韓國受精卵移植學會誌(2005) 第 20 卷 第 2 號
Korean J. Emb. Trans. (2005) Vol. 20, No. 2, pp. 123~128

Effects of Kinds and Concentrations of Cryoprotectants, Trehalose, Sucrose-Addition in Cryoprotectants on the Survival Rates of Vitrification-Thawed Porcine IVM/IVF Embryos

J. G. Lim[1], J. H. Quan, K. S. Lee[1] and S. K. Kim[†]

College of Veterinary Medicine, Chungnam National University

돼지 난포란의 내동제의 종류와 농도 및 Trehalose, Sucrose 등의 첨가가 Vitrification 동결 융해 후 생존율 및 수정율에 미치는 영향

임정훈[1] · 전연화 · 이규승[1] · 김상근[†]
충남대학교 수의과대학

SUMMARY

This study examines the effects of kinds and concentrations of cryoprotectants on the survival rate of vitrification-thawed porcine oocytes, together with the effects on survival, *in vitro* fertilization and development of immature oocytes.

1. The developmental rate of oocytes to M Ⅱ and diploid stage when the vitrification-thawed of recovered immature oocytes cultured for 0, 15, 30 and 40 h were cultured for 0, 15, 30 and 40 h were 56.7%, 53.3% 63.3%, 65.0% and 23.3%, 18.3%, 10.0%, 3.3%, respectively. The *in vitro* development to M Ⅱ stage were lower than the control group (78.2%), but higher for diploid stage (5.5%).
2. When the vitrification of immature oocytes after being culture for 0, 15, 30 and 40 hours, the survival rate were 34.0%, 26.0%, 18.0% and 10.0% respectively. This result was lower than that of the control group (60.0%).
3. When the fertilization of the vitrified immature oocytes after being culture for 0, 15, 30 and 40 hours, the *in vitro* fertilization rate were 60.0%, 54.0%, 48.0%, 38.0%, and developmental rates were 26.0%, 18.0%, 8.0%, 4.0%, respectively. This results were lower than the control group (78.0% and 38.0%).
4. When the fertilization of the immature oocytes after being culture for 0~15 hours vitrified with EDS and ETS, the fertilization and developmental rates were 50.0%, 22.0% and 46.0%, 18.0%, respectively. This results were lower than the control group (74.0% and 38.0%).

(Key words : vitrification of immature oocytes, survival and fertilization rate)

INTRODUCTION

Research on embryo cryopreservation about survival of embryos (Schmidt *et al.*, 1993; Leibo, 1993) or oocytes (Suzuki and Nishikata, 1992; Robinski *et al.*, 1991; van Blerkom, 1989) after frozen-thawed

[1] College of Agriculture and Life Science, Chungnam National University
[†] Correspondence : E-mail : kskkim@cnu.ac.kr

have been reported but there was much difference between the reporters and their results. Recently the study of embryo vitrification are being conducted because the embryos are kept in over-cooling while preventing water from hydrating and ice crystal formation with the addition of high concentrations of cryoprotectants in vitrification solution (Rall and Fahy, 1985; Kasai *et al.*, 1990; Vaita *et al.*, 1998; Cuello *et al.*, 2004). Rall (1992) and Hamlett *et al.* (1989) reported that embryo cells exposed with cryoprotectants during freezing in metaphase I or II stages had damage of the spindle fiber and external granule. Mazur (1970) reported that during freezing, the main reason that the cells died was because the in the cell had ice crystallization and thawing influence and because of this have reported that reasonable equilibrium time is needed. Renard *et al.* (1984) reported that freezing with short equilibrium time was capable of two step freezing with the addition of the non-permeable sucrose. Rall and Fay (1985) reported that freezing the early immature oocytes at different developmental stages was more appropriate than the mature G-V stage (van der Elst *et al.*, 1993; Candy *et al.*, 1994; Toth *et al.*, 1994). In vitrification of the oocytes, the factors that influence on the survival are the toxicity of the cryoprotectants, the composition of the vitrification solution and the freezing and thawing speed (Cuello *et al.*, 2004). If oocytes that have high fertilization rates and *in vitro* developmental rates after oocyte vitrification can be preserved, it could be assumed that it will be highly utilized for *in vitro* fertilization and. other fields of biotechnology. However, an urgent subject need to be increasement of the survival rates of porcine immature oocytes or embryos are lower than that of experimental animals and other animals.

The present work examines the effects of the kinds and concentration of cryoprotectants on the survival rate of vitrification-thawed porcine oocytes, together with the effects on survival, *in vitro* fertilization and development of immature oocytes.

MATERIALS AND METHODS

1. Recovery and Culture of Oocytes

Ovaries were collected immediately after slaughter and were kept at 25℃ saline containing 100 IU/ml penicillin G and 100 μg/ml streptomycin sulfate. Upon arrival at the laboratory, ovaries were washed three times with saline. Follicular fluids was collected by 18 g syringe from 2~5 mm follicles. Afterwards morphologically excellent oocytes were recovered under a stereomicroscope (40 ×). The follicular oocytes cultured in TCM-199 medium supplemented with FCS (Sigma, U.S.A.), 1 μg/ml FSH (Sigma, U.S.A.), 2 IU/ml hCG (Sigma, U.S.A.), 1 μg/ml β-estradiol (Sigma, U.S.A.), 100 IU/ml penicillin G and 100 μg/ml streptomycin sulfate were used for the experiment.

2. Vitrification and Thawing of Oocytes

The immature oocytes were cultured for 0, 10, 14 and 20 hours, and then vitrification was performed with the use of EDS (20% ethylene glycol + DMSO 16.5% + 0.5 M sucrose + 10% FCS). Vitrification embryos are cultured in VS_1 solution for 1 min., afterwards transferred to a 20 μl drop VS_2 solution, and then quickly added to the EDS solution to expose for 1 minute. The oocytes were sealed in a 1.0mm OPP straw (Vajta *et al.*, 1998a) and placed in a LN_2 container. Frozen oocytes were rapidly thawed in a water bath at 30~35℃, and then placed in 0.5 M sucrose, 0.5 M galactose and 0.5 M trehalose for 5 min each. After being washed for 2~3 times, using fresh medium the oocytes were cultured in a 10% FAC +TCM-199 medium.

3. Maturation and Fertilization of Vitrification-Thawed oocytes

After vitrification-thawed 5 oocytes were cultured in 50 μl maturation media for 4~24 h. A 2

μl aliquot of the capacitated spermatozoa suspension (1~5 × 10^6 ml) was added to the oocytes, covered with mineral oil, and incubated for 7~10 hours at CO_2 incubator, and investigate *in vitro* fertilization rate.

4. The Assessment of Maturation and Survival Rate

The oocytes were treated with 0.2% hyaluronidase (Sigma, U.S.A.) for 2 min to remove cumulus cells and denuded oocytes were fixed in acetic acid : ethanol (1 : 3) solution for 24 h then stained using 1% aceto-orcein (Sigma, U.S.A.) or 10 μg/ml bisbenzimide (Hoechst 33342, Sigma, U.S.A.). The judgement of oocytes maturation *in vitro* was carried out depending on the criteria of maturation by cell and nucleus division, and survival rate or *in vitro* development by investigating embryo development and fluorescence diacetate (FDA)-test.

5. Statistical Analysis

The results were expressed by treatment as mean ±SD. For comparison of means, Duncans's multiple verification was performed using SAS package of General Linears Model (GLM) procedures (SAS Institute, 1996).

RESULTS AND DISCUSSION

1. *In Vitro* Maturation Rate Vitrification-Thawed Oocytes

Developmental rates for MI, M Ⅱ and diploid when the vitrification-thawed of recovered immature oocytes cultured for 0, 15, 30 and 40 h are shown on Table 1.

The developmental rate of oocytes to M Ⅱ and diploid stage when the vitrification-thawed of recovered immature oocytes cultured for 0, 15, 30 and 40 h were cultured for 0, 15, 30 and 40 h were 56.7%, 53.3% 63.3%, 65.0% and 23.3%, 18.3%, 10.0%, 3.3%, respectively. The *in vitro* development to M Ⅱ stage were lower than the control group (78.2%), but higher for diploid stage (5.5%). *In vitro* maturation rates of early stage of oocytes was higher than that of mature oocytes. This result was much higher than that of Luna *et al.* (2001) who reported that *in vitro* maturation to diploid of bovine oocytes were 2.0~35.4%, and was highest after maturation for less than 8 hours. This result was much lower than that of Kasai *et al.* (1990) who reported that survival rate of frozen-thawed mouse embryos were 80~85%. This result was much agreed than that of van der Elst *et al.* (1993), Candy *et al.* (1994) and Toth *et al.* (1994) who reported that development to G-V stage of immature oocytes were more appropriate than other stages.

2. Survival Rate of Vitrification-Thawed Oocytes

When the vitrification of immature oocytes after

Table 1. *In vitro* maturation rate of vitrification-thawed various stages of porcine oocytes

Time of culture (h)	No. of oocytes		M I (%)	M Ⅱ (%)	Diploid (%)
	Freeze	Survived			
Control	55	50 (90.9)	7 (12.7)	43 (78.2)	3 (5.5)
0*	60	41 (68.3)	2 (3.3)	34 (56.7)	14 (23.3)[a]
15	60	40 (66.7)	5 (8.3)	32 (53.3)	11 (18.3)[a]
30	60	40 (66.7)	8 (13.3)	38 (63.3)	6 (10.0)[b]
40	60	39 (65.0)	13 (21.7)	39 (65.0)	2 (3.3)[b]

* Culturing period was hours after thawing to complete *in vitro* maturation.

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

being culture for 0, 15, 30 and 40 hours, the survival rates of the oocytes are shown on Table 2.

When the vitrification of immature oocytes after being culture for 0, 15, 30 and 40 hours, the survival rate were 34.0%, 26.0%, 18.0% and 10.0% respectively. This result was lower than that of the control group (60.0%). This results showed that the vitrification of immature oocytes were different on survival rate by the condition of oocytes, freezing method, the kinds and concentration of the cryoprotectants and equilibrium time (Robinski *et al.*, 1991).

3. *In Vitro* Fertilization Rate of Vitrification-Thawed Oocytes Cultured for 0~40 Hours

When the fertilization of the vitrified immature oocytes after being culture for 0, 15, 30 and 40 hours, the *in vitro* fertilization and developmental rates of the oocytes are shown on Table 3.

When the fertilization of the vitrified immature oocytes after being culture for 0, 15, 30 and 40 hours, the *in vitro* fertilization rate were 60.0%, 54.0%, 48.0%, 38.0%, and developmental rates were 26.0%, 18.0%, 8.0%, 4.0%, respectively. This results were lower than the control group (78.0% and 38.0%). The results was different were compared to Caroll *et al.* (1989), Kon *et al.* (1991) and Shaw *et al.* (1992) since the testing animal and the freezing method was different.

Table 3. *In vitro* fertilization and developmental rates of vitrification-thawed porcine oocytes cultured in medium for 0~40 hours

Time of culture (h)	Time of maturation (h)	No. of oocytes		
		Freeze	Fertilized (%)	Cleavaged (%)
Control	50	50	39 (78.0)	19 (38.0)[a]
0*	50	48	30 (60.0)	13 (26.0)
15	50	49	27 (54.0)	9 (18.0)
30	50	47	24 (48.0)	4 (8.0)[b]
40	50	45	19 (38.0)	2 (4.0)[b]

* Culturing period was hours after thawing to complete *in vitro* maturation.

[a,b] Values with different superscripts within column were significantly different ($p<0.01$).

4. *In Vitro* Fertilization Rate of Oocytes Vitrification-Thawed with EDS and ETS

In vitro fertilization and developmental rates of porcine oocytes vitrification-thawed with EDS (40% EG + 20% DMSO +0.4M sucrose), ETS (20% EG + 20% DMSO +0.3M trehalose) + TCM-199 + 10% FCS are shown on Table 2.

When the fertilization of the immature oocytes after being culture for 0~15 hours vitrified with

Table 2. Survial rate of immature of vitrification thawed porcine oocytes

Time of culture (h)	No. of oocytes examined	Degree of FDA test						Survival rate (%)
		A	B	C	D	E	F	
Control	50	1	8	10	8	12	11	30 (60.0)
0*	50	2	7	11	9	12	10	17 (34.0)[a]
15	50	4	10	12	9	9	6	13 (26.0)[a]
30	50	6	12	12	10	6	4	9 (18.0)[b]
40	50	8	14	11	10	5	2	5 (10.0)[b]

* Culturing period was hours after thawing to complete *in vitro* maturation.

[a] Values with different superscripts within column were significantly different ($p<0.05$).

Table 4. *In vitro* fertilization and developmental rate of porcine oocytes vitrification-thawed with EDS and ETS cryoprotectants

Kinds of cryoprotectants	No. of embryos (%)		
	Vitrified	Fertilized	Cleavaged
Control	50	37 (74.0)[a]	19 (38.0)
EDS	50	25 (50.0)	11 (22.0)
ETS	50	23 (46.0)[b]	9 (18.0)

* EDS (40% EG + 20% DMSO +0.4M sucrose), ETS (20% EG + 20% DMSO +0.3M trehalose) +TCM-199 + 10% FCS.

[a,b] Values with different subscripts within columnin were significantly different ($P<0.05$).

EDS and ETS, the fertilization and developmental rates were 50.0%, 22.0% and 46.0%, 18.0%, respectively. This results were lower than the control group (74.0% and 38.0%). This result was significantly lower than that of Vajta *et al.* (1998a), Kasai *et al.* (1990) who reported that development and cleavage rate of mouse embryos when vitrification-thawed using EFS and EPS were 85.0~95.0%, 80~85% and 90.0~95.0%, respectively. The fertilization and development rate of embryos when vitrification-thawed using EFS (35.0%, 25.0%) was little higher than that of EPS (30.0%, 22.5%), and this result was similar that of Vajta *et al.* (1998a) and Kasai *et al.* (1990).

적 요

본 연구는 돼지 난포란의 vitrification 동결 시 내동제의 종류 및 농도가 생존율에 미치는 영향과 수정 후 체외발생율을 조사하고자 수행하였다.

1. 0, 15, 30 및 40시간 성숙 배양시킨 난포란을 vitirfication 동결보존 후의 MⅡ로의 발생율은 각각 56.7%, 53.3% 63.3%, 65.0%였으며, diploid로의 발생율은 23.3%, 18.3%, 10.0%, 3.3%로서 대조군의 MⅡ 단계의 78.2%에 비해 낮게 나타났으며 diploid 단계의 5.5%에 비해서는 높은 체외성숙율을 나타냈다. 체외발생율은 초기의 미숙 난포란일수록 높은 체외성숙율을 나타냈다.
2. 미성숙 난포란을 회수 후 0, 15, 30 및 40시간 성숙 배양시킨 난포란을 vitirfication 동결 후 융해하였을 때 생존율은 각각 34.0%, 26.0%, 18.0% 12.0%, 10.0%로서 비동결 난포란의 60.0%에 비해 낮게 나타났지만 비교적 양호한 생존율을 나타냈다.
3. 0, 15, 30 및 40시간 성숙 배양시킨 미성숙 난포란을 vitrification 동결 융해 후 수정시켰을 때 체외수정율은 60.0%, 54.0%, 48.0%, 38.0%였으며, 배반포로의 체외발생율은 각각 26.0%, 18.0%, 8.0%, 4.0%로서 비동결 대조군의 78.0%와 38.0%에 비해 낮은 체외수정율과 체외발생율을 나타냈다.
4. 돼지 난포란을 EDS와 ETS 액으로 vitrification 동결융해 후 0~15 시간 배양한 다음 체외수정시켰을 때 체외 수정율과 발생율은 각각 50.0%, 22.0% 및 46.0%, 18.0%로서 대조군의 74.0%와 38.0%에 비해 낮게 나타났다.

REFERENCES

van Blerkom J. 1989. Maturation at high frequency of germinal vesicle-stage mouse oocyte after cryopreservation : alterations in cytoplasmic, nuclear, nucleolar and chromosomal structure and organization associated with vitrification. Hum. Rerpod., 4: 883-898.

Candy CJ, Wood M, Whittingham DG, Merriman JA. and Choudhury N. 1994. Cryopreservation of immature mouse oocytes. Hum. Reprod., 9:1738-1742.

Caroll J, Warnes GM and Matthews CD. 1989. Increase in digyny explains polyploidy after *in vitro* fertilization of frozen-thawed mouse embryos. J. Reprod. Fert., 85:489-494.

Cuello C, Sntonia MG, Parrilla I, Tornel J, Vazquez JM, Roca J, Berthelot F, Martinat-Botte F and Martinez EA. 2004. Vitrification of

porcine embryos at various development stages using different ultra-rapis cooling procedures. Theriogenology, 62:353-361.

van der Elst JC, Nerinckx SS and van Steirteghem AC. 1993. Slow and ultrarapid freezing of fully grown germinal vesicle-stage mouse oocytes: optimization of survival rate outweighted by defective blastocyst formation. J. Assist. Reprod. Gene, 10: 202-212.

Hamlett DK, Franken DR, Cronje HS and Luus H. 1989. Murine oocyte cryopreservation : Comparison between fertilization success rates of fresh and frozen metaphase I and II oocytes. Arch. Andol., 23:27.

Kasai M, Komi JH, Takakamo A, Tssnoda 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.

Kono T, Kwon OY and Nakahara T. 1991. Development of vitrified mouse oocytes after *in vitro* fertilization. Cryobiology, 28:50-54.

Leibo SP and Oda K. 1993. High survival of mouse zygote and embryos cooled rapidly or slowly in ethylene glycol plus polyvinylpyrrolidone. Cryo-Letters, 14:133-144.

Mazur P. 1972. Cryobiology ; the freezing of biological systems. Sci. Washington DC, 168:939-949.

Rall WF. 1992. Cryopreservation of oocytes and embryos : methods and applications. Anim. Reprod., 28:237-245.

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

Rall WF and Wood MJ. 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.

Renard JP, Nguyen BX and Garnier V. 1984. Two-step freezing of two-cell rabbit embryos after partial dehydration at room temperature. J. Reprod. Fertil., 71:573-580.

Robinski B, Arav A and Devires AL. 1991. Cryopreservation of oocytes using directional cooling and antifreeze glycoproteins. Cryo-Lettters, 12: 93-106.

Schilling E, Niemann H and Smidt D. 1982. Evaluation of fresh and frozen cattle embryos by fluorescence microscopy. Cryobiology, 15:245-248.

Schmidt M, Hyttle P, Grece T and Avery B. 1993. Ultrastructure of frozen thawed bovine *in vitro* matured oocytes. Theriogenology, 39:304.

Shaw PW, Benarde AG, Fuller BJ, Hunter JH and Shaw RW. 1992. Vitrification of oocytes using short cryoprotectant exposure : Effects of varying exposure times on survival. Mol. Reprod. Dev., 33:210-214.

Suzuki T and Nishikata Y. 1992. Fertilization and cleavage of frozen thawed bovine oocytes by one step dilution methods *in vitro*. Theriogenology Abst., 37:306.

Toth TL, Jones HW, Baka SC, Muasher S, Veeck LL and Lanzendorf SE. 1994. Fertilization and *in vitro* development of cryopreserved human prophase I oocytes. Fertil. Steril., 61: 891-894.

Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen A, 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.

(접수일: 2005. 5. 20 / 채택일: 2005. 7. 5)