韓國家畜繁殖學會誌 22(3) : 207~211(1998)
Korean J. Animal Reprod.

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

Kim, S. K., S. S Yoo and Y. S. Kim
College of Veterinary Medicine, Chungnam National University

소 동결 미성숙 난자의 생존율과 체외수정율에 관한 연구

김상근 · 유상식 · 김용섭
충남대학교 수의과대학

요 약

본 연구는 소 미성숙 난자의 동결에 있어서 미성숙 난자의 1, 2, 4, 8 시간 성숙배양한 다음 동결 융해 후 체외배양하였을 때 체외수정율과 생존율을 조사하고저 수행하였다.

미성숙 난자를 1~8시간 성숙배양후 동결융해 및 체외수정시켰을 때 수정율은 12.8~30.9%로서 단시간의 체외성숙 배양이 높게 나타났으며(23.8~28.8%), 또한 체외배양시 생존율은 11.8~29.9%를 나타냈다.

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

Ⅰ. INTRODUCTION

The development of simplified cryopreservation procedures for cattle blastocysts has great potential in facilitating the use of embryo transplantation in research and practical breeding programs.

Since Whittingham et al.(1972) developed the techniques for cryopreservation of mouse embryos, various research studies on mammals such as rabbits(Nagashima and Ogawa, 1981), sheep, cattle(Renard et al., 1983; Schellander et al., 1988; Schmidt et al., 1993; Suzuki et al., 1990) etc. were reported by many workers. 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 determine suitable conditions for freezing of bovine immature oocytes, together with the effects on survival and *in vitro* fertilization after frozen-thawing of immature oocytes.

Ⅱ. MATERIALS AND METHODS

1. Follicular oocytes and culture media

Ovaries were taken from Korean native cows 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. 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 24~30 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℃) for 2~3 hours.

Frozen semen was thawed in water bath at 35℃. 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 1000 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. Freezing and thawing procedures

The follicular oocytes were frozen in 0.25 ml straws(I.M.F., France) employing procedures based on those of Takeda 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 10% 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 Cell-freezer(Tokyo Co., Japan). 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.

4. Survival rate of frozen-thawed oocytes and embryos

The rate of maturation and fertilization was determined according to the method described by Shea et al.(1976) and Ball et al.(1983). The frozen oocytes were washed in three changes of PBS, and these oocytes were divided into 6 groups according to the morphologies observed under a fluorescence microscope by FDA-test (Schilling et al., 1982).

Ⅲ. RESULTS AND DISCUSSION

1. *In vitro* fertilization survival rates of frozen-thawed immature oocytes

1) *In vitro* maturation and fertilization

Follicular oocytes were matured *in vitro* for 1, 2, 4, and 8 hours after recovery and 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

Table 1. Effects of cryoprotectants on *in vitro* maturation and fertilization rate of frozen immature bovine oocytes

Culture of frozen oocytes	No. of oocytes examined	No. of oocytes matured(%)	No. of oocytes fertilized(%)
Control*	40	29(72.5)	22(55.0)
Immature oocytes(1 hr.)	81	44(54.3)	25(30.9)
Immature oocytes(2 hr.)	84	40(47.6)	29(23.8)
Immature oocytes(4 hr.)	80	31(38.8)	16(20.0)
Immature oocytes(8 hr.)	86	23(26.7)	11(12.8)

* Control group was not freezed bovine immature oocytes

capacitated spermatozoa, then the *in vitro* fertilization rates were examined. The results are presented in Table 1.

The *in vitro* fertilization rates observed decreased to 12.8~20.0% depending on the maturation time, with rates being relatively high for those matured for a short period of time(23.8~30.9%). However, these rates were lower than the control of unfrozen oocytes. *In 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).

2) Survival rate

Follicular oocytes were matured by the similar procedures as in the previous *in vitro* fertilization experiment for 1, 2, 4, and 8 hours, and were 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 were *in vitro* fertilized with the capacitated spermatozoa, and their survival rates were determined by the FDA test. The results are shown in Table 2.

The survival rates observed were 12.0~30.8% 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

Table 2. Effects of cryoprotectants in the freezing medium on the survival rate of frozen immature bovine oocytes

Cryoprotectant[a]	Culture period before freezing	No. of oocytes examined	Degree of FDA test						Survival rate(%)
			A	B	C	D	E	F	
Control		40	0	5	3	1	4	5	22(55.0)
1.5 M D +	1 hr.	78	1	8	7	10	15	15	22(30.8)
2.0 M G +	2 hr.	82	2	8	12	18	9	12	21(25.6)
0.25 M S	4 hr.	84	5	10	15	19	10	10	15(17.9)
	8 hr.	80	7	15	20	12	8	9	9(11.3)
2.0 M D +	1 hr.	87	1	5	8	15	18	15	25(28.7)
1.5 M G +	2 hr.	88	1	9	10	17	13	17	21(23.9)
0.25 M S	4 hr.	84	6	14	13	15	11	9	16(18.0)
	8 hr.	83	12	19	15	12	9	6	10(12.0)

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

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

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 freezing of bovine immatur oocytes the concentration of cryoprotectants, culture time in freezing medium were examined in relation to the survival and *in vitro* fertilization rates of frozen-thawed immature occytes.

The results obtained are as follows ;

The *in vitro* fertilization rates of frozen-thawed follicular oocytes which matured *in vitro* for 1, 2, 4 and 8 hours were 12.8~30.9% 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 11.3~30.8% depending on the maturation time.

Ⅴ. REFERENCES

1. Ball, G. D., M.L. Leibfried, R. W. Lenz, R. L. Ax, B. D. Bavister and N. L. First. 1983. Factors affecting successful *in vitro* fertilization of bovine follicular oocytes. Biol. Reprod., 28:717-725.
2. Carroll, J., G. M. Warnes and C. D. Matthews. 1989. Increase in digyny explains polyploidy after *in-vitro* fertilization of frozen-thawed mouse oocytes. J. Reprod. Fert., 85:489-494.
3. Didion, B. A., D. Pomb, N. J. Martin, G. F. Homanics and C. L. Markert. 1990. Observation on the cooling and cryopreservation of pig oocytes at the germinal vesicle stage. J. Anim. Sci., 68:2803-2810.
4. Friedler, S., L. C. Giudice and E. J. Lamb. 1988. Cryopreservation of embryos and ova. Fertil. and Steril., 49:743-764.
5. Kim, S. K., Y. Y. Nam and B. H. Hyun. 1997. Effects of equilibration of cryoprotectants temperature and time of thawing and 1 step straw method on *in vitro* developmental rates of embryos. Kor. J. Animal Reprod., 21(2):103-109.
6. Kim, S. K., Y. Y. Nam, B. H. Hyun and H. B. Seok. 1997. Effects of kinds, concentration and freezing method of cryoprotectants on *in vitro* developmental rates of embryos. Kor. J. Animal Reprod., 21(2) :96-102.
7. Kono, T., O. Y. Kwon and T. Nakahara. 1991. Development of vitrified mouse oocytes after *in vitro* fertilization. Cryobio., 28:50-54.
8. Nagashima, H. and S. Ogawa. 1981. Studies on the developmental potential and survival after the deep freezing of microsurgically dichotomized morula embryos in rats and rabbits. Jap. J. Anim. Reprod., 27:12-19.
9. Renard, J. P., Y. Heyman, P. Leymonie and J. C. Plat. 1983. Sucrose dilution ; A technique for field transfer of bovine embryos frozen in the straw. Therio., 19:145.
10. Schellander, K., B. K. Brackett, F. Fuher and W. Schleger. 1988. *In vitro* fertilization of frozen-thawed cattle oocytes. Proc. 11th Congr. on Anim. Reprod. and A. I., June, Dublin Ireland, 26-30.
11. Schilling, E., H. Niemann and D. Schmidt. 1982. Evaluation of fresh anf frozen cattle embryos by fluoresence microscopy. Cryobio., 15:245-248.
12. Schmidt, M, P. Hyttle, T. Greve and B.

Avery. 1993. Ultrastructure of frozen-thawed bovine *in vitro* matured oocytes. Therio., 39:304.

13. Shaw, P. W., A. G. Benarde, B. J. Fuller, J. H. Hunter and R. W. Shaw. 1992. Vitrification of oocytes using short cryoprotectant exposure : Effects of varying exposure times on survival. Mol. Reprod. Develop. 33:210-214.
14. Shea, B. F., J. P. A. Latour, K. N. Berdin and R. D. Baker. 1976. Maturation *in vitro* and the subsequent fertilizability of extra follicular bovine oocytes. J. Anim. Sci., 43:809-815.
15. Suzuki, T., M. Yamamoto, M. Ooe, A. Sakata and K. Matsuoka. 1990. Comparison of one step sucrose dilution and direct transfer of frozen bovine embryos in glycerol and 1, 2-propanediol. Therio., 33:334.
16. Whittingham, D. G., S. P. Leibo and P. Mazur. 1972. Survival of mouse embryos frozen to −196℃ and −269℃. Science, 178:411-414.
(접수일자 : 1998. 6. 1. /채택일자 : 1998. 7. 20.)