大韓不妊學會雜誌: 第 24 卷 第 1 號 1997
Kor. J. Fertil. Steril., Vol. 24, No. 1, 1997, 4

In Vitro Developments of Porcine Embryos Produced by *In Vitro* Fertilization and Parthenogenetic Activation

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체외수정과 단위발생된 돼지 난자의 체외발달 양상의 비교

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= 국문초록 =

본 연구는 단위발생과 체외수정으로 생산된 돼지 난자의 발달양상과 inner cell mass (ICM) 그리고 trophectoderm (TE)의 세포배열을 조사하기 위하여 실시하였다. 단위발생은 ethanol 단독처리 (haploid) 혹은 ethanol과 cytochalasin B을 공동처리 (diploid) 하였던바, 단위발생란은 체외수정란에 비하여 배반포까지의 발달이 저조하였지만, 단위발생에 있어서 ethanol과 cytochalasin B을 공동처리한 군이 ethanol 단독처리한 군보다 배반포까지 발달이 촉진되었다. 또한 단위발생란의 경우 total 세포수와 ICM 수에 있어서 체외수정란에 비하여 현저하게 감소되었지만, ethanol과 cytochalasin B을 공동처리한 단위발생란이 ethanol 처리된 단위발생란보다는 현저하게 높은 total 세포수와 ICM 수가 조사되었다. 이상의 결과로, 돼지의 착상전 배발달 양상과 ICM와 TE의 세포배열에 있어서 ploidy가 영향을 미친다는 것을 알수 있었다.

INTRODUCTION

Parthenogenetic activation of mammalian oocytes can be induced by various stimuli. Activation of porcine oocytes matured *in vitro* has been by calcium ionophore (Funahashi *et al.*, 1994), electrical shock (Hagen *et al.*, 1991), CaCl2 (Machaty *et al.*, 1996), G protein stimulation (Machaty *et al.*, 1995), protein kinase inhibitors (Mayes *et al.*, 1995), and cycloheximide treatment (Nussbaum and Prather, 1995). Fukui *et al.* (1992) reported that bovine oocytes matured *in vitro* and treated with ethanol and cytochalasin B can develop to the blastocyst satge *in vitro* and embryos have the ability to pregnancy up to the preimplantation stage. Cytochalasin B is an inhibitor of microfilament polymerization. Treatment of cytochalasin B following parthenogenetic stimulation enhanced developmental competence in cattle (Presicce and Yang, 1994; Fukui *et al.*, 1992), rabbit (Collas and Robl, 1990) and sheep (Smith and Wilmut, 1989). This may be due to the action of cytochalasin B in preventing the release of the second polar body after activation of the oocytes, which result in the diploid development (Fukui *et al.*, 1992). It was known that in the mouse the developmental potential of parthenogenetic

mouse oocytes with two pronuclei was much better than that of the oocytes with one pronucleus (Kaufman *et al.*, 1983).

In the mouse, less haploid than diploid parthenotes reached the blastocyst stage and the cell number of haploid parthenotes reduced as compared to that of diploid (Kaufman, 1983). It is also known that haploid mouse parthenotes appear to develop *in vitro* more slowly than diploid parthenotes during the preimplantation period (Kaufman and Sachs, 1975; Henery and Kaufman, 1992). Furthermore, haploid parthenotes contain few or no inner cell mass cells (Kaufman, 1978). However, very little information is available on the developmental pattern and cell allocation to the ICM and TE of haploid and diploid parthenotes for any species other than the mouse. Therefore, the present study was undertaken to determine the developmental pattern and cell allocation to the ICM and TE in these parthenotes and *in vitro* fertilized embryos.

MATERIALS AND METHODS

1. Recovery of immature oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in saline (35 to 39℃) within 1hr. The oocyte-cumulus complexes (OCCs) were recovered by aspiration from the follicles (2-6 mm in diameter) using a 18-gauge needle and a 10 ml disposable syringe. The OCCs were washed three times with TL-HEPES (1 mg/ml BSA) and the maturation medium, respectively.

2. *In vitro* maturation (IVM)

The oocytes (about 50 oocytes) were transferred into a 0.5 ml of maturation medium equilibrated for 2 hr in 5% CO_2 and 95% O_2 incubator under warm mineral oil in a four well culture dish. The maturation medium consisted of TCM-199 (with Earle's salts: Gibco, USA) supplemented with 25 mM $NaHCO_3$ (Sigma, USA), 0.6 mM cysteine, 0.2 mM pyruvate (Sigma, USA), p-FSH (Schering-Plough Animal Health, USA), and 25 μg/ml gentamycin (Sigma, USA). Culture was carried out at 39℃ in 5% CO_2 in air for 44 hr.

3. Activation of oocytes

Porcine oocytes following IVM for 44 hr were used for activation in this study. Cumulus-free oocytes (CFOs) were obtained by removal of cumulus cells with 0.1% hyaluronidase in calcium-free DPBS-PVP for 5 min. and pipetting. The CFOs were activated by ethanol or combination of ethanol and cytochalasin B. Briefly, oocytes were activated by bathing them for 5 min. in 7% ethanol in NCSU (BSA-free) medium, followed by washing them in NCSU 23 (0.4% BSA) medium. These oocytes were then incubated for 5 hr in NCSU 23 (0.4% BSA) medium containing 10 μg/ml cytochalasin B, followed by washing them in NCSU 23 (0.4% BSA) medium.

4. Sperm capacitation and *in vitro* fertilization (IVF)

Semen were collected from cauda of epididymis obtained from a a local slaughterhouse. Semen (1 ml) were diluted into Sp-TALP (5 ml), and the extender was removed by washing two times (1,000 rpm) for 5 min. Then the sperm pellet was resuspended with Sp-TALP, and motile sperm were collected by swimup separation after incubation for 10 min.

Highly motile sperm were added into the fertilization medium containing about 50 oocytes/0.5 ml to make a final concentration of 2.5×10^5 sperm/0.5 ml. Incubation conditions for IVF were 5% CO_2 in air with saturated humidity at 39℃. The fertilization medium was consisted of fatty acid free bovine serum albumin (FAF-BSA, 6 mg/ml), 0.2 mM pyruvate, 2 μg/ml heparin, 18.2 μM penicillamine, 1.8 μM hypotaurine, 9.1 μM epinephrine, and 25 μg/ml gentamycin. The oocytes and sperm were incubated for 30 hr at

5% CO_2 in air with saturated humidity at 39℃.

5. *In vitro* development (IVD)

Activated or inseminated oocytes were washed 3 times in NCSU 23 (0.4% BSA). Washed oocytes were then transferred to previously prepared culture dish containing of NCSU 23 (0.4 % BSA) medium covered with mineral oil and cultured at 5% CO_2 in air with saturated humidity at 39℃. The medium was not changed during the culture period. The blastocysts of embyos were assessed at 6 days after insemination. The blastocysts were stained by differential labelling of ICM and TE nuclei.

6. Differential labelling of ICM and TE nuclei

Total, TE and ICM nuclei of blastocysts were differentially labelled by using a method of Papaioannou and Ebert (1988) with some modifications. Briefly, TE nuclei are labelled first specifically with fluorochrome PI (Sigma). This fluorochrome was excluded from viable ICM cell but labelled TE cells undergoing antibody-mediated complement lysis during immunosurgery. The whole embryo is rapidly fixed and both the TE and ICM nuclei labelled with bisbenzimide. The protocol was as follows; embryo zona was removed in 0.5% pronase (Sigma) solution. and allowed to recover for 10 min. in TL-Hepes. Embryos were incubated on ice for 10~15 min. in 15 mM TNBS (Sigma) containing 4 mg/ml PVP (Sigma) in TL-Hepes. After washing completely, embryos were incubated in 0.1 mg/ml anti-DNP-BSA (ICN Immunobiological.) in TL-Hepes for 10 min. at 37℃. After washing sufficiently in TL-Hepes, the embryos were incubated in 0.01 mg/ml PI and 10% (v/v) guinea pig complement (Sigma) in TL-Hepes for 15~30 min. at 37℃. After 15 min. observed them until even lysis of the outer TE cells was seen and then transferred them into 0. 05 mM bisbenzimide in absolute alcohol. After overnight storage at 4℃, the embryos were washed in absolute alcohol for at least 1 hr, and mounted in glycerol under a coverslip on a slide glass. Labelled nuclei were observed under ultra violet excitation filter incorporated fluorescent microscope and TE nuclei labelled with PI and bisbenzimide appeared pink or red, ICM nuclei labelled with bisbenzimide appeared blue or unlabelled.

7. Analysis

At least four replicate trials were conducted

Table 1. Cleavage of activated or fertilized porcine oocytes

Time	Treatment	Total	Cleaved	2 cell	3-4 cell	6-8 cell
18 hr	Control	260	3 (1.2)[a]	3	0	0
	Ethanol	236	5 (2.1)[ab]	2	3	1
	Ethanol+CB	236	10 (4.2)[b]	6	4	0
24 hr	Control	260	34 (13.1)[a]	16	18	1
	Ethanol	236	18 (7.6)[b]	7	9	2
	Ethanol+CB	236	27 (11.4)[ab]	12	12	3
30 hr	Control	260	70 (11.4)[a]	31	31	8
	Ethanol	236	42 (17.8)[b]	10	12	10
	Ethanol+CB	236	49 (20.8)[ab]	21	20	8
40 hr	Control	260	139 (53.5)[a]	36	74	29
	Ethanol	236	72 (30.5)[b]	21	22	29
	Ethanol+CB	236	81 (34.3)[b]	27	36	18

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

Table 2. *In vitro* development of porcine oocytes activated by ethanol and CB

Time	Treatment	Total	Cleaved	Blastocyst			
				Total	early	middle	expand
4 day	Control	260	174 (66.9)[a]	4 (2.3)	4	0	0
	Ethanol	236	128 (54.2)[a]	0 (0.0)	0	0	0
	Ethanol+CB	236	152 (64.4)[a]	2 (1.3)	2	0	0
5 day	Control	260	174 (66.9)[a]	24 (13.8)[a]	15	8	1
	Ethanol	236	128 (54.2)[b]	2 (1.6)[b]	2	0	0
	Ethanol+CB	236	152 (64.4)[a]	14 (9.2)[a]	13	1	0
6 day	Control	260	174 (66.9)[a]	53 (30.5)[a]	31	10	12
	Ethanol	236	128 (54.2)[b]	11 (8.6)[b]	10	1	0
	Ethanol+CB	236	152 (64.4)[a]	31 (20.4)[c]	20	9	2

[a-c]Different superscripts within column were significantly different ($p<0.05$).

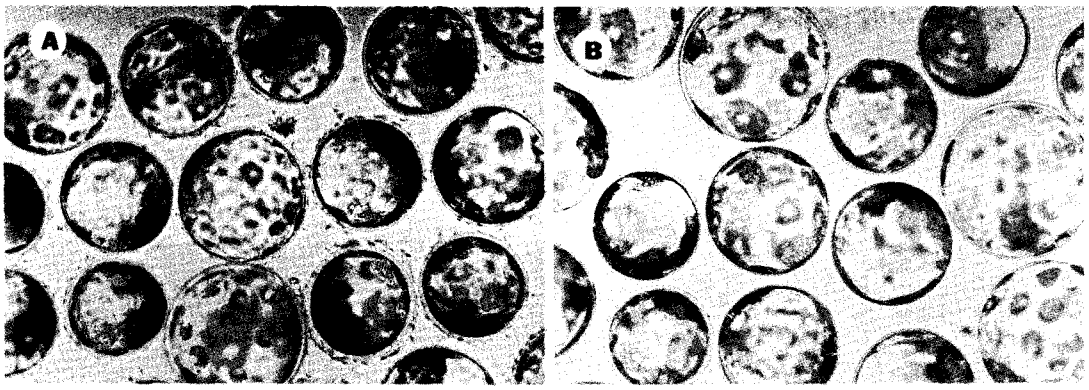

Fig. 1. A, Pig blastocysts produced by IVM/IVF/IVC (6 day after IVF). Many sperm attach on zona pellucida (×150). **B,** Pig blastocyst produced by parthenogenetic activation (6 day after activation, ×150). Nothing attaches on zona pellucida.

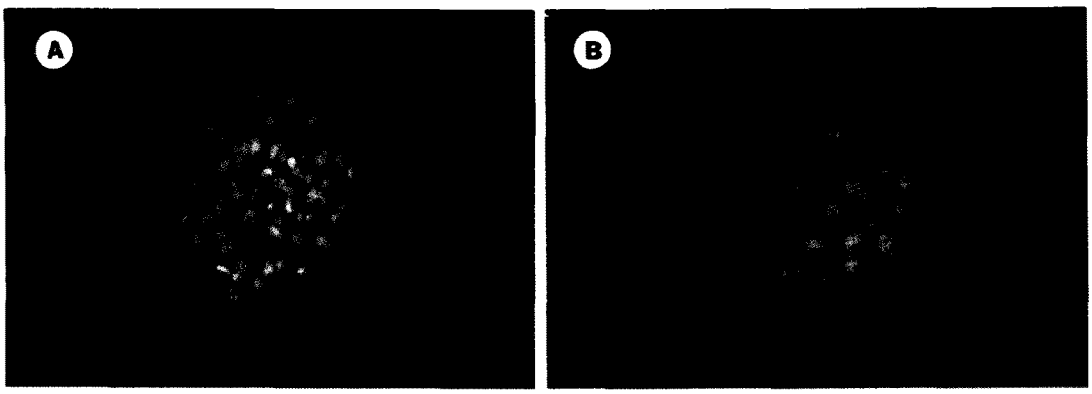

Fig. 2. A, Differential staining of a pig blastocyst produced by IVM/IVF/IVC (6 day after IVF). The blastocyst contains 16 ICM and 52 TE. **B,** Differential staining of a pig blastocyst produced by parthenogenetic activation (6 day after activation, ×300). The parthenote contains 6 ICM and 17 TE (×300).

Table 3. Number of total and ICM cells (mean ± SEM) in blastocysts of porcine embryos produced *in vitro* fertilization or parthenogenetic activation

Treatment of maturation	No. of blastocyst	Total cells		ICM		
		No. of cells	Range	No. of cells	Range	Proportion (%)
Control	15	45.1 ± 6.2^{a}	21-93	8.9 ± 4.2^{a}	3-16	22.5 ± 3.2
Ethanol	6	16.0 ± 3.2^{b}	9-29	2.2 ± 0.5^{b}	0-3	16.8 ± 4.4
Ethanol+CB	12	26.7 ± 3.6^{c}	11-50	5.7 ± 0.9^{c}	2-12	22.8 ± 2.6

[a-c]Different superscripts within column were significantly different ($p<0.05$).

for each experiment. A chi-square test was used to ascertain statistical differences between treatments. A p value of less 0.05 was considered statistically significant.

RESULTS

The cleavage rates of fertilized or activated oocytes examined during 18 to 40 hr after fertilization or activation (Table 1). The incidence of cleavage was significantly different between fertilized and activated embryos after 40 hr ($p < 0.05$), but not different between ethanol and combination of ethanol and CB treatments. However, the formation of blastocoel observed at the combined of ethanol and CB treatment and fertilized embryos after 4 days, and the development of blastocyst rates of them those were significantly higher than ethanol treated embryos ($p < 0.05$, Table 2). The morphology of blastocysts between fertilized and activated embyos was not significantly different, but many sperm attached to the zona pellucida of blastocysts of fertilized embryos (Fig. 1). The differential labelling showed obvious distinction between the pink TE and ICM nuclei (Fig. 2). Significant reduction in the average number of total cells and ICM was observed in the ethanol treatment alone as compared with combined ethanol and CB treatment or fertilized embryos ($p < 0.05$, Table 3).

DISCUSSION

While a high percent pronuclear formation is readily achievable, development of parthenotes to the blastocysts was limited in the pig (Funahashi *et al.*, 1994; Kim *et al.*, 1996; Machaty *et al.*, 1995). The reason for the low rate of *in vitro* developmental competence following activation in pig remain unknown. In the present study, treatment with cytochalasin B followed by ethanol stimulation induced diploid parthenotes which appear to more confidently develop to the blastocysts than the haploid.

Diploid parthenotes more fastly developed to the blastocysts than of haploid parthenotes. This result is similar with previous reports in the mouse that haploid embryos developed significantly slower than diploid embryos (Kaufman, 1983; Henery and kaufman, 1992). This result suggests that the lack of one genetic component (haploid parthenote) may increase the duration of the cell cycle and consequently slow down their development, while the presence of two maternally derived genetic components does not appear to do so. Interestingly, in the amphibian, the haploid, which were half the volume and half the mass of their diploid counterparts, had an S-phase which occupied the same relative position in the cell cycle, and lasted for the same period as in the diploids. Edwards *et al.* (1958) examined the relationship between cell number and the mean cleavage number in the mouse.

The haploid embryos had slightly fewer cells than the diploid embryos, and this was attributed to a delay in their time of entry into the first cleavage, rather than to different rates of cleavage in the mouse. Collectively, present study suggests that ploidy could affect the pattern of embryonic development after parthenogenetic activation. The exact mechanism in which the diploid genetic material enhances *in vitro* development is not known at present.

There have been numerous evidences that ICM cells in blastocysts will differentiate into all tissues of the developing fetus, in contrast, TE cells are responsible for attachment to the uterine endometrium and formation of the fetal placenta. The allocation of these cells could influence embryos for the developmental competence following implantation (Papaioannou and Evert, 1988; Giles and Foote, 1995). In the present study we employed the differential labelling to identify inner cell mass and trophoblastic cells in the blastocysts. The differential staining procedure used to identify inner cell mass and trophoblastic cells in the mouse (Handyside and Hunter, 1984), pig (Papaioannou and Ebert, 1988), and bovine embryos (Iwasaki *et al.*, 1989). Previously Papaioannou and Ebert (1988) observed that in the pig the ratio of ICM/total cells was approximately 0.22, 0.25, and 0.17 for early, expanded, and hatched blastocysts. In the present study, the parthenotes following ethanol treatment alone has few or no ICM cells. Previously, Kaufman (1978) observed that haploid mouse embryos clearly have a reduced total number of ICM cells. Curiously, some haploid parthenogenetic morulae that initially appeared to compact normally go though a 'decompaction phase' before progressing to form normal blastocysts. This 'decompaction phase' was not observed in the diploid parthenotes or normally fertilized embryos.

In conclusion, our results suggested that the parthenotes appear to develop more slowly than fertilized embryos. Haploid porcine parthenotes have significantly more reduced to the development of blastocyst and number of total and ICM cells number than diploid parthenotes, suggesting that ploidy affect the developmental pattern and cell allocation to the ICM and TE for blastocyst formation.

SUMMARY

The objective of this study is to determine developmental pattern and cell allocation to the ICM and TE in haploid and diploid of embryos following parthenogenetic activation and *in vitro* fertilization. The incidence of development to blastocyst was lower in the combined treatments of ethanol stimulation and cytochalasin B as compared to the control. However, the combined ethanol stimulation and cytochalasin B treatment (diploid) accelerated development to the blastocyst as compared to the ethanol treatment alone (haploid). Significantly reduction in the average number of total cells and ICM was observed in the parthenotes alone as compared to fetilized embryos, but those of combined ethanol stimulation and cytochalasin B treatment embryos were significantly increased as compared to ethanol alone embryos. These results suggested that the ploidy affects preimplantation developmental pattern and cell allocation to the ICM and TE in the porcine.

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