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Study on the Additive Effect of Epidermal Growth Factor (EGF) and Expression of EGF-Receptor (EGF-R) on IVM/IVF Bovine Embryo Development

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체외 생산된 소 수정란의 발달에 있어서 EGF 첨가제 효과와 EGF-R 발현에 관한 연구

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요 약

본 연구는 EGF가 체외성숙과 수정에 의해 생산된 소 수정란의 발달과 inner cell mass (ICM)와 trophectoderm (TE) 세포수에 미치는 영향 및 공동배양시의 첨가효과를 조사하고 그와 더불어 간접 면역 형광법을 이용하여 EGF-R 단백의 발현 유무를 조사하기 위해 실시하였다. 그 결과를 요약하면 다음과 같다. EGF 1, 10, 100 ng/ml의 농도로 처리되었던 4-세포기와 8-세포기 배는 대조군에 비하여 유의차는 인정되지 않았으나 양호한 배반포기 배 발달과 ICM과 TE 세포수 증가 양상을 나타내었다. 특히, 발달단계에 따른 EGF (10 ng/ml) 효과를 조사하였던 바, 8-세포기 이후 배에서 대조군에 비하여 배반포기까지 유의한 배 발달을 유도하는 것을 확인할 수 있었지만 ($p<0.05$), ICM과 TE 세포수 증가에는 유의한 영향을 미치지 못하는 것을 알 수 있었다. 또한, 간접 면역 형광에 의한 EGF-R의 발현 유무를 조사한 결과, EGF-R는 4-세포기 이후에 발현되며 그 강도는 발달단계가 진행되면서 다양하게 나타난다는 것을 알 수 있었다. 한편, 수정란과 난구세포 공동배양 군은 EGF의 첨가 유무에 상관없이 대조군에 비하여 유의한 배 발달과 총세포수의 증가를 나타내며, 공동배양군에 대한 EGF 첨가는 수정란과 난구세포와의 공동배양 효과를 증진시키는 것으로 나타났다. 따라서, EGF는 착상전 소 수정란의 4-세포기 이후에 발현되는 EGF-R에 반응하여 배 발달을 유기하고, 공동배양시의 배 발달에 유용한 물질 형성을 촉진시키지만, 배반포기 배의 ICM과 TE 세포수 증가에는 유의한 영향을 나타내지 못한다는 것을 알 수 있었다.

I. INTRODUCTION

Many growth factors are synthesized by embryo itself and local tissue environment and act for the control of growth and differentiation during mammalian embryogenesis as paracrine or autocrine hormones (Rappolee et al., 1988; Zhang et al., 1994; Gandolfi, 1994; Harvey et al., 1995). Among the growth factors, epidermal

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growth factor (EGF) is one of the most biologically potent mitogen (Carpenter and Cohen, 1979). However, the transcript for EGF is not detected even at the blastocyst stage (Nexo et al., 1980; Rappolee et al., 1988). Nontheless, EGF stimulates growth and protein synthesis in the preimplantation embryo (Wood and Kaye, 1989; Werb, 1990) and compensates for the dilution effect that occurs when single embryos are cultured in large volumes (Paria and Dey, 1990). EGF directly affect the rate of blastocoel expansion through the EGF-R (Dardik and Schultz, 1991). At present, it is known that EGF-R is expressed at increasing levels on mouse preimplantation embryos (Wiley et al., 1992) and presented in both the inner cell mass (ICM) and trophectoderm (TE) cells (Adamson and Meek, 1984; Dardik et al., 1992; Brison and Shultz, 1996). In our previous study, we demonstrated that EGF is expressed after 4-cell stage in mouse IVF preimplantation embryo (Kim et al., 1996b). On the other hand, many of the same growth factors and receptors are expressed in both murine and bovine embryos, the embryonic stages at which transcripts can be detected are distinctly different. This pattern may correlate with the later stage of bovine embryonic genome activation compared with that of the mouse. In fact, in cattle, only a few study on the expression of EGF and EGF-R is reported yet (Watson et al., 1992).

The objective of this study was to investigate the effect of EGF on the development of IVM/IVF bovine embryos and their ICM and TE cell number. In addition, we examined the combined effect of EGF and coculture on the bovine embryo development and the expression of EGF-R protein in preimplantation embryonic stage by indirect immunofluorescence.

Ⅱ. MATERIALS AND METHODS

1. Oocytes maturation and *in vitro* fertilization

Bovine ovaries were transported from a slaughterhouse to the laboratory suspended in saline (0.9% NaCl; 32 ± 2℃) in container. Cumulus oocyte complexes (COCs) were collected from visible follicles (2~6 mm) of ovaries. The COCs were washed three times with TL-HEPES (low carbonate TALP; Parrish et al, 1988) medium containing 1mg/ml of bovine serum albumin (BSA; Fraction V, Sigma). The basic medium for maturation was tissue culture medium (TCM-199). The additives were fetal bovine serum (FBS; 10%), sodium pyruvate (0.2 mM), FSH (1 μg/ml), estradiol-17β (1 μg/ml), and gentamycin (25 μg/ml). Ten COCs were placed in each maturation drop (50 μl), and then cultured for 22~24 h at 39℃, 5% CO_2 incubator.

In vitro fertilization was carried out by a method of Sirard et al. (1988) with some modifications. Briefly, highly motile bull spermatozoa were recovered from frozen-thawed semen separated on a discontinuous percoll column and resuspended with Sp-TALP (Rosenkrans et al., 1993) at a concentration of 2.5×10^7 cells/ml. After 22~24 h *in vitro* maturation, COCs were washed three times with Sp-TALP, and transferred into 44 μl fertilization drops (Fert-TALP; Rosenkrans et al., 1993). Ten COCs were moved to each fertilization drop, followed by 2 μl of motile sperm (5×10^4 cells/50 μl drop), 2 μl of heparin (2 μg/ml), and 2 μl of PHE (18.2 μM Penicillamine, 9.1 μM Hypotaurine and 1.8 μM Epinephrine) stock (Rosenkrans et al., 1993).

2. Embryo culture experiments

Cleaved embryos were selected at an appropriate time and then cultured in group of 5 embryos in 25 μl of CR1 medium (control medium which was basically supplemented with fatty

acid free BSA 3 mg/ml) with or without EGF (recombinant human gene, Sigma) under mineral oil. In all experiments, embryos were scored on day 8 after *in vitro* fertilization. The cell number of blastocysts was determined by differential labelling method.

Experiment Ⅰ: Effect of EGF on the *in vitro* development and cell number of IVM/IVF bovine embryo

Four- to 8-cell embryos were selected at 48 ± 2 h post insemination (hpi), they were cultured in CR1 medium added with 0, 1, 10 and 100 ng/ml of EGF. Medium was not changed for culture duration.

Experiment Ⅱ: Effect of 10 ng/ml of EGF on the *in vitro* development and cell number of IVM/IVF bovine embryo with different stages

Embryos were recovered at different stages as follows ; 2-cell stage at 30 to 35 h, 4-cell stage at 40 to 44 h, 8-cell stage at 50 to 54 h, ≤ 16-cell stage at 88 to 92 h and morula stage at 118 to 124 hpi. Each groups were cultured in CR1 medium with or without EGF (10 ng/ml). Medium was not changed for culture duration.

Experiment Ⅲ: Effect of EGF or/and coculture on the *in vitro* development and cell number of IVM/IVF bovine embryo

Four- to 8-cell embryos were selected at 48 ± 2 hpi, they were cultured in CR1 medium with or without EGF (10 ng/ml) and/or cumulus cell coculture. Coculture plates were prepared as follows; Briefly, cumulus cell masses were cutted off from the mature-appearing oocytes at 22 h after IVM using a syringe fitted with a 28 gauge needle. They were vortexed in 0.5% hyaluronidase solution diluted with CR1 medium in 1.5 ml eppendorf tube for 10min., allowed to sediment until large clumps were settled down. And then, upper part was washed 2 times by centrifugation at 500 × g for 5 min., resuspended in CR1 medium and then cumulus cells (1×10^4) were seeded in 20μl drop. Most cumulus cells were attached after 16~20 h. In this experiment, CR1 medium was supplemented with 1.5 mg/ml of fatty acid free BSA and 5% FBS and also not changed for culture duration.

3. Differential labelling of ICM and TE nuclei

Bovine blastocysts on day 8 were stained by a method of Kim et al (1996a). Briefly, TE nuclei are labelled first specifically with fluorochrome propidium iodide (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 ICM and TE nuclei labelled with bisbenzimide. Finally, TE nuclei labelled with PI and bisbenzimide appeared pink or red, ICM nuclei labelled with bisbenzimide appeared blue or unlabelled.

4. Detection of EGF-R on embryonic stage by indirect immunofluorescence

Study on expression of EGF-R protein of bovine embryo was carried out by the method of Kim et al. (1996b) with some modifications. Briefly, zona removed embryos were treated with human EGF (Sigma), Goat anti-human EGF (Upstate Biotechnology Incorporated) and/or FITC-conjugated donkey anti-goat IgG (UBI). Preimplantation embryos at 2-, 4-, 8-, 16-cell, morula, and blastocyst stages were recovered on days 2~8 after IVF. Embryo zona was removed in 0.5% pronase (Sigma) solution and returned in CR1 medium for at least 1 h prior to assay. Assays were performed at 4℃. Living embryos were processed through the following steps; (1) 200 ng/ml EGF in PBS for 1 h; (2) washing sufficiently; (3) antibody to

EGF diluted 1:50 with PBS for 2 h; (4) washing completely; (5) FITC-conjugated donkey anti-goat IgG diluted 1:50 with PBS for 4 h. And then they were washed completely, transferred to a drop of PBS on a slide glass and observed immediately with an inverted phase-contrast microscope fitted with epifluorescence illumination.

5. Statistical analysis

Difference in rate of blastocyst and number of cells between development groups was compared using the Chi-square test and Student's t-test, respectively.

Ⅲ. RESULTS

To determine the additive effect of EGF on IVM/IVF bovine embryo development and their cell number, four- to 8-cell embryos were treated with EGF at concentrations of 0, 1, 10, 100 ng/ml (Table 1). As shown in Table 1, embryos cultured in the EGF treatment group showed improved development to blastocyst and increased pattern of ICM and TE cell number compared with control, although there was not significantly different (Fig. 1; K). In EGF treatment group, the best result was obtained in 10 ng/ml of EGF at concentration. The addition effect of 10 ng/ml of EGF according to the development level was examined at 2-, 4-, 8-, 16-cell and morula stage of IVM/IVF bovine embryos (Table 2). In Table 2, EGF treatment group showed more improved development rate to blastocyst than control, although there was no significant difference in cell numbers. Especially, the stimulating effect of EGF was significantly increased after 8-cell stage ($p<0.05$). However, it was confirmed that expression of EGF-R on the bovine embryonic stage by indirect immunofluorescence presents after 4-cell stage and the intensity of the EGF-R staining was variable with the development progression (Fig. 1; A-J). In addition, to determine the combined effect of EGF and coculture on bovine embryo development and their cell number, four- to 8-cell embryos were cultured in 10 ng/ml of EGF or/and cumulus cell monolayer drop (Table 3). As presented in Table 3, coculture group embryo added either with or without EGF commonly indicated the significant difference in development rate to blastocyst and Total cell number compared with control embryo.

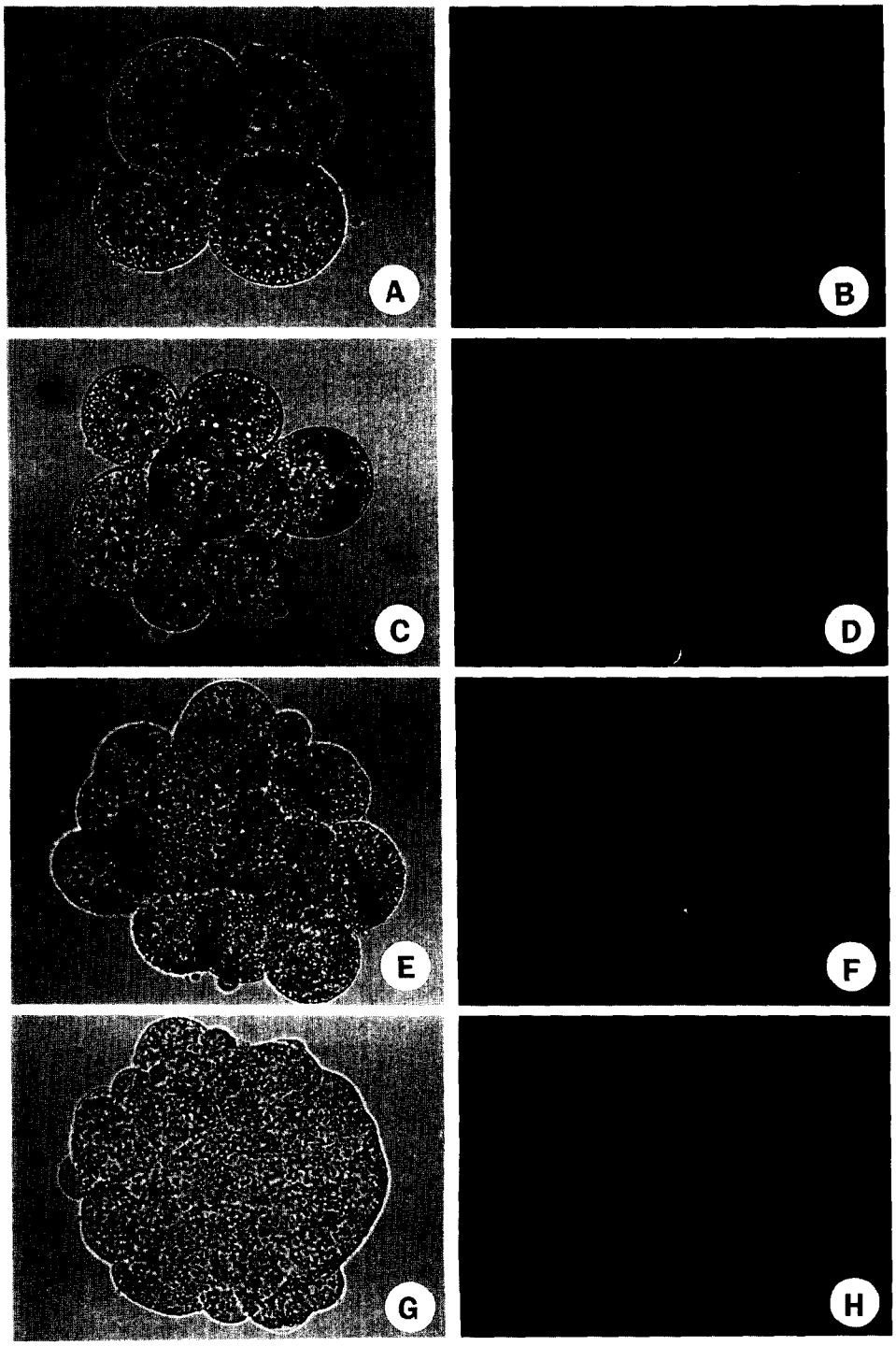
Ⅳ. DISCUSSION

This study was undertaken to determine the stimulating effect of EGF on IVM/IVF bovine embryo development and the possibility of EGF as a supplement to the embryo culture medium. In our previous study (Kim et al., 1996b), we already demonstrated the similar data in mouse.

Table 1. Effect of EGF on the *in vitro* devlopment and cell number of IVM/IVF bovine embryo

EGF (ng/ml)	No. of 4- to 8-cell embryos	Rate (%) of blastocyst	Cell number (Mean ± SEM)		
			ICM*	TE**	Total
0	69	14 (20.3)	19.5 ± 3.3	60.0 ± 3.0	80.5 ± 8.4
1	69	15 (21.7)	23.7 ± 2.3	68.6 ± 2.1	94.3 ± 7.4
10	70	21 (30.0)	25.8 ± 3.8	70.1 ± 2.9	95.8 ± 9.8
100	63	17 (27.0)	23.9 ± 2.7	69.3 ± 1.7	95.2 ± 9.8

* ICM: inner cell mass, ** TE: Trophectoderm.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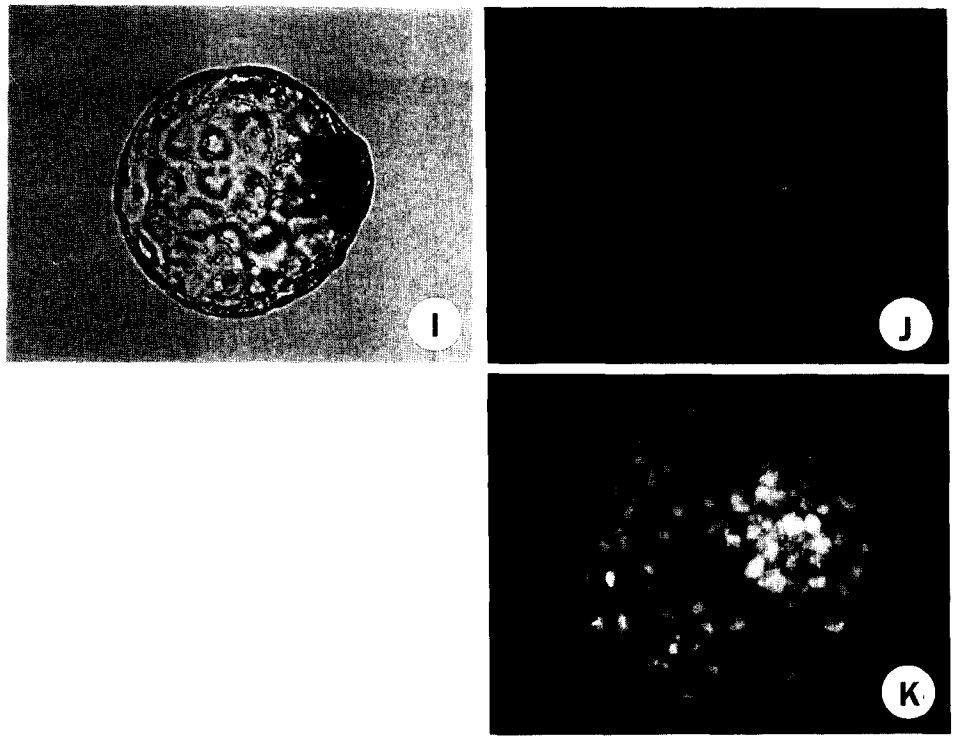

Fig. 1. Indirect immunofluorescence (IIF; A-J) and differential labelling (DFL; K) of IVM/IVF bovine embryos. IIF assays demonstrated the staining results according to binding between EGF and EGF-R (B, D, F, H and J), and corresponding phase micrographs (A, C, E, G and I). A and B; 4-cell embryo at 44 hpi, C and D; 8-cell embryo at 54 hpi, E and F; 16-cell embryo at 92 hpi, G and H; morulae at 120 hpi and I and J; blastocyst at 192 hpi. DFL presented the nuclei of ICM and TE of blastocyst recovered at 192 hpi which is differentially stained with bisbenzimide and propidium iodide. Blue ICM nuclei and red TE nuclei are easily distinguished (ICM: 48, TE: 86). × 300.

Until now, the study on the role of growth factor in embryo have mainly been done in mouse. It has been known that the addition of physiological levels of EGF gene family to the culture medium of early mouse embryos results in a broad range of effects that include stimulation of RNA and protein synthesis, increased rate of cell division, increase of the cell number in blastocysts, and increase in the percentage of cultured embryos that hatch from the zona pellucida (Wood and Kaye, 1989; Paria and Dey, 1990; Werb, 1990). The present study also showed the positive role of EGF on the development of IVM/IVF bovine embryos to blastocysts. Especially, when examined the effect of EGF on the ICM and TE cell number of bovine blastocyst using differential labelling, the increases of ICM and TE cell numbers were found in the EGF treatment group although there was no significant difference.

On the other hand, it was reported that EGF-R mRNA was increased after the 4-cell stage in mouse embryos (Wiley et al., 1992) and EGF binding was firstly detected at the 8-cell stage (Paria and Dey, 1990). Wood and Kaye (1989) reported that EGF enhances compaction

Table 2. Effect of EGF (10 ng/ml) on the *in vitro* development and cell number of IVM/IVF bovine embryo at different stages

*Stage of embryos cultured	Treatment	No. of embryos cultured	Rate(%) of blastocyst	No. of cells per Blastocyst (Mean ± SEM)	
				ICM	Total
2-cell	Control	44	12 (27.3)	19.3 ± 3.1	85.6 ± 14.0
	EGF	45	17 (37.8)	22.6 ± 1.1	95.5 ± 4.7
4-cell	Control	53	12 (22.6)	21.5 ± 3.2	88.3 ± 9.1
	EGF	56	18 (32.1)	24.3 ± 2.1	93.4 ± 11.0
8-cell	Control	48	15 (31.3)	22.2 ± 1.7	92.0 ± 12.0
	EGF	48	21 (43.8)	25.3 ± 2.0	98.2 ± 7.7
≤16-cell	Control	43	18 (41.9)[a]	23.3 ± 2.8	91.2 ± 13.0
	EGF	46	29 (63.0)[b]	27.9 ± 3.8	96.2 ± 9.2
Morula	Control	38	18 (47.3)[c]	25.6 ± 2.6	90.6 ± 11.2
	EGF	40	28 (70.0)[d]	28.3 ± 3.4	98.2 ± 13.1

* 2-cell, 4-cell, 8-cell, ≤16-cell and morula embryos were selected at 30 to 35 h, 40 to 44 h, 50 to 54 h, 88 to 92 h and 118 to 124 h post insemination, respectively.

[a~b, c~d] Different superscripts are significantly different ($P<0.05$).

Table 3. Effect of EGF and/or coculture on the *in vitro* development and cell number of IVM/IVF bovine embryo

Treatment*	No. of 4- to 8-cell embryos	Rate (%) of blastocyst	Cell number	
			ICM	Total
Control	62	13 (21.0)[a]	20.2 ± 2.3	78.1 ± 10.7[c]
EGF	64	22 (34.4)[a,b]	25.4 ± 2.0	96.5 ± 7.7[c,d]
Coculture	64	26 (40.6)[b]	26.3 ± 2.8	108.1 ± 9.1[d]
Coculture + EGF	65	32 (49.2)[b]	25.7 ± 3.1	106.2 ± 11.3[d]

[a~b, c~d] Different superscripts are significantly different ($P<0.05$).

* EGF; 10 ng/ml of EGF, Coculture; cumulus cell monolayer, Coculture + EGF; cumulus cell monolayer with 10 ng/ml of EGF.

and blastulation in murine embryos upon completion of the transition from maternal to the embryonic control of development. In cattle, study on the transcripts of EGF and EGF-R is limited. Yang et al. (1993) demonstrated that growth factors may be especially important during the fourth cell cycle as the embryonic genome assumes full control. In the viewpoint, we investigated the effect of EGF according to the development level and the expression of EGF-R on the embryonic stage. In this experiment, EGF significantly stimulated the embryonic development after 8-cell stage ($P<0.05$), but embryos cultured in earlier stage than 8-cell showed slightly improved development to blastocyst in EGF treatment group. However, we confirmed that EGF-R presented after 4-cell stage by indirect immunofluorescence using EGF and anti-EGF. This result indicates that transcription of EGF-R mRNA is carried out from the earlier stage than 4-cell in bovine IVM/IVF embryo and these gene products may be originated

from the maternal genome. Furthermore, it was detected that the intensity of EGF-R staining was variable with the developmental progression. Recently, it was reported that EGF-Rs are expressed at similar levels both the ICM and TE of mouse blastocysts (Brison and Shultz, 1996). In our previous result, we indicated that proportion of ICM cell in the EGF treatment group was higher than that of TE cell in mouse (Kim et al., 1996b). The present study showed that there is no significant effect of EGF to the increase of ICM and TE cell number of bovine blastocyst although expression of EGF-R presents from the early development stage. In addition, many researchers reported that the use of trophoblastic vesicle (Camous et al., 1984), cumulus cell (Goto et al., 1989) and oviductal cell (Eyestone and First, 1989) for emryo co-culture has been beneficial effect for the development of bovine embryos. When the combined effect of EGF and cumulus cell coculture on the *in vitro* development of IVM /IVF bovine embryo and their cell number was examined, the best result was obtained in EGF-added coculture group (Table 3). In coculture treated with EGF, cumulus cells significantly may be alter metabolite levels in the embryo culture environment. Also, these changes affect embryo development and are responsible for the improved embryo development observed with coculture. However, our results indicated that embryos cultured in coculture group added either with or without EGF commonly showed the significant difference in development rate to the blastocyst and total cell number compared with control.

Therefore, It can be concluded that EGF could promote preimplantation bovine embryo development by binding with expressed EGF-R after 4-cell stage, and stimulate on the production of embryotrophic factors from coculture environment. Also, the present study showed that there was no significant effect of EGF to the increase of ICM and TE cell number, although the rate of blastocyst significantly increased when treated with EGF after 8-cell stage.

V. SUMMARY

The objective of this study was to determine the effect of EGF on the development of IVM /IVF bovine embryos and their ICM and TE cell number. In addition, we examined the combined effect of EGF and coculture to the bovine embryo development and the expression of EGF-R protein on bovine embryos by indirect immunofluorescence. The results obtained in these experiments were summarized as follows; When the IVM / IVF 4- to 8-cell embryos were treated at 0, 1, 10, 100 ng /ml of EGF, EGF treatment group showed improved development to blastocyst and increased pattern of ICM and TE cell number compared with control, although there is not significantly different. The stimulating effect of EGF (10 ng /ml) to the development level of IVM /IVF bovine embryos significantly increased development rate to blastocyst after 8-cell stage ($p<0.05$), although there is no significant effect to the increase of ICM and TE cell numbers. Also, expression of EGF-R on the bovine embryonic stage by indirect immunofluorescence presents after 4-cell stage and the intensity of the EGF-R staining was variable with the development progression. On the other hand, embryos cultured in coculture group added either with or without EGF commonly indicated the significant difference in development rate to blastocyst and Total cell number compared with control. These results suggest that the addition of EGF to the coculture may stimulate the coculture effect between IVM /IVF bovine embryos and cumulus cells. Therefore, EGF could promote preimplantation bovine embryo

development by binding with expressed EGF-R after 4-cell stage, and stimulate the production of embryotrophic factors from the coculture environment. Also, the present study showed that there was no significant effect of EGF to the increase of ICM and TE cell number although the rate of blastocyst significantly increased when treated with EGF after 8-cell stage ($p<0.05$).

Ⅵ. 인용문헌

1. Adamson, E. D and J. Meek. 1984. The ontogeny of epidermal growth factor receptors during mouse development. Dev. Biol., 103:62-70.
2. Brison, D. R. and R. M. Schultz. 1996. RT-PCR based method to localize the spatial expression of genes in the mouse blastocyst. Mol. Reprod. Dev., 44:171-178.
3. Camous, S., Y. Heyman, W. Weziou and Y. Menezo. 1984. Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblastic vesicles. J. Repord. Fert., 72p:479-485.
4. Carpenter, G. and S. Cohen. 1979. Epidermal growth factor. Ann. Rev. Biochem., 48: 193-216.
5. Dardik, A. and R. M. Schultz. 1991. Blastocoel expansion in the preimplantation mouse embryo: Stimulatory effect of TGF-α and EGF. Development, 113:919-930.
6. Dardik, A., R. M. Smith and R. M. Schultz. 1992. Colocalization of transforming growth factor-α and a functional epidermal growth factor receptor (EGF-R) to the inner cell mass and preferential localization of the EGF-R on the basolateral surface of the trophectoderm in the mouse blastocyst. Dev. Biol., 154:396-409.
7. Eyestone, W. H. and N. L. First. 1989. Co-culture of early cattle embryos to the blastocyst stage with oviductal tissue or in conditioned medium. J. Reprod. Fert., 85: 715-720.
8. Gandolfi, F. 1994. Autocrine, paracrine and environmental factors influencing embryonic development from zygote to blastocyst. Theriogenology, 41:95-100.
9. Goto, K., M. Koba, Y. Takuma, Y. Nakanishi and K. Ogawa. 1989. Co-culture of bovine embryos with cumulus cells. Asian-Australas J. Anim. Sci., 2:595-598.
10. Harvey, M. B., K. J. Leco, M. Y. Arcellana-Panlilio, X. Zhang, D. R. Edwards and G. A. Shultz. 1995. Roles of growth factors during peri-implantation development. Mol. Reprod. Dev., 10:712-718.
11. Kim, E. Y., S. J. Uhm, S. E. Kim, S. H. Yoon, S. P. Park, K. S. Chung and J. H. Lim. 1996a. ICM-trophectoderm cell numbers of bovine IVM/IVF/IVC blastocysts. Kor. J. Anim. Reprod., 20(1):27-34.
12. Kim, E. Y., S. J. Uhm, M. K. Kim, S. H. Yoon, S. P. Park, K. S. Chung and J. H. Lim. 1996b. Effects of epidermal growth factor (EGF) on mouse IVF embryo development and their cell number. Kor. J. Fertil. Steril., 23(3), in press.
13. Nexo, E., M. D. Hollenberg, A. Figueroa and R. M. Pratt. 1980. Detection of epidermal growth factor-urogastrone and its receptor during fetal mouse development. Proc. Natl. Acad. Sci. USA, 77:2782-2786.
14. Paria, B. C. and S. K. Dey. 1990. Preimplantation embryo development *in vitro*: Cooperative interactions among embryos and role of growth factors. Proc. Natl. Acad. Sci. USA., 87:4756-4760.
15. Parrish, J. J., J. Susko-Parrish, M. A. Winer and N. L. First. 1988. Capacitation of bovine sperm by heparin. Biol. Reprod., 38: 1171-1180.

16. Rappolee, D. A., C. A. Brenner, R. Schultz, D. Mark and Z. Werb. 1988. Developmental expression of PDGF, TGF-α and TGF-β genes in preimplantation embryos. Science, 241:1823-1825.
17. Rosenkrans, C. F. Jr., G. Q. Zeng, G. T. McNamara, P. K. Schoff and N. L. First. 1993. Development of bovine embryos *in vitro* as affected by energy substrates. Biol. Reprod., 49:459-462.
18. Sirard, M. A., J. J. Parrish, C. B. Ware, M. L. Leibfried-Rutledge and N. L. First. 1988. The culture of bovine oocytes to obtain developmentally competent embryos. Biol. Reprod., 39:546-552.
19. Watson. A. J., A. Hogan, A. Hahnel, K. E. Wiemer and G. A. Schultz. 1992. Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. Mol. Reprod. Dev., 31:87-95.
20. Werb. Z. 1990. Expression of EGF and TGF-α genes in early development. Mol. Reprod. Dev., 27:10-15.
21. Wiley, L. M., J. X. Wu, I. Harari and E. D. Adamson. 1992. Epidermal growth factor receptor mRNA and protein increase after the four-cell preimplantation stage in murine development. Dev. Biol., 149:247-260434.
22. Wood, S. A. and P. L. Kaye. 1989. Effects of epidermal growth factor on preimplantation mouse embryos. J. Reprod. Fertil., 85:575-582.
23. Yang, B. K., X. Yang and R. H. Foote. 1993. Effect of growth factors on morula and blastocyst development of *in vitro* matured and *in vitro* fertilized bovine oocytes. Theriogenology, 40:521-530.
24. Zhang, X., A. Watson, G. A. Schultz and D. T. Armstrong. 1994. Possible roles of insulin-like growth factors in preimplantation development: investigation of gene expression by RT-PCR. J. Reprod. Fertil., 100:375-382.