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

IV. Effect of Epidermal Growth Factor on *In Vitro* Development

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Epidermal Growth Factor가 돼지 미성숙난포란의 체외성숙에 미치는 영향
IV. 체외 배발달에 미치는 Epidermal Growth Factor의 효과

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= 국문초록 =

본 연구는 epidermal growth factor (EGF)가 처리된 돼지 체외성숙란의 체외수정 후 배발달을 조사하기 위하여 실시하였다. 난구세포가 치밀한 미성숙란은 TCM 199 배양액에 ① 무처리군 ② 10 ng/ml EGF 처리군 ③ 10 μg/ml FSH와 10% FBS 처리군 ④ 10 ng/ml EGF와 10 μg/ml FSH 그리고 10% FBS 처리군으로 나누어 42시간 동안 배양하였다. 실험 1은 이들 처리군을 수정 후 NCSU (0.4% BSA) 배양액에서 배양하여 후기 배발달을 조사하였다. 그 결과 ③과 ④ 처리군 (13.4, 18.3%)의 배반포로의 발달율이 ② 처리군(5.2%, $p < 0.005$)보다 현저하게 높았지만, ② 처리군의 경우 ① 처리군(1.2%, $p < 0.005$)의 배반포로의 발달보다는 현저하게 높았다. 실험 2는 수정 이후 6일째 이들 처리군에서 생산된 배반포기배를 double staining (propidium iodide and bisbenzimide) 방법을 이용하여 세포수를 조사하였다. 그 결과 ④ 처리군 (58.80 ± 11.90)의 배반포의 total 세포수가 ②와 ③ 처리군 (42.17 ± 9.97, 49.07 ± 9.77, $p < 0.05$)보다 현저하게 많았으며, 배반포의 ICM 수에 있어서도 ④ 처리군 (11.69 ± 5.56)이 ②와 ③ 처리군 (5.00 ± 4.24, 6.77 ± 4.92, $p < 0.05$)보다 현저하게 많았다. 더우기 ④ 처리군 (19.0 ± 1.6)의 total 세포수에 대한 ICM의 비율이 ②와 ③ 처리군 (11.1 ± 3.0, 12.7 ± 2.1)보다 높았다. 이들의 결과로서, EGF가 단독 처리된 돼지 체외성숙란은 비록 낮았지만 배반포로 발달할 수는 있으나, 체외성숙시 FSH와 FBS의 추가 첨가는 높은 배반포로의 발달과 total 세포수와 ICM의 증가를 가져올 수 있음을 알수 있었다.

INTRODUCTION

During *in vitro* oocyte maturation, the nuclear events, germinal vesicle breakdown, and polar body formation appear to occur normally, but fertilization, cleavage, and early embryonic development may not be successful (Leibfried and Bavister, 1983; Fleming *et al.*, 1985). This interruption of development might be the result of incomplete cytoplasmic maturatuion. Oocytes are much more successfully matured

in vivo than *in vitro* (Leibfried *et al.*, 1987), which might suggest that hormonal and/or follicular factors are required to improve maturation to obtain normal fertilizability and development rate. Therefore, a number of researchers have tested possible factors which may dominate events during the late stages of follicular development which may enhance the fertilizability and developmental capability of *in vitro*-matured oocytes. Fukushima and Fukui (1985) reported that the addition of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E_2) to the medium improved the fertilizability of extrafollicular bovine oocytes cultured *in vitro*. Several investigators have demonstrated the ability of porcine oocytes matured and fertilized *in vitro* to develop normally (Niwa, 1993; Nagai, 1994), and the birth of piglets from embryos produced *in vitro* has been reported (Mattioli *et al.*, 1989; Yoshida *et al.*, 1993). However, the *in vitro* development of *in vitro*-matured and -fertilized porcine oocytes to the blastocyst stage is poor.

Porcine oocyte-cumulus complexes (OCCs) expansion *in vitro* is stimulated by EGF (Ding and Foxcroft, 1994) and a trend toward increased maturation and fertilization has been seen as well. In previous report, we studied that EGF affects on nuclear maturation of pig oocytes (Uhm *et al.*, 1995a,b). Harper and Brackett (1993) reported that treatment of EGF for *in vitro* maturation (IVM) of bovine oocytes affected the development to blastocyst stage.

This investigation, using the double dye technique for differential staining is the first to examine the effects of EGF on inner cell mass (ICM) and trophectoderm (TE) in pig blastocysts produced *in vitro*. This technique was refined by using two different fluorochromes: propidium iodide (PI) for labelling TE nuclei and bisbenzimide to identify the nuclei of ICM cells (Handyside and Hunter, 1984). Here, we report experiments carried out to determine; 1) whether EGF alone or in concert with FSH and FBS during IVM might influence oocyte quality as reflected by resulting proportions of ova reaching blastocyst stage after *in vitro* fertilization (IVF), 2) and cell allocation to the total and ICM cells in these blastocysts.

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 were used for this study (Uhm *et al.*, 1995a,b).

2. *In vitro* maturation (IVM)

The oocytes were transferred into a 4-well dish (NUNC) containing maturation medium (50 oocytes/0.5 ml) 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), 0.2 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 for 42-44 hr (Uhm *et al.*, 1995a,b).

3. 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 (1000 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. 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 28-30 hr at 5% CO_2 in air with saturated humidity at 39℃.

4. *In vitro* development (IVD)

Inseminated oocytes were washed 3 times in TL-HEPES. 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 7 days after insemination. The blastocysts were stained by differential labelling of ICM and TE nuclei.

5. 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 is 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 1hr, 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.

6. Design and analysis

The experiment was designed to examine the effect of cleavage and blastocyst development following IVF of *in vitro*-matured porcine oocytes treated with EGF. The treatment groups for maturation were separated the three groups by 10 ng/ml EGF, combination of 10 μg/ml FSH and 10% FBS, or combination of 10 ng/ml EGF, 10 μg/ml FSH and 10% FBS. The cell number (ICM, TE, and Total cells) of blastocysts produced by three maturation treatment methods examined by differential labelling method. At least three trials were conducted 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

Differences in proportions of oocytes un-

dergoing cleavage or development to the blastocyst stage were significantly different as a result of IVM with EGF alone as compared with combination FSH and FBS or combination of EGF, FSH, and FBS treatments ($p < 0.05$, Table 1). However, the matured oocytes produced by EGF treatment alone for IVM can be developed to the blastocyst stage, and the cleavage and blastocyst rate of them was higher than those of control. The blastocysts produced by this experiment are shown in Fig. 1. The double dye technique allowed a reliable and obvious distinction between the pink TE nuclei and intact ICM cells in which the nuclei appear blue. The finding of at least one blue nucleus was taken as a measure for the presence of the ICM. The blastocyst stained by differential labelling method is shown in Fig. 2. The average number of total cells and ICM of blastocysts produced by combination EGF, FSH, and FBS treatment for IVM was significantly higher than that of blastocysts by EGF alone or combination FSH and FBS treatments ($p < 0.05$, Table 2), but that of EGF treatment alone was lower than that of blastocyst by combination FSH and FBS treatment.

DISCUSSION

To assess full maturation of oocytes, it is necessary to examine fertilization and early embryonic development potential, both of which require proper signals from the medium during maturation; LH (Brackett *et al.*, 1989), steroids (Zhang and Armstrong, 1989), FSH (Younis *et al.*, 1989), fetal calf serum (FCS) and estrus cow serum (ECS) (Schellander *et al.*, 1990), bovine serum albumin (BSA) (Leibfried *et al.*, 1986), and prolactin (Yoshimura *et al.*, 1989) have been added individually to medium to im-

Table 1. Development of porcine embryos produced by various treatments for IVM

Treatment of maturation	Total of matured oocytes	Cleaved (≥2-cell)	Blastocyst			
			Total	Early	Middle	Expand
Control	256	63 (24.6)[a]	3 (1.2)[a]	1	1	1
E group[1]	250	119 (47.6)[b]	13 (5.2)[b]	4	1	8
FF group[2]	254	118 (74.0)[c]	34 (13.4)[c]	9	7	18
EFF group[3]	268	221 (82.5)[c]	49 (18.3)[c]	8	22	19

[1]EGF 10 ng/ml added on maturation medium. [2]Combination of FSH 10 μg/ml and FBS 10% on maturation medium. [3]Combination of EGF 10 ng/ml, FSH 10 μg/ml, and FBS 10% on maturation medium. [a-b]Different superscripts within columns were significantly different ($p<0.05$).

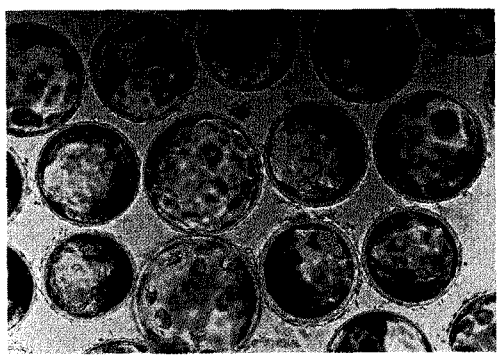

Fig. 1. Pig IVM/IVF/IVC blastocysts (6 day after IVF, ×150).

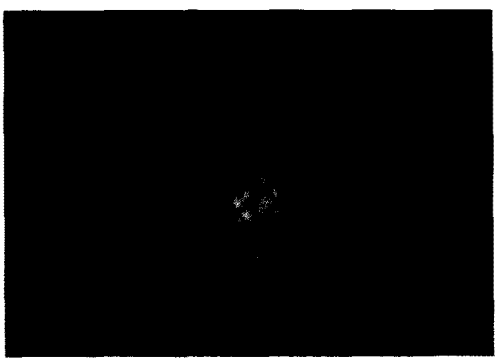

Fig. 2. Differetial staining of a pig IVM/IVF/IVC blastocyst (6 day after IVF). The blastocyst contains 16 ICM (blue) and 52 TE (red, ×300).

Table 2. Number of total and ICM cells (mean±SEM) in blastocysts of porcine embryos produced by various treatments for IVM

Treatment of maturation	No. of blastocyst	Total cells		ICM		
		No. of cells	Range	No. of cells	Range	Proportion (%)
E group[1]	5	42.4±5.2[a]	31-58	5.0±1.9[a]	2-12	11.1±3.0
FF group[2]	13	49.6±2.8[a]	34-68	6.8±1.4[a]	2-18	12.7±2.1
EFF group[3]	13	58.8±3.3[b]	44-82	11.7±1.5[b]	5-22	19.0±1.6

[1]EGF 10 ng/ml on maturation medium. [2]Combination of FSH 10 μg/ml and FBS 10% on maturation medium. [3]Combination of EGF 10 ng/ml, FSH 10 μg/ml, and FBS 10% on maturation medium. [a-b]Different superscripts within columns were significantly different ($p<0.05$).

prove fertilization and embryonic development. In the present study, we examined the acquisition of developmantal capacity by oocytes matured in the presence of EGF in serum-free, chemically defined medium *in vitro*. Although the above all contribute essential information about the effects of EGF on the reproductive system, the physiological significance of EGF is not yet clear. It is possibly that EGF plays a regulatory role in follicular development in a paracrine/autocrine manner, or it might be one of the signaling factors for the resumption of meiosis in oocytes. Complete cytoplasmic and nuclear oocyte maturation relies upon a complex interaction of gonadotropins, steroids, and follicular signals (Moor *et al.*, 1981). Growth factors have been shown to bind to high-affinity receptors and promote the generation of signals and second messengers in the membrane and cytoplasm (Hill, 1989). Binding of EGF to its receptor induces the activation of tyrosine kinase, an essential primary event in the EGF pathway. Tyrosine kinase activation initiates phosphorylation of several cellular proteins as well as the receptor itself (Rozengurt, 1983; Carpenter and Cohen, 1990). Several specific tyrosine kinase substrates have been identified that may be important signals in initiating completion of oocyte maturation. These include phospholipase C1, which allows release of profolin and inositol triphosphate (Goldschmidt-Clermont *et al.*, 1991), PI-3 kinase, GTPase activating protein and membrane-associated protein kinase, and raf kinase (Carpenter and Cohen, 1990), any or all of which may be involved in the signaling pathway leading to protein synthesis and phosphorylation. Such changes in protein synthesis and protein phosphorylation as have been shown to be integral to bovine oocyte maturation provide support for a physiological role of growth factors in oocyte maturation. Because EGF has been demonstrated in follicular fluid (Hsu *et al.*, 1987) and also has been shown to exert a beneficial influence on oocyte maturation *in vitro* (Coskun *et al.*, 1991; Das *et al.*, 1991; Dekel *et al.*, 1985; Ding and foxcroft, 1994; Downs, 1989; Harper and brackett, 1991), it is highly probable that EGF is one growth factor capable of regulating oocyte maturation through secondary effects after binding to cumulus cell receptors.

In previousely study, we reported that EGF affects on nuclear maturation of porcine oocytes, and combination of EGF with FSH and FBS promotes fertilization of porcine oocytes (Uhm *et al.*, 1995a,b,1996). Coskun *et al.* (1992) reported that EGF alone could improve the developmental potential of *in vitro* matured bovine oocytes. Harper and Brackett (1993) showed that combination of EGF with low concentrations of gonadotropins during IVM enabled subsequent blastocyst development in proportions comparable to those afforded by high concentrations of FSH or LH. In the porcine, the present study showed that the oocytes treated with EGF alone for IVM are significantly lower than the development

to blastocyst of oocytes treated with combination of EGF, FSH, and FBS for IVM (Table 1), and the cell number of total cells and ICM is significantly reduced (Table 2). However, the oocytes treated EGF alone for IVM are significantly higher than the development to blastocyst of oocytes of control (oocytes untreated anything factors for IVM). The differential staining procedure used to identify ICM and TE cells was successfully used in mouse (Handyside and Hunter, 1984), pig (Papaioannou and Ebert, 1988), frozen-thawed bovine and pig embryos (Iwasaki *et al.*, 1994a,b), and *in vitro* produced bovine embryos (Iwasaki *et al.*, 1990). Tao *et al.* (1995) reported that the average number of total and ICM cells in pig blastocyst produced in vivo was 74.7 and 16.4 and the proportion of ICM for total cells was 22.0%. However, the average number of cells for pig blastocysts produced *in vitro* by IVM/IVF was lower than that produced in vivo, and these data was the first to examine for IVM of pig blastocyst produced *in vitro*. Collectively, these data indicate that EGF alone for IVM can affect on nuclear maturation of oocytes, but the interaction of EGF with materials like FSH and unkown factors within FBS can affect to cleavage and formation of blastocyst as well as on cytoplasmic maturation of oocytes. Therefore, these results suggested that *in vitro*-matured porcine oocytes treated with EGF alone can be developed to the blastocyst stage, but high proportion on development to the blastocyst stage and the cell number of total cells and ICM can be obtained when supplemented with FSH and FBS.

SUMMARY

The objective of this study was to examine the effect of embryos development following IVF of *in vitro*-matured porcine oocytes treated with epidermal growth factor (EGF). When cumulus-enclosed oocytes were incubated in TCM 199 medium supplemented with ① control group, ② 10 ng/ml EGF, ③ 10 μg/ml FSH and 10% FBS, or ④ 10 ng/ml EGF, 10 μg/ml FSH, and 10% FBS for 42 hr, the late developmental rates on NCSU (0.4% BSA) medium after fertilization were higher in ③ and ④ groups (13.4, 18.3%) than in ② group (5.2%, $p < 0.005$), but ② group is significantly higher than the development to blastocyst of oocytes of ① group (1.2%). Also, when the cell number of total, ICM, and TE of those blastocysts at 6 day produced *in vitro* was investigated by double staining (PI and bisbenzimide), total cell number of ④ group (58.80 ± 11.90) was higher than that of ② and ③ groups (42.17 ± 9.97, 49.07 ± 9.77, $p < 0.05$). ICM cell number of blastocysts of ④ group (11.69 ± 5.56) was higher than that of ② and ③ groups (5.00 ± 4.24, 6.77 ± 4.92, $p < 0.05$). Furthermore, the proportion of ICM in ④ group (19.0 ± 1.6) was higher than that in ② and ③ groups (11.1 ± 3.0, 12.7 ± 2.1). These results suggested that *in vitro*-matured porcine oocytes treated with EGF alone can be developed to blastocyst, but high proportion on the development to blastocyst and number of total cell and ICM in blastocyst can be obtained when supplemented with additional FSH and FBS.

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