韓國家畜繁殖學會誌 23(1) : 85~92(1999)
Korean J. Animal Reprod.

Effect of Cryoprotectant Kinds and Cell Stages on the Viability of Mouse Embryos Cryopreserved by OPP Vitrification

Kong, I. K., S. G. Cho and S. K. Cho*

Department of Animal Science, Sunchon National University, Sunchon

동결보호제의 종류 및 배발달단계가 OPP Vitrification 동결보존시 생쥐수정란의 생존성에 미치는 영향

공일근 · 조성균 · 조성근*

순천대학교 농과대학 동물자원과학과

요 약

본 연구는 동결동결보호제의 종류와 배발달단계가 생쥐의 OPP vitrification 동결방법에 미치는 영향을 알아보고자 실시하였다. 동결속도, 동결보호제 및 배발달단계는 vitrification 방법에 따른 수정란의 생존성에 영향을 미칠 수 있다. 본 연구에 사용된 동결보존액은 40% (v /v) ethylene glycol, 18% (w/v) Ficoll , 0.3 M sucrose와 5% FCS가 첨가된 D-PBS (EFS) 및 16.5% ethylene glycol, 16.5% dimethyl sulfoxide, 0.5 M sucrose와 5% D-PBS (EDS)을 이용하였다. 배반포기배는 hCG 처리후 90시간째에 자궁으로부터 채취하여 실험 1에 이용하였고, 실험 2와 3에서는 zygote를 hCG 처리후 18시간에 난관에서 채취하여 mHTF 배양액에 5% CO_2, 37℃조건하에 배양하면서 2-, 4-, 8-cell, compacted morula, 또는 blastocyst를 이용하였다. 실험 1에서 배반포기배의 적당한 동결보존액을 결정하기 위하여 EFS 또는 EDS로 OPP vitrification을 실시하였다. 재확장배반포기에 의한 생존율은 대조군과 EDS 처리군 (100, 100%)이 EFS 군 (95.0%)보다 유의적 ($P<0.05$)으로 높게 나타났으나, 부화배반포기에서는 EFS군 (90.0%)이 대조군 (100%) 및 EDS군 (95.0%)보다 유의적으로 낮은 발달율을 보였다. 실험 2에서는 zygote, 2-, 4-, 8-cell, 상실배 및 배반포기 등의 초기배에서도 OPP vitrification 동결방법이 적당한지를 판단하기 위하여 실시하였다. Zygote (70.0%)는 동결융해 후 배발달율이 2, 4, 8, 상실배 및 배반포기배에 비하여 유의적으로 낮은 발달율을 보였다 (89.7, 90.0, 92.8, 97.6 및 97.5%) ($P<0.05$). 또한 동결융해란의 할구수에서는 대조군 및 배반포기배 (35.7±2.98 및 39.6±2.81)에서 zygote, 2-, 4- 8-cell, 상실배 (29.8±3.21, 31.3±3.83, 29.3±3.58, 28.9±3.21 및 30.8±2.93)보다 유의적으로 높게 나타났다 ($P<0.05$). 실험 3에서는 zygote의 VS1에 노출시간에 따른 생존율을 조사한 결과 융해후 2-cell (91.6, 88.5 및 88.9%) 및 배반포기 (83.3, 74.3 및 69.4%)까지 배발달율은 1, 2 및 3분간의 노출시간에 따른 유의적인 차이를 보이지 않았다. 또한 융해후 노출시간에 따른 할구수에서도 유의적인 차이를 보이지 않았다 (36.4±4.76, 32.4±4.67 및 27.6±4.52).

이상의 결과에서 OPP vitrification 방법은 EFS 또는 EDS 동결보존액에 따른 유의적인 차이 없이 이용될 수 있는 것으로 판단된다. 배발달단계에 따른 생존율은 zygote의 초기배는 2, 4, 8, 상실배 및 배반포기보다 유의적으로 저조한 생존율을 보였다. Zygote의 VS1에 노출시간에 따른 생존율도 1분간의 노출시간에서 높은 배발달율을 보였다. OPP vitrification 동결보존방법으로 생쥐수정란의 동결보존에 유용하게 이용가능한 것으로 판단된다.

(Key words : Cryopreservation, Mouse, Embryo, OPP)

* 경상대학교 축산학부 (Department of Animal Science, Gyeongsang National University)

I. INTRODUCTION

Vitrification is the solidification of the solution brought about not by crystalization but by elevation in viscosity during cooling. Since Rall and Fahy (1985) first demonstrated that the vitrification is an effective cryopreservation procedure of mouse embryos (8-cell stage), vitrification has been widely used and is now regarded as a potential alternative to transitional slow-rate freezing. There was many reports of successful vitrification with various cryoprotectants in several species embryos, in which was used as VS3a method (Rall and Wood, 1994; Dinnyes et al., 1995), EFS method (Kasai et al., 1990; Tachikawa et al., 1993), ethylene glycol/PVP (Leibo and Oda, 1993) and ethylene glycol/DMSO (Vajta et al., 1998a; Lane et al., 1999). When embryos are cryopreserved by vitrification, ice crystal formation is prevented by use of high concentration of cryoprotectants and high cooling and warming rates. Acceleration of the speed of temperature changes may offer two advantages: decreased cryoprotectant concentration would be required with consequent lower osmotic and toxic effects, and less severe chilling injury as a result of the rapid passage through the "dangerous" temperature zone (Vajta et al., 1998a).

The efficiency of vitrifying embryos has been markedly improved by increasing the speed of cooling and warming. Three techniques have been established for this purpose: direct immersing into LN_2 (Landa and Tepla, 1990; Riha et al., 1991), using an electron microscopy grid to provide a support (Martino et al., 1996; Kim et al., 1998), OPS method (Vajta et al., 1998b) and OPP method (Kong et al., 1999). The preimplantation mouse embryos were very sensitivity to vitrification solution and stage-dependent viability (Tada et al., 1993).

The OPP vitrification method has recently been developed in our laboratory. The purpose of the present work was to establish the vitrification method which can be used with the same incubation parameters for EFS or EDS vitrification solutions and for preimplantation stages of mouse embryos.

II. MATERIALS AND METHODS

All chemicals except those otherwise indicated were Sigma products.

1. Animals and embryo collection

Immature (3- to 5 week-old) male and female C57BL/6 mice were purchased from Chemical Research Institute (Daeduk, Korea). Males were housed in individual cages and female in groups of six to eight in a light-controlled room (14 h light on at 06:00) in which was placed in the facilities of the Experimental Station in Department of Animal Science, Sunchon National University. Zygotes were collected from immature female that were induced to superovulate with 5 IU PMSG (Sankyozoki Co, Japan) followed 48 h later 5 IU hCG (Yuhan Co, Korea). The female were mated with fertile C57BL/6 males immediately after the hCG injection, and were sacrificed by cervical dislocation 20 h later. The zygotes were flushed from the excised oviduct, and removed the cumulus by exposing in hyaluronidase for 2 min and rinsed three times with sterile D-PBS supplemented with 0.3% BSA (Whittingham, 1974). All of the zygotes collected were cultured in mHTF supplemented with 10% FCS to 2-, 4-, 8-cell, compacted morula and blastocyst stage for 21, 34, 45, 69 and 76 h.

2. Making of OPP straw

OPP straws were also made with capillary glass pipette (outer/inner diameter: 1.0/0.8 mm, thin microcapillary pipette; Drummond Sci Co., USA) by the minor modification of Vajta et al. (1998a) and Kong et al. (1999). The capillary glass pipette were pulled by puller until the inner diameter of central part decreased from 1.0 mm to approximately 0.3 mm. The OPP straws were cooled in air, then cut at the narrowest point with diamond tip. The OPP straws were sterilized with 70% ethanol, which was flushing with ethanol and dried in clean bench.

3. Vitrification procedure

Embryos were vitrified as reported previously using EFS (Kasai et al., 1993) and EDS solution (Vajta et al., 1998a). For EFS vitrification, embryos were suspended in EFS solution on a dish and then washed in the solution twice more. They were loaded into OPP straw 1 min after exposure in EFS. For EDS vitrification, embryos were first incubated in VS1 for 1 min, and then transferred in approximately 1 to 2 μl of solution into a 20 μl droplet of VS2. Embryos were mixed quickly by pipetting, then another drop containing approximately 1 to 2 μl solution and embryos was made using a 10 μl automatic pipette. The time between the contact of the embryos with the concentrated cryoprotectant solution and cooling did not exceed 25 sec. The OPP method have to control the capillary reaction with finger by carefully, because OPP method was very sensitive of capillary phenomenon. If 1 to 2 μl VS2 containing embryos could be loading into the wide column, it might be decrease the survival rate after vitrification because of reducing of freezing speed.

4. Warming and cryoprotectant dilution

Warming was performed by immersion the end of the straw containing the embryos into 1.2 ml of 0.25 M sucrose in HM prepared in the bottom of the 4 well dish. After 1 min, all embryos were transferred into 1.0 ml 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 controlled approximately 35℃.

5. Assessment of embryo viability

The survival rates of vitrified embryos were determined by development to expanded blastocysts at 92, 71, 47, 19, and 12 h after post-thaw in vitro culturing according to zygote, 2, 8, compacted morula and blastocyst stage, respectively. The embryos were cultured in mHTF supplemented with 10% FCS under sterile silicone oil (Junsei Chem Co., Japan) at 37℃ in a humid atmosphere of 5% CO_2.

6. Statistical analysis

Data were analyzed by chi-square tests with SAS program.

Ⅲ. RESULTS

1. Survival rates according to kind of cryoprotectants

To determine the optimal cryoprotectants in OPP vitrification method, the blastocysts were vitrified by OPP methods with EFS or EDS vitrification solution. As shown in Table 1, the post-thaw survival rates at re-expanded stage rates were significantly different between EFS and EDS (95.0 vs 100%), but at hatching stage was not different between EFS and EDS (90.0 vs 95.0%). The OPP vitrification method could be useful in mouse blastocyst stage either EFS or EDS vitrification solution.

2. Effect of Cell stage on survival rates

To determine the sensitivity of various cell

stage in mouse embryos, zygote, 2-, 4-, 8-cell, compacted morula or blastocysts were vitrified by OPP method with EDS solution. As shown in Table 2, the development rates to expanded blastocyst in zygote (70.0%) were significantly lower rather than those in 2-, 4-, 8-cell, compacted morula or blastocyst (89.7, 90.0, 92.8, 97.6 and 97.5%), respectively. The early stage in mouse embryos as zygote was more sensitivity than that in late stage embryos in OPP vitrification with EDS vitrification solution. However, the cell number of post-thaw developed to expanded blastocyst in blastocyst and control blastocyst stage (39.6±2.81, 35.7±2.98) were significanty higher than those in zygote, 2-, 4-, 8-cell, compacted morula (29.8±3.21, 31.3±3.83, 29.3±3.58, 28.9±3.21 or 30.8±2.93).

3. Development capacity of zygote following various exposed time in VS1

In our system, the duration of exposure to a VS1 is one of the key factors affecting the survival of vitrified mouse 1-cell embryos (Tada et al., 1993). Thus, we investigated the survival rates of mouse zygotes embryos vitrified using OPP method with EDS solution, after exposure to them for 1, 2, or 3 min at room temperature. As shown in Table 3, the cleavage rates (91.6, 88.5, 88.9%) and developmental rates to blastocyst (83.3, 74.3 and 69.4%) depends on the exposed time in VS1 were not significantly different among 1, 2, or 3 min, respectively. The cell number also were not significantly different among exposed time in VS1.

Ⅳ. DISCUSSION

This study showed a rapid and efficient cryo-

Table 1. Effect of cryoprotectant kinds on survival of mouse blastocyst following OPP vitrification

Kind of cryoprotectants	No. of embryos (%)		
	Vitrified	Re-expanded	Hatching
Control	40	40(100)^{ab}	40(100)^{a}
EFS	60	57(95.0)^{b}	54(90.0)^{b}
EDS	60	60(100)^{a}	57(95.0)^{ab}

* No. of vitrified in control was not vitrified, but just cultured for 24 h as same time.

* Values with different superscripts in same column were denoted significantly different ($P<0.05$).

Table 2. Effect of cell stage on survival rates and cell number of mouse embryo after OPP vitrification

Cell stage	No. of embryos (%)		No. of blastomeres
	Vitrified	Exp blastocyst	Counted (Mean±S.E.) /embryo
Zygote	40	28(70.0)^{b}	29.8±3.21 /10^{b}
2-cell	39	35(89.7)^{a}	31.3±3.83 / 7^{ab}
4-cell	40	36(90.0)^{a}	29.3±3.58 / 8^{b}
8-cell	42	39(92.8)^{a}	28.9±3.21 / 9^{b}
Comp morula	42	41(97.6)^{a}	30.8±2.93 /12^{b}
Blastocyst	40	39(97.5)^{a}	39.6±2.81 /13^{a}
Control blast	40		35.7±2.98 / 7^{a}

* Values with different superscripts in same column were denoted significantly different ($P<0.05$).

Table 3. Effect of exposed time in VS1 on post-that survival of zygote following OPP method

Exposed time in VS1 (min)	No, of zygotes developed to (%)			No. of blastomeres
	Vitrified	2-cell	Blastocyst	Counted (Mean±S.E.) /Embryos
1	36	33(91.6)	30(83.3)	36.4±4.76 / 9
2	35	31(88.5)	26(74.2)	32.4±4.67 / 9
3	36	32(88.9)	25(69.4)	27.6±4.52 /10

* No siginificantly different in the same column.

preservation of mouse preimplantation embryos at zygote, 2-, 4-, 8-cell, compacted morula or blastocyst stages by the OPP vitrification using EFS or EDS vitrification solution, and also demonstrated that zygotes showed a maximal survival rates for 1 min VS1 exposing time rather than 2 or 3 min and very sensitivity in VS1 exposure time compared with other cell stage embryos. The OPP vitrification methods with EFS or EDS vitrification solution were determined to be very successful for cryopreservation of mouse blastocysts. The OPP methods at blastocyst can get over at least 95.0% in re-expanding or 90.0% in hatching rates. Kong et al. (1999) reported that OPP method does not need any cap to protect floating of the straw after LN_2 immersion as well as increase the freezing speed by diminish the pipette size and column of embryos including of vitrification solution. The OPP straws were optimal for commercial use in animal fields, because it could be made easily and cheaper rather than OPS straw. If we could increase the freezing speed, the survival rates might be improved following vitrification of any species of embryos. The size of OPP glass pipette could be decreased approximately 0.3 mm o.d. easily. The weight of OPP straw was a little heavy rather than OPS straw (0.098 vs 0.070 g). The volume of OPP straw was 19 times smaller rather than OPS straw (0.14 vs 2.68 mm^3), when the volume loaded was calculated by loading into 10 mm narrow column (Kong et al., 1999). We guess that under 4 embryos might be applicable to commercial use in farm animal as well as human embryo transfer. The embryos loaded into OPP straw were limited under 4 embryos. Vajta et al. (1998a) demonstrated that the open pulled straw (OPS) method, which renders very high cooling and warming rates (over −20,000℃ /min) and short contact with concentrated cryoprotective additives (less than 30 sec) offers a possibility to circumvent chilling injury and to decrease toxic and osmotic damage. OPP method can get more rapid freezing speed and heat conductivity, because it made of the capillary glass pipette. The only disadvantage of OPP method as well as OPS, electron microscopy grid is the potential hazard of contamination as the embryo holding medium is directly in contact with liquid nitrogen (Tedder et al., 1995; Vajta et al., 1998a). It has been shown that the viability of vitrified embryos depends on their stage of development in mouse (Scheffen et al., 1986; Nakagata, 1989; Li and Trounson, 1991). Tada et al. (1993) reported that exposure of 1-cell embryos to DPS for 15 sec at room temperature and subsequent vitrification in DPS yielded high cryosurvival rate (80%). However, longer exposure (30 sec) greatly reduced the cryosurvival rate (33%), suggesting that mouse 1-cell embryos may be more sensitive to the vitrification process we used than embryos any other advanced stages of development. Similar tendency was also observed in our system, zygotes for 1 min exposed time (83.3%) were better post-thaw survival rate rather than

that in 2 or 3 min exposed time (74.2 or 69.4%), even it was not significantly different among the exposed time.

In conclusion, the OPP method has been established to increase the speed of freezing and warming, survival rates, and decrease embryo damage by reducing of straw size. Thus, the OPP method can be very useful in cryopreserving mouse preimplantation embryos either with EFS or EDS vitrification solution. On the other hand, vitrification of zygotes will need further improvement.

V. SUMMARY

This study was designed to determine effect of cryoprotectant kinds and cell stages on OPP vitrification method in mouse embryos. The freezing speed, cryoprotectants and cell stage could affect of embryo viability following various vitrification methods. The vitrification solution used were consisting of 40% (v/v) ethylene glycol, 18% (w/v) Ficoll , 0.3 M sucrose solution in holding medium (D-PBS supplemented with 5% FCS: HM) (EFS) or 16.5% ethylene glycol, 16.5% dimethyl sulfoxide, 0.5 M sucrose in HM (EDS). The embryos were collected from oviduct at 18 h after hCG injection and then washed and cultured in mHTF medium until use. **In experiment 1**, the blastocysts were vitrified by OPP straw to determine the optimal vitrification solution of EFS or EDS. The post-thaw survival rates at re-expanded stage rates were significantly different between EFS and EDS (95.0 vs 100%), but at hatching stage was not different between EFS and EDS (90.0 vs 95.0%). respectively. **In experiment 2**, zygotes, 2-, 4-cell, morula and blastocysts were vitrified by OPP method to determine the acceptable of early stage embryos. The development rates to expanded blastocyst in zygote (70.0%) were significantly lower rather than those in 2-, 4-8-cell, compacted morula or blastocyst (89.7, 90.0, 92.8, 97.6 or 97.5%), respectively. However, the cell number of post-thaw developed to expanded blastocyst in blastocyst and control blastocyst stage (39.6±2.81, 35.7±2.98) were significanty higher than those in zygote, 2-, 4-, 8-cell, compacted morula (29.8±3.21, 31.3±3.83, 29.3±3.58, 28.9±3.21 or 30.8±2.93). **In experiment 3**, the zygotes were exposed in VS1 for 1, 2, and 3 min to the optimal exposed time. The cleavage rates (91.6, 88.5, 88.9%) and developmental rates to blastocyst (83.3, 74.3 and 69.4%) depends on the exposed time in VS1 were not significantly different among 1, 2, or 3 min, respectively. The cell number also were not significantly different among exposed time in VS1. respectively.

These results indicate that OPP method could be useful for vitrification either EFS or EDS vitrification solution. The post-thaw survival rates at zygote were significantly lower than those at 2-, 4-, 8-cell, morula or blastocyst, respectively. The zygote stage were more sensitive rather than late stage embryos. The exposing time in VS1 for 1 min was better than that for 2 or 3 min, even it was not significantly different. The OPP vitrification method could be useful of mouse embryos either with EFS or EDS vitrification solution.

VI. REFERENCES

1. Kasai, M., J. H. Komi, A. Takakamo, H. Tsunoda, T. Sakurai and T. Machida. 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.
2. Kim, E.Y., N.H. Kim, B.K. Yi, S.H. Yoon, S.P. Park, K.S. Chung and J.H. Lim.

1998. Developmental capacity of bovine follicular oocytes after ultra-rapid freezing by electron microscope grid. II. Cryopreservation of *in vitro* matured bovine oocytes. Korean J. Anim. Reprod., 22:1-9.

3. Kong, I.K., S.K. Cho, S.I. Lee and S.K. Cho. 1999. Comparison of open pulled straw (OPS) vs open pulled capillary glass pipette (OPP) vitrification in mouse embryos. 32nd Annual Meeting of SSR at Washington State Univ, Pullman, Washington, USA, 1999 (inpress).
4. Landa, V. and O. Tepla. 1990. Cryopreservation of mouse 8-cell embryos in microdrops. Folia. Biologica. (Praha) 36:153-158.
5. Lane, M., K.T. Forest, E.A. Lyons and B.D. Bavister. 1999. Live births following vitrification of hamster embryos using a novel containerless techniques. Theriogenology, 51: 167(abstr).
6. Leibo, S.P. and K. Oda. 1993. High survival of mouse zygotes and embryos cooled rapidly or slowly in ethylene glycol plus polyvinylpyrrolidone. Cryo-Letters, 14:133-144.
7. Li, R. and A. Trounson. 1991. Rapid freezing of the mouse blastocysts: Effects of cryoprotectants and of time and temperature of exposure to cryoprotectant before direct plunging into liquid nitrogen. Reprod. Fertil. Dev., 3;175-183.
8. Martino, A., N. Songsasen and S.P. Leibo. 1996. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol. Reprod., 54:1059-1069.
9. Mazur, P. 1984. Freezing of living cells: mechanisms and implications. Am. J. Physiol., 247:C125-C142.
10. Nakagata, N. 1989. Survival of mouse embryos derived from *in vitro* fertilization after ultrarapid freezing and thawing. J. Mamm. Ova. Res., 6:23-26.
11. Niemann, H. 1991. Cryopreservation of ova and embryos from livestock: Current status and research needs. Theriogenology,, 35:109-124.
12. Rall, W.F. 1992. Cryopreservation of oocytes and embryos: methods and applications. Anim. Reprod., 28:237-245.
13. Rall, W.F. and G.M. Fahy. 1985. Ice-free cryopreservation of mouse embryos at −196℃ by vitrification. Nature, 313:573-575.
14. Rall, W.F. and M.J. Wood. 1994. High *in vitro* and *in vivo* survival of Day 3 mouse embryos vitrified or frozen in a non-toxic solution of glycerol and albumin. J. Reprod. Fertil., 101:681-688.
15. Riha, J., V. Landa, J. Kneissl, J. Matus, J. Jindra and Z. Kloucek. 1991. Vitrification of cattle embryos by direct dropping into liquid nitrogen and embryo survival after non-surgical transfer. Zivoc. Vir., 36:113-120.
16. Scheffen, B., P. Van Der Zwalmen and A. Massip. 1986. A simple and efficient method for preservation of mouse embryos by vitrification. Cryo-Letter, 7:260-269.
17. Tachikawa, S., T. Otoi, S. Kondo, T. Machida and M. Kasai. 1993. Successful vitrification of bovine blastocysts, derived by *in vitro* maturation and fertilization. Mol. Reprod. Dev., 34:266-271.
18. Tada, N., M. Sato, E. Amann and S. Ogawa. 1993. Stage-dependent viability of mouse preimplantation embryos vitrified with sugar-containing solutions. J. Reprod. Dev., 39:139-144.
19. Tedder, R.S., M.A. Zuckerman, A.H. Goldstone, A.E. Hawkins, A. Fielding, E.M. Briggs, D. Irwin, S. Blair, A.M. Gorman, G. Patterson, D.C. Linch, J. Heptonstall and N.S. Brink. 1995. Hepatitis B transmission from contaminated cryopreservation

tank. Lancet, 346:137-140.
20. Vajta, G., P. Holm, M. Kuwayama, P.J. Booth, A. Jacobsen, T. Greve and H. Callesen. 1998a. Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol. Reprod. Dev., 51:53-58.
21. Vajta, G., I.M. Lewis, M. Kuwayama, T. Greve and H. Callesen. 1998b. Sterile application of the open pulled straw (OPS) vitrification method. Cryo-Letters, 19:389-392.
22. Whittingham, D.G. 1980. Principles of embryo preservation. In Ashwood-Smith MJ and Farrant J (eds), Low Temperature Preservation in Medicine and Biology. Pitman. Medical. Ltd, UK, 1980;65-83.
(접수일자 : 1999. 2. 12. /채택일자 : 1999. 3. 5.)