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Effect of Epidermal Growth Factor on *In Vitro* Maturation in Pig Immature Oocytes
II. Effect of Epidermal Growth Factor on GVBD

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Epidermal Growth Factor가 돼지 미성숙난포란의 체외성숙에 미치는 영향
II. GVBD에 미치는 Epidermal Growth Factor의 효과

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= 국문초록 =

본 연구는 돼지 미성숙난포란의 체외배양시 EGF가 핵성숙의 GVBD와 MⅡ에 미치는 효과를 조사하였다. 실험 1에서는 EGF를 첨가하였을 때, 난포란의 배양경과 시간 (0, 16, 24, 42시간)에 따른 핵성숙도를 조사하였던바, EGF 10ng/*ml*이 첨가된 군이 무첨가된 군에 비해서 24시간 이후에 GVBD가 유의하게 높았다 ($p < 0.001$). 실험 2에서는 난포란의 배양시 EGF 노출시간에 따른 난포란의 핵성숙의 효과를 조사하였던바, 배양 초기 (0-24시간)와 배양 전시간 (0-42시간) 동안 EGF를 배양액내에 첨가한 군의 경우 최종 성숙단계인 MⅡ까지의 핵성숙율은 72.8과 84.8%로써 배양 후기 (24-42시간)에 EGF가 첨가된 군의 53.5%와 배양 전시간 (0-42시간) 동안 무첨가한 군의 26.1%보다 유의하게 높았다 ($p < 0.001$). 그리고 실험 3에서는 난구세포 부착 난포란과 난구세포가 제거된 난포란에 있어서 EGF의 효과를 조사하였던바, EGF가 첨가된 난구세포 부착 난포란 군의 경우 핵성숙율 (MⅡ)은 84.6%로써 EGF가 첨가된 난구세포 제거군의 53.0%와 무첨가 난구세포 부착군의 27.6%, 난구세포 제거군의 44.2%보다 유의하게 높았다 ($p < 0.001$). 따라서 이상의 결과로 미루어보아 EGF 단독만으로도 돼지 미성숙난포란의 체외성숙의 핵성숙에 있어서 GVBD와 MⅡ을 유도할 수 있다고 사료된다.

INTRODUCTION

Fully grown mammalian oocytes, surrounded by a compact mass of somatic cumulus cells, are maintained in the immature, germinal vesicle stage *in vivo* until a preovulatory gonadotropin surge provokes a dramatic physiological response. In the hours following the ovulation stimulus, the oocyte resumes nuclear maturation, manifested initially by germinal vesicle breakdown (GVBD). Dekel and Beers (1980) hypothesized that meiotic arrest is maintained within the follicle by the transfer of the cyclic adenosine monophosphate (cAMP) from follicle cells to the oocyte via the gap junctional coupling pathway and that termination of gap junctional communication between the oocyte and follicle cells following the gonadotropin surge should therefore interrupt the flow of cAMP to the oocyte and result in meiosis resumption.

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However, when metabolic coupling in oocyte-cumulus complexes was examined at the time of GVBD in sheep and mice, GVBD occurred before a detectable loss in coupling (Moor *et al.*, 1981; Eppig, 1982; Downs *et al.*, 1988).

A study by Downs *et al.*, (1988) implicated the cumulus cell investment as the site of action of epidermal growth factor (EGF) and suggested that the cumulus cells could provide a positive maturational signal for the oocyte. A recent study in pig oocytes also concluded that the action of EGF is mediated via the cumulus cells and gap junctions with the oocyte (Coskun and Lin, 1992). EGF prevented the inhibition of rat oocyte maturation *in vitro* by Mullerian inhibiting substances (Ueno *et al.*, 1988). In addition, this growth factor induced GVBD in isolated cumulus enclosed oocytes from mice when meiotic arrest was maintained with purines, cAMP analog, dibutyryl cAMP (dbcAMP), or the cAMP phosphodiesterase inhibitor, isobutylmethylxanthin (IBMX) (Downs *et al.*, 1988). Thus, maturation was stimulated despite the presence of substances that normally maintain meiotic arrest. Moreover, maturation was stimulated in a manner suggesting the generation of a positive stimulus of cumulus cell origin, since EGF was not stimulatory to cumulus free (denuded) oocytes (Downs *et al.*, 1988).

The present study was undertaken to examine: 1) the effect of EGF on nuclear maturation of pig oocyte cumulus complexes (OCCs) accoding to different maturational times, 2) the effect of duration of exposure of pig OCCs to EGF supplement on nuclear maturation, and 3) the effect of EGF on nuclear maturation of pig cumulus-enclosed (CEOs) and free oocytes (CFOs).

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 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 compacted cumulus cell mass or cumulus cell denuded were used for this study. CFOs from compacted cumulus cell oocytes were obtained by removal of cumulus cells with 0.1% hyaluronidase for 5 min and pipetting (Uhm *et al.*, 1995).

2. *In vitro* maturation (IVM)

The oocytes were transferred into a 50 μl droplet of maturation medium equilibrated for 2 hr in 5% CO_2 and 95% O_2 incubator under warm paraffin 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 μg/ml estradiol-17β (Sigma, USA), and 25 μg/ml gentamycin (Sigma, USA). EGF (Sigma, USA) was added to culture according to the experimental designs. Culture was carried out at 39℃ in 5% CO_2 in air (Uhm *et al.*, 1995).

3. Examination of nuclear status

After maturation culture (0, 16, 24, or 42hr) according to experimental purpose, oocytes intended for direct fixation were stripped 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 status as Ger-

Table 1. Effect of EGF on nuclear maturation of pig immature oocytes according to different maturational times

Treatment (EGF 10 ng/ml)	Oh	16h		24h		42h	
		-	+	-	+	-	+
No. of eggs examined	140	116	121	185	201	137	114
No. of GV	118	87	76	116	41	76	10
(%)	(84.3)	(75.0)	(62.8)	(62.7)[a]	(20.4)[b]	(55.5)[a]	(8,8)[b]
No. of GVBD	22	24	36	40	53	12	5
(%)	(15.7)	(20.7)	(29.8)	(21.6)	(26.4)	(8.8)	(4.4)
No. of M I	-	5	9	21	82	11	4
(%)		(4.3)	(7.4)	(11.4)	(40.8)	(8.0)	(3.5)
No. of M II	-	-	-	8	25	38	95
(%)				(4.3)	(12.4)	(27.7)	(83.3)

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

Table 2. Effect of duration of exposure of pig oocyte cumulus complexe (OCCs) to EGF supplement in maturation medium

	EGF (10 ng/ml)			
Oh-24h	-	-	+	+
24h-42h	-	+	-	+
No. of eggs examined	134	127	136	145
No. of GV	77	31	14	7
(%)	(57.5)	(24.4)	(10.3)	(4.8)
No. of GVBD	16	9	4	3
(%)	(11.9)	(7.1)	(2.9)	(2.1)
No. of M I	6	19	19	12
(%)	(4.5)	(15.0)	(14.0)	(8.3)
No. of M II	35	68	99	123
(%)	(26.1)[a]	(53.5)[b]	(72.8)[c]	(84.8)[d]

[a-d]Different superscripts indicate that percentages were significantly different at $p< 0.001$ and 0.05

minal Vesicle (GV), Germinal Vesicle Breakdown (GVBD), Metaphase I (M I), and Metaphase II (M II) was identified (Uhm *et al.*, 1995).

4. Design and analysis

The experiment was designed by using an effective dose (10 ng/ml) of EGF for nuclear maturation of pig immature OCCs previously reported by Uhm *et al.* (1995). The oocytes were randomly divided into cuture dish (8-10 oocytes per dish). Experiment 1 examined to the effect of EGF on nuclear maturation of pig immature oocytes according to different maturational times (0, 16, 24, or 42hr). Experiment 2 treated to the effect of duration of exposure (0, 24-42, 0-24, or 0-42hr) of pig OCCs to EGF supplement in maturation medium. Experiment 3 investigated to the effect of EGF on nuclear maturation of pig immature CEOs or CFOs. A chi-square test was used to ascertain statistical differences between treatments. A p value of less 0.001, 0.005, and 0.05 were considered statistically significant.

RESULTS

Experiment 1. Influences of EGF according to time course during IVM

Resuts of experiment 1 (Table 1) revealed significantly differences in the proportions of

Table 3. Effect of EGF on nuclear maturation of pig immature oocytes according to different maturational times

Treatment (EGF 10 ng/ml)	CEOs		CFOs	
	-	+	-	+
No. of eggs examined	156	123	156	168
No. of GV	87	7	47	38
(%)	(55.8)	(5.7)	(30.1)	(22.6)
No. of GVBD	22	3	9	11
(%)	(14.1)	(2.4)	(5.8)	(6.5)
No. of M I	4	9	31	30
(%)	(2.5)	(7.3)	(19.9)	(17.9)
No. of M II	43	104	69	89
(%)	(27.6)[a]	(84.6)[b]	(44.2)[c]	(53.0)[c]

[a-c]Different superscripts indicate that percentages were significantly different at $P< 0.001$ and 0.005

OCCs undergoing nuclear maturation between untreated- and 10 ng EGF/ml treated groups according to time course during IVM. Immediately after recovery of OCCs, nuclear configuration was shown the majority GV stage (84.3%), and 10 ng EGF/ml treated groups were shown 62.8, 20.4, and 8.8% (GV stage) according to time course during IVM. But GV stage of untreated groups (75.0, 62.7, and 55.5%) were higher than that of treated groups. Specifically, two groups (un- and treatment) were significantly different ($p < 0.001$) at the GV stage after 24hr. Also, GVBD of OCCs in medium supplemented with FSH and FBS for 24hr was shown 84.1% (95/113, not data shown) and this was similar to EGF treatment group (EGF added during 24hr)

Experiment 2. Influences of exposure of EGF during IVM

As shown in Table 2, nuclear maturation of duration of exposure of OCCs to EGF supplement in maturation medium was tested during 0, 24-42, 0-24, or 0-42hr, respectively. Nuclear maturation of these was shown 26.1, 53.5, 72.8, and 84.8% to M II, respectively. Specifically, the supplement of EGF at the initiation of OCCs maturation was shown significantly higher than untreatment and the supplement of EGF at 24hr ($p < 0.001$). Also, nuclear maturation (M II) between 0-24 and 0-42hr supplemented with EGF at the initiation of maturation was significantly different ($p < 0.05$), and between untreatment and 24-42hr added EGF during maturation were different on nuclear maturation ($p < 0.001$).

Experiment 3. Influences of EGF on cumulus-enclosed and free oocytes

In Table 3, the effect of EGF on nuclear maturation of CEOs or CFOs was indicated. In CEOs, 84.6% of nuclear maturation (M II) of EGF treated group was significantly higher than 27.6% of untreated group ($p < 0.001$). The nuclear maturation reached M II in CFOs was shown 44.2 or 53.0% in EGF untreated or treated group, respectively, and these two groups were not different on nuclear maturation. Also, nuclear maturation between CEOs (27.6%) and CFOs (44.2%) of untreated groups were significantly different ($p < 0.005$). On the other hand, EGF treated group of CEOs was significantly higher than two groups of CFOs ($p < 0.001$), but EGF untreated group of CEOs was significantly lower than two groups of CFOs ($p < 0.005$).

DISCUSSION

In the study, the effect of EGF supplement

in maturation medium was examined on nuclear maturation during *in vitro* maturation of pig immature oocytes, and this factor was affected at GVBD and M Ⅱ on nuclear maturation of oocytes. Use was made of a serum-free media for *in vitro* maturation in order to explain the relationship between growth factors and the regulation of nuclear maturation, while effectively ruling out the influence of unknown serum factor(s). Sera contain many components, including hormones, trace nutrients, and growth factors such as EGF; in pig, reported serum EGF levels are 8 ng/ml and in human, 1.20-3.75 ng/ml (Harper and Brackett, 1993). EGF on nuclear maturation during maturation of oocytes was shown different at GVBD according to maturational times (Table 1). Specifically, EGF treated group on nuclear maturation after 24hr was significantly revealed the high percentage at GVBD. Also, when the duration of exposure of OCCs to EGF supplement in maturation medium on nuclear maturation was treated during 0-24h or 0-42hr, these groups were significantly revealed the high percentage to M Ⅱ on nuclear maturation (Table 2). Pig oocytes complete GVBD *in vivo* and *in vitro* about 20hr after hCG injection (Hunter and Polge, 1966; Ainsworth *et al.*, 1980) or culture (Naito and Toyoda, 1991). Both cumulus-enclosed and free pig oocytes resume meiosis under suitable culture conditions and during 20 to 42hr undergo GVBD (Motlik *et al.*, 1976). It appears therefore that the requirement of hormone supplementation was limited to the GVBD phase. *In vivo*, concentrations of gonadotropins and steroids in follicular fluid change dramatically during oocyte maturation (Moor, 1974; McNatty *et al.*, 1975; Hunter *et al.*, 1976; Ainsworth *et al.*, 1980; Lenton *et al.*,1988). EGF was shown to stimulate GVBD of follicle- and cumulus-enclosed mouse oocytes (Downs *et al.*, 1988; Dekel and Sherizly, 1985). But nuclear maturation at untreated group (Table 1, 2, and 3) was significantly shown the low percentage of M Ⅱ as well as GVBD during maturation of oocytes. Mattioli *et al.* (1991) reported that oocyte complexes cultured in medium not supplemented with gonadotropins or EGF had a low nuclear maturation rate (about 18.1% of M Ⅱ), and the majority (62.9%) of the oocytes remained at the GV stage after 47hr of the culture. These results (Table 1 and 2) suggest that EGF on nuclear maturation of pig OCCs was affected to M Ⅱ as well as GVBD stimulating in early phase (during 0-24hr).

In Table 3, nuclear maturation rates of pig CEOs of EGF treated group were significantly higher than CFOs. EGF effects on oocytes seem to be mediated at least in partly follicular (cumulus) cells, since denuded murine and porcine oocytes showed no significant increase in GVBD when treated with EGF (Downs *et al.*, 1988; Coskun and Lin, 1992). However, Das *et al.*, (1991) reported that EGF stimulated both GVBD and first polar body formation in both denuded and cumulus-enclosed murine and human oocytes. Mechanisms capable of mediating EGF effects via actions of follicular cells clearly exist. EGF receptors have been demonstrated specific binding of EGF to bovine cumulus and small antral granulosa cells (Rose *et al.*, 1991), and to pig granulosa cells (Fujinaga *et al.*, 1992). Also, recent study using immunohistochemical staining by Ding and Foxcroft (1994) showed that both EGF and its receptor were synthesized by the oocyte and somatic follicular cells of the pig ovary.

In summary, these results implicated that EGF alone can stimulate GVBD as well as M Ⅱ on the nuclear maturation in pig cumulus oocyte complexes.

SUMMARY

The objective of this experiment was to test the effect of EGF on GVBD and M Ⅱ of nuclear maturation of pig immature oocytes *in vitro*. Experiment 1 examined to the effect of

EGF on nuclear maturation of pig immature oocytes according to different maturational times. The percentage of GVBD of EGF 10ng/ml treated groups were significantly higher than untreated groups after 24hr ($p < 0.001$). Experiment 2 examined to the effect of duration of exposure of oocytes to EGF supplement in maturation medium. Nuclear maturation rates (M Ⅱ) of EGF treated groups (during 0-24: 72.8% and 0-42hr: 84.8%) were significantly higher than 53.5 and 26.1% of EGF treated group (during 24-42hr) and untreated group ($p < 0.001$). Also, experiment 3 examined to the effect of EGF on nuclear maturation of CEOs or CFOs. Nuclear maturation rate (M Ⅱ) 84.6% of EGF treated group of CEOs was significantly higher than 53.0, 27.6, and 44.2% of EGF treated group of CFOs and untreated groups of CEOs and CFOs ($p < 0.001$). These results conclude that EGF alone can stimulate GVBD and M Ⅱ of nuclear maturation in pig immature oocytes.

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