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난자성숙시간에 따른 처녀발생유기 돼지난자의 초기발생

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Early Development of Parthenogenetically Activated Porcine Oocyte after *In Vitro* Maturation for Various Periods

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SUMMARY

In vitro development of parthenogenetic embryo was examined after ethanol treatment of follicular oocytes matured *in vitro* for 42, 48, 54 and 60h in the pig. The follicular oocytes were matured in TCM 199 containing 15% FCS and gonadotrophins in an atmosphere of 39 ℃ 5% CO_2. The cumulus-free oocytes were activated by 10% ethanol treatment in M2+4mg/ml BSA for 10 min. The ethanol-activated oocytes were washed and further cultured in TCM199+20%FCS containing granulosa cell monolayer. Maturation rates at 42, 48, 54 and 60h of IVM were 75.0, 86.5, 81.6 and 87.9%, respectively. Thus the oocytes maturated *in vitro* for longer periods did not improve nuclear maturation much. Pronuclear formation rates at 18h post-activation in ethanol-activated oocytes were 21.9, 25.0, 47.4 and 32.6%. The cytoplasmic maturation leading to pronuclear formation upon activation increased when the IVM period was extended from 42 to 54h. When the activated oocytes were cultured for 96~120h to analyse early development of the activated oocytes, the rates of embryonic development upto ≤ 5~8 cell were 5.3, 5.8, 12.0 and 11.7% among the cultured embryos. The result indicate that earlier development of activated porcine oocyte is dependent on the duration of oocyte maturation, and that better development could be obtained from the oocyte matured for 54h.

Key Words ; ethanol activation, porcine oocytes, early development

INTRODUCTION

Parthenogenetic activation of metaphase II mammalian oocytes has been induced in a number of ways mainly in mouse oocytes (Kaufman, 1983). In farm animals, parthenogenetic activation is not well characterized yet. (Nagai, 1987, 1992 ;Lee *et al*. 1991). However parthenogenetic activation of the oocyte is necessary procedure for cloning of embryonic cells into unfertilized, enucleated oocyte rather than basic understanding of embryogenesis in farm animals(Gordon and Lu, 1990 ; Yoo *et al*., 1993).

With the available *in vitro* technology of embryo production it become now possible to produce *in vitro* matured oocytes and early embryos from slaughter house ovaries. The quality of the matured oocyte is critical for subsequent development for sucessful cloning. Many treatments

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have been made to produce better quality of the oocyte.

The aim of this study was to determine the proper IVM period leading to completion of both nuclear and cytoplasmic maturation by examining the response to parthenogenetic activation.

Materials and Methods

1. *In vitro* maturation (IVM) of porcine follicular oocytes

The ovaries were recovered from the gilts at a slaughterhouse and transferred to the laboratory in 0.85% physiological saline at 39℃ within an hour. After the ovaries were washed with saline and the blood were wiped with 70% EtOH-soaked gauze. The oocytes were collected from the follicle that had diameters of 2~6mm by puncturing with fine forceps. The oocytes with compact cummulus cells were recovered and washed three times in M2+4% FCS (Fulton and Whittingham, 1978). They were cultured for 42, 48, 54, 60 h in TCM 199 (with 25mM HEPES buffer, Gibco, USA) supplemented with 15 % (v/v) inactivated fetal calf serum, 10 IU/ml PMSG and 10 IU/ml hCG (Intervet, Holland) in a plastic culture dish (15 × 60 mm, Falcon) under paraffin oil in an atmosphere of 5% CO_2 in air at 39 ℃.

2. Parthenogenetic activation of the oocytes

After *in vitro* maturation, the oocytes were suspended in M2 containing 3% sodium citrate to disperse cumulus cells and were freed from the cells by vortexing (Kinis *et al.*, 1989) The denuded oocytes were washed 3 times with M2 + 4mg/ml BSA and incubated for 10 min at 39 ℃ in M2 containing 10 % (v/v) ethanol to induce parthenogenetic activation as previously described(Yoo et al., 1993).

3. Granulosa cell monolayer (GCM) culture

After oocyte collection, large masses of granulosa cells were collected by a flame-polished Pasteur pipette, and spun down at 500 × g for 10 min. The cells were then washed 3 times in M2+20% FCS. The supernatant was removed prior to resuspending the cells in TCM 199+20% FCS. The cell pellet was aspirated 5 times using 1ml syringe with a 18 gauge needle. After gently mixing, the cell concentration was adjusted either by additon of cells or dillution to 1×10^6/ml with medium. 50㎕ of GC suspension drops were made in a plastic culture dish under paraffin oil. The following day, the blood cells and cell debris were removed and the medium was replaced at every 48h. The ethanol-treated oocytes were washed 3 times in TCM 199+15% FCS, transferred into GCM culture drop and left for 120h in CO_2 incubator.

4. Assessment of activation

1) Pronuclei (PN) formation

The *in vitro* matured oocytes for different periods were analysed for chromosomal stage by rapid staining method (Byun *et al.*, 1991). Activated oocytes were also analysed for PN formation at appropriate times. Early development of the activated oocytes was determined using the similar method by scoring the number of cell nuclei.

2) Cortical granule exocytosis (CGE)

After activation, the oocytes were processed for the analysis of CGE by fluorescein isothiocyanate conjugated to Ulex europaeus I (FITC-UEA I, Sigma) staining. The oocytes

were incubated in drops of 200㎍ FITC-UEA I /ml in PBS + BSA for 2h at 37℃ in the dark. After incubation, the oocytes were washed in PBS + BSA to remove unbound FITC-UEA I and free FITC molecules (Lee *et al.*, 1988 ; Choi *et al.*, 1994) Activated oocytes showed uniform distribution of both labeled spots at the upper surface and a fluorescent ring at equatorial focal plane. Photographs were taken with automatic photomicrographic system (PM-10ADS, Olympus).

Result and Discussion

1. Nuclear maturation at different IVM periods

IVM periods from 42 to 60 h did not appear to improve greatly maturation rate of metaphase II stage. 75.0, 86.5, 81.6 and 87.9% of maturated oocytes were obtained at 42, 48, 54 and 60 h of IVM, respectively. Although there may be a slight difference by increasing IVM period from 42 to 48 h (Table 1). However, it was noted that cummulus expasion was gradually prominent in longer periods. In the meantime, the chromosomal configuration was normal even in the 60 h group. Thus the aging process was not found at least in the chromosomal configuragon.

2. Pronuclear formation in activated oocytes

Demonstration of normal fertilization and early development is a proper way of testing the developmental capacity of the IVM oocyte. However, the *in vitro* fertilized porcine oocytes do not develop fully in culture systems reported so far. Involvement of various factors derived from spermatozoa may also cause complex interpretation further. Therefore more simple method was used to analyse develpmental capacity of IVM oocytes. Pronuclear formation in ethanol-activated oocytes was measured indirectly to determine cytoplasmic maturation, which can be found in ovulated, maturated oocytes. The pronuclei formation gradually increased in longer IVM periods (Table 2). Thus the cytoplasmic maturation leading to pronuclear formation increased nearly twice when the IVM period was extended from 42 to 54h. Cytoplasmic maturation appeared to depend on the IVM period although nuclear maturation did not greatly improve. When all the chromosomal activation, i. e, anaphase or telophase II, was included the overall activation rates were much higher. Therefore prolonged incubation of the oocytes may bring proper maturation in the whole.

3. Early development of parthenogenetic embryos

Table 1. Effects of IVM period on the proportion of metaphase II Culture period for of IVM (h)

Culture Period for of IVM(h)	No. of the oocytes used	No. of the oocytes at the following stages1 (%)				
		GV	M I	A I	T I	M II
42	40	2(5.0)	7(17.5)	1(2.5)	–	30(75.0)
48	37	1(2.7)	3(8.1)	–	1(2.7)	32(86.5)
54	38	1(2.6)	6(15.8)	–	–	31(81.6)
60	33	–	4(12.1)	–	–	29(87.9)

1. Abbreviations are GV, germinal vesicle; M I, metaphase I ; A I, anaphase I ; T I, telophase I and M II, metaphase II, respectively.

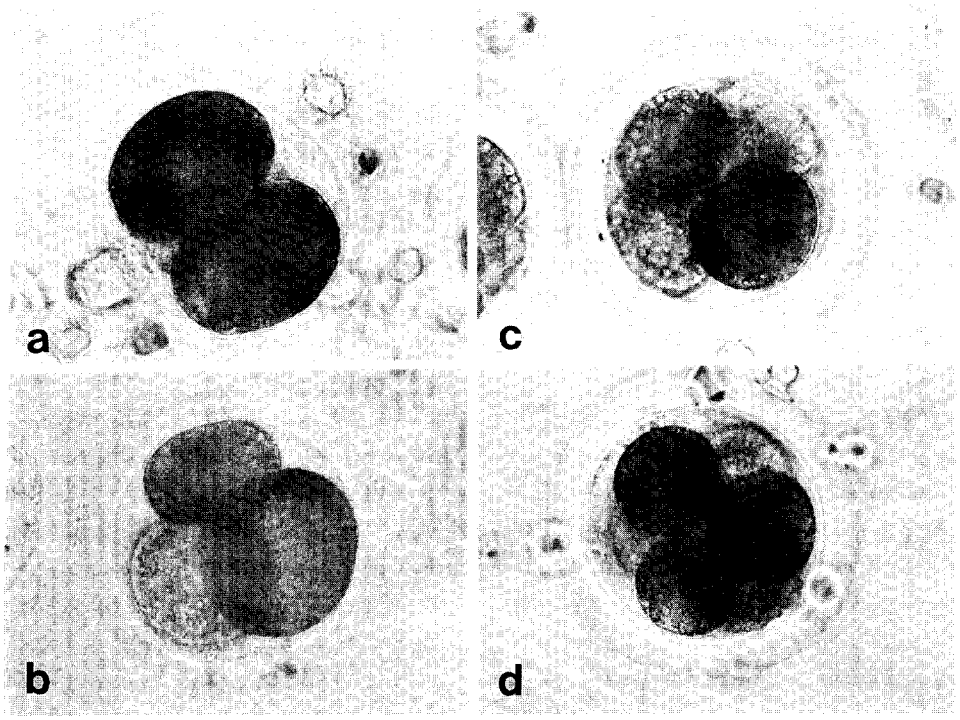


Fig. 1. Morphology of porcine cultured during 120h after activation *in vitro*.
(a) 2-cell stage oocyte;
(b) 3-cell stage oocyte;
(c) 4-cell stage oocyte;
(d) 8-cell stage oocyte.
Magnifications are × 200.

To demonstrate further developmental capacity with GCM. The cleavage indice at 48 h were 4.7, 14.3, 14.3 and 15.2 at 42, 48, 54 and 60 h IVM period, respectively. There was no clear difference amongst the groups except for 42 h (Table 3). The number of nuclei was analysed in all embryos for the accurate assessment, since the morphological examination which is not alway correct due to fragmentation. the typical embryonic stage were shown in Fig. 2. Although the limited development was found in subsequent culture, the rates of 2-4 cell embryos were similar among the groups. However, the rate of 5-8 cell embryos became higher in longer IVM periods (54 and 60h) (Table 4). It was also interesting to note that fragmentation was the least in 54 h group.

Since Cuthberson (1983) reported that mouse eggs were easily activated with brief alcohol treatment *in vitro*, this method has been used by many laboratories in mouse, cattle and pig eggs. (Kaufman, 1982; Surani *et al.*, 1984; Mann and

Table 2. Pronuclear formation at 18 h post-activation in ethanol activated oocytes matured *in vitro* for various periods

Culture period for IVM(h)	No. of the oocytes used	No. of the oocyte showing the folowing nuclear stages1 (%)							No. of activated oocytes(%)	No. of PN oocytes (%)
		M I	A I	M II	A II	T II	1PN	2PN		
42	64	5	3	30	1	8	13	1	23(35.9)	14(21.9)
48	32	2	–	16	2	4	4	4	14(43.8)	8(25.0)
54	38	6	–	9	2	3	3	1	23(60.5)	18(47.4)
60	43	1	–	10	8	8	8	3	30(69.8)	14(32.6)

1. Abbreviations are PN, pronucleus. See also the footnotes of Table 1.
2. Activation rates were obtained by including A II, T II oocytes and pronucleate embryos.

Table 3. Early cleavage of ethanol-activated oocytes matured *in vitro* for various periods[1]

Culture period for IVM(h)	No. of the oocytes used	No. of the eggs showing the following stages[2] (%)												No. of the oocytes cleaved(%)
		M I	A I	T I	M II	A II	T II	1PN	2PN	3PN	2-cell	3-cell	4-cell	
42	43	6	2	1	14	6	8	1	3	–	2	–	–	2(4.7)
48	28	2	–	–	8	–	1	7	5	1	3	1	-	4(14.3)
54	49	3	–	–	11	2	6	13	7	–	6	1	–	7(14.3)
60	66	5	–	–	18	1	–	18	13	1	7	2	1	10(15.2)

1. The cleavage rates were assessed at 48 h post-activation.
2. Proportion of 2PN increased according to the IVM period, showing 3/4,5/13,7/20 and 13/31, respectively.

Table 4. Early development of parthenogenetically activated porcine oocytes matured *in vitro* for various period at 120 h post-activation

Culture period for IVM (h)	No. of the oocytes used	No. of eggs at the following stages (%)			
		1-cell	2~4-cell	5~8-cell	fragmented
42	152	60(39.5)	42(27.6)	8(5.3)	42(27.6)
48	241	122(50.6)	83(34.4)	14(5.8)	22(9.1)
54	158	99(62.7)	37(23.4)	19(12.0)	3(1.9)
60	60	22(36.7)	22(36.7)	7(11.7)	9(15.0)

1. Abbreviation was Frag, fragmentation.

Table 5. CG exocytosis of activated oocytes matured in vitro for different periods

Culture period for IVM (h)	No. of the experiment	No. of the oocytes used	No. of the oocytes released CG contents	Rate of cortical granule exocytosis(%)
42	2	17	1	2.9±4.16
48	3	36	19	51.5±13.54
54	2	30	9	27.51±4.85
60	2	28	4	13.67±4.31

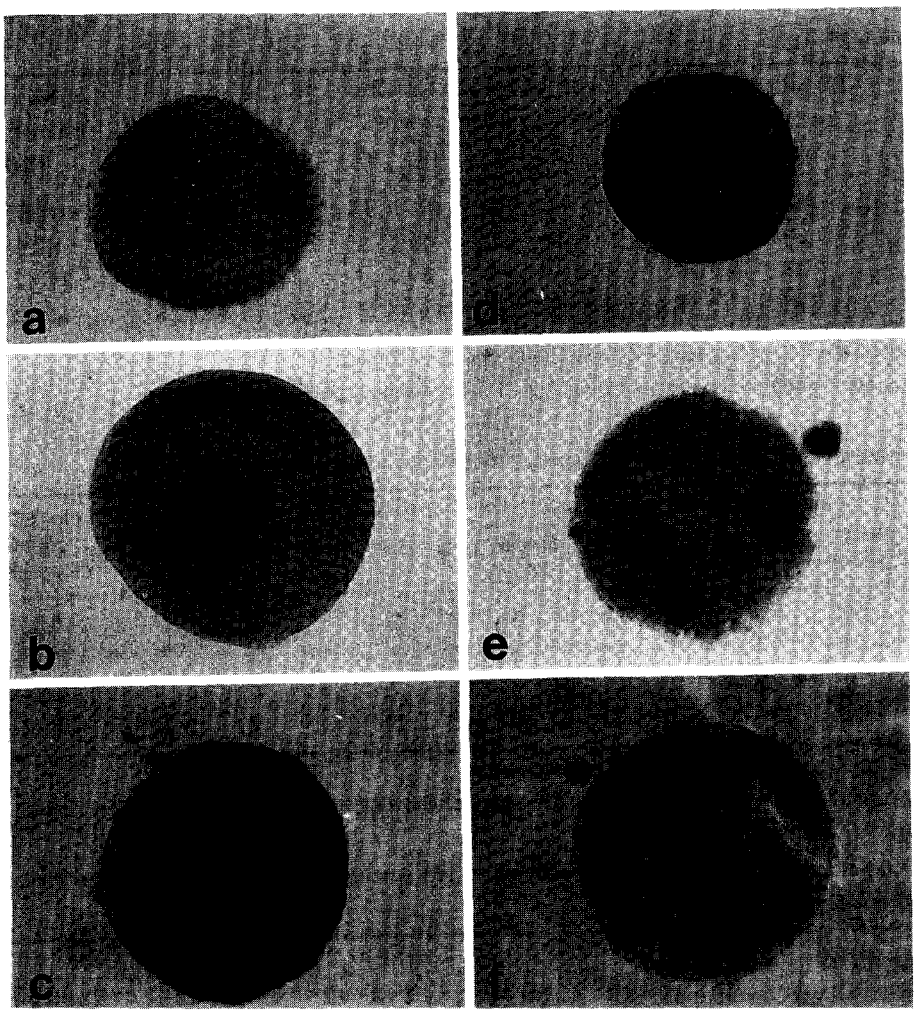


Fig. 2. Chromosome configuration of porcine oocyte cultured during 120h after activation *in vitro* stained by rapid staining method. (a) 2-cell stage oocyte; (b) 3-cell stage; oocyte; (c) 4-cell stage oocyte; (d) 4-cell stage oocyte; (e) 6-cell stage oocyte; (f) 7-cell stage oocyte; (g) 8-cell stage oocyte. Magnifications are × 200.

Lovell-Badge, 1984). However, the activation rates have been reported to be low (10%) with porcine oocytes (Didion, 1990). Although the reason for these low rates is not clear, aging of oocytes seems to be necessary for the parthenogenetic activation of porcine follicular oocytes. The matured oocytes showed the capability of early parthenogenetic development as well as pronucleus formation upon ethanol activation.

Cortical granule exocytosis (CGE) experiment also showed that longer IVM period may be beneficial for the appropriate cytoplasmic maturation as shown by membrane response (Table 5

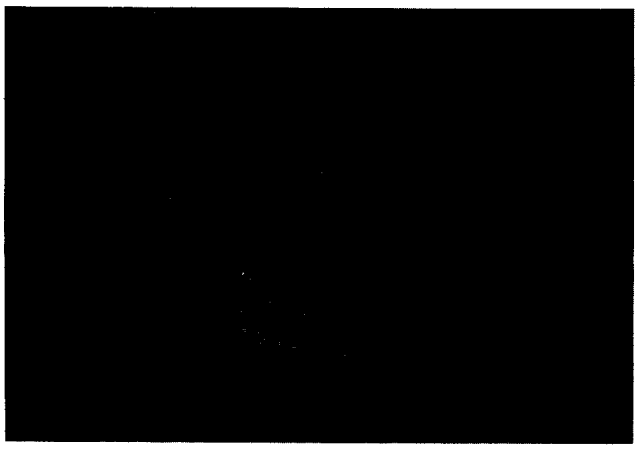
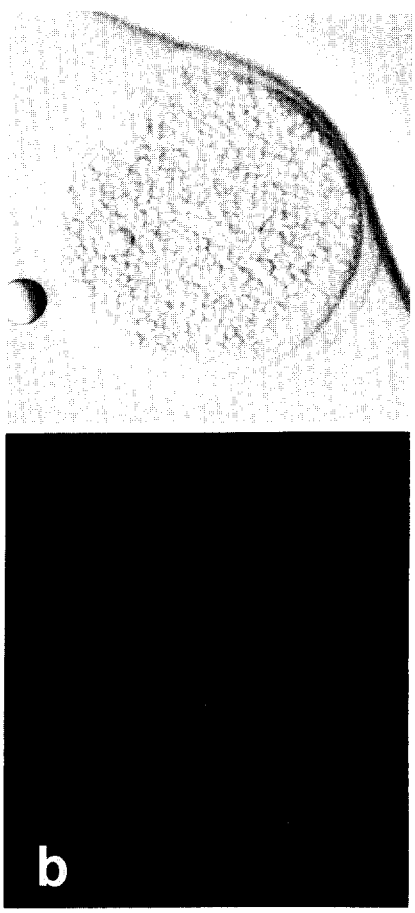


Fig. 3. Comparative morphologies between activated and unactivated porcine oocytes. Magnifications are × 200.



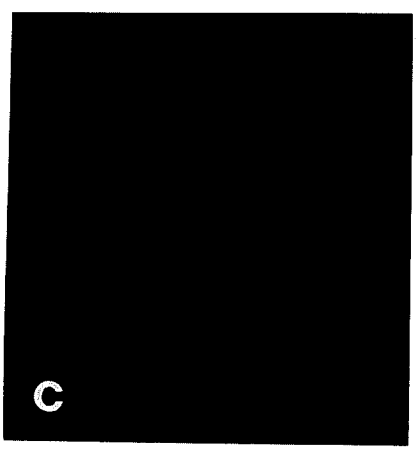


Fig. 4. Binding pattern of FITC-UEA I at 2-cell parthenogenetic porcine oocyte. At 18h postactivation the oocytes were labelled with FITC-UEA I and observed under UV light. Porcine oocytes with immediate cleavage was shown under light microscope (a) and fluorescent microscope (PM-10ADS, Olympus) with a large single aggregate in the cleavage (b) ,(c), respectively. Magnifications are × 200.

and Fig. 1) upon ethanol activation, which is a similar response found in ovulated, mature oocyte.

In conclusion cytoplasmic maturation could be achieved by longer IVM. And these matured oocytes also showed early develpment after parthenogenetic activation. The method of activation described here is highly reproducible and can be used as a biological assay system for the test of proper maturation of the follicular oocytes. These oocytes matured for longer period may respond rapidly to the normal sperm penetration, thus exerting block to polyspermy.

적 요

처녀발생 유기에 의한 체외성숙 돼지난포란의 초기발생 능력을 조사하였다. 난자는 42, 48, 54, 60시간동안 15% FCS와 성선 자극 호르몬이 첨가된 TCM 199에 39°C 5% CO_2 조건하에서 성숙시켰다. 난구세포를 제거한 난자는 10% 에탄올이 첨가된 M2+4mg/ml BSA 에서 10분간 활성화시켰다. 활성화된 난자는 48시간 전에 TCM 199+20% FCS에 공배양시킨 각각의 50㎕ 과립막세포단층으로 옮긴 후 활성화된 난자는 TCM199+20% FCS에 공배양된 과립막 세포단층에서 배양하였다.
42, 48, 54 그리고 60시간의 체외성숙 처리구에서 성숙율은 75.0, 86.5, 81.6 그리고 87.9%였다. 다양한 기간동안 체외성숙된 초기배에서 큰 차이는 볼 수가 없었다. 활성화된 난자에서 18 시간 후 전핵형성율은 21.9, 25.0, 47.4 그리고 32.6%였다. 체외성숙기간이 42~54시간까지 증가될 때 전핵형성을 이끄는 체세포성숙은 증가되었다. 활성화된 난자의 초기발생을 분석하기 위하여 96~120시간동안 배양할 때 5~8 세포기 이상 난할된 초기배의 비율은 3, 5.8, 12.0 그리고 11.7%였다. 이같은 결과는 활성화된 돼지난자의 초기발생은 난자의 체외성숙 기간에 의존하며 가장 높은 발생능력은 54시간 체외성숙시킨 난자에서 얻을 수 있었다. 아울러 본실험에서는 54시간의 성숙기간이 난자 세포질 성숙과 노화의 분수령이 됨을 보여 주고 있다.

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