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Limited Development of *In Vitro* Fertilization or Chemically Activated Porcine Follicular Oocytes†

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체외수정 또는 처녀발생 유기된 돼지 난포란의 제한된 발달†

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요 약

10% ethanol에 의한 처녀발생유기 및 체외수정된 돼지 난포란을 CZB와 CR_{1aa}에서 배양하여 배발달률을 조사하였다. 또한 CZB에 각기 다른 농도의 cholesterol (0㎍/mL, 2㎍/mL, 5㎍/mL, 10㎍/mL)을 첨가한 후 체외수정된 돼지 난포란을 배양하여 배발달률을 조사하였다. CZB 구는 BOEC와 공배양하였다. 처녀발생유기 48시간 후 2~8 세포기로 발달한 난자의 비율은 CZB 구가 32.2%, CR_{1aa} 구가 16.8%였으며, 192시간 후 상실배로 발달한 난자의 비율은 CZB 구가 5.75%, CR_{1aa} 구가 0%였다. 체외수정 48시간 후 2~8 세포기로 발달한 난자의 비율은 CZB 구가 42.4%, CR_{1aa} 구가 17.6%였으며, 192시간 후 상실배로 발달한 난자의 비율은 CZB 구가 5.81%, CR_{1aa} 구가 0%였다. 각기 다른 농도의 cholesterol이 첨가된 CZB 구에서 상실배로 발달한 난자의 비율은 각각 0, 10, 0.45, 0%였다.

INTRODUCTION

Recent progress in the development of co-culture system with oviductal, trophoblastic or follicular cells has enabled *in vitro* development of zygotes beyond the block stage to blastocysts. Thus, various *in vitro* culture systems are now being used as routine procedures for assessing viability of embryos produced by *in vitro* fertilization in sheep and cattle (Goto *et al.*, 1988). Substantial progress has been also made in devising *in vitro* culture conditions for porcine zygotes and cleavage stage embryos. As described in two recent reviews (Reed *et al.*, 1992; Petters and Wells, 1993), three types of culture condition have been studied: (i) organ culture, in which the embryos are grown within oviducts either *in vivo* or *in vitro*; (ii) co-culture with somatic cells, with oviductal cells, or in media supplemented with oviductal fluid; (iii) culture in defined media. Early porcine embryos develop into morulae or blastocysts with varying degrees of success in all three systems. Only organ culture or co-culture systems appear to support the development into blastocysts of zygotes and embryos earlier than the four-cell stage (Archibong *et al.*, 1989; Prather *et al.*, 1991). Nevertheless, because simple, defined culture media are easiest to replicate, and because the influence of the addition or elimination of individual components can be studied easily in simple solut-

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ions, considerable attention has been paid to devising such media.

The objective of the present study was to compare the efficacy of two culture media in supporting development of porcine zygotes into expanded and hatched blastocysts. The aim was to identify media that could be used for *in vitro* culture to assess embryo viability. The media tested were CZB, as first described by Chatot *et al.* (1989), and CR_{1aa}, as described by Rosenkrans and First (1991), both of which require high CO_2 to maintain a neutral pH. CZB medium was found to be effective for culturing mouse zygotes, particularly from strains early embryos of which exhibited the so-called 'two-cell block' (Chatot *et al.*, 1990), bovine embryos (Ellington *et al.*, 1990) and porcine embryos, in a preliminary study (Misener *et al.*, 1991). CR_{1aa} was found to be effective for culturing porcine embryo (Uhm *et al.*, 1994). In addition, we examined the effect of a lipid, cholesterol on development of porcine embryos produced *in vitro*.

MATERIALS AND METHODS

1. Embryo culture systems

The following culture systems using microdroplets of two different media with or without co-culture cells were examined. Media were adjusted to pH 7.4 and sterilized through 0.22μm Millipore filters. All the droplets of media were covered with liquid paraffin oil in a 35mm plastic petri dishes and maintained in an atmosphere of 5% CO_2 in humidified air at 39℃.

1. CZB medium supplemented with 15% FCS was used in 50μL droplets with BOEC.
2. CR_{1aa} containing essential and non-essential amino acids, SIT and 4mg BSA/mL was used in 50μL droplets without BOEC.

2. Preparation of oocytes

Ovaries were collected from maturing gilts at a local slaughterhouse and transported to the laboratory within 1~1.5 h in 0.8% (w/v) NaCl solution at 37~39℃. Oocytes were aspirated from antral follicles (3~5mm in diameter) with an 18-gauge needle fixed to a 1-mL disposable syringe. Immature oocytes were washed three times and with TCM 199 containing 20% FCS, 10 IU hCG/mL and 10 IU PMSG/mL. Ten oocytes with compact cumulus were transferred to 50μL of the same medium, which had been previously covered with warm paraffin oil in a 35mm plastic petri dishes and equilibrated in an atmosphere of 5% CO_2 in air for about 3 h, cultured for 48 h at 39℃ in the same atmospheric condition (Yoo *et al.*, 1993).

3. Sperm preparation and *in vitro* fertilization

Spermatozoa were capacitated in heparin-containing Capa medium, which had been previously equilibrated in an atmosphere of 5% CO_2 in air for about 3 h. A 10μL sample was introduced into 50μL of Fert medium that included the ten oocytes, and the mixture was incubated at 39℃ in an atmosphere of 5% CO_2 in air. The mixture gave final concentrations of $1\sim2\times10^6$ spermatozoa/mL. After incubation for 18 h with the spermatozoa, the inseminated oocytes were washed with culture medium and transferred to embryo culture droplets indicated above.

4. Chemical activation of oocytes matured *in vitro*

After *in vitro* maturation, the oocytes were suspended in 3% sodium citrate solution to disperse cumulus cells and were freed from the cells by vortexing (Kinis *et al.*, 1989). The denuded oocytes were washed 3 times with TCM 199 containing 15% (v/v) FCS and incubated for 10 min at 39℃ in TCM 199 containing 15% (v/v) FCS and 10% (v/v) ethanol to induce parthen-

ogenetic activation (Kim *et al.*, 1994).

5. Culture of embryos

The *in vitro* fertilized or parthenogenetically activated oocytes were cultured in either CZB medium with BOEC or CR_{1aa} medium without BOEC. Some embryos fertilized *in vitro* were cultured in CZB medium containing different concentrations of cholesterol (0μg/mL, 2μg/mL, 5μg/mL and 10μg/mL) with BOEC to examine whether the lipid in medium support early development. The media were changed at every 48 h, and the morphology of the *in vitro* cultured embryos in CZB and CR_{1aa} were examined at 48 h and 192 h under a microscopy. The *in vitro* cultured embryos were stained with Hoechst 33258 and examined using a fluorescence microscopy (200×), and nuclei were counted for the accurate assessment of the embryonic development (Fig. 1). Rapid staining method was also used in some embryos.

RESULTS AND DISCUSSION

Development of the *in vitro* fertilized embryos were presented in Table 1. The cleavage rate of the *in vitro* cultured embryos in CR_{1aa} was lower than in CZB at 48 h (17.6 vs 42.4%, respectively). And the ratios of morula were 5.81 and 0% in CZB and CR_{1aa} at 192 h, respectively.

Development of the *in vitro* ethanol-activated embryos were presented in Table 2. The cleavage rate of the *in vitro* cultured embryos in CR_{1aa} was lower than in CZB at 48 h (16.8 vs 32.2%, respectively). And the ratios of morula were 5.75 and 0% in CZB and CR_{1aa} at 192 h, respectively. However, no blastocyst was found in all the experimental groups of fertilized or activated oocytes. Although the 'earlier-cell block' was overcome in our study in co-culture with BOEC, development of embryos cultured *in vitro* were much lower compared with *in vivo*.

From the results of both fertilized and activated oocytes, it was evident that porcine embryos did not grow properly in the chemically-defined medium, CR_{1aa} without BOEC and FCS. However, it is concluded that the CZB

Table 1. Early development of porcine oocytes matured and fertilized *in vitro*[1]

Culture media[2]	No. of the oocytes used	No. of embryos cleaved at 48 h (%)	No. of embryos developed to the following stage			
			at 48 h (%)		at 192 h (%)	
			2~3-cell	4-cell	Morula	Total
CZB	344	146 (42.4)	101 (69.7)	27 (18.6)	9 (5.81)	155 (45.1)
CR_{1aa}	306	54 (17.6)	40 (74.1)	9 (16.7)	-	61 (19.9)

[1], Capacitated epididymal spermatozoa were coincubated with the oocyte matured for 48 h.

[2], CZB medium + 15% FCS + BOEC, and CR_{1aa} medium containg 4mg BSA/mL + SIT + essential and non-essential amino acids.

Table 2. Early development of porcine oocytes matured and ethanol-activated *in vitro*[1]

Culture media[2]	No. of the oocytes used	No. of embryos cleaved at 48 h (%)	No. of embryos developed to the following stage			
			at 48 h (%)		at 192 h (%)	
			2~3-cell	4-cell	Morula	Total
CZB	214	69 (32.2)	47 (68.1)	14 (20.3)	5 (5.75)	87 (40.7)
CR_{1aa}	202	34 (16.8)	27 (79.4)	6 (17.6)	-	43 (21.3)

[1], Oocyte activation was performed at 54 h of maturation *in vitro*.

Table 3. Early development of porcine oocytes cultured *in vitro* in the presence of cholesterol at different concentrations[1]

Concentration of cholesterol (μg/mL)	No. of the oocytes used	No. of embryos cleaved at 48 h (%)	No. of embryos developed to the following stage at 48 h (%)		at 192 h (%)
			2~3-cell	4-cell	Morula
0	39	16 (41.0)	14 (87.5)	2 (12.5)	-
2	40	20 (50.0)	13 (65.0)	7 (35.0)	2 (10.0)
5	42	22 (52.4)	15 (68.2)	7 (31.8)	1 (0.45)
10	38	14 (36.8)	11 (78.6)	3 (21.4)	-

[1], CZB + 15% FCS + BOEC was used as a basic medium.

medium may be used for the production of pre-blastocyst embryos either fertilized or activated until an adequate culture system is found. The early embryos produced in this way can be served as adequate materials for various biotechnology including rapid predetermination of sex by PCR in porcine.

Development of the embryos cultured *in vitro* in the presence of cholesterol at different concentrations were presented in Table 3. The cleavage rates of the *in vitro* cultured embryos in treatment 1 (2μg cholesterol/mL), 2 (5μg cholesterol/mL) were higher than those in control (0μg cholesterol/mL) and treatment 3 (10μg cholesterol/mL). From this result, it was suggested that cholesterol at a concentration of 2~5μg/mL may help development of porcine embryos cultured *in vitro*. It has been reported that porcine early cleavage-stage embryos survived cryopreservation following removal of cytoplasmic lipid and developed up to blastocyst under a similar condition used in this study (Hiroshi *et al.*, 1994). It is supposed that lipids may be very important in development of embryos cultured *in vitro*. The effects of various fatty acids on development of embryos cultured *in vitro* perhaps will provide a clue for the improvement of porcine embryonic development *in vitro*. Such experiments are under progress in our laboratory.

SUMMARY

In the present experiment, development *in vitro* after chemical activation and fertilization *in vitro* were examined when porcine oocytes were matured in simple media. Porcine cumulus-oocyte complexes were cultured in TCM 199 supplemented with 20% (v/v) FCS and hormonal supplements (10 IU hCG/mL, 10 IU PMSG/mL) for 24 h. They then were cultured without hormonal supplements for an additional 24 h. After activation by 10% ethanol or fertilization, oocytes were co-cultured in CZB medium supplement with 15% FCS with BOEC, or in CR_{1aa} medium supplement with BSA+A.A+SIT. After activation or fertilization, development of the oocytes was observed every 48 h up to 192 h (8 days). At 48 h after activation, 32.2% of the oocytes co-cultured in CZB medium with BOEC, and 16.8% of the oocytes cultured in CR_{1aa} medium developed to the 2- to 8-cell stages. When oocytes were cultured for 8 days after activation, the percentages of oocytes that developed to the morula stages were higher in the CZB group compared with the CR_{1aa} group (5.75% vs 0%, respectively). All oocytes cultured in the both media didn't developed to the blastocyst stages. At 48 h after fertilization, 42.4% of the oocytes co-cultured in CZB medium with BOEC, and 17.6% of the oocytes cultured in

CR_{1aa} medium developed to the 2- to 8-cell stages. When oocytes were cultured for 8 days after activation, the percentages of oocytes that developed to the morula stages were higher in the CZB group compared with the CR_{1aa} group (5.81% vs 0%, respectively). When oocytes were cultured in CZB medium containing different concentrations of cholesterol (0, 2, 5, and 5μg /mL) with BOEC, the ratios of morula were 0, 10, 0.45 and 0%, respectively. All oocytes cultured in the both media didn't developed to the blastocyst stages. It is concluded that the CZB group is more competent to support development of porcine zygotes compared with the CR_{1aa} group. It is also considered that chemical activation is very available for the predetermination of sex in animals using PCR as control.

REFERENCES

Archibong AE, Petters RM and Johnson BH. 1989 Development of porcine embryos from one- and two-cell stages to blastocysts in culture medium supplemented with porcine oviductal fluid. Bio. Reprod., 41: 1076-1083.

Chatot CL, Lewis JL, Torres I and Ziomek CA. 1990. Development of 1-cell embryos from different strains of mice in CZB medium. Bio. Reprod., 42:432-440.

Chatot CL, Ziomek CA, Bavister BD, Lewis JL and Torres I. 1989. An improved culture medium supports development of random -bred 1-cell mouse embryos *in vitro*. J. Reprod. Fert., 86:679-688.

Ellington JE, Carney EW, Farrell PB, Simkin ME and Foote RH. 1990. Bovine 1~2-cell embryo development using a simple medium in three oviduct epithelial cell coculture systems. Bio. Reprod., 43:97-104.

Goto K, Kajihara Y, Kosaka S, Koba M, Nakanishi Y and Ogawa K. 1988. Pregnancy after co-culture of cumulus cells with bovine embryos derived from *in vitro* fertilization of *in vitro* matured follicular oocytes. J. Reprod. Fert., 83:753-758.

Hiroshi N, Naomi K, Rodney JA, Christopher GG, Robert FS and Mark BN. 1994. Removal of cytoplasmic lipid enhances the tolerance of porcine embryos to chilling. Bio. Reprod., 51:618-622.

Kim SB, Lee H, Byun TH, Jeon JT, Lee SH and Song, HB. 1994. Early development of parthenogenetically activated porcine oocyte after *in vitro* maturation for various periods. Korean J. Emb. Trans., 9:117-125.

Kinis A, Vergos V, Gallagher M and Gordon A. 1989. Use of sodium citrate in the denudation of bovine oocytes prior to *in vitro* fertilization. Proc. 5th Conference of European Embryo Transfer Association, p. 162 (Abstract).

Misener M, Pollard JW and Metzger K. 1991. *in vitro* culture of porcine embryos in CZB medium. Theriogenology, 35:244 (Abstract).

Naito K, Fukuda Y and Ishibashi I. 1989. Developmental ability of porcine ova matured in porcine follicular fluid *in vitro* and fertilized *in vitro*. Theriogenology, 31:1201-207.

Petters RM and Wells KD. 1993. Culture of pig embryos. J. Reprod. Fert. Suppl., 48:61-73.

Prather RS, Sims MM and First NL. 1991. Culture of porcine embryos from the one- and two-cell stage to the blastocyst stage in sheep oviducts. Theriogenology, 35:1147 -1151.

Reed ML, Illera MJ and Petters RM. 1992. *In vitro* culture of pig embryos. Theriogenology, 37:95-109.

Rosenkrans CFJr and First NL. 1991. Culture of bovine zygotes to the blastocyst stage : effects of amino acids and vitamins. Theriogenology, 35:266.

Uhm SJ, Chung HM, Park JK, Lee JH, Lee HT and Chung KS. 1994. Effect of mouse leukemia inhibitory factor on the development of *in vitro* produced pig embryos. Korean J. Animal Reprod., 18(3):207-216.

Yoo HJ, Choi SC and Lee SH. 1993. Maximization of the number of follicular oocytes recovered from the bovine ovaries. Korean J. Anim. Reprod., 17:149-157.