大韓不妊學會雜誌: 第24卷 第2號 1997
Kor. J. Fertil. Steril., Vol. 24, No. 2, 1997, 8

In Vivo Development of Mouse IVF/IVC Embryo Treated with Epidermal Growth Factor (EGF)

E.Y. Kim, M.K. Kim, B.K. Yi, H.S. Lee, S.H. Yoon[1], S.P. Park, K.S. Chung[2] and J.H. Lim[1]

Maria Infertility Medical Institute, Maria OB/GYN, Seoul[1], College of Animal Husbandry, Kon-Kuk University[2]

EGF 처리를 받은 체외생산된 생쥐배의 체내 발달

마리아 산부인과 기초의학연구소, 마리아 산부인과[1], 건국대학교 축산대학[2]

김은영 · 김묘경 · 이봉경 · 이현숙 · 윤산현[1] · 박세필 · 정길생[2] · 임진호[1]

= 국문초록 =

본 연구는 EGF가 체외수정 및 체외배양에 의해 생산된 생쥐배의 체내 발달에 미치는 영향을 조사하고자 실시하였다. 본 실험에 사용된 난자는 체외수정 후 얻어진 2-세포기배를 EGF 첨가유무에 따라 배양 (5-6 embryos/25μl/drop)하여 얻어진 4일령의 배반포기배로서, 각 처리군의 배반포기배는 가임신 3일된 대리모의 자궁내에 이식되어졌다. 그 결과를 요약하면 다음과 같다. 1. 2-세포기배를 EGF의 첨가유무에 따라 배양하여 배반포기배로의 발달율과 세포수를 조사하였던 바, 처리군간의 유의한 차이는 나타나지 않았다. 2. 하지만, 각 처리군에서 회수된 배반포기배의 체내 발달을 조사하였던 바, 총 수태율의 결과에 있어서는 대조군과 EGF 처리군 각각 64.4%와 69.8%로서 두 군간에 유의한 차이를 나타내지 않았지만, 정상태아 발생율에 있어서는 EGF 처리군 (51.2%) 이 대조군 (31.1%) 보다 매우 높게 나타냈다. 따라서, 비록 EGF 처리군이 대조군과 비교하여볼 때 체외수정 및 체외배양에 의해 생산된 난자의 유의한 발달은 나타내지 않았지만 난자의 질적인 향상을 통해서 체내발달을 증진시킬 수 있을 것으로 사료된다.

INTRODUCTION

Growth factors are produced by the preimplantation embryo and/or reproductive tract (Zhang *et al.*, 1994; Harvey *et al.*, 1995). Also, control of growth and differentiation during mammalian embryogenesis may be regulated by those growth factors (Rappolee *et al.*, 1988). Addition of physiological levels of growth factors 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 in the cell number in blastocysts, and increase in the percentage of cultured embryos that hatch from the zona pellucida (Heyner *et al.*, 1989; Wood and Kaye, 1989; Harvey and Kaye, 1990; Paria and Dey, 1990). Among the growth factors, EGF is one of the most biologically potent mitogen (Carpenter and Cohen, 1979). In previous study (Kim *et al.*, 1997a), we reported that EGF could stimulate preimplantation mouse embryo development by binding with EGF-receptor (EGF-R) expressed after 4-cell stage and affect to the cell number of inner cell mass (ICM) and trophectoderm (TE) of blastocyst. In addition, we confirmed that

EGF-R was expressed on cell membrane of ICM (Kim *et al.*, 1997b). Thus, there is strong supporting evidence that exogenous EGF and expression of EGF-R in early embryonic stage play important roles in development of the preimplantation mouse embryo. Also, it has known that the role of growth factors continued for the implantation and fetal growth as well as preimplantation embryo development and EGF modulates trophoblast differentiation and function (Hill, 1989).

Therefore, this study was carried out to examine the effect of EGF to the *in vivo* development of mouse embryo which was produced from the EGF culture environment and transferred to the pseudopregnant recipient.

MATERIALS AND METHODS

1. Mouse IVF

The procedures employed in the production of mouse IVF embryos were as outlined by Kim *et al.* (1997a). Oocytes were recovered from four to six week old F1 female mice (C57BL/6 × CBA) which had been superovulated by i.p. injection with 7.5 IU PMSG (Sigma) and followed by i.p. injection of 7.5 IU of hCG (Sigma) 50 hr later. At 13.5 hr post hCG injection, cumulus-oocyte complexes (COCs) were transferred into 50 μl drop of M16 medium supplemented with 0.4% BSA until insemination using epididymal sperm obtained from adult F1 male mice (C57BL/6 × CBA). At 14 hr post hCG injection, insemination was carried out with final concentration of 1×10^6 sperm/ml.

2. Embryo culture and experimental design

Cleaved 2-cell embryos were cultured in M16 medium (5-6 embryos/25μl/drop) with or without EGF for 72 hr. On the basis of previous study (Kim *et al.*, 1997a), 10 ng/ml of EGF was used in the treatment group. Blastocysts recovered from control and EGF treatment group were counted and examined cell number by differential labelling method (Experiment 1) or transferred to the pseudopregnant recipient (Experiment 2).

3. Differential labelling of ICM and TE nuclei

The procedure was used as described by Kim *et al.* (1996). Briefly, zona-removed blastocysts were treated through the following steps; (1) 10 mM trinitrobenzene sulfonic acid in TL-PVP on ice for 10~15 min.; (2) washing sufficiently; (3) 0.1 mg/ml anti-DNP-BSA (ICN Immunobiological.) in TL-PVP at 37℃ for 10 min.; (4) washing completely; (5) 0.01 mg/ml propidium iodide (PI) and 10% (v/v) guinea pig complement (Sigma) in TL-PVP at 37℃ for 20 min.; (6) 0.05 mM bisbenzimide in absolute alcohol at 4℃ for overnight. And then they were washed in absolute alcohol at least for 1 hr, and mounted in glycerol under a coverslip on a slide glass. Labelled nuclei were observed under fluorescence microscope fitted with ultra violet excitation filter and TE nuclei labelled with PI and bisbenzimide appeared pink or red, ICM nuclei labelled with bisbenzimide appeared blue or unlabelled.

4. Embryo transfer

Blastocysts recovered from control and EGF treatment group were transferred surgically one uterine horns (6-8 embryos/horn) of ICR recipient female mice on day 3 of pseudopregnancy. The day on which a copulation plug was found was designed day 1 of pseudopregnancy. All recipients were examined on days 15 of gestation to score the total number of fetuses including the resorption sites.

5. Statistical analysis

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

Table 1. Effect of W/Wo EGF on the development rate of mouse IVF/IVC embryo and their cell number

Treat.*	No. of ≥2-cell	Rate (%) of blastocyst	Cell number of blastocyst at day 4		
			ICM		Total (M ± SEM)
			M ± SEM	Proportion (%)	
Control	81	61 (75.3)	9.9 ± 1.5	26.4	37.5 ± 3.4
EGF	82	65 (79.3)	11.6 ± 1.0	27.8	41.7 ± 2.1

* Control; M16 supplemented 0.4% BSA, EGF; 10 ng/ml of EGF added in M16 + 0.4% BSA.

Table 2. Effect of EGF on *in vivo* development of mouse IVF/IVC embryo

Treat*	No. (%) of embryos transferred	No. of recipient	No. (%) of			
			Preg./Recipi.	Resorp. sites	Normal fetus	Total
Control	51	8	6/8	15 (33.3)	14 (31.1)	29 (64.4)
EGF	49	8	8/8	8 (18.6)	22 (51.2)	30 (69.8)

* Control; M16 supplemented 0.4% BSA, EGF; 10 ng/ml of EGF added in M16 + 0.4% BSA.

RESULTS

To determine the effect of EGF to the *in vivo* development, firstly, we examined the *in vitro* development and cell number of blastocysts produced from the culture of 2-cell embryos in culture environment supplemented w/ wo EGF (10 ng/ml). As shown in Table 1, *in vitro* development to the blastocysts and their cell number of ICM and TE of EGF treatment group showed not significant difference compared with control, although there are increases of development rate and cell number. Secondly, day 4 blastocysts recovered from each treatment group were transferred into the uteri of recipients of pseudopregnant day 3. As indicated in Table 2, production of the normal fetus (51.2%) against transferred embryos in EGF treatment group was very higher than that (31.1%) in control group, although total implantation was not significantly different between treatment group (control: 64.4%, EGF: 69.8%).

DISCUSSION

This study presents that EGF can affect to the *in vivo* development of IVF/IVC embryos. In fact, the transcripts for EGF is not detected even at the blastocyst stage in preimplantation embryos (Nexo *et al.*, 1980; Rappolee *et al.*, 1988; Watson *et al.*, 1992). Nontheless, many researchers reported that EGF stimulates growth and protein synthesis in preimplantation embryo through the EGF-R expressed from the early embryonic stage (Dardik and Schultz, 1991; Wiley *et al.*, 1992). However, the result of this study indicated that the significant additive effect of EGF was not presented in development of 2-cell embryos cultured in group compared with control. This result may be caused by autocrine effect of growth factors secreted by the embryos themselves due to culture of embryos in group (Paria and Dey, 1990). The EGF concentration used in this study was 10 ng/ml as described by Wood and Kaye (1989). In the results, they presented that 10 ng/ml is the maximum concentration of stimulating the protein synthesis of mouse morula. Also, in previous study (Kim *et al.*, 1997a), we demonstrated that EGF-R presents after 4-cell stage, although low level of expression of EGF-R detects in fluorescence level at late 2-cell stage (30 hr after IVF). In the results, we

also indicated that increased pattern of hatching blastocyst was showed in the EGF treatment group, although there is not significantly different. In addtion, we confirmed that the EGF-R was expressed on cell membrane of ICM as well as TE (Kim *et al.*, 1997a,b). Therefore, we concluded that EGF could stimulate the *in vivo* development of mouse embryo through the improving of embryo quality. On the other hand, successful implantation of the blastocyst produced from IVF/IVC culture environment is dependent on the synchrony between the developmental programme of the embryo itself and the complex series of molecular and cellular events such as endocrine-hormone, cytokine, uterine environment, *etc* (Linda, 1994; Harvey *et al.*, 1995). Especially, in respect to the role of EGF/EGF-R in peri-implantation level, it has known that EGF modulates the differentiation of epithelial structures in the fetus and neonate (Hill, 1989). Also, receptors for EGF are abundunt in human placenta, especially on the microvillus plasma membranes in contact with the maternal circulation, and on the basolateral membranes adjacent to the fetal circulation (Roa *et al.*, 1984). This distribution implies an interaction between EGF and the syncytio-trophoblast, and studies with isolated trophoblasts and placental cultures have shown that EGF modulates trophoblast differentiation and function (Hill, 1989). However, in IVF-ET program, firstly, it is important to obtain the healthy embryo. As mentioned earlier, EGF has ability on the improvement of embryo quality *in vitro*, if it is supplemented as a growth factor to the culture medium. Also, as indicated in Table 2, it presented that production of the normal fetus against transferred embryos in EGF treatment group was very higher than that in control group, although total implantation was not significantly different between treatment group. Generally, development to the normal fetus of transferred embryos means the possibility of delivery of live birth. Therefore, we concluded that EGF can affect to the *in vivo* development of IVF/IVC embryos through the improvement of embryo quality.

SUMMARY

The objective of this study was to examine the effect of EGF to the *in vivo* development of mouse IVF/IVC embryo. The 2-cell embryos were cultured in medium (5-6 embryos/ 25 μl/drop) w/wo EGF (10 ng/ml) and day 4 blastocysts recovered from each treatment group were transferred into the uteri of recipients of pseudopregnant day 3. The results obtained in this experiment were summarized as follows: 1. When the effect of EGF to the *in vitro* development and cell number of blastocysts produced from the culture of 2-cell embryos in w/wo EGF was determined, those results of EGF treatment group showed not significant difference compared with control. 2. However, when the effect of EGF to the *in vivo* development of blastocysts recovered from each treatment group was examined, production of the normal fetus against transferred embryos in EGF treatment group (51.2%) was very higher than that in control group (31.1%), although total implantation was not significantly different between treatment group (control: 64.4%, EGF: 69.8%). Therefore, this result suggested that EGF can affect to the *in vivo* development of IVF/IVC embryos through the improvement of embryo quality, although EGF treated embryos showed not significant development rate compared with control.

REFERENCES

Carpenter G, Cohen S: Epidermal growth factor. *Ann Rev Biochem* 1979, 48, 193-216.

Dardik A, Schultz RM: Blastocoel expansion in the preimplantation mouse embryo: Stimulatory effect of TGF-α and EGF. *De-*

velopment 1991, 113, 919-930.

Harvey MB, Leco KJ, Arcellana-Panlilio MY, Zhang X, Edwards DR, Shultz GA: Roles of growth factors during periimplantation development. *Mol Reprod Dev* 1995, 10, 712-718.

Harvey MB, Kaye PL: Insulin increases the cell number of the inner cell mass and stimulates morphological development of mouse blastocysts *in vitro*. *Development* 1990, 110, 963-967.

Heyner SL, Rao V, Jarett L, Smith RM: Preimplantation mouse embryos internalize maternal insulin via receptor-mediated endocytosis: Pattern uptake and functional correlations. *Dev Biol* 1989, 134, 48-58.

Hill DJ: Growth factors and their cellular actions. *J Reprod Fertil* 1989, 85, 723-734.

Kim EY, Kim SE, Uhm SJ, Yoon SH, Park SP, Chung KS, Lim JH: ICM-trophectoderm cell numbers of mouse IVF/IVC blastocyst Kor J Fertil Steril 1996, 23(1), 25-32.

Kim EY, Uhm SJ, Kim MK, Yoon SH, Park SP, Chung KS, Lim JH: Study on epidermal growth factor (EGF) and expression of EGF-receptor (EGF-R) in mouse IVF/IVC embryo; I) Additive effects of EGF and expression of EGF-R on mouse IVF embryo development. *Kor J Fertil Steril* 1997a, 24(3), 13-20.

Kim EY, Kim MK, Yoon SH, Park SP, Chung KS, Lim JH: Study on epidermal growth factor (EGF) and expression of EGF-receptor (EGF-R) in mouse IVF/IVC embryo; II) Expression of EGF-R on the inner cell mass (ICM) of mouse IVF/IVC blastocyst. *Kor J Fertil Steril* 1997b, 24(3), 21-26.

Linda CG: Growth factors and growth modulators in human uterine endometrium: their potential relevance to reproductive medicine. *Fertility and sterility* 1994, 61(1), 1-17.

Nexo E, Hollenberg MD, Figueroa A, Pratt RM: Detection of epidermal growth factor-urogastrone and its receptor during fetal mouse development. *Proc Natl Acad Sci USA* 1980, 77, 2782-2786.

Paria BC, Dey SK: Preimplantation embryo development *in vitro*: Cooperative interactions among embryos and role of growth factors. *Proc Natl Acad Sci USA* 1990, 87, 4756-4760.

Rappolee DA, Brenner CA, Schultz R, Mark D, Werb Z: Developmental expression of PDGF, TGF-α and TGF-β genes in preimplantation embryos. *Science* 1988, 241, 1823-1825.

Roa CV, Carman FR, Ghegini N, Schults GS: Binding sites for epidermal growth factor in human fetal development. *J Clin Endocr Metab* 1984, 58, 1034-1042.

Watson AJ, Hogan A, Hahnel A, Wiemer KE, Schultz GA: Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. *Mol Reprod Dev* 1992, 31, 87-95.

Wiley LM, Wu JX, Harari I, Adamson ED: Epidermal growth factor receptor mRNA and protein increase after the four-cell preimplantation stage in murine development. *Dev Biol* 1992, 149, 247-260.

Wood SA, Kaye PL: Effects of epidermal growth factor on preimplantation mouse embryos. *J Reprod Fertil* 1989, 85, 575-582.

Zhang X, Watson A, Schultz GA, Armstrong DT: Possible roles of insulin-like growth factors in preimplantation development: Investigation of gene expression by RT-PCR. *J Reprod Fertil* 1994, 100, 375-382.