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Effect of Epidermal Growth Factor on *In Vitro* Maturation in Pig Immature Oocytes
Ⅰ. Effect of Epidermal Growth Factor on Nuclear Maturation

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Epidermal Growth Factor가 돼지 미성숙난포란의 체외성숙에 미치는 영향
Ⅰ. 핵성숙에 미치는 Epidermal Growth Factor의 효과

엄상준 · 김선의 · 김은영 · 윤산현* · 박세필 · 정길생 · 임진호***
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요 약

본 연구는 돼지 미성숙난포란의 체외배양시 핵성숙에 미치는 EGF의 효과를 검토하고자 실시하였다. 기초 배양액으로는 TCM-199에 0.2 mM pyruvate, 1μg /ml estradiol-17β, 25μg /ml gentamycin을 첨가하였으며, 이 배양액에 EGF, FSH와 FBS을 처리하였다. 실험 1에서는 난포란 성숙에 있어서 FSH와 0, 1, 10, 100 ng EGF /ml의 농도에 따른 효과를 조사하였던 바, 1, 10, 100 ng EGF /ml가 처리된 군의 핵성숙율은 83.0, 86.7, 87.5%로서 무처리군 27.3%와 FSH 처리군 60.3%보다 유의하게 높았다 ($p<0.001$). 실험 2에서는 EGF, FSH와 FBS의 상호작용에 대해서 조사하였다. EGF 단독, EGF에 FSH 첨가, EGF에 FBS 첨가, FSH에 FBS 첨가와 EGF와 FSH에 FBS가 첨가된 군의 핵성숙율은 86.7, 90.2, 87.1, 89.6, 92.6%로서 무처리 군 22.3%, FSH와 FBS가 각각 단독 처리된 군의 52.2, 42.3%보다 유의하게 높았다 ($p<0.001$). 또한, 정상적인 난구세포의 팽창은 FSH에 FBS, FSH에 EGF 혹은 FSH, EGF와 FBS가 첨가된 군에서 나타났으며, EGF가 첨가된 군에서는 대부분의 난구세포가 난포란으로부터 분리되었지만, 일부는 덩어리로 난포란에 부착되어 있었다. 따라서, EGF는 돼지 미성숙난포란에 있어서 핵성숙을 유도할 수 있다고 사료된다.

Ⅰ. INTRODUCTION

Immature pig oocytes *in vivo* remain arrested in the first meiotic prophase until maturation is induced by the follicular secretions are contained stimulating factors. These stimulating factors during oocyte maturation involve not only acquisition of meiotic competency, but also cytoplasmic maturation (Motlik et al., 1986). Yoshida et al. (1992) reported that the stimulatory effect of follicular fluid on oocyte nuclear maturation was lost after heating at 56℃ for 30 min, but there was no significant decrease

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after defatting. These results suggest that at least some of the stimulatory factors may be polypeptides.

Recent studies show that growth factors found within the ovary may act as both autocrine and paracrine regulators of ovarian function (Hammond et al., 1988; Carson et al., 1989). Culture ovarian cells in a number of species have been shown to synthesize growth factors (cattle, Neufeld et al., 1987; pig, Hammond et al., 1985; rat, Skinner et al., 1987). Epidermal growth factor (EGF) has been found in pig ovarian follicles and there are binding sites for EGF within the ovary (Balwant et al., 1995). EGF has been shown to stimulate DNA synthesis and cell proliferation in pig granulosa cells while inhibiting differentiation (May et al., 1988). *In vitro* studies showed that EGF could stimulate nuclear maturation in rats (Dekel and Sherizly, 1985; Fening et al., 1987; Ueno et al., 1988), mice (Downs, 1989; Das et al., 1991), cattle (Harper and Brackett, 1993), pigs (Ding and Foxcroft, 1994), and human (Das et al., 1991). The developmental potential of *in vitro* matured bovine oocytes to the eight-cell stage (Coskun et al., 1991) and to the blastocyst stage (Harper and Brackett, 1993) was also stimulated by EGF. These results suggest that EGF might be one of the major follicular factors responsible for stimulating oocyte cytoplasmic, as well as nuclear maturation. The objective of this study was to examine possible beneficial effects of EGF on nuclear maturation of pig oocytes *in vitro*.

Ⅱ. 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 follicular 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 PVP) and the maturation medium, respectively. Oocytes possessing a compact cumulus cell mass and evenly granulated ooplasm were used for this study.

2. *In vitro* maturation (IVM)

The OCCs were transferred into a 50 ㎕ droplet of maturation medium equilibrated for 2 hr in 5% CO_2 and 95% O_2 incubator under warm mineral oil in a polystyrene culture dish (60×10 mm). The maturation medium consisted of TCM-199 (with Earle's salts: Gibco, USA) supplemented with 25 mM $NaHCO_3$ (Sigma, USA), 0.2 mM pyruvate (Sigma, USA), 1 ㎍/ml estradiol-17β (Sigma, USA), and 25 ㎍/ml gentamycin (Sigma, USA). FSH (Schering-Plough Animal Health, USA), EGF (Sigma, USA), and fetal bovine serum (FBS: Sigma, USA) were added to culture according to the experimental designs. Culture was carried out at 39℃ in 5% CO_2 in air for 42 hr.

3. Examination of nuclear status

After 42 hr maturation culture, oocytes intended for direct fixation were denuded of cumulus cells by incubation with 1% hyaluronidase for 5 min and pipetting. After the cumulus cells were removed, the oocytes were fixed for a minimum of 10 min in a buffered 2% formalin solution, the oocytes were then placed on a slide with a drop of mounting medium consisting of 1:1 glycerol:phosphate-buffered saline, containing 2.5 mg/ml sodium azide and 2.5 mg/ml Hoechst 33342 DNA label (Sigma, USA). A cover slip was placed on top of the oocytes, and the edges were sealed with fingernail polish. Nuclear stat-

us (GV, Diakinesis, M I, M II) was identified (Fig. 2).

4. Design and analysis

The experiment was designed to determine an effective dose of EGF for oocyte nuclear maturation in our culture system. OCCs were randomly divided into cuture dish (8~10 OCCs per 50 μl drop). In experiment 1. the effects of 0, 1, 10 or 100 ng EGF/ml added to medium were compared to the effects of the same treatment with 10 μg/ml FSH added. In experiment 2, the interactive effects of 10 ng/ml EGF, 10 μg/ml FSH or 10% FBS during IVM. A chi-square test was used to ascertain statistical differences between treatments. A p value of less 0.001 was considered statistically significant.

III. RESULTS AND DISCUSSION

Experiment 1.

Influences of differnt EGF concentration during IVM

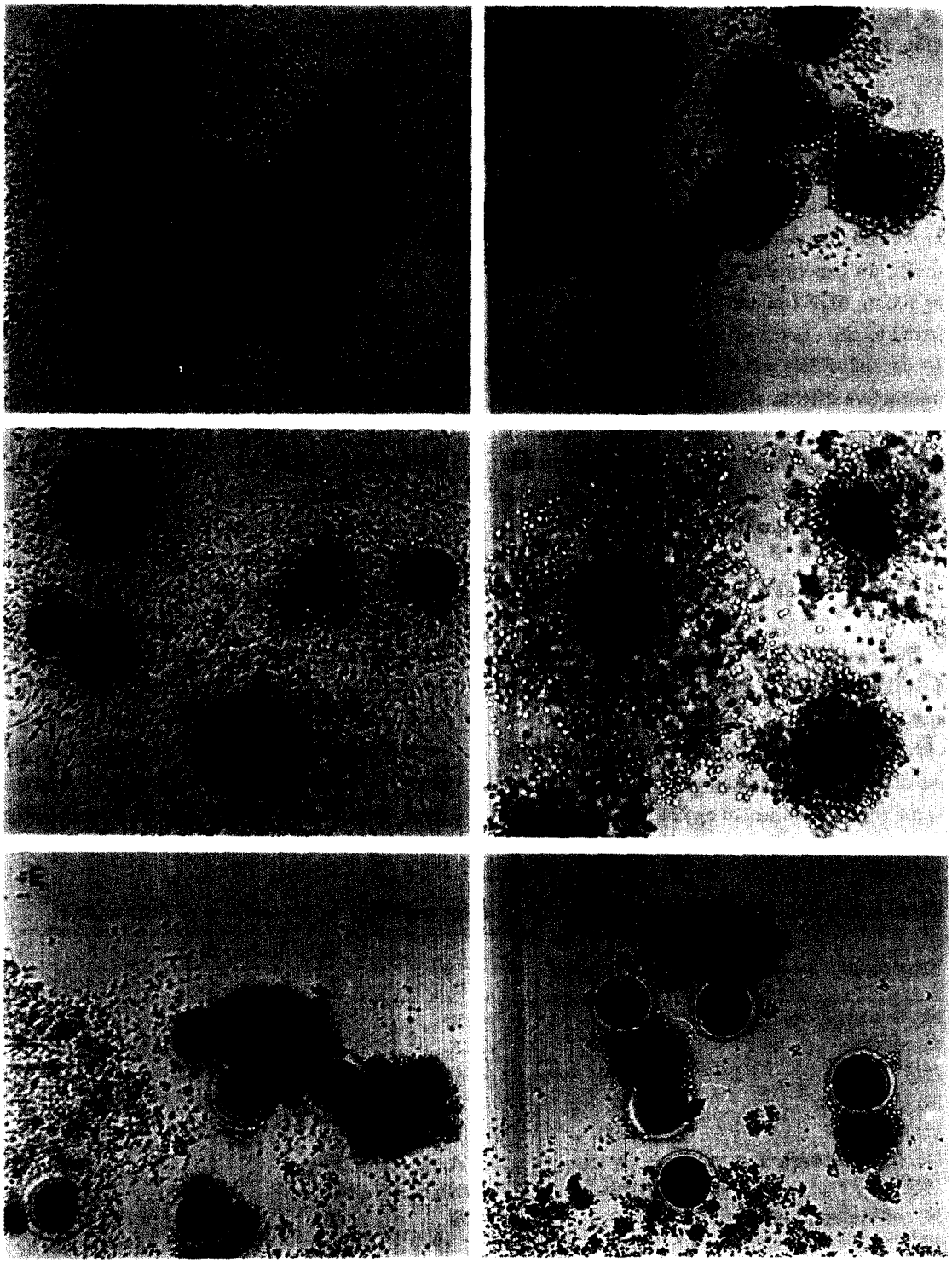
Nuclear maturation of OCCs in 0, 1, 10 or 100 ng EGF/ml added medium was compared to the same treatment with 10 ng/ml FSH added (see Table 1). EGF significantly stimulated nuclear maturation. All three EGF-treated groups had higher rates of nuclear maturation (M II) than the EGF control group (no EGF and no FSH ; 27.3%). Also, EGF-treated group (83.0%, 86.7%, 87.5%) and FSH-treated group (60.3%) had higher rate of nuclear maturation (M II) than the EGF control group. But nuclear maturation rates were not significantly different among the 1, 10 and 100 ng EGF/ml groups.

Result of experiment 1 revealed significant differences in the morphological evaluation of OCCs undergoing cumulus cells expension between the EGF control group and 1, 10 and 100 ng EGF/ml in IVM treatments, and also FSH-treated group was not expanded of cumulus cells. Normal cumulus cells expansion during culture was characterized by expanded cumuli and dispersed cumulus cells loosely surrounding the oocytes, and had mucification. Most of the cumulus cells were still clumped together in EGF-treated groups although they had separated from, or only loosely maintained contact with, oocytes. They were revealed of cumulus cells disaggregation, and cumulus cells of 10 ng EGF/ml treatment (Fig. 1-F) were disaggregated more than 1 ng EGF/ml treatment (Fig. 1E). These had lost almost all their cumulus cel-

Table 1. *In vitro* maturation of pig immature oocytes according to the addition of FSH or EGF

Treatment	FSH (10 μg/ml)	EGF (ng/ml)			
		0	1	10	100
No. of eggs examined	126	110	118	128	128
No. of GV	28	55	7	6	2
(%)	(22.2)	(50.0)	(5.9)	(4.7)	(1.6)
No. of GVBD	19	14	3	1	5
(%)	(15.1)	(12.7)	(2.5)	(0.8)	(3.9)
No. of Metaphase I	3	11	10	10	9
(%)	(2.4)	(10.0)	(8.5)	(7.8)	(7.0)
No. of Metaphase II	76	30	98	111	112
(%)	(60.3)[a]	(27.3)[b]	(83.1)[c]	(86.7)[c]	(87.5)[c]

[a-c] Different superscripts indicate that percentages were significantly different at $p<0.001$.



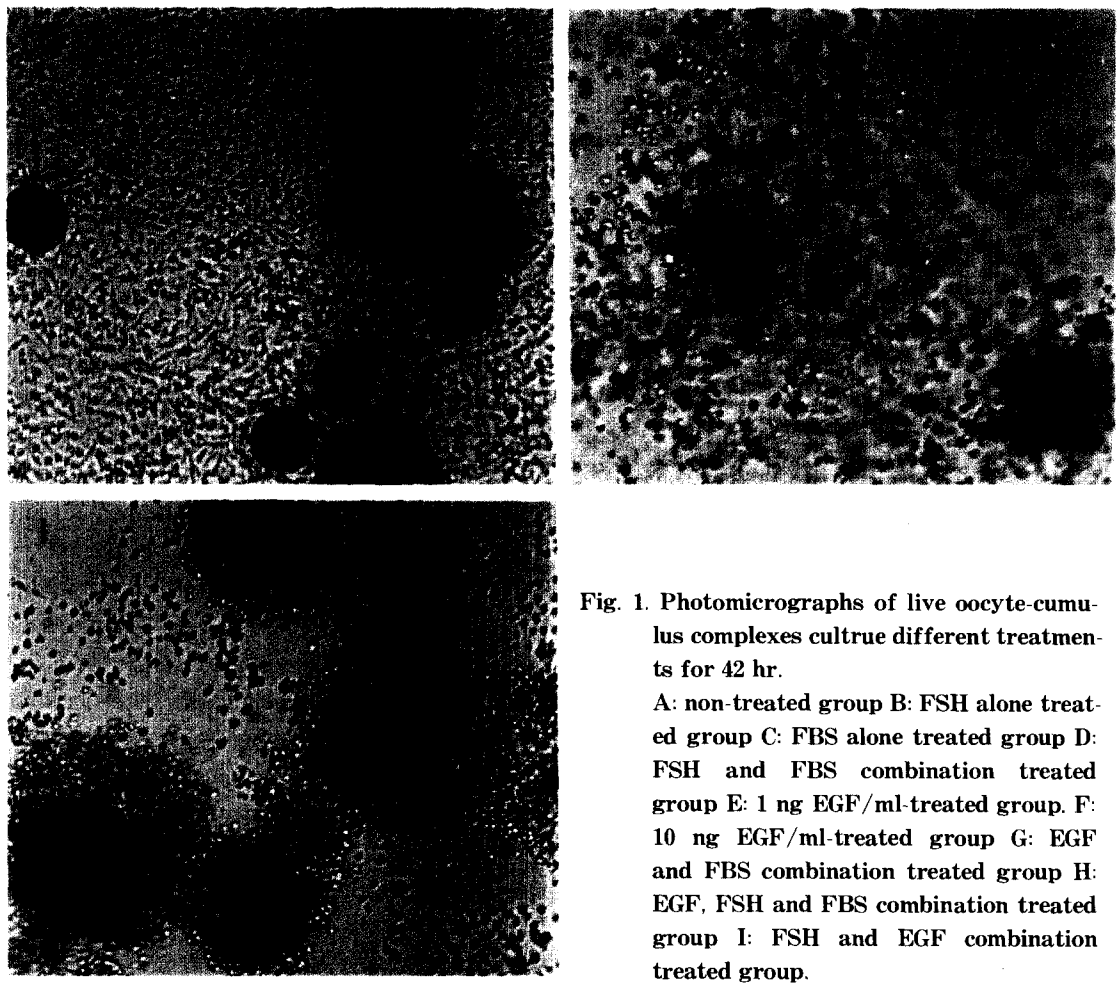


Fig. 1. Photomicrographs of live oocyte-cumulus complexes cultrue different treatments for 42 hr.
A: non-treated group B: FSH alone treated group C: FBS alone treated group D: FSH and FBS combination treated group E: 1 ng EGF/ml-treated group. F: 10 ng EGF/ml-treated group G: EGF and FBS combination treated group H: EGF, FSH and FBS combination treated group I: FSH and EGF combination treated group.

ls including the corona radiata were classified as cumulus-disaggregated oocytes. Cumulus cells were dark-looking with no mucification and was easily removed from the oocyte by pipetting a few times. Oocytes from the EGF control group were showed no cumulus cells expansion or disaggregation, and cumulus cells were compact and dark-looking with no mucification and was removed with difficulty from the oocyte by repeated pipetting (Fig. 1-A). Also, cumulus of oocyte complexes from FSH-treated group were very sticky with mucification and was very difficult to remove from oocytes by repeated pipetting through a fine bore pipette (Fig. 1-B). The fact that we postulate that EGF may accelerate the disruption of gap junctions between cumulus cells and between cumulus cells and the oocytes, which probably provide one of mechanisms by which EGF stimulates nuclear maturation of oocytes. This data was similar to Ding and Foxcroft (1994) reported that although the addition of EGF to the *in vitro* maturation media stimulated nuclear maturation of oocytes, EGF alone did not stimulate cytoplasmic maturation, but EGF interacted with gonadotropins in stimulating cytoplasmic maturation (male pronuclear forma-

tion). Coskun et al. (1991) reported that EGF could improve the developmental potential of *in vitro* matured bovine oocyte. But culture condition (condition medium added serum) of these results was different our data, that nuclear maturation of our FSH-treated group was lower than that of them. However, this data indicate that, at least in the pig, EGF is a potential stimulator of nuclear maturation of oocytes.

Experiment 2.

Influences of EGF, FSH or FBS during IVM

Effects of EGF, FSH or FBS on nuclear maturation are shown in Table 2. The proportion of oocytes maturating beyond M II after 42hr maturation culture were significantly affected by EGF alone (E group: 86.7%), EGF plus FSH (F group: 90.2%) EGF plus FBS (G group: 87.1%), FSH plus FBS (D group: 89.6%), and EGF plus FSH added FBS (H group: 92.6%) treatments. The proportion of oocytes maturating beyond M II by FBS (B group: 42.3%) and FSH (C group: 52.2%) alone-treated groups were higher than non-treated (A group: 22.3%) group. Nevertheless, these two groups (B and C groups) were significantly lower than from D to H groups oocytes reaching M II. No significant differences were observed from D to H groups oocytes reaching M II, but these were significantly differnt with B and C treatments.

Significant differences were observed for OCCs showing cumulus cells expansion among all groups. Normal cumulus cells expansion was observed on FSH plus FBS treatment (Fig. 1-D). Cumulus cells of EGF plus FBS treatment (Fig. 1-G) were observed disaggregation and dark-looking with no mucification. Especially, cumulus cells debris separated from OCCs on FSH treatments were not or a few observed in drop, but many cumulus cells debris on others treatments were observed in dish. Cumulus cells debris in drop of FBS-treated groups were attached on dish, but cumulus cells debris in drop of non- and EGF alone-treated groups were floated on dish. Also, cumulus cells of FBS alone-treated group (Fig. 1-C) were not expanded of cumulus cells, but were separated from oocyte and attached on dish. Cumulus cells of

Table 2. The interactive effects of EGF, FSH or FBS during *in vitro* maturation of pig immature oocytes

	Treatments							
	A	B	C	D	E	F	G	H
EGF (10 ng /ml)	−	−	−	−	+	+	+	+
FSH (10 μg /ml)	−	−	+	+	−	+	−	+
FBS (10%)	−	+	−	+	−	−	+	+
No. of eggs examined	121	123	113	106	105	133	101	148
No. of GV	62	34	27	0	4	2	3	1
(%)	(51.2)	(27.6)	(23.9)	(0.0)	(3.8)	(1.5)	(3.0)	(0.6)
No. of GVBD	18	14	20	1	2	2	2	4
(%)	(14.9)	(11.4)	(17.7)	(0.9)	(1.9)	(1.5)	(2.0)	(2.7)
No. of Metaphase I	14	23	7	10	8	9	8	6
(%)	(11.6)	(18.7)	(6.2)	(9.5)	(7.6)	(6.8)	(7.9)	(4.1)
No. of Metaphase II	27	52	59	95	91	120	88	137
(%)	(22.3)[a]	(42.3)[b]	(52.2)[b]	(89.6)[c]	(86.7)[c]	(90.2)[c]	(87.1)[c]	(92.6)[c]

[a c] Different superscripts indicate that percentages were significantly different at $p<0.001$.

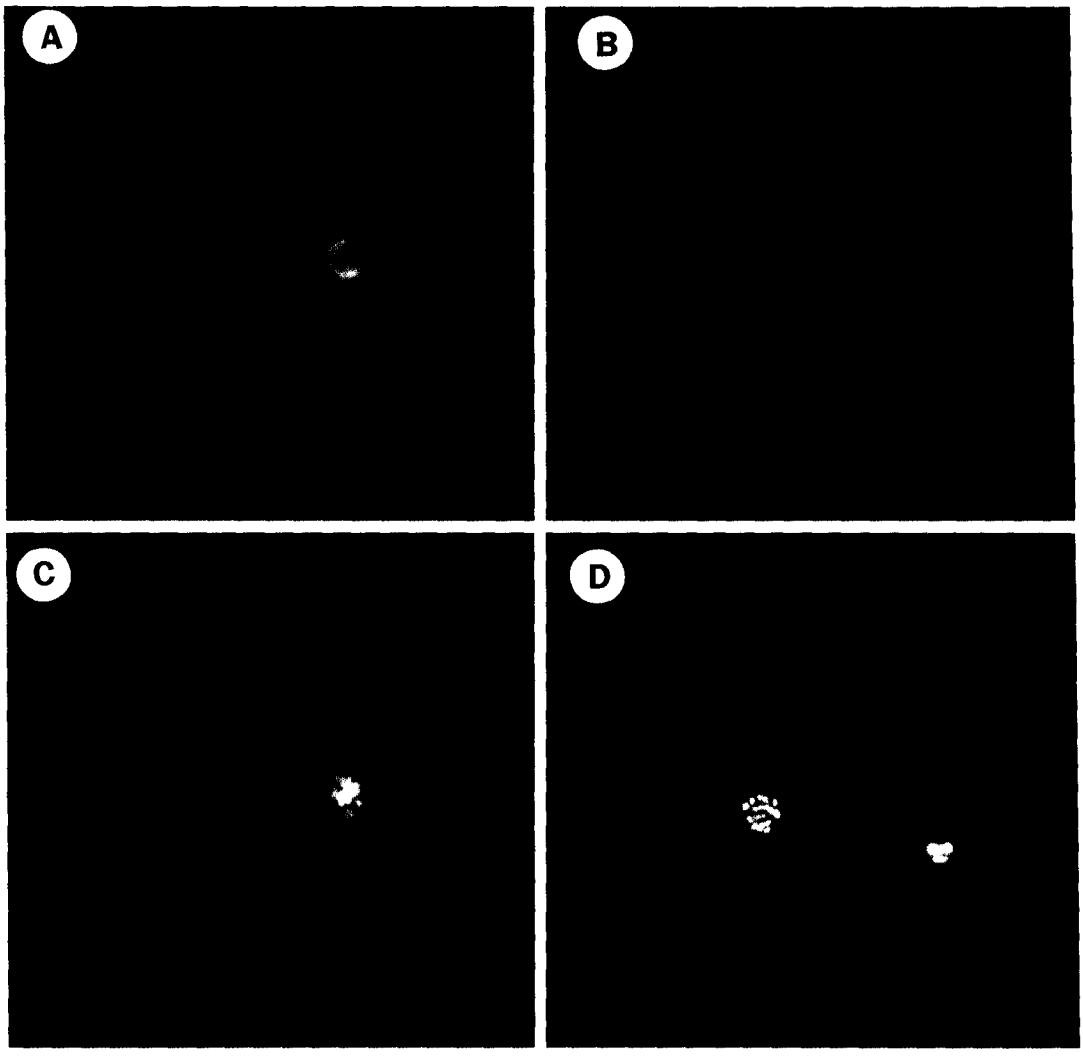


Fig. 2. Hoechst 33342 staining of pig oocyte nuclear after 42 hr.
A. Germinal Vesicle (GV) **B. Diakinesis**
C. Metaphase I (MI) **D. Metaphase II (MII)**

FSH, EGF and FBS combination treatment (Fig. 1-H) were shown normal cumulus cells expansion, but many cumulus cells debris were attached on dish. Especially, cumulus cells of EGF plus FSH treated group (Fig. 1-I) were shown normal cumulus cells expansion, but this was observed less than cumulus cells expansion of FSH plus FBS treated group. Ding and Foxcroft reported (1994) that cytoplasmic maturation of pig oocyte was affected by gonadotropin. EGF was shown high nuclear maturation rate of pig oocyte, but was not shown cytoplasmic maturation. The fact that disaggregation of cumulus cells by EGF affect the interruption of gap

junctions between cumulus cells and between cumulus cells and oocyte, can be associated one of mechanisms by which EGF stimulates nuclear maturation of oocytes (Ding and Foxcroft, 1994). The cumulus cells expansion seems to be consistent with the fact that in vivo meiosis is reinitiated when the gap junctions are rapidly eliminated in the entire cumulus cells interruption of oocyte maturation inhibitors transport to the oocyte (Mattioli et al., 1988b). Normal cumulus cells expansion of FSH treated group was shown mucification, and these were observed cytoplasmic as well as nuclear maturation (Ding and Foxcroft, 1994). Probably, the role of mucification is the transport of cytoplasmic maturation factors in the oocyte after the meiotic resumption, and this is very important on cytoplasmic maturation. These results were in contrast to Harper and Brackett (1993) reported that bovine oocyte complexes of gonadotropin treatment not added serum were shown not only cumulus expansion, but also higher nuclear maturation. Also, further studies were examined that pig oocytes were shown significant enhancement of cytoplamsic maturation (indicated by the high male pronuclear formation rate) when cumulus-enclosed oocytes were cocultured with follicular shells or cultured in the follicular shell-conditioned medium or in the medium supplemented with follicular fluid (Ding and Foxcroft, 1992, 1993a,b; Mattioli et al., 1988a, 1989; Nagai et al., 1993; Naito et al., 1988, 1989; Yoshida et al., 1992; Zheng and Sirard, 1993). However, these results implicated that normal maturation of pig oocyte complexes firstly necessitate factor like EGF for the meiotic resumption, and gonadotropin necessitate the transport of factors in order to continue cytoplasmic maturation. Also, serum has various factors as well as EGF, and these associate cytoplasmic maturation as well as nuclear maturation by interaction with gonadotropin.

Ⅳ. SUMMARY

The objective of this experiment was to test the effect of EGF on nuclear maturation of pig immature oocytes *in vitro*. Basic medium used TCM-199 supplemented with 0.2 mM pyruvate, 1 μg/ml estradiol-17β and 25 μg/ml gentamycin, this medium treated with EGF, FSH and FBS. Experiment 1 examined to the effect according to the addition of FSH or EGF (0, 1, 10 and 100 ng EGF/ml) in oocytes maturation. Nuclear maturation rates (M Ⅱ %) of 1, 10 and 100 ng EGF/ml (83.0, 86.7 and 87.5%) treatments were significantly higher than those of non- and FSH-treated groups (27.3 and 60.3%, $p < 0.001$). Experiment 2 examined to the interactive effects of EGF, FSH or FBS during oocytes maturation. Nuclear maturation rates (M Ⅱ %) of EGF alone, EGF plus FSH, EGF plus FBS, FSH plus FBS, and EGF plus FSH added FBS treatments (86.7, 90.2, 87.1, 89.6% and 92.6%) were significantly higher than those of non, FSH, and FBS alone treatments (22.3, 52.2 and 42.3%, $p < 0.001$). Also, cumulus cells expansion of oocytes maturation was examined to total treatments. Normal cumulus cells expansion was shown by FSH plus FBS, EGF or EGF with FBS combination treatments, but cumulus cells of oocyte complexes were still clumped together in EGF-treated groups although they had separated from oocytes. However, EGF showed a positive on nuclear maturation. These results conclude that EGF alone can stimulate nuclear maturation in pig immature oocytes.

Ⅴ. REFERENCES

1. Balwant, S., J. M. Rutledge, and A. T. Armstrong. 1995. Epidermal growth factor

and its receptor gene expression and peptide localization in porcine ovarian follicles. Mol. Reprod. Dev. 40:391-399.
2. Carson, R. S., Z. Zhang, L. A. Hutchinson, A. C. Herington, and J. K. Findlay. 1989. Growth factors in ovarian function. J. Reprod. Fertil. 85:735-746.
3. Coskun, S., A. Sanbuissho, Y. C. Lin and Rikihisa. 1991. Fertilizability and subsequent developmental ability of bovine oocytes matured in medium containing epidermal growth factor (EGF). Theriogenology 25:485-494.
4. Das, K., G. E. Tagatz, L. E. Stout, W. R. Phipps, H. C. Hensleigh, and B. S. Leung. 1985. Direct positive effect of epidermal growth factor on the cytoplasmic maturation of mouse and human oocytes. Fertil. Steril. 55:1000-1004.
5. Dekel, N. and I. Sherizly. 1985. Epidermal growth factor induces maturation of rat follicle enclosed oocytes. Endocrinology 116: 46-49.
6. Ding, J. and G. R. Foxcroft. 1992. Follicular heterogeneity and oocyte maturation *in vitro* in pigs. Biol. Reprod. 47:648-655.
7. Ding, J. and G. R. Foxcroft. 1993a. FSH stimulated follicular secretions enhance oocyte maturation in pigs. Theriogenology 41:14 73-1481.
8. Ding, J. and G. R. Foxcroft. 1993b. Conditioned media produced by follicular shells with different maturity affect oocyte maturation in the pig. Biol. Reprod., 156:1377-1384.
9. Ding, J. and G. R. Foxcroft. 1994. Epidermal growth factor enhances oocyte maturation in pigs. Mol. Reprod. Dev. 39:30-40.
10. Downs, S. M. 1989. Specificity of epidermal growth factor action on maturation of the murine oocyte and cumulus oophorus *in vitro*. Biol. Reprod. 41:371-379.
11. Downs, S. M., S. A. J. Daniel, and J. J. Eppig. 1988. Introduction of maturation in cumulus cell-enclosed mouse oocytes by follicle-stimulating hormone and epidermal growth factor: evidence for a positive stimulus of somatic cell orgin. J. Exp. Zool. 245:86-96.
12. Fening, P., M. Knecht, and K. Catt. 1987. Hormonal control of epidermal growth factor receptors by gonadotropins during granulosa cell differentiation. Endocrinology 120:1121-1126.
13. Hammon, J. M., J. L. S. Baranao, D. Skaleris, A. B. Knight, J. A. Romanus, and M. M. Rechler. 1985. Production of insulin-like growth factors by ovarian gronulosa cells. Endocrinology 117:2553-2555.
14. Hammond, J. M., C. J. Hsu, J. S. Mondschein, and S. F. Canning. 1988. Paracrine and autocrine functions of growth factors in the ovarian follicle. J. Anim. Sci. 66(suppl 2) :21-31.
15. Harper, K. M and B. G. Brackett. 1993. Bovine blastocyst development after *in vitro* maturation in a defined medium with epidermal growth factor and low concentrations of gonadotropins. Biol. Reprod. 48:409-416.
16. Mattioli, M., G. Galeati, and E. Seren. 1988-a. Effect of follicle somatic cells during pig oocyte maturation on egg penetrability and male pronuclear formation. Gamate Res. 20:177-183.
17. Mattioli, M., M. L. Bacci, G. Galeati, and E. Seren. 1988b. Follicular factors influence oocyte fertilizability by modulating the intercellular cooperation between cumulus cells and oocytes. Gamate Res. 21:223-232.
18. Mattioli, M., M. L. Bacci, G. Galeati, and E. Seren. 1989. Developmental competence of pig oocytes matured and fertilized *in vitro*. Theriogenology 31:1201-1207.
19. May, J. V., J. P. Frost, and D. W. Schom-

berg. 1988. Differential effects of epidermal growth factor, somatomedin-C /insulin growth factor I, and transforming factor-beta on porcine granulosa cell deoxyribonucleic acid synthesis and cell proliferation. Endocrinology 123:168-179.

20. Motlik, J. and J. Fulka. 1986. Factors affecting meiotic competence in pig oocytes. Theriogenology 25:87-96.
21. Nagai, T., J. Ding, and R. M. Moore. 1993. Effect of follicular cells and steroidogenesis on maturation and fertilization *in vitro* of pig oocytes. J. Exp. Zool. 266:146-151.
22. Naito, K., Y. Fukuda, and Y. Toyoda. 1988. Effects of porcine follicular fluid on male pronuclear formation in porcine oocytes matured *in vitro*. Gamete Res. 21:289-295.
23. Naito, K., Y. Fukuda, and I. Ishibashi. 1989. Developmental ability of porcine ova matured in porcine follicular fluid *in vitro* and fertilized *in vitro*. Theriogenology 31:1049-1057.
24. Neufeld, G., N. Ferrara, L. Schweigerer, R. Mitchell, and D. Gospsdarowicz. 1987. Bovine granulosa cells produce basic fibroblast growth factor. Endocrinology 121:597-603.
25. Skinner, P., D. Lobb, and J. H. Dorrington. 1987. Ovarian theca-interstitial cells produce an epidermal growth factor-like substance. Endocrinology 121:1892-1899.
26. Ueno, S., T. F. Manganaro, and P. K. Donahoe. 1988. Human recombinant mullerian inhibiting substance of rat oocyte meiosis is reversed by epidermal growth factor *in vitro*. Endocrinology 123:1652-1659.
27. Yoshida, M., Y. Ishizaki, H. Kawagishi, K. Bamba, and Y. Kojima. 1992. Effect of pig follicular fluid on maturation of pig oocytes *in vitro* and on their subsequent fertilizing and developmental capacity *in vitro*. J. Reprod. Fertil. 95:481-488.
28. Zheng, Y. S. and M. A. Sirard. 1992. The effect of sera, bovine serum albumin and follicular cells on the *in vitro* maturation and fertilization of porcine oocytes. Theriogenology 37:779-790.