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Studies on the Survival and *In Vitro* Fertilization Rates of Frozen-Thawed Porcine Embryos and Immature Oocytes

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돼지 수정란 및 미성숙 난자의 동결융해 후의 생존율에 관한 연구

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요 약

본 연구는 돼지 수정란의 동결에 있어서 내동제의 종류, sucrose 농도, 평형시간 및 융해온도가 생존율에 미치는 영향과 미성숙 난자의 체외배양과 동결융해 후 생존율과 체외수정율을 조사하고져 수행하였다.

1. 수정란의 급속동결에 있어서 1.5 M glycerol, 2.0 M DMSO, 2.5 M ethyleneglycol 및 2.0 M propanediol 농도에서 생존율이 유의하게 높았으며, 내동제에 첨가된 sucrose농도는 0.25 M 첨가가 다른 농도에 비해 생존율이 높게 나타났다. 동결수정란은 30℃에서 융해시 생존율은 33.3~40.6%로서 25℃ 및 37℃의 융해온도에 비해 높게 나타났으며, 동결시 1.5 M 및 2.0 M glycerol + 0.25 M sucrose가 첨가된 내동액으로 짧은 평형시간(2.0~5.0분)이 긴 평형시간 (10~15분)보다 높은 생존율을 나타냈다.
2. 미성숙란을 1~8시간 체외배양시킨 다음 동결융해 후 체외수정시켰을 때 수정율은 6.7~26.7%로서 단시간의 체외성숙 배양이 높게 나타났으며, 또한 체외배양시 생존율은 10.0~30.0%를 나타냈다.

(Key words : Cryoprotectants, Equilibration time, Survival rates, *In vitro* fertilization rates)

I. INTRODUCTION

The advant of recent reproductive technologies including multiple ovulation and embryo transfer and *in vitro* fertilization(IVF) has brought animal breeders a promising potential for acceleration of genetic improvement.

Deep freezing and transport of embryos is now well established and commonly employed in cattle. The freezing and thawing methods used are based on experiments done with shee and mouse embryos to −30℃ to −60℃ and below in the presence of molar concentrations of a cryoprotective additive. Thawing has to be slow or rapid depending on the degree of dehydration reached by the animal embryos(Whittingham et al. 1972; Nagashima and Ogawa, 1981), sheep, cattle(Renard et al., 1983; Schellander et al., 1988; Schmidt et al., 1993; Suzuki et al., 1990). In recent years a rapid freezing method has been commonly practiced together with cryoprotectant dilution, employing glycerol, dimethyl sulfoxide(DMSO), and sucrose. However, the results appeared to be ambiguous according to the investigators because the viabilities were

affected by various factors such as the concentration and composition of cryoprotectants, conditions for equilibration, seeding procedures for freezing and thawing. The survival rates of rapidly frozen embryos are generally lower than that of the low rate freezing, and especially porcine embryos are known to be suseptible to low temperatures.

The present work examines the effects of the kind and concentration of cryoprotectants, sucrose addition, equilibration time and thawing temperature on the survival of rapidly frozen porcine embryos, together with the effects on survival and *in vitro* fertilization of immature oocytes.

Ⅱ. MATERIALS AND METHODS

1. Follicular oocytes and culture media

Ovaries were taken from sows(Yorkshire, body weight 120~180 kg) immediately after slaughter, and then were transported to the laboratory within 1 hour in saline at 38℃ containing 100 IU/ml of penicillin G and 100 μg/ml of streptomycin sulfate. Follicular fluids were aspirated from follicles of 2~5 mm in diameter using a 19 gauge needle on a 10 ml syringe, and the oocytes which were morphologically normal were collected from the fluid under a stereomicroscope. The basal culture media for the maturation and fertilization of oocytes *in vitro* was TCM-199(Sigma, USA) which contained 10% (v/v) of FCS(Sigma, USA), 1 μg/ml of FSH (Sigma, USA), 2 IU/ml of hCG(Sigma, USA) 1 μg/ml of β-estradiol (Sigma, USA) and 100 mg/ml of streptomycin sulfate. It was filtered with a 0.2 μm millipore before use.

2. *In vitro* maturation and fertilization

The follicular oocytes were washed three times with the culture media and were immersed 5 per drop of the liquid, then incubated for 40~48 hours. The liquid was prepared by covering a 45 μl aliquot of the culture media with mineral oil (Squibb, USA) and preincubating in a CO_2 incubator(5% CO_2, 95% air, 100% RH, 38℃; Sanyo Co., Japan) for 2~3 hours.

Testis were collected from a boar immediately after slaughter and were transported to the laboratory within 1 hour while keeping at 4℃. The epididymis was then separated from the testis. A 0.02 ml aliquot of sperm suspension which was aspirated from the epididymis through a 18 gauge needle and 2 ml of BO solution were mixed in a tube and swimmed up for 1 hour at 38℃ in the CO_2 incubator. After incubation, the top 0.5 ml was taken and centrifuged for 10 minutes at 1,000 rpm, using a bench-top centrifuge, to obtain the sperm pellet. The sperm pellet was mixed with the same volume of heparin solution(100 mg/ml, Sigma, USA) and kept in the CO_2 incubator.

After maturation, the fresh or frozen follicular oocytes were washed with the fertilization media three times, and were placed in a drop(45 μl) of the media. 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 6~7 hours at 38℃ in the CO_2 incubator.

3. Judgement of maturation and fertilization

The follicular oocytes were treated with 0.3% hyaluronidase(Sigma, USA) to remove cumulus cells within 24 hours after fertilization and were fixed on a slide glass with ethanol-acetic acid solution(1:3) for 48 hours. These were stained with 1% acetic-orcein solution, then head expansion of spermatozoa, formation of male or female prenucleus, and tail existence of spermatozoa in cytoplasm were investigated. The rate of maturation and fertilization was determined

according to the method described by Shea et al.(1976) and Ball et al.(1983).

4. Freezing and thawing procedures

The follicular oocytes and embryos were frozen in 0.25 ml straws(I.M.F., France) employing procedures based on those of Taketa et al. (1987). The fraction of freezing medium, air, oocytes or embryos in the freezing medium were aspirated into the straw. TCM-199 containing 0. 25 M sucrose and 20% FCS was used as the freezing medium. The straw was heat-sealed and labeled. After equilibration for 2, 5, 10, 15 and 20 minutes, the straws were frozen by placing them horizontally in the vapour about 1 cm above the surface of liquid nitrogen for 5 minutes and were then transferred into the liquid nitrogen. The straw were thawed in a waterbath (38℃) after standing at room temperature for 30 seconds, then were shaken. After 10 minutes the contents were pushed out into petri dishes and washed 2~3 times with the fresh culture medium.

5. Survival rate of frozen-thawed oocytes and embryos

The frozen oocytes or embryos were washed in three changes of PBS, and placed in the solution which was prepared by melting 1 mg of fluorescence diacetate(FDA) in 1 ml acetone then by diluting this in TCM-199(1 : 600,000, pH 7.0~7.4). These oocytes or embryos were divided into 6 groups according to the morphologies observed under a fluorescence microscope, and the survival rates were analysed by the method by Schilling et al.(1982).

Ⅲ. RESULTS AND DISCUSSION

1. Survival of frozen-thawed embryos

Survival rates according to the kinds and coccentrations of cryoprotectants were investigated and the results are presented in Table 1.

As shown in the table, survival rates of porcine embryos obtained from the experiment employing DMSO, glycerol, ethylene glycol and propanediol were in the range of 16.7~40.0%, of which the highest survival of 40% was obtained when the freezing medium contained 2.0 M DMSO, and the lowest of 16.7% resulted by adding 4.0 M DMSO or 3.0 M ethylene glycol.

The results show that the level of viability of porcine embryos was markedly lower than those of mouse embryos, because viabilities reported for the mouse were 84.7% for 2.0 M glycerol (Williams and Johnson, 1985); and 95% for 3.0 M glycerol(Szell and Shelton, 1987). However, the optimal concentration of cryoprotectants in the freezing media for mouse embryos varied depending on the investigator : from 3.0 M (Trounson et al., 1987) to 4.5 M (Wilton et al., 1989) for DMSO; from 2.0 M(Kasai et al., 1981;

Table 1. Effects of cryoprotectants concentration on the survival rate of frozen porcine embryos

Concent.	DMSO		Glycerol		Ethylene glycol		Propanediol	
of cryopro.	Frozen	Surv.(%)	Frozen	Surv.(%)	Frozen	Surv.(%)	Frozen	Surv.(%)
1.0 M	33	7(21.2)	33	7(21.2)	31	6(19.4)	30	6(20.0)
1.5 M	33	8(24.2)	28	10(35.7)	36	11(30.6)	28	8(28.6)
2.0 M	25	10(40.0)	26	9(34.6)	38	9(23.7)	33	11(33.3)
2.5 M	29	8(27.6)	35	8(22.9)	26	7(26.9)	34	7(20.6)
3.0 M	35	7(20.0)	28	5(17.9)	30	5(16.7)	34	6(17.6)
4.0 M	24	4(16.7)	29	5(17.2)	24	5(20.8)	33	6(18.2)

Table 2. Effects of sucrose concentration in the feezing medium on the survival rate of frozen porcine embryos

Cryo-protectant (D+P+G)*	Sucrose concentration									
	0.00		0.25		0.50		0.75		1.00	
	Frozen	Sur.(%)	Frozen	Sur.(%)	Frozen	Sur.(%)	Frozen	Sur.(%)	Frozen	Sur.(%)
1.0 M	28	4(14.3)	31	9(29.0)	30	9(30.0)	30	6(20.0)	29	3(10.3)
1.5 M	31	3(9.7)	31	11(35.5)	27	6(22.2)	30	7(23.3)	28	3(10.7)
2.0 M	28	2(7.1)	26	10(38.5)	28	6(21.4)	28	5(17.9)	27	2(7.4)
2.5 M	27	1(3.7)	27	8(27.6)	27	5(18.5)	26	4(15.4)	28	2(7.1)
3.0 M	28	1(3.6)	26	7(21.9)	26	4(15.4)	30	2(6.7)	22	1(4.5)

* : D+P+G : DMSO + Propanediol + Glycerol.

Table 3. Effects of equilibration time of cryprotectants on the survival rate of frozen porcine embryos

Equili-bration time(Min.)	Cryoprotectants					
	1.5 M Glycerol + 0.25 M Sucrose			2.0 M Glycerol + 0.25 M Sucrose		
	Freezing	Recovery(%)	Survival(%)	Freezing	Recovery(%)	Survival(%)
2.5	21	20(95.2)	7(33.3)	20	8(90.0)	5(25.0)
5.0	21	18(85.7)	5(23.8)	20	19(95.0)	6(30.0)
10.0	20	18(90.0)	5(25.0)	23	21(95.5)	4(17.4)
15.0	21	20(95.2)	5(23.8)	20	19(95.0)	4(20.0)
20.0	20	18(90.0)	3(15.0)	22	21(95.5)	2(9.1)

Miyamoto et al., 1986) to 3.0 M(Szell and Shelton, 1987) for glycerol; 1.2 M(Kojima et al., 1984) for ethylene glycol; and 2.0~3.0 M for propanediol(Hernandez-Ledezma and Wright, 1990; Ko and Threlfall, 1988, Rall and Polge, 1984). The optimal concentrations of cryoprotectants for porcine embryos are considered to be 1.5 M glycerol, 2.0 M DMSO, 2.5 M ethylene glycol, and 2.0 M propanediol. Survival rates according to sucrose concentration were studied when the freezing media containing 1.0~3.0 M DMSO + propanediol + glycerol was used, and the results are shown in Table 2.

For mouse embryos the effects of sucrose addition to glycerol have been reported, however the suitable concentrations appeared to differ among the workers, with 0.25 M(Szell and Shelton, 1987, Wilton et al., 1989)-1.0 M or 1.5 M (Bielanski et al., 1984; Andrede and Rodrigues, 1987). The effects of equilibration time on survival rate of porcine embryos were examined when the embryos were equilibrated for certain periods in 1.5 or 2.0 M glycerol containing 0.25 M sucrose. The results are shown in Table 3. The periods of 2.5 and 5.0 minutes revealed the higher survival rates for both media when compared to 10 and 15 minutes.

It was reported that 2 minutes of equilibration time was adequate for mouse embryos in 2.0 M DMSO containing 0.2 M sucrose by Trounson et al.(1987) and that 2~5 minutes was adequate by Kasai et al.(1990) and Takahashi and Kanagawa(1990).

Porcine embryos were rapidly frozen in TCM -199 + 20% FCS + 0.25 M sucrose containing various concent rations of the cryoprotectants

Table 4. Effects of thawing temperature on the survival rate of frozen porcine embryos

Cryoprotectants (+0.25M S) (Min)	Thawing temperature(℃)					
	25		30		37	
	Frozen	Survival(%)	Frozen	Survival(%)	Frozen	Survival(%)
1.0M D+G+P	30	9(30.0)	29	11(37.9)	27	10(37.0)
1.5M D+G+P	27	11(40.7)	30	11(36.7)	32	11(34.4)
2.0M D+G+P	30	10(33.3)	30	10(33.3)	30	9(30.0)
2.5M D+G+P	29	9(31.0)	32	13(40.6)	30	10(33.3)
3.0M D+G+P	30	8(26.7)	30	12(40.0)	31	12(38.7)

S : Sucrose, D : Dimethyl sulfoxide, G : Glycerol, P : Propanediol.

Table 5. Effects of cryoprotectants on *in vitro* maturation and fertilization rate of rapidly frozen immature procine oocytes

Culture of frozen oocytes	No. of oocytes examined	No. of oocytes matured(%)	No. of oocytes fertilized(%)
Control	20	13(65.0)	7(35.0)
Immature oocytes(1 hr.)	30	14(46.7)	8(26.7)
Immature oocytes(2 hr.)	30	12(40.0)	6(20.0)
Immature oocytes(4 hr.)	30	10(33.3)	4(13.3)
Immature oocytes(8 hr.)	30	7(23.3)	2(6.7)

DMSO + glycerol + propanediol. The frozen embryos were thawed at 25, 30, or 37℃ and their survival rates were investigated. The results are presented in Table 4.

The survival rates appeared to relatively higher at a 30℃ thawing temperature with the lowest of 33.3% in 2.0 M and the highest of 40.6% in 2.5 M of the cryoprotectants. The suitable thawing temperatures reported for mouse embryos were 30℃ for 1 minute by Bielanski et al. (1984) and Mapletoft et al.(1987), 37℃ for 30 seconds by Robertson et al.(1989) and 23 ± 3℃ by Andrede and Rodrigues(1987).

2. Survival of frozen-thawed immature oocytes

Follicular oocytes were matured *in vitro* for 1, 12, 24, and 48 hours after recovery and rapidly frozen and thawed in the medium containing 1.5 M DMSO + 2.0 M glycerol + 0.25 M sucrose. These frozen-thawed oocytes were fertilized with the capacitated spermatozoa, then the *in vitro* fertilization rates were examined. The results are presented in Table 5.

The *in vitro* fertilization rates observed decreased to 6.7% depending on the maturation time, with rates being relatively high for those matured for a short period of time. However, these rates were lower than the control of unfrozen oocytes. *In vitro* vitro fertilization of mouse oocytes was reported by Friedler et al., (1988) Caroll et al.(1989), Kono et al.(1991) and Shaw et al.(1992).

Follicular oocytes were matured by the similar procedures as in the previous *in vitro* fertilization experiment for 1, 12, 24, and 48 hours, and were rapidly frozen and thawed in the medium containing 1.5 M DMSO + 2.0 M glycerol + 0.25 M sucrose or 2.0 M DMSO + 1.5 M glycerol + 0.25 M sucrose. The frozen-thawed oocytes

Table 6. Effects of cryoprotectants in the freezing medium on the survival rate of rapidly frozen immature porcine oocytes

Cryoprotectant[a]	Culture period before freezing(hr)	No. of oocytes examined	Degree of FDA test						Survival rate(%)
			A	B	C	D	E	F	
	Control	20	0	7	3	1	4	5	9(45.0)
1.5 M D +	1 hr.	20	1	8	4	2	3	2	5(21.7)
2.0 M G +	2 hr.	23	2	8	5	3	3	2	5(21.7)
0.25 M S	4 hr.	30	2	10	8	5	3	2	5(16.7)
	8 hr.	29	3	10	9	4	2	1	3(10.3)
2.0 M D +	1 hr.	20	1	5	4	4	4	2	6(30.0)
1.5 M G +	2 hr.	30	1	10	7	7	3	2	5(16.7)
0.25 M S	4 hr.	30	2	11	8	5	3	1	4(13.3)
	8 hr.	30	2	9	9	6	2	2	4(13.3)

[a] D+G+S : Dimethyl Sulfoxide + Glycerol + Sucrose.

[b] Oocytes were fertilized without freezing after *in vitro* culture for 24~30 hours.

were *in vitro* fertilized with the capacitated spermatozoa, and their survival rates were determined by the FDA-test. The results are shown in Table 6.

The survival rates observed were 10.3~30.0% depending on the *in vitro* maturation time, however the differences between the cryoprotectants applied were not significant. Didion et al. (1990) reported that the viability of pig oocytes at the germinal vesicle stage following cooling or cryoperservation at −196℃ was 57% based on morphological appearance of the cumulus cell whereas it was 0% as evidenced by trypan blue uptake and lack of fluorescence. High viabilities for other mammals were reported as 88.9% for bovine oocytes(Schellander et al., 1988) and 83% for mouse oocytes(Kono et al., 1991).

Ⅳ. SUMMARY

In order to determine suitable conditions for rapid freezing of porcine embryos, the kind and concentration of cryoprotectants, sucrose concentrations, equilibration time and thawing temperature in freezing medium were examined in relation to the survival of frozen-thawed occytes and embryos.

The results obtained are as follows :

1. The suitable concentrations of cryoprotectant in the freezing medium which consisted of TCM-199 + 20% FCS were 1.5 M for glycerol, 2.0 M for DMSO, 2.5 M for ethylene glycol, and 2.0 M for propanediol. The sucrose concentration of 0.25 M in the medium was found to optimal because the survival rate was markedly higher at this concentration when compared to the others. The survival rate was relatively high when the frozen embryos were thawed at 30℃ in the freezing medium containing 2.5 M cryoprotectants. The equilibration periods of 2.0 and 5.0 minutes revealed the higher survival in the media containing 1.5 or 2.0 M glycerol when compared to 10 and 15 minutes.
2. The fertilization rates of frozen-thawed follicular oocytes which matured *in vitro* for 1, 12, 24 and 48 hours were 6.7~26.7% depending on the maturation time, and the rates were relatively high for those matured

for a short period of time. The survival rates of frozen-thawed oocytes which matured *in vitro* for certain periods and fertilized were 10.0~30.0% depending on the maturation time.

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