(received: 2012. 10. 16 / revised: 2012. 10. 17 / accepted: 2012. 10. 26)