韓國家畜繁殖學會誌 27(2) : 169~177 (2003)
Korean J. Animal Reprod.

Apoptosis and Apoptosis Related Gene Expression in Preimplantation Porcine Diploid Parthenotes Developing *In Vitro*

Cui, X. S., I. H. Kim and N. H. Kim†

Department of Animal Sciences, Chungbuk National University, Research Center for Transgenic Cloned Pigs

착상전 이배체 단위발생 돼지난자의 체외 배양에서 세포사멸과 세포사멸에 관여하는 유전자의 발현에 관한 연구

최향순 · 김일화 · 김남형†

충북대학교 축산학과 / 형질전환복제돼지 연구센터

ABSTRACT

This study was conducted to determine effects of polyvinyl alcohol (PVA), fetal bovine serum (FBS) bovine serum albumin (BSA) and epidermal growth factor (EGF) on blastocoel formation, total cell number, apoptosis and apoptosis-related gene expression of porcine diploid parthenotes developing *in vitro*. The addition of 0.4% BSA to the culture medium enhanced the development of 2-cell stage parthenotes to the blastocysts stage ($P<0.01$). FBS reduced cell numbers of blastocysts ($P<0.01$) and increased percentage of apoptosis in the blastocysts ($P<0.001$). However, while BSA increased cell numbers, it did so only when EGF was present. Either agent on its own had no effect. Similarly, apoptosis in the blastocysts was not influenced by either agent on its own but was reduced when both BSA and EGF were present. Furthermore, semi-quantitative reverse-transcriptase polymerase chain reaction revealed that EGF enhanced the mRNA expression of Bcl-xL in the presence of 0.4% BSA but BSA and EGF alone had no effect, and EGF and/or BSA did not influence Bak gene expression in the blastocyst stage parthenotes. However FBS reduced Bcl-xL mRNA expression ($P<0.05$) and enhanced Bak expression. This result suggests that apoptosis related genes expression is significantly affected by supplements, which may result in alteration of apoptosis and embryo viability of porcine embryos developing *in vitro*.

(Key words : Porcine embryo, BSA, FBS, EGF, Apoptosis, Blastocyst)

Ⅰ. INTRODUCTION

Systems to culture mammalian embryos *in vitro* have been developed to elucidate early embryonic development and to produce viable embryos. The basic culture system used to develop the pre-implantation mammalian embryo employs bovine serum albumin (BSA) as a common constituent of

* 본 연구는 한국과학재단 우수연구센터(R11-2002-100-02002-0) 지원으로 수행되었음.

† Correspinding author : Nam-Hyung Kim, Ph.D. Department of Animal Sciences, Chungbuk National University, Gaesin-dong, Cheongju, Chungbuk. 361-763, South Korea, TEL: 043-261-2546, Fax: 043-272-8853, E-mail : nhkim@chungbuk.ac.kr

the culture medium. BSA seems to provide beneficial factors such as energy substrates or scavenger ions and small molecules (Bavister et al., 1983; Maurer et al., 1992). However, medium-containing BSA is still poorly defined, and it contains many unknown proteins and growth factors which may affect directly or indirectly embryo development.

Preimplantation embryos express a variety of growth factor receptors. These include epidermal growth factor (EGF) receptors, which are expressed during the preimplantation stage by murine (Wiley et al., 1992), porcine (Wei et al., 2001) and human (Chia et al., 1995) embryos. Due to the expression of such receptors, many aspects of embryo development can be modulated by the presence of exogenous growth factors in the culture medium. In particular, exogenous EGF enhances the developmental rate and mitogenesis of preimplantation murine and bovine embryos (Paria and Dey, 1990; Lee and Fukui, 1995; Adamson, 1993; Yang et al., 1993).

Programmed cell death or apoptosis is crucially involved in development and differentiation. Environmental stresses such as those imposed by *in vitro* culturing can induce unscheduled apoptosis in cultured embryos, which may lead to arrest or abnormal development and lower viability of embryos (Hardy et al., 1989; Jurisicava et al., 1998; Byrne et al., 1999). Cory and Adams (Cory and Adams, 1998) reported, members of the Bcl-2 gene family play key roles in regulating apoptosis, and at least 15 mammalian Bcl-2 gene family members have been identified and categorized into two subgroups, anti-apoptotic (Bcl-2, Bcl-w, Bcl- xL, A1, Mcl-1) and pro-apoptotic (Bax, Bak, Box, Bik, Blk, Hrk, BNIP3, Bim, Bad, Bid, Bcl-xS). Effects of different culture systems on mRNA expression pattern of apoptosis-related genes have been determined in human (Jurisicova et al., 2003), mouse (Jurisicova et al., 1998; Warner et al., 1998) and bovine (Cory and Adams, 1998) embryos, but not in porcine embryos.

It is difficult to obtain pig embryos of homogeneous quality due to the relatively high incidence of polyspermy during *in vitro* fertilization. Therefore, diploid parthenotes have frequently been used to study early development in the pig (Van et al., 2002). In the present study we determined effects of PVA, FBS, BSA and EGF on the developmental ability and apoptosis, of porcine 2-cell (30 h after activation) developing *in vitro*. We additionally determined relative amounts of gene expression of Bcl-2 and Bak in porcine parthenotes developed from same culture conditions using sensitive semi-quantitative Reverse transcriptase Polymerase Chain Reaction.

Ⅱ. MATERIALS AND METHODS

1. *In vitro* Porcine Oocyte Maturation and Parthenogenesis Activation

Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25 C in Dulbeccos phosphate-buffered saline (PBS) supplemented with 5.54 mM D- glucose, 0.33 mM sodium pyruvate, 75 g/ml potassium penicillin G and 50 g/ml streptomycin sulphate (mDPBS). Cumulus-oocyte complexes (COC) were aspirated from follicles 3 to 6 mm in diameter with an 18-gauge needle into a disposable 10 ml syringe. The COC were washed 3 times with Hepes-buffered Tyrodes medium containing 0.1% (w/v) polyvinyl alcohol (Hepes-TL-PVA). Each group of 50 COC was matured in 500 l tissue culture medium (TCM)-199 supplemented with 0.57 mM cysteine (Sigma, St Louis, MO), 10 ng/ml epidermal growth factor (EGF, Sigma), 10 IU/ml PMSG (Sigma) and 10 IU/ml hCG (Sigma) under paraffin oil at 39℃ for 44 h. Following maturation, cumulus cells were removed by pipetting in the presence of

1 mg/ml hyaluronidase for 2～3 min. For parthenogenetic activation, oocytes were activated by 2 direct pulses of 140 V/mm for 50 s in 0.28 mol/L mannitol supplemented with 0.1 mmol/L $MgSO_4$, and 0.05 mmol/L $CaCl_2$. After 3 h of culture in North Carolina State University (NCSU) 23 medium containing 7.5 g/ml cytochalasin B (CB, Sigma), embryos were washed three times in NCSU 23 medium with 0.4 % (w/v) BSA and cultured in the same medium for 24h at 39℃ in an atmosphere of 5% CO_2 and 95% air.

2. Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Assay

Fragmented blastocysts were washed three times in PBS (pH 7.4) containing polyvinylpyrolidone (PVP, 1 mg/ml). This was followed by fixation in 3.7% paraformaldehyde in PBS for 1 h at room temperature (RT). After fixation, the embryos were washed in PBS/PVP and permeabilized by incubation in 0.3% Triton X-100 for 1 h at RT. The embryos were then washed twice in PBS/PVP and incubated with fluorescein-conjugated dUTP and the terminal deoxynucleotidyl transferase enzyme (Roche, USA) in the dark for 1 h at 37É. After being counterstained with 50 g/ml RNase A in 40 g/ml propidium iodide (PI) for 1 h at 37 E to label all nuclei, embryos were washed in PBS/ PVP, mounted with slight coverslip compression, and examined under an Olympus fluorescence microscope.

3. Semi-Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Embryos were cultured (from 30 h) in vitro and harvested at the blastocyst stage on day 7 or produced *in vivo*. Single embryos were washed in Ca^{2+} – and Mg^{2+} – free PBS, snap frozen in liquid nitrogen, and stored at 70℃. Messenger RNA was extracted using the Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway) according to the manufacturers instructions. In all experiments, â-actin was used as an internal standard. First, standard cDNA synthesis was achieved by reverse transcription of the RNA by using the random hexamers and the Superscript reverse transcriptase enzyme (Invitrogen Co., Grand Island, NY). The mRNAs of Bcl-xL, Bak and â-actin species were then detected by RT-PCR with specific primer pairs (Table 1) using reagents supplied in a Taq DNA polymerase kit (Takara Korea Biomedical Inc., Seoul, Korea). The PCR products were visualized under ultraviolet light on 1.5% agarose (Invitrogen Co., Grand Island,

Table 1. Primer sequences and cycling conditions used in RT-PCR

Genes	Genbank accession No.	Primer sequence	Position in sequence	Cycle number/ /Annealing temperature	Product size (base pairs)
β-actin	X04751	5′GCAGCCACGGTGGCGAGTAT	241～260	35/55℃	257
		3′GTGGGACAGGAGCTTGAAAT	555～657		
Bcl-xL	AF216205	5′GGAGCTGGTGGTTGACTTTC	30～ 49	35/55℃	518
		3′CTAGGTGGTCATTCAGGTAAGG	527～547		
Bak	AJ001204	5′CTAGAACCTAGCAGCACCAT	46～ 65	35/55℃	151
		3′CGATCTTGGTGAAGTACTC	178～196		

NY) gels in 1 × TAE buffer containing 1 g/ml ethidium bromide (Sigma). The intensity of each band was assessed by densitometry using an image analysis program (LabWorks; UVP Inc., Upland, CA). The relative amount of each mRNA species was calculated by dividing the intensity of the bands by the intensity of the corresponding â-actin band.

4. Experimental Design and Embryo Culture

To obtain embryos of homogenous quality, good quality 2-cell stage parthenotes were selected 24 h after activation. The embryos were randomly allocated into experimental groups.

Experiment 1 examined the effect of different protein supplements on porcine parthenotes development. Diploid parthenotes were recovered after 24 h of culture in NCSU 23 medium containing 0.4% BSA, presumptive 2-cell stage (30 h) embryos were collected and washed three times in NCSU 23 medium without (control) or with 0.1% PVA (w/v, P-1763, Sigma), 10% FBS (v/v, 16140-071, Gibco), 0.4% BSA (w/v, A-8806, Sigma), 0.1% PVA added 10 ng/ml murine EGF (Sigma, St. Louis, USA) or 0.4% BSA added 10 ng/ml murine EGF and then randomly cultured in the same medium containing none (control), 0.1% PVA, 10% FBS, 0.4% BSA, 0.1% PVA+10 ng/ml EGF or 0.4% BSA+10 ng/ml EGF. The embryos were cultured for 7 days at 39℃ and 5 % CO_2 in air. On day 7, development to blastocysts were recorded.

Experiment 2 was conducted to determine the effect of protein supplements on the apoptosis and total cell numbers in the blastocysts. *In vitro* cultured embryos obtained as described in experiment 1 were harvested at the blastocyst stage on day 7 and then used in the TUNEL assay of apoptosis. Total nuclei numbers in these embryos were also counted.

Experiment 3 evaluated the effect of protein supplements on Bcl-xL and Bak gene expression in the porcine parthenotes and *in vivo* produced embryos. Presumptive diploid parthenotes obtained as described in experiment 1 were harvested at the blastocyst stage on day 7, washed in PBS and stored at 70 C until RT-PCR analysis.

5. Statistical Analysis

The scores in stage of blastocyst, mean cell number of blastomeres, percentage of apoptosis and relative abundance of gene expression were subjected to analysis of variance using the general linear model (PROC-GLM) in SAS program (Anon, 1992). When the significance of the main effectswas detected in each experimental parameter, the treatment effects were compared by the least square method. Differences of $P<0.05$ were considered significant.

Ⅲ. RESULTS

1. Effect of Different Supplements on Parthenotes Development (Experiment 1)

More 2-cell embryos develop into morulae and blastocyst at day 7 when BSA was present than its absent ($P<0.01$, Table 2). However, when absence of BSA in the medium, FBS, PVA and PVA+EGF did not significantly increase development of porcine diploid parthenotes.

2. Effect of Different Supplements on Cell Numbers and Apoptosis (Experiment 2)

The results of experiment 2 are presented in Table 3. Mean cell number of blastocysts resulting from control, PVA, PVA+EGF and BSA groups did not differ from 2-cell parthenotes cultured. However, in the presence of FBS, total cell number significantly decreased at the day 7 ($P<0.01$), and EGF enhanced total cell numbers at the blastocysts stage in the NCSU 23 medium complements BSA ($P<0.05$).

Table 2. Developmental ability of porcine parthenotes after 7 days cultured in the NCSU 23 medium added different supplements

Supplement To medium	No. of embryos examined (r)	Percentage of embryos developed to	
		Morulae	Blastocysts
Control(none)	350 (5)	28.0±2.1[b]	35.9±2.4[b]
FBS	352 (5)	27.6±2.3[b]	35.5±3.1[b]
PVA	356 (5)	28.2±2.0[b]	39.3±2.4[b]
PVA+EGF	351 (5)	28.7±2.8[b]	40.9±3.4[b]
BSA	359 (5)	48.5±2.1[a]	60.6±2.4[a]
BSA+EGF	355 (5)	47.8±2.4[a]	61.6±3.1[a]

Table 3. Number of cells per blastocyst and apoptosis at day 7 that cultured in the NCSU 23 medium added different supplements

Supplement To medium	No. of embryos examined (r)	Cell numbers	Percentage of apoptosis
Control(none)	68 (4)	39.8±3.6[b]	4.7±0.7[b]
FBS	64 (4)	24.9±2.3[c]	7.3±0.4[a]
PVA	65 (4)	40.6±2.9[b]	4.5±0.5[b]
PVA+EGF	64 (4)	40.2±2.1[b]	4.4±0.7[b]
BSA	68 (4)	46.1±2.6[b]	4.7±0.6[b]
BSA+EGF	66 (4)	61.8±2.1[a]	2.1±0.6[c]

Table 4. Relative abundance of mRNA expression in porcine parthenotes at day 7 that cultured in the NCSU 23 medium added different supplements

Supplement To medium	No. of embryos examined (r)	Genes	
		Bcl-xL	Bak
Control(none)	8 (8)	0.24±0.02[b]	0.14±0.01[b]
FBS	8 (8)	0.18±0.02[c]	0.34±0.02[a]
PVA	8 (8)	0.24±0.03[b]	0.17±0.02[b]
PVA+EGF	8 (8)	0.25±0.02[b]	0.16±0.03[b]
BSA	8 (8)	0.26±0.03[b]	0.16±0.02[b]
BSA+EGF	8 (8)	0.44±0.04[a]	0.17±0.02[b]

The DNA fragments resulting from the apoptotic nicking of genomic DNA in individual embryos were measured by the TUNEL assay. There were no difference in apoptosis (fragmented cell number/total cell number) of blastocysts in the control and PVA, PVA+EGF or BSA-derived embryos.

However addition of FBS increased percentage of apoptosis ($P<0.05$), but in the presence of BSA, EGF significantly reduced the degree of apoptosis in the blastocysts ($P<0.001$).

3. Effect of Different Supplements on Apoptotic Gene Expression (Experiment 3)

To investigate whether different supplements modulate mRNA expression of apoptotic-related genes in diploid porcine parthenotes developing *in vitro*, mRNA was prepared from single blastocysts cultured in the NCSU 23 added different supplement was subjected to RT-PCR for Bcl-xL and Bak transcripts (Table 4). In the PVA−, PVA+EGF and BSA-supplemented medium, the relative abundance of Bcl-xL mRNA expression similar to control-derived blastocysts and higher than FBS-derived embryos ($P<0.05$). However in the presence of BSA, EGF enhanced relative abundance of Bcl-xL mRNA expression ($P<0.01$).

There was no difference in Bak mRNA expression in control, PVA, PVA+EGF, BSA and BSA+EGF derived embryos, but significantly enhanced in FBS-derived embryos ($P<0.005$).

Ⅳ. DISCUSSION

In the present study we demonstrated the effects of exogenous protein supplements on porcine blastocyst production. The experiments were performed with embryos of homogenous quality by selecting good quality porcine diploid parthenotes at the 2-cell stage. We found that porcine presumptive diploid parthenotes at 30h after activation developed to the blastocyst stage at a relatively high rate in the presence of BSA as compared to those in the presence of either PVA or none-supplement control. However, BSA did not increase the cell numbers of blastocysts. We found that BSA increased the cell numbers of the cultured blastocysts but that this effect was manifested only when EGF was present. EGF on its own had no effect. That the effect of EGF is observed only when BSA is present suggests a synergic effect between EGF and BSA on the cell numbers of the cultured blastocysts. EGF has been shown to stimulate both the cellular proliferation and differentiation of various somatic cells (Carpenter and Cohen, 1979; Fisher and Lakshmanan, 1990). In the mouse, EGF receptor mRNA and protein levels are increased after the 4-cell stage (Wiley et al., 1992). Similarly, porcine embryos express the EGF receptor mRNA in the morula and blastocyst stages (Wei et al., 2001). Thus, exogenous EGF may act as a mitogen during early embryo development by working through its receptor. Our study showed EGF decreased apoptosis, but only in combination with BSA. EGF or BSA on their own had no effect on apoptosis. These observations are in line with the effect of BSA and EGF on cell numbers, as blastocyst cell numbers are the end product of a mix of cell proliferation and cell death processes and reflect which process dominates.

In the present study FBS decreased cell numbers of blastocysts cultured from 24h. The ratio of TUNEL-labeled DNA to total DNA area of FBS-derived blastocysts was greater than in its absence medium-derived counterparts (7% vs. 4%). This result is similar to the previous results in cattle. Exposure of early cattle embryos (2 to 8 cell) to FBS significantly reduced the formation of blastocoel, blastocysts cell numbers (Lawitts and Biggers, 1991; Schults et al., 1981; Eckert and Niemann, 1995; Hagemann et al., 1998). Byme et al. (1999) also reported that a higher apoptotic cell death index was one consequence of culturing 2-cell embryos to blastocysts with serum (10% v/v) instead of albumin (0.8% BSA). Collectively, undefined factors in the serum induce apoptosis in early cleavage mammalian embryos, which result in the

lower incidence of blastocysts.

Supporting the idea that EGF reduces apoptosis and FBS enhances it are our observations on the expression of two apoptosis-related genes during blastocyst development *in vitro*. Supporting the idea that EGF reduces apoptosis induced by BSA factors are our observations on the expression of two apoptosis-related genes during blastocyst development *in vitro*. Entry and progression in the apoptotic pathway seem to be achieved by the balance of the expression of several conserved genes that have either a pro- or anti-apoptotic effect. The Bcl gene family is known to include anti-apoptotic genes that protect a variety of cell types against apoptotic cell death. In contrast, another group of highly conserved genes are positive regulators of apoptosis. These include the Bak proteins in the pig. In the present study, we found that EGF together with BSA enhanced Bcl gene expression, while EGF and BSA on their own had no such effect. We also found FBS decreased Bcl-xL gene expression in blastocysts developing *in vitro*. This observation may reflect the ability of FBS to reduce the apoptotic process in pig blastocysts. Bcl-xL is a very potent cell death suppressor, in embryos that are destined to develop, increases in expression of cell death inducers genes would be inhibited cell protector expression (e.g., Bcl-xL) would be increased, augmenting maternal stores (e.g., Bcl-xL, Jurisicova et al., 1998). Pro-apoptotic gene Bak was higher in blastocysts produced in the presence of FBS than produced in its absence embryos, which would be consistent with the notion that mRNA levels for this transcript are higher in bad-quality embryos.

In conclusion, our data indicate that conditions of supplementing PVA, FBS, BSA or EGF in culture medium, affect on developing, cell numbers and apoptosis. BSA enhanced *in vitro* development of porcine diploid parthenotes, while FBS reduce embryo viability. However, addition of EGF to the culture medium in the presence of BSA reduce apoptosis and increase cell number of porcine presumptive diploid parthenotes. The effect of EGF and FBS are probably mediated by their ability to regulate Bcl-xL and Bak genes expression, which affect to increase or decrease cell numbers.

Ⅴ. 요 약

본 연구는 착상전 이배체 단위발생 돼지난자를 체외 배양시 우태아혈청 (FBS), 우혈청 알부민 (BSA) 및 상피세포성장인자 (EGF)를 배양액에 첨가하였을 때 배반포, 총 세포수, 세포사멸 및 세포사멸에 관여하는 유전자의 발현을 조사하고자 수행하였다. 0.4% BSA를 배양액에 첨가하였을 때 2세포기 단위발생 난자의 배반포까지의 발달율이 증가되었다($P<0.01$). FBS는 배반포의 총세포수를 감소시켰고 세포사멸을 증가하였다($P<0.01$). 그리고 EGF는 BSA가 존재하는 조건하에서 배반포의 총세포수를 증가하였는데 EGF와 BSA가 각각 단독으로 존재할 때는 이런 작용이 없었다. 세포사멸도 이와 이슷한 경향을 보였는데 EGF와 BSA가 각각 존재할 때에는 비처리군과 차이가 없었지만 함께 존재할 때에는 세포사멸을 감소시켰다. RT-PCR의 결과에 의하면 EGF는 BSA가 존재하는 배양액에서 Bcl-xL 유전자의 상대적 발현량을 증가시키고 Bak 유전자의 상대적 발현량에는 영향을 주지 않는 과정을 통하여 세포사멸을 감소시키는 것 같다. 반면에 FBS는 Bcl-xL의 발현량을 감소시키고 Bak 유전자의 상대적 발현량을 증가시킨다. 이러한 결과는 세포사멸에 관여하는 유전자의 발현은 배양액의 첨가물에 따라 유의적으로 영향을 받으며, 체외배양시 배아의 초기발달에 관여함을 시사한다.

Ⅵ. REFERENCES

1. Adamson, E. D. 1993. Activities of growth factors in preimplantation embryos. J. Cell Bio-

Chem. 53:280-287.
2. Anon. SAS Users Guide. Statistics. Cary, NC: Statistical Analysis System Institute 1992.
3. Bavister, B. D., Leibfried, M. L. and Lieberman, G. 1983. Development of preimplantation embryos of the golden hamster in a defined culture medium. Biol. Reprod. 28:235-247.
4. Byrne, A. T., Southgate, J., Brison, D. R. and Leese, H. J. 1999. Analysis of poptosis in the pre-implantation bovine embryo using TUNEL. J. Reprod. Fertil. 117:97- 105.
5. Carpenter, G. and Cohen, S. 1979. Epidermal growth factor. Annu. Rev. Biochem. 48:193-216.
6. Chia, C. M., Winston, R. M. L. and Handyside, A. H. 1995. EGF, TGFa, and EGFR expression in human preimplantation embryos. Development. 121:299-307.
7. Cory, S. and Adams, J. M. 1998. Matters of life and death: programmed cell death at cold spring harbor. Biochim. Biophys. Acta. 1377:R25-44.
8. Eckert, J. and Niemann, H. 1995. *In vitro* maturation, fertilization and culture to blastocysts of bovine oocytes in protein-free media. Theriogenology. 43:1211-1225.
9. Fisher, D. A. and Lakshmanan, J. 1990. Metabolism and effects of epidermal growth factor and related growth factors in mammals. Endocr. Rev. 11:418-442.
10. Hagemann, L. J., Weilert, L L., Beaumont, S. E. and Tervit, H. R. 1998. Development of bovine embryos in single *in vitro* production (sIVP) systems. Mol. Reprod. Dev. 51:143-147.
11. Hardy, K., Handyside, A. H. and Winston, R. M. L. 1989. The human blastocyst, cell number, death and allocation during later preimplantation development. Development. 107: 597-604.
12. Jurisicova, A., Antenos, M., Varmuza, S. and Tilly, J. L. 2003. Casper RF. Expression of poptosis-related genes during human preimplantation embryo development: potential roles for the harakiri gene product and caspase-3 in blastomere fragmentation. Molecular. Human. Reproduction. 9:133-141.
13. Jurisicova, A., Latham, K. E., Casper, R. F. and Varmuza, S. L. 1998. Expression and regulation of genes associated with cell death during murine pre-implantation embryo development. Mol. Reprod. Dev. 51:243-253.
14. Lawitts, J. A. and Biggers, J. D. 1991. Overcoming the 2-cell block by modifying components in a mouse embryo culture medium. Biol. Reprod. 45:245-251.
15. Lee, E. S. and Fukui, Y. 1995. Effect of various growth factors in a defined culture medium on vitro development of bovine embryos matured and fertilized *in vitro*. Theriogenology. 44:71-83.
16. Maurer, H. R. 1992. Towards serum-free, chemically defined media for mammalian cell culture. In: Freshney RI (ed.), Animal Cell Culture: A Practical Approach. Oxford: Oxford University:15-46.
17. Paria, B. C. and Dey, S. K. 1990. Preimplantation embryo development *in vitro*: Cooperative interactions among embryos and role of growth factors. Proc. Natl. Acad. Sci. U. S. A. 87:4756-4760.
18. Schults, G. A., Kaye, P. L., McKay, D. J. and Johnson, M. H. 1981. Endogenous amino acid pool sizes in mouse eggs and preimplantation embryos. J. Reprod. Fertil. 61:387-393.
19. Van, T. N., Harayama, H. and Miyake, M. 2002. Characteristics of preimplantational development of porcine parthenogenetic diploids relative to the existence of amino acids *in vitro*. Biol. Reprod. 67:1688-1698.
20. Warner, C. M., Exley, G. E., Mcelhinny, A. S. and Tang, C. 1998. Genetic regulation of preimplantation mouse embryo survival. The Jour-

nal of Experimental Zoology. 282:272-9.

21. Wei, Z., Park, K. W., Day, B. N. and Prather, R. S. 2001. Effect of epidermal growth factor on preimplantation development and its receptor expression in porcine embryos. Mol. Reprod. Dev. 60:457-462.
22. Wiley, L. M., Wu, J. X., Harari, I. and Adamson, E. D. 1992. Epidermal growth factor receptor mRNA and protein increase after four-cell preimplantation stage in murine development. Dev. Biol. 149:247-260.
23. Yang, B. K., Yang, X. and Foot, R. H. 1993. Effect of growth factors on morula and blastocyst development of *in vitro* matured and *in vitro* fertilized bovine oocytes. Theriogenology. 40:521-530.

(접수일자: 2003. 5. 7. / 채택일자: 2003. 5. 20.)