J. Emb. Trans. (2013) Vol. 28, No. 2, pp. 141~147
http://dx.doi.org/10.12750/JET.2013.28.2.141

Development of New Vitrification Method for Preimplantation Mouse Embryo

A-Na Ha[1], Md. Fakruzzaman[1], Kyeong-Lim Lee[1], Erdan Wang[1], Jae-Ik Lee[1], Chan-Sik Min[3] and Il-Keun Kong[1,2,*]

[1]Division of Applied Life Science (BK21), Graduate School of Gyeongsang National University, Jinju 660-701, Republic of Korea
[2]Institute of Agriculture and Life Science, Jinju 660-701, Republic of Korea
[3]Gyeongsangnamdo Agriculture Research & Extension Services, Jinju 660-985, Republic of Korea

ABSTRACT

The purpose of this study was attempted to new methods in mammalian embryos vitrification. This method was affected to increase of the embryo vitrification efficiency and it would be applied to the field of embryo transfer to recipient by modified loading method of embryo into 0.25 ml plastic straw. The frozen mouse embryos were carried out warmed from two different cell stages (8-cell and blastocyst, respectively) by attachment of an embryo in the vitrification straw (aV) method. All groups were cultured in M-16 medium to determine the development and survivability for 24 h, respectively. Results shown that, the survivability of two different groups were significantly different (94.8% vs. 70.9%). Total cell number was not significantly different the non-frozen blastocyst (99.7 ± 12.4) compared to the post-thaw blastocyst (94.8 ± 15.1). From the 8-cell embryo, total cell number of frozen blastocysts were significantly lower than others groups (74.7 ± 14.6, $p<0.05$). In the case of cell death analysis, the blastocysts from non-frozen and frozen-thawed 8-cell group were not different (0.0 ± 0.0 vs. 1.9 ± 3.1, $p>0.05$). However, the apoptotic nuclei of blastocyst were significantly observed the frozen-thawed group (5.4 ± 4.4) compared to non-frozen group ($p<0.05$). Therefore, this new method of embryos using in-straw dilution and direct transfer into other species would be more simple procedure of embryo transfer rather than step-wise dilution method and cryopreservation vessels, so we can be applied in animal as well as human embryo cryopreservation in further.

(Key words : vitrification, mouse embryos, step-wise, direct transfer, cell number)

INTRODUCTION

In various stages, many studies were developing for cryopreservation of mammalian. The cryopreservation of mouse embryos is meaningful as well as useful models for the cryopreservation of livestock and human embryos (Quinn and Kerin, 1986). The first successful frozen of mammalian embryos was achieved with 8-cell mouse embryos (Rall and Fahy, 1985). Also, many various techniques of cryopreservation were developed such as conventional freezing method compared with slow freezing, rapid freezing, ultra-rapid freezing and vitrification. The vitrification is the solidification of the solution brought about not by crystallization but by elevation in viscosity during cooling (Kong *et al.,* 2000). Vitrification may freeze very fast and easily using the small volume. However, a major disadvantage of vitrification is the requirement for a high concentration of cryoprotectant and its accompanying embryos toxicity. To improve the efficiency of the vitrification method, many different cryoprotectants and vitrification using the various tools have been studies. The most commonly used extracellular cryoprotectants for mammalian embryo vitrification including of DMSO, glycerol, ethylene glycol, propylene glycol (Kasai *et al.,* 1990; Yang *et al.,* 2007b). Frozen of intra cellular part used non-permeability cryoprotectants as sucrose, trehalose and propylene-pyrrolidone. Moreover, the vitification was used to various modified straws which is droplets methods, OPS (Open pulled straw) (Vajta *et al.,* 1998), CPS (close pulled straw) (Chen *et al.,* 2000), GPS (Glass pulled straw) (Kong *et al.,* 2000), and Hemi-straw (Vanderzwalmen *et al.,* 2003).

† This work was partly supported by Next-Generation BioGreen 21 Program (Grant No. PJ009587022013), IPET (Grant No. 110020-5 and 112020-3) and a scholarship from the BK21 program. A-Na Ha and Kyeong-Lim Lee were supported by BK21 fellowship in Graduate School of Gyeongsang National University, Republic of Korea.
* Correspondence : E-mail : ikong7900@gmail.com

During the cell frozen, the water of extent inner cell may be formed ice crystal. In cryopreservation process, damage of embryos can be induced by several factors, including of extracellular and intracellular ice formation, solution effects, osmotic activity, or physical damage by growing ice crystals (Van Den Abbeel and Van Steirteghem, 2000). During cryopreservation, the addition and removal of penetrating cryoprotective agents may create an osmotic imbalance across the cell membrane. This imbalance may alter the function, morphology and cytoskeletal organization (Hotamisligil *et al.,* 1996). Although excessive volumetric changes can be reduced by adding and removing cryoprotectants in a step wise fashion, prolonged exposure of cells to cryoprotectants at non-freezing temperature may induce toxic effects (Rall and Fahy, 1985). When cryopreserving embryos, cryoprotective component in the freezing medium appears to be indispensable. Cryoprotectants of low molecular weight such as glycerol, ethylene glycol permeate the cell membrane and exert intracellular cryoprotective action (El-Gayar *et al.,* 2008).

Freezing and thawing during vitrification, reduce the exposure time of cells to cryoprotectants at non-freezing temperature and may reduce toxic stress to oocytes and embryos. Moreover, vitrification and thawing also minimize chilling injury, as the cells are exposed to critical temperature zones for comparatively short interval (Dhali *et al.,* 2009; Vajta *et al.,* 1998). Leibo *et al.,* (1984) demonstrated that on a one-step dilution method which was direct thawing with sucrose in straw after frozen with glycerol in bovine. Also, frozen embryos were transferred directly into recipients after thawing without to dilution the cryoprotectants in bovine (Dochi *et al.,* 1998).

In many studies demonstrated, the pregnancies of many animals have developed after step-wise removal of the cryoprotectants thought in straw dilution methods (Kuwayama *et al.,* 1992). For prevention to pathogenic infection of cross-contamination by LN_2, embryo vitrification used to 'straw in straw' system was adopted in human embryos (Isachenko *et al.,* 2005).

Direct transfer following in-straw dilution of cryoprotectants has become a widely adopted practice for the transfer of bovine embryos frozen by conventional slow rate (Dochi *et al.,* 1998) and has the advantage that manipulation of embryos, through dilution media, at the time of transfer is not necessary (Pugh *et al.,* 2000). These simplified regimens of thawing and in-straw diluting of vitrified embryo offers the possibility to conduct a direct transfer of embryos without the necessity of a microscope and other laboratory equipment.

Therefore, this study is carried out to establish the straw loading vitrification method for improve the post-thaw viability efficiency of cryopreserved embryo and apply in assisted reproductive technology (ART) of human and domestic animal species.

MATERIALS AND METHODS

All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

1. Animal Care and Embryos Collection

ICR mice were housed and bred in the animal installation of Gyeongsang National University. The mice were kept in light and temperature controlled condition as follows light and dark for 12 h at 22 ± 2℃ temperature. The age of female and male mice used 4 to 8 and 10 to 14 weeks, respectively. Donor mice were induced superovulation by intraperitoneal injection of 5 IU of PMSG (Daesung Microbiological Labs. Co. Ltd. Republic of Korea) and 5 IU of hCG (Daesung Microbiological Labs. Co. Ltd. Republic of Korea) 48 h apart of PMSG. Synchronization of recipient was done injected with hCG after1-day delay of donor treatment. After 3～4 days of superovulation, mice were killed by cervical dislocation and oviducts were removed. The 8-cell embryos and blastocysts were collected from oviduct and cervix by flushing.

2. Vitrification and Thawing Procedure

The vitrification procedure was done according to (Yang *et al.,* 2007a) with minor modification. Briefly, the embryos were washed in D-PBS added with 0.3% BSA (DB) media several times for equilibratrion. Embryos were putted in DB added 10% ethylene glycol (EG) for 30 sec and then putted in 3% Ficoll and 0.5 M sucrose (FS solution) in DB added 40% ethylene glycol for 30 sec. Finally, embryos were loaded in 0.25 ml straw (FHK, Japan) by aV method. All of thawing procedure was described by Inaba*et al.,* (2011) with minor modification. The frozen straw was thawed at the 20℃ water for 5 sec and shakes up-down side slowly to dilution, and then cut out the top sealing part with straw cutter. After pour embryos in the dish, the post-thawed embryos were washed in 0.5 M sucrose solution for 2.5 min and 0.25 M sucrose for 2.5 min. Finally, the post-thawed blastocysts and 8-cell embryos

were transferred into 20 μl droplets of M-16 medium, and then cultured at 37℃ in a humidified atmosphere of 5% CO_2 for 24 h, respectively.

3. Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining

To analysis the total and apoptosis cells, post-thawed 8-cell and blastocysts were washed 2～3 times in PBS-PVP and fixed in a well of 4-well dish containing 700 μl of 4% (w/v) paraformaldehyde in PBS-PVP for 1 h at room temperature. After fixation, embryos were stored at 4℃ until TUNEL assay was performed. The TUNEL assay was performed according to the manufacturer protocol using the In Situ Cell Death Detection Kit (Fluorescein, Roche Diagnostics Corp.; USA; Cat. 1684795). Briefly, the fixed embryos were washed twice with PBS-PVP before being incubation in permeabilization solution [0.5% (v/v) Triton X-100, 0.1% (w/v) sodium citrate] for 30 min at room temperature. After permeabilization, the embryos were washed twice in PBS-PVP. The embryos were then incubated in fluorescence-conjugated dUTP and terminal deoxynucleotide transferase (TUNEL reagent, Roche) for 1 hrs at room temperature in the dark. The TUNEL stained embryos were then washed in PBS-PVP and counterstained with 10 μg/ml Hoechst 33342 in PBS-PVP for 10 min at room temperature in the dark to label all nuclei. The blastocysts were then washed twice in PBS-PVP to remove excess Hoechst 33342 and mounted on glass slides under coverslips to evaluate the nuclear configuration. Cell numbers of each blastocyst was counted using an epifluorescence microscope (Olympus IX71, Japan) equipped with a mercury lamp. The positive cell was indicated by a bright red fluorescence indicating apoptotic cells and the total cell number was determined by green/blue fluorescence.

4. Statistical Analysis

Embryo survivability and the rates of re-expansion were expressed as percentages (%). The number of total and apoptotic cells was expressed as mean ± S.D. Statistical differences were analyzed by one-way ANOVA (SPSS Inc., Chicago, IL, USA). Significant differences between groups was performed by Duncan's multiple range test. Statistical significance was taken at $p<0.05$.

RESULTS

1. Developmental Competence of Post-thawed Embryo Vitrified by Two Different Cell Stages

Table 1. Effect of cell stage on the development competence of vitrified embryo by different cell stage

Cell stage	No. & (%) of Embryos			No. (%) of blastocyst developed to expanded.
	Frozen	Recovery	Survival	
Non-frozen blastocyst	-	-	-	54 (100.0)[a]
8-cell	62	55 (88.7)	42 (76.4)	39 (70.9)[b]
Blastocyst	60	58 (96.7)	55 (94.8)	55 (94.8)[a]

* [a,b] Values with different superscripts in same column were significantly different ($p<0.05$).

* Non-frozen blastocyst group was cultured in M-16 medium for 24 h.

Total 6 biological replications were used for developmental competence of post-thawed embryos. The development rate of post-thawed vitrified blastocyst was highest in 8-cell and blastocyst groups (Table 1). The post-thaw re-expansion rate in control was not significantly higher than blastocyst group, but significantly higher than in 8-cell groups (non-frozen blastocyst, 100% vs. blastocyst, 94.8% vs. 8-cell, 70.9 %, $p<0.05$).

2. Assessment of Embryo Quality among Two Different Cell Stages

In Table 2, the total cell numbers of post-thaw blastocyst was reduced regardless of the embryo stage used (control, 99.7 ± 12.4 vs. blastocyst, 94.8 ± 15.1 and 8-cell, 74.7 ± 14.6, $p<0.05$). Even total cell numbers in blastocyst group was the

Table 2. Comparison of total and apoptosis cell number of vitrified 8-cell and blastocyst with non-frozen blastocyst

Treatments	No. of embryos stained	Total cell no. (Mean ± S.D.)	Apoptotic cell no. (Mean ± S.D.)
Non-frozen blastocyst	15	99.7 ± 12.4[a]	0.0 ± 0.0[c]
Post-thaw 8-cell	15	74.7 ± 14.6[b]	1.9 ± 3.1[cd]
Post-thaw blastocyst	15	94.8 ± 15.1[a]	5.4 ± 4.4[d]

* [a~d] Values with different superscriptsin same column were significantly different ($p<0.05$).

higher compared with 8-cell ($p<0.05$), but no significantly higher than in control groups ($p>0.05$). On the other hand, the number of apoptotic cells was significant difference between non-frozen blastocyst and blastocyst (0.0 ± 0.0 vs. 5.4 ± 4.4). Also, post-thaw 8-cell group was no significant difference other groups (1.9 ± 3.1, $p>0.05$).

DISCUSSION

In mammalian embryos, one-step dilution method was developed. However, this method is very limited apply for direct embryo transfer in the field yet. Although, many studies according to frozen of mouse embryos were developed, but they needed another control steps at lab. In this study, post-thaw survivability of aV method was very efficient compared with other methods and also more simple and fast for processing.

Vieira *et al.,* (2008a) demonstrated that the open pulled straw (OPS) system straw was directly putted into the container. In this method straw was exposed to air for 3～4 sec and then after warming at 35℃. If the operator fails to insert the OPS into the straw, the warming rate will be reduced, which may cause embryo damage by ice crystal and cryoprotectants toxicity of high concentration. These methods were inconvenient processing that need cryo-tool as OPS, Cryotop.

In general, conventional vitirification methods were require gradual cryoprotectants dilution at high concentration (Ishimori *et al.,* 1993; Saito *et al.,* 1994). This is the main difficulty to perform vitrification. However, recently some vitfirication methods have been reported as suitable for in-straw dilution of embryos. These methods may simplify the embryo transfer procedure such an extent that may be used to transfer vitrified embryos on farms at the same level of complexity as carrying out an artificial insemination with frozen-thawed semen (Inaba *et al.,* 2011).

Since the implementation of one-step dilution of conventional cryopreserved mammalian embryos exposed to sucrose solution in many studies (Saha *et al.,* 1996a, 1996b; Taniguchi *et al.,* 2007; Vajta *et al.,* 1996; Yang *et al.,* 2007a). Dilution solutions usually used as non-permeable cryoprotectants of high osmolarity to prevent rehydration from taking place too vehemently leading to excessive swelling and cell damage. Rehydration and cryoprotectant loss are occurring simultaneously while the embryos are exposed to solutions of decreasing sucrose concentration until iso-osmotic conditions have been restored (El-Gayar *et al.,* 2008).

In the present study, the short exposure time was insured low intra-cellular cryoprotectant concentrations that maintained the osmotic differential between the embryos and their environment never became high enough to cause serious osmotic damage. However, straw-paste method was perfectly overcome to osmotic damage.

To direct plunging into liquid nitrogen, cooling rate and velocity was verified micro-droplet methods (～2,500℃/min) (Palasz and Mapletoft, 1996), electron microscope grids (50,000～180,000℃/min) (Martino *et al.,* 1996), and OPS methods (over 20,000℃/min) (Vajta *et al.,* 1998). However, these strawpaste methods were used very small volume of cryoprotectant such as the OPS technique used 1～2 μl volume into 0.25 ml straw (Vajta *et al.,* 1998). The small volume of cryoprotectant was helpful for success of pregnancy through embryos transfer of human as well as animals (Green *et al.,* 2009; Stanger *et al.,* 2012).

OPS technique was proven as successful for in-straw dilution following vitrification of bovine embryos (Vajta *et al.,* 1999). This technique was allowed to thawing and dilution of vitrified embryos in one-step dilution. However, recently studies desired to allow direct transfer to recipients without use of a microscope or other laboratory equipment. This technique for direct transfer of vitrified embryos requires skillful operators for proper handling of the embryo during in straw warming. Like this, a novel vitrification through cryotop device was innovated similar method with OPS (Kitzato bio pharama, Fuinomiya, Japan) (Inaba *et al.,* 2011).

In the current study, the post-thaw survivability of 8-cell and blastocysts stage were up to 70.9% and 94.8% (Table 1). Whereas, the cell number of blastocysts and apoptosis index is an important indicator of embryo quality. In our investigation, the total number of cells was significantly higher in non-frozen blastocyst and post-thaw blastocyst compared with post-thaw 8-cell. The apoptosis of 8-cell and blastocyst was not significantly different compared with control group as shown Table 2. The aV method was high frozen velocity and temperature than other methods as OPS, Cryotop. This had low affected to frozen-damage about cryoprotectants toxicity. Also, this was improved post-thaw survivability of 8-cell and blastocyst embryos and reduced apoptotic cell number compared with non-frozen blastocyst stage (Table 2, Fig. 1).

The important factors related to successful vitrification are including the decrease of frozen temperature and the increase

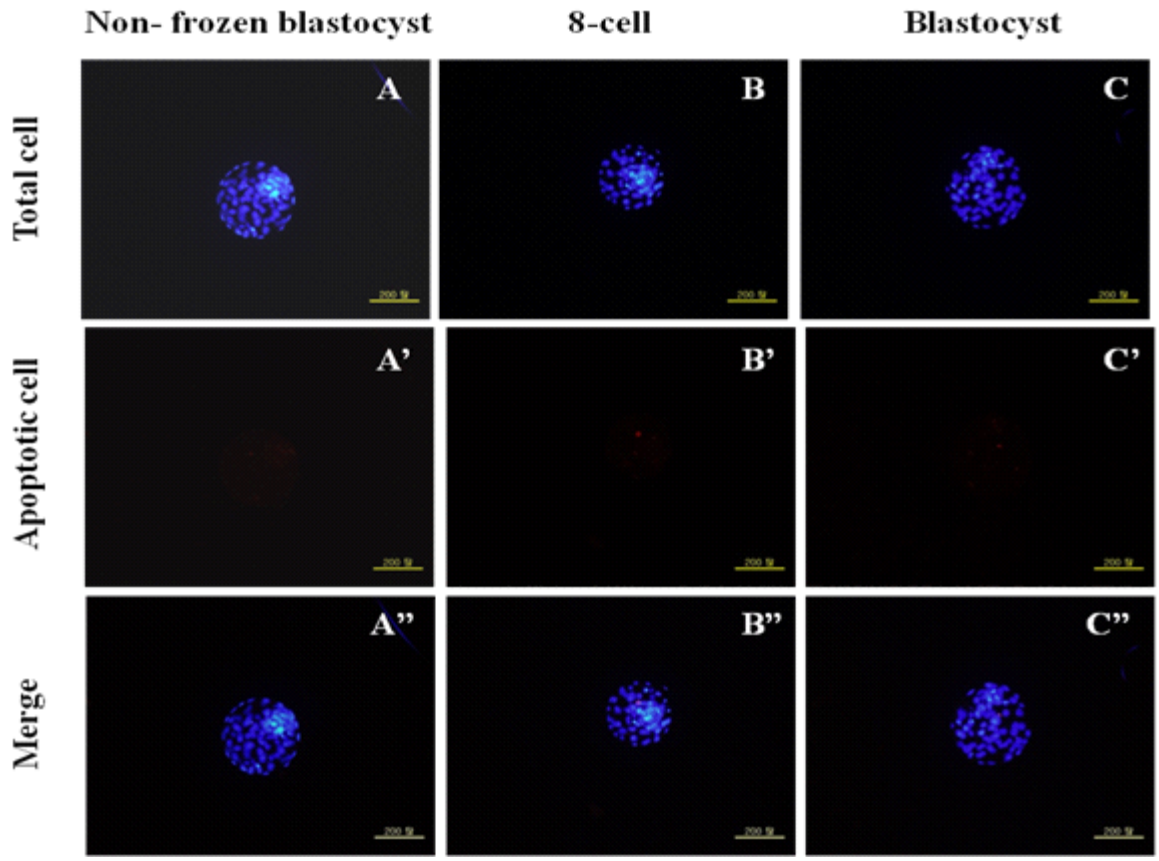

Fig. 1. The photographics shown apoptosis incidence of non-frozen blastocyst, post-thawed blastocysts and 8-cell stage of mouse. All treatments ware appeared according to the order of total cell, apoptotic cell and Merge photograph. Non-frozen blastocyst is A, A', A'', frozen blastocyst group is B, B', B'', and frozen 8-cell stage is C, C', C''.

of cooling rates of vitrification solutions. An important role in cooling rate of solution is attributed to the freezing container, thermal conductivity and the volume of vitfirication solution (Tamada *et al.,* 2008). Hredzak *et al.,* (2005) demonstrated that different carrier systems (containers) into which the embryos in a cryoprotectant solution are loaded. In addition, various cross-contamination factors are important factor for hazard of embryos. These factors are infected through mediator as donors, serum, culture medium, culture cells, incubators as culture system and liquid nitrogen (Kuleshova and Shaw, 2000). The various bacteria and pathogen have existed in LN_2. It is recognized that pathogen was tolerated and hold at low temperature of LN_2. That was very harmful to embryonic development. The advantage of the majority systems, the embryo is not direct contact with liquid nitrogen which can't become a source of its contamination by vitrification solution (Bielanski, 2012). In this experiment, production of mice pups after transfer of post-thaw embryos was highly efficient rather than other methods. The dilution of cryoprotectant agents use plastic straw allowed embryos to be promptly prepared for direct embryo transfer without the need of laboratory equipment in bovine (Vieira *et al.,* 2008). Therefore, the vitrification of embryos using the paste in straw methods for in-straw dilution and direct transfer into recipients compared with step-wise removal of the cryoprotectant solution would be simplified an embryo transfer procedures and can be applied in assist reproductive technology in mouse and human embryos.

REFERENCES

Bielanski A. 2012. A review of the risk of contamination of semen and embryos during cryopreservation and measures to limit cross-contamination during banking to prevent disease transmission in ET practices. Theriogenology 77:

467-482.

Chen SU, Lien YR, Chen HF, Chao KH, Ho HN and Yang YS. 2000. Open pulled straws for vitrification of mature mouse oocytes preserve patterns of meiotic spindles and chromosomes better than conventional straws. Hum. Reprod. 15:2598-2603.

Dhali A, Anchamparuthy VM, Butler SP, Pearson RE, Mullarky IK and Gwazdauskas FC. 2009. Effect of droplet vitrification on development competence, actin cytoskeletal integrity and gene expression in *in vitro* cultured mouse embryos. Theriogenology 71:1408-1416.

Dochi O, Yamamoto Y, Saga H, Yoshiba N, Kano N, Maeda J, Miyata K, Yamauchi A, Tominaga K, Oda Y, Nakashima TandInohae S. 1998. Direct transfer of bovine embryos frozen-thawed in the presence of propylene glycol or ethylene glycol under on-farm conditions in an integrated embryo transfer program. Theriogenology 49:1051-1058.

El-Gayar M, Gauly M and Holtz W. 2008. One-step dilution of open-pulled-straw (OPS)-vitrified mouse blastocysts in sucrose-free medium. Cryobiology 57:191-194.

Green RE, Santos BF, Sicherle CC, Landim-Alvarenga FC and Bicudo SD. 2009. Viability of OPS vitrified sheep embryos after direct transfer. Reprod. Domest. Anim. 44:406-410.

Hotamisligil S, Toner M and Powers RD. 1996. Changes in membrane integrity, cytoskeletal structure, and developmental potential of murine oocytes after vitrification in ethylene glycol. Biol. Reprod. 55:161-168.

Hredzak R, Ostro A, Maracek I, Kacmarik J, Zdilova V and Vesela J. 2005. Influence of slow-rate freezing and vitrification on mouse embryos. Acta. Vet. Brno. 74: 23-27.

Inaba Y, Aikawa Y, Hirai T, Hashiyada Y, Yamanouchi T, Misumi K, Ohtake M, Somfai T, Kobayashi S, Saito N *et al*. 2011. In-straw cryoprotectant dilution for bovine embryos vitrified using Cryotop. J. Reprod. Dev. 57:437-443.

Isachenko V, Montag M, Isachenko E, Zaeva V, Krivokharchenko I, Shafei R and van der Ven H. 2005. Aseptic technology of vitrification of human pronuclear oocytes using open-pulled straws. Hum. Reprod. 20:492-496.

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.

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

Kong IK, Lee SI, Cho SG, Cho SK and Park CS. 2000. Comparison of open pulled straw (OPS) vs. glass micropipette (GMP) vitrification in mouse blastocysts. Theriogenology 53:1817-1826.

Kuleshova LL and Shaw JM. 2000. A strategy for rapid cooling of mouse embryos within a double straw to eliminate the risk of contamination during storage in liquid nitrogen. Hum. Reprod. 15: 2604-2609.

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.

Martino A, Songsasen N and Leibo SP. 1996. Development into blastocysts of bovine oocytes cryopreserved by ultrarapid cooling. Biol. Reprod. 54:1059-1069.

Palasz AT and Mapletoft RJ. 1996. Cryopreservation of mammalian embryos and oocytes: recent advances. Biotechnol. Adv. 14:127-149.

Pugh PA, Tervit HR and Niemann H. 2000. Effects of vitrification medium composition on the survival of bovine *in vitro* produced embryos, following in straw-dilution, *in vitro* and *in vivo* following transfer. Anim. Reprod. Sci. 58:9-22.

Quinn P and Kerin JF. 1986. Experience with the cryopreservation of human embryos using the mouse as a model to establish successful techniques. J. *In Vitro* Fert. Embryo Transf. 3:40-45.

Rall WF and Fahy GM. 1985. Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. Nature 313:573-575.

Saha S, Otoi T, Takagi M, Boediono A, Sumantri C and Suzuki T. 1996a. Normal calves obtained after direct transfer of vitrified bovine embryos using ethylene glycol, trehalose, and polyvinylpyrrolidone. Cryobiology 33:291-299.

Saha S, Rajamahendran R, Boediono A, Sumantri C and Suzuki T. 1996b. Viability of bovine blastocysts obtained after 7, 8 or 9 days of culture in vitro following vitrification and one-step rehydration. Theriogenology 46:331-343.

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.

Stanger J, Wong J, Conceicao J and Yovich J. 2012. Vitrification of human embryos previously cryostored by either slow freezing or vitrification results in high pregnancy rates. Reprod. Biomed. Online 24:314-320.

Tamada T, Siriaroonrat B, Subramaniam V, Hamachi M, Lin LK, Oshida T, Rerkamnuaychoke W and Masuda R. 2008. Molecular diversity and phylogeography of the Asian leopard cat, *Felis bengalensis*, inferred from mitochondrial and Y-chromosomal DNA sequences. Zoology Sci. 25:154-163.

Taniguchi M, Ikeda A, Arikawa E, Wongsrikeao P, Agung B, Naoi H, Nagai T and Otoi T. 2007. Effect of cryoprotectant composition on *in vitro* viability of *in vitro* fertilized and cloned bovine embryos following vitrification and in-straw dilution. J. Reprod. Dev. 53:963-969.

Vajta G, Holm P, Greve T and Callesen H. 1996. Factors affecting survival rates of *in vitro* produced bovine embryos after vitrification and direct in-straw rehydration. Anim. Reprod. Sci. 45:191-200.

Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T and Callesen H. 1998. Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol. Reprod. Dev. 51:53-58.

Vajta G, Rindom N, Peura TT, Holm P, Greve T and Callesen H. 1999. The effect of media, serum and temperature on *in vitro* survival of bovine blastocysts after Open Pulled Straw (OPS) vitrification. Theriogenology 52:939-948.

Van Den Abbeel E and Van Steirteghem A. 2000. Zona pellucida damage to human embryos after cryopreservation and the consequences for their blastomere survival and *in-vitro* viability. Hum. Reprod. 15:373-378.

Vanderzwalmen P, Bertin G, Debauche C, Standaert V, Bollen N, van Roosendaal E, Vandervorst M, Schoysman R and Zech N. 2003. Vitrification of human blastocysts with the Hemi-Straw carrier: application of assisted hatching after thawing. Hum. Reprod. 18:1504-1511.

Vieira AD, Forell F, Feltrin C and Rodrigues JL. 2008. Calves born after direct transfer of vitrified bovine *in vitro*-produced blastocysts derived from vitrified immature oocytes. Reprod. Domest. Anim. 43:314-318.

Yang QE, Hou YP, Zhou GB, Yang ZQ and Zhu SE. 2007a. Stepwise in-straw dilution and direct transfer using open pulled straws (OPS) in the mouse: a potential model for field manipulation of vitrified embryos. J. Reprod. Dev. 53: 211-218.

Yang ZQ, Zhou GB, Hou YP, Yan CL and Zhu SE. 2007b. Effect of in-straw thawing on *in vitro*- and *in vivo*-development of vitrified mouse morulae. Anim. Biotechnol. 18: 13-22.

(접수: 2013. 4. 29 / 심사: 2013. 4. 30 / 채택: 2013. 5. 24)