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. 129~135

Effects of Kinds and Concentrations of Cryoprotectants, PVP on Survival Rate of Vitrified Porcine Embryos

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내동제의 종류와 농도, PVP 첨가가 돼지 수정란의 Vitrification 동결 융해 시 생존율에 미치는 영향

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SUMMARY

The present study examines the effects of kinds and concentrations of cryoprotectants, PVP and sucrose and trehalose on the survival rate of vitrified porcine embryos.

1. The developmental stages for the embryos used in vitrification were 245 (23.0%) for 2 cell stage, 256 (24.1%) for the blastocyst, 234 (22.0%) for the early blastocyst 221 (20.8%) from the expanded blastocyst and 107 (10.1%) from hatching blastocyst out of 1,063 embryos.
2. The survival rate of morula, early blastocyst and expanded blastocyst vitrification-thawed with EDT and EGS were 69.1%, 70.3%, 69.8% and 62.5%, 61.7%, 63.6%, respectively. The expanded blastocyst treated with EDS showed the highest survival rate compared with the other cryoprotectants.
3. The survival rate of early blastocyst, expanded blastocyst and hatching blastocyst vitrification-thawed with EDS diluted in medium + 10% FCS were 61.1%, 27.8%, 16.7%, respectively. This result were lower than the control group (92.3%, 71.2%, 55.8%).
4. The survival rate of embryos vitrified with EDS and EDT supplemented with 10% and 20% PVP were 74.3%, 77.5% and 79.4%, 71.1%, respectively. The survival rate of vitrified embryos cultured for 24~48 hours were 37.1%, 40.0% and 35.3%, 31.6% which were significantly lower than that of non-cultured embryos. The survival rate of embryos vitrified with EDS and EDT supplemented between 10% or 20% PVP did not have a significant difference.
5. The survival rate of embryos vitrification-thawed with EDS to morula, early blastocyst, expanded blastocyst and hatching blastocyst were 58.2%, 36.4%, 14.5% to morula, 62.5%, 45.8%, 20.8% to early blastocyst, 74.1%, 61.1%, 29.6% to expanded blastocyst and 60.0%, 40.0%, 14.0% to hatching blastocyst.

(Key words : porcine embryos, vitrification, survival rate, cryoprotectants)

INTRODUCTION

Recently the study of embryo vitrification are being conducted because the embryos are kept in

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over-cooling while preventing hydration and ice crystal formation with the addition of high concentrations of cryoprotectants in vitrification (Rall and Fahy, 1985; Kasai *et al.*, 1990; Vajta *et al.*, 1998b; Cuello *et al.*, 2004).

The factors that affect the survival of the frozen-thawed embryo are the kinds of cryoprotectants, the addition or removal or cryoprotectants, the developmental stage the embryo is in thawing speed and the composition of the cryoprotectant. After vitrification the factors that effect porcine embryos are the embryos developmental stage, the toxicity of the cryoprotectant, composition of the vitrification solution and freezing and thawing speed etc. (Cuello *et al.*, 2004). Research about embryo vitrification was first reported by Rall and Fay (1985) and from then on much research has been done but there are still many improvements needed to be made. Cryoprotectants like glycerol (Iwasaki, 1994; Saito *et al.*, 1994), ethyleneglycol (Ishimori *et al.*, 1991; Saha *et al*, 1994; Voelkel and Hu, 1992) propylene glycol (Kasai *et al.*, 1991), DMSO (dimethylsulfoxide) were being used for vitrification. Each cryoprotectant has different toxicities or has poor permeabilities which are their weak points. Although, when permeable cryoprotectants like glycerol, DMSO, Ethleneglycol etc. are mixed to non- permeable cryoprotectants sucrose and trehalose, less cell damage occur during the steps of equilibrium and thawing (Massip *et al.*, 1993; Saha *et al.*, 1996; Saito *et al.*, 1994). When cells are treated with the non-permeable macro-molecule sucrose or trehalose and high concentrations of cryoprotectants, it prevents ice crystal formation, protecting the cell wall resulting in not only high survival but also making it easier to thaw the 1st stage (van der Elst *et al.*, 1993; Candy *et al.*, 1994; Toth *et al.*, 1994). The vitrification methods that have been reported are 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), EDS (Vajta *et al.*, 1998a; Lane *et al*, 1999). These researchers gained high survival rates by treating high concentrations of cryoprotectants to the vitrified embryos which also prevents ice crystal formation. Holm *et al.* (1999) and Kuwayama *et al.* (1997) have reported that survival rates were 33~90% when vitrified hatching blastocyst were used. Cuello *et al.* (2004) have reported that freezing speed decreases freezing damage and raises the permeability of the cryoprotectants, but also reported that OPS (open pulled straw) has shown an 8 times more higher cooling ratio than that of 0.25ml straw. To raise the OPS freezing time a reduced inside diameter and thicker wall straw was used.

The present study examines the effects of kinds and concentrations of cryoprotectants, PVP, sucrose and trehalose on the survival rate of vitrified porcine embryos.

MATERIALS AND METHODS

1. Recovery and Incubation 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. Follicular fluids was collected by 18 g. syringe from 2~5 mm follicles. Afterwards morphologically excellent oocytes were recovered under a stereo microscope (40 ×). The recovered oocytes were cultured in TCM-199 medium supplemented with 10% (v/v) 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 or 44~ 48 hours in an incubator (38.5℃, 5% CO_2).

2. Preparation of Embryos

The oocytes were washed 3 times with fertilization medium. Five oocytes were transferred to 50 ul of maturation medium covered with mineral oil.

Frozen-thawed sperm of 0.01 ml were added in 2 ml of BO solution. After swim-up treatment in a CO_2 incubator, the supernatant was added to fertilization medium, and centrifuged at 500 rpm for 10 min. The sperm pellets were cultured for 15 minutes with diluted 100 ug/ml heparin (Sigma Co., USA) solution. 2 ul of capacitation-sperm suspension ($1\sim5\times10^6$ ml) was added in the fertilization medium afterwards cultured for 7~10 hours in a CO_2 incubator.

3. Vitrification and Thawing of Embryos

During vitrification of embryos were diluted with EDS (20% EG + 20% DMSO +0.4M sucrose), EDT (20% EG + 20% DMSO +0.3M trehalose) + 10% FCS solutions. EDS vitrification is when embryos are washed 2 times and suspended with EDS solution, and EDT vitrification is when the embryos are cultured in VS_1 solution for 1 minute, and rapidly transferred to a 20 ul drop then mixed with EDT solution, and were sealed in 1.0 mm OPP straw (Vajta *et al.*, 1998a) and exposed in air for 1 minute, then plunged in a LN_2 container.

4. The Judgement of Development Vitrification-Thawed Embryos

Frozen embryos were thawed for 10 sec in a 30 ℃ water-bath and was shaken upside down for 2 min. The thawed embryos were then transferred to a petri dish and washed 2~3 times with fresh maturation medium. The judgement was carried out depending on the criteria survival rate and *in vitro* development by investigating embryo development and fluorescence diacetate (FDA)-tests.

5. Statistical Analysis

Data were expressed as mean ± standard deviation. For comparison of means, Duncan's multiple verification was performed using SAS package of General Linear Model (GLM) procedures (SAS institute, 1996).

RESULTS AND DISCUSSION

1. Survival Rate of Vitrified Embryos with Different Kinds of Cryoprotectants

The survival rate of vitrified porcine embryos at various stages and kinds of cryoprotectants are shown in Table 1.

The developmental stages for the embryos used in vitrification were 245 (23.0%) for 2 cell stage, 256 (24.1%) for the blastocyst, 234 (22.0%) for the early blastocyst, 221 (20.8%) from the expanded blastocyst and 107 (10.1%) from hatching blastocyst out of 1,063 embryos.

The survival rates of different stages of embryos vitrification-thawed with optimal kinds of cryoprotectants are shown in Table 2.

The survival rates of morula, early blastocyst and expanded blastocyst vitrification-thawed with EDS and EDT were 69.1%, 70.3%, 69.8% and 62.5%, 61.7%, 63.6%, respectively. The expanded blastocyst treated with EDS showed the highest survival rate compared with the other cryoprotectants. Although different animal embryos were used in vitrification, this results were similar to that of Saha *et al.* (1996) and Kasai *et al.* (1990) who reported that the survival rates of porcine embryos vitrified with EDS and EDT were high.

Table 1. *In vitro* fertilized porcine oocytes were used for the experiment

No. of oocytes	≧ 2 cell (%)	No. of oocytes developed to (%)			
		≧ B.	Early B.	Expended B.	Hatching B.
1,063	245 (23.0)	256 (24.1)	234 (22.0)	221 (20.8)	107 (10.1)

Table 2. Survival rate of porcine embryos vitrified with kinds of cryoprotectants

Cryprotectants	Stage of embryos	No. of embryos examined	No. of embryos survived	
			Normal (%)	Degenerate (%)
EGT	M	55	21 (41.8)	32 (58.2)
	EB	56	27 (48.2)	29 (51.8)
	B	62	35 (56.5)	27 (43.5)
EGS	M	65	32 (49.2)	33 (50.8)
	EB	66	34 (51.5)	32 (48.5)
	B	65	36 (55.4)	29 (44.6)
EDS	M	55	38 (69.1)	17 (30.9)
	EB	64	45 (70.3)	19 (29.7)
	B	63	47 (74.6)	16 (25.4)
EDT	M	48	30 (62.5)	18 (37.5)
	EB	60	37 (61.7)	23 (38.3)
	B	66	45 (68.2)	21 (31.8)

* EGT (10% EG + 20% G + 0.3M trehalose), EGS (20% EG + 20% G +0.3M sucrose), EDS (20% EG + 20% DMSO +0.4M sucrose), EDT (20% EG + 20% DMSO +0.3M trehalose).

** M; morula, EB; early blastocyst, EB; expended embryos.

2. Survival Rate of Vitrified Embryos at Different Stages

The survival rate of porcine embryos in various stages after being cultured, then vitrified with EDS diluted in medium + 10% FCS are shown in Table 3.

The survival rate of early blastocyst, expanded blastocyst and hatching blastocyst vitrification-thawed with EDS diluted in medium + 10% FCS were 61.1%, 27.8%, 16.7%, respectively. This result were lower than the control group (92.3%, 71.2%, 55.8 %). This results were similar to that of Takagi *et al.* (1994) who reported that the survival rates of vitrified blastocyst cultured for 7~8 days were highest. However, the survival rates were different to that of Saha *et al.* (1996) who reported that the survival rate of vitrified blastocyst cultured for 7 days were higher than that of blastocyst cultured for 8~9 days.

3. Survival Rate of Embryos Vitrified using EDS and EDT Supplemented with PVP

The survival rate of embryos vitrified with EDS and EDT supplemented with 10% and 20% PVP are shown in Table 4.

The survival rate of embryos vitrified with EDS

Table 3. Survival rate of porcine blastocysts exposed or vitrified with EDS solution

Treatment	No. of embryos examined	No. of embryos survived (%)		
		Early B.	Expended B.	Hatching B.
Control	52	48 (92.3)	37 (71.2)	29 (55.8)
Exposed	56	40 (71.4)	23 (41.1)	14 (25.0)
Vitrified	54	33 (61.1)	15 (27.8)	9 (16.7)

Table 4. Survival rate of porcine embryos vitrified with EDS and EDT supplemented with different PVP concentrations

Cryorpro-tectants	Concent. of PVP	No. of frozen embryos	No. of embryos (%) after thawing		No. of embryos (%) 24~48 h culture	
			Normal	Degenerated	Normal	Degenerated
EDS	10%	35	26 (74.3)	9 (25.7)	13 (37.1)	22 (62.9)
	20%	40	31 (77.5)	9 (22.5)	16 (40.0)	24 (60.0)
EDT	10%	34	27 (79.4)	7 (20.6)	12 (35.3)	22 (64.7)
	20%	38	27 (71.1)	11 (28.9)	12 (31.6)	26 (68.4)

and EDT supplemented with 10% and 20% PVP were 74.3%, 77.5% and 79.4%, 71.1%, respectively. The survival rate of vitrified embryos cultured for 24~48 hours were 37.1%, 40.0% and 35.3%, 31.6% which were significantly lower than that of non-cultured embryos. The survival rate of embryos vitrified with EDS and EDT supplemented between 10% or 20% PVP did not have a significant difference. The results were lower than that of Saha *et al.* (1996) who reported that the survival rates of blastocyst vitrified with EDS + PVP were 70% but hatching blastocyst cultured for 24~48 hours decreased to 43%. Embryos vitrified with EDT supplemented with trehalose prevents toxicity of the cell, detriment temperature and cell damage but also prevents excess penetration to increase survival rates (Clark *et al.*, 1984; Fahy *et al.*, 1984; Sutton, 1982; Utsumi *et al.*, 1982).

4. Survival Rate of Embryos at Different Stages Vitrified with EDS +PVP

The survival and developmental rate of embryos cultured for 7 days vitrified with EDS supplemented with 10% PVP are shown in Table 5.

The survival rate of embryos vitrification-thawed with EDS supplemented with 10% PVP to morula, early blastocyst, expanded blastocyst and hatching blastocyst were 58.2%, 36.4%, 14.5% to morula, 62.5%, 45.8%, 20.8% to early blastocyst, 74.1%, 61.1%, 29.6% to expanded blastocyst and 60.0%, 40.0%, 14.0% to hatching blastocyst. This results were similar to and higher than that of Massip *et al.* (1993) and Mamoudzadeh *et al.* (1995) who reported that the survival rates of various stage of vitrified blastocyst were 19% and 25%, respectively.

적 요

본 연구는 돼지 수정란의 동결에 있어서 vitrification 동결 융해 후 내동제의 종류와 농도, PVP 및 sucrse와 trehalose의 첨가가 생존율에 미치는

Table 5. Survival rate of porcine blastocysts at the different stage vitrified with EDS supplemented with 10% PVP

Embryo stage	No. of embryos examined	No. of embryos survived (%)		
		Early B.	Expended B.	Hatching B
Morula	55	32 (58.2)	20 (36.4)	8 (14.5)
Early B.	48	30 (62.5)	22 (45.8)	10 (20.8)
Expended B.	54	40 (74.1)	33 (61.1)	16 (29.6)
Hatching B.	50	30 (60.0)	20 (40.0)	7 (14.0)

영향을 조사하고자 수행하였다.

1. Vitrification 동결에 이용된 각 발생단계의 체외수정란은 1,063개 중 2세포기는 245 (23.0 %)개, 배반포는 256 (24.1%), 초기 배반포는 234 (22.0%), 확장 배반포는 221개 (20.8%), hatching 배반포는 107개 (10.1%)이었다. 상실배, 초기 배반포 및 확장배반포를 EDS와 ETS로 희석 후 vitrification 동결 융해했을 때 생존율은 각각 69.1%, 70.3%, 69.8% 및 62.5%, 61.7%, 63.6%로서 EDS군에서 확장 배반포군에서 가장 높은 생존율을 나타냈다.
2. 각 발생단계의 수정란을 vitrification 동결 융해했을 때 생존율은 초기 배반포는 61.1%, 확장 배반포는 27.8%, hatching 배반포는 16.7%로서 대조군의 92.3%, 71.2%, 55.8%에 비해 낮았지만 높은 생존율을 나타냈다.
3. 수정란을 EDS와 EDT 내동제에 10% 및 20% PVP 액을 첨가하여 희석 후 vitrification 동결 융해했을 때 정상적 발생을 나타내는 수정란은 74.3%, 77.5% 및 79.4% 및 71.1%였다. 동결 융해한 수정란을 24~48시간 배양했을 때 37.1%, 40.0% 및 35.3%, 31.6%로서 생존율이 현저하게 감소하였다. 수정란에 EDS와 EDT와 10% 및 20%의 PVP를 첨가한 내동제를 이용하여 동결 융해했을 때 PVP 농도간의 생존율은 유의한 차이가 없었다.
4. 각 발생단계의 수정란을 EDS 내동제로 vitrification 동결 융해 후 배양했을 때 발생율은 상실배는 58.2%, 36.4%, 14.5%였고, 초기 배반포는 62.5%, 45.8%, 20.8%였고, 확장 배반포는 74.1%, 61.1%, 29.6%였고, hatching 배반포는 60.0%, 40.0 %, 14.0%였다.

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(접수일: 2005. 5. 26 / 채택일: 2005. 7. 10)