Effect of Epidermal Growth Factor (EGF) on Meiotic Maturation and Pronuclear Formation of Porcine Oocytes Produced *In Vitro*

Song, S. H.[1], J. G. Kim, H. J. Song, B. Mohana Kumar, S. R. Cho[2], C. Y. Choe[2], S. H. Choi, G. J. Rho[†] and S. Y. Choe

Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, Chinju

ABSTRACT

The objective of this study was to examine the effect of EGF on meiotic maturation and pronuclear (PN) formation of porcine oocytes. Prepubertal gilt cumulus-oocyte-complexes (COCs) aspirated from 2~6 mm follicles of abbatoir ovaries were matured in TCM199 containing 0.1 mg/ml cysteine, 0.5 ug/ml FSH and LH, and EGF (0, 5, 10, 20, 40 ng/ml) for 22 hr at 39°C in a humidified atmosphere of 5% CO_2 in air. They were then cultured for an additional 22 hr without hormones. In Experiment 1, to examine the nuclear maturation at 44 hr of culture, the expanded cumulus cells were removed by vortexing for 1 min in 3 mg/ml hyaluronidase. The oocytes were fixed in acetic acid : methanol (1:3, v/v) at least for 48 hr and stained with 1% orcein solution for 5 min. Nuclear status was classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), prophase-metaphase I (PI-MI), and PII-MII under microscope. In Experiment 2, to investigate PN formation, oocytes were fertilized with Percoll-treated freshly ejaculated sperm (1 × 10^5 cells/ml) in mTBM with 0.3% BSA and 2 mM caffeine for 5 hr, and cultured in NCSU-23 medium with 0.4% BSA. At 6 hr of culture, the embryos were fixed in 3.7% formaldehyde for 48 hr and stained with 10 ug/ml propidium iodide for 30 min. PN status was classified as no or one PN (unfertilized), 2 PN (normal fertilized) and ≥ 3 PN (polyspermy). Differences between groups were analyzed using one-way ANOVA after arc-sine transformation of the proportional data. The rate of oocytes that had reached to PII-MII were significantly (*P*<0.05) higher in all groups added EGF than that of non-treated group (67%), but it did not differ among the all added groups (86%, 85%, 79% and 81%, in 5, 10, 20 and 40 ng/ml EGF, respectively). No differences on the incidence of 2PN were observed in all treated groups (25%, 30%, 33%, 29% and 29%, in 0, 5, 10, 20 and 40 ng/ml EGF, respectively), however, in non-treated group, polyspermy tended to be increased (66% vs. 58%, 54%, 52% and 55%, 0 vs. 5, 10, 20, 40 ng/ml EGF, respectively). These results suggest that EGF can be effectively used as an additive for enhancing oocyte maturation and reducing the incidence of polyspermy in pig.

(Key words: Pig oocytes, *In vitro* maturation, Pronuclear formation, EGF)

INTRODUCTION

The development of efficient reproductive technologies in pigs has been increasingly required, as pigs have become important in the field of biomedical research and transgenics. Despite recent progress in the *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC), the rates of blastocyst formation and the birth of piglets are still low (Prather et al., 2003; Lee et al., 2005). The conditions for IVM of oocytes and IVC of embryos significantly affect the quality and it is further complicated by the relatively high incidence of polyspermy during IVF (Wang et al., 1999; Cui and Kim, 2003). However, many attempts have been successful in improving the developmental competence of IVM and IVF embryos to the blastocyst stage by maturing pig oocytes in the presence of energy substrates (Lee et al., 2003; Kim et al., 2004) or growth factors (Abeydeera et al., 1998a; Abeydeera et al., 2000; Wei et al., 2001; Cui and Kim, 2003), or in different culture media (Ding and Foxcroft, 1994a; Funahashi et al., 1997). Furthermore, piglets have been obtained by transfer of embryos derived after IVF of oocytes matured in the presence of follicular cells (Abey-

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[1] Department of Pet Care, Changwon College, Changwon.

[2] National Live Stock Research (RDA), Namwon.

[†] Corresponding author : Gyu-Jin Rho, DVM, PhD, Associate Professor, Department of Obstetrics and Theriogenology, College of Veterinary Medicine, Gyeongsang National University, 900 Gazwa, Chinju, 660-701, Phone: 82-55-751-5824, Fax: 82-55-751-5803, E-mail: jinrho@gsnu.ac.kr

deera et al., 1998b).

Expression of variety of growth factor receptors in preimplantation embryos has shown that, many aspects of embryo development can be modulated by the presence of exogenous growth factors in the culture medium (Cui and Kim, 2003). It has been reported that, epidermal growth factor (EGF) has an important effect during preimplantation embryo development in mammals (Wei et al., 2001). It has also been suggested that, the EGF system is related to early embryo development in an autocrine and/or paracrine manner (Paria et al., 2001). Furthermore, the presence of mRNA for EGF and its receptor (EGFr) in the oocyte, cumulus and granulosa cells has indicated EGF synthesis by these tissues. Thus, in pigs EGF alone can stimulate nuclear maturation and can interact with gonadotropins to enhance cytoplasmic maturation (Ding and Foxcroft, 1994b). It is envisaged that, supplementation of EGF during IVM may improve the cytoplasmic maturation of pig oocytes with a higher developmental competence.

In the study reported here, we examined the effect of EGF supplementation during IVM on meiotic maturation and pronuclear (PN) formation of porcine oocytes.

MATERIALS AND METHODS

Culture Media

Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, MO). The basic medium used for oocyte maturation was TCM-199(Gibco BRL) with 0.1mg/ml cysteine as washing solution. The media for IVM drop were modified TCM-199 containing 0.5 ug/ml FSH and LH, and EGF (0, 5, 10, 20, 40 ng/ml, seperately) were cultured for 22 hr in humidified atmosphere of 5% CO_2 in air. The basic medium used for IVF was essentially as used by Abeydeera and Day (1997) with a minor modification. This medium, designated as modified Tris-buffered medium (mTBM), consists of 113.1mM NaCl, 3mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris (cystallized free base; Fisher Scientific, Fair Lawn, NJ), 11 mM glucose, 5 mM sodium pyruvate, 0.3% bovine serum albumin (BSA, A-7888), and 2 mM caffeine. The embryo culture medium for embryo development was North Carolina State University (NCSU) 23 medium containing 0.4% BSA (A-8022).

Oocyte Collection and *In Vitro* Maturation

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 1hr in phosphate buffered saline (PBS) containing 75 ug/ml potassium penicillin G and 50 ug/ml streptomycin sulphate maintained at 37℃. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles of 3~6 mm in diameter with an 18 guage needle fixed to a 10ml syringe. The COCs for *in vitro* maturation (IVM) were washed two times with Ham's F-10, after that washed three times with TCM-199 medium without hormonal supplements and one time with hormonal supplements. Then, 50 oocytes were transferred into each well of a Nunc 4-well multidish (Nunc, Roskilde, Denmark) containing 500 ul of TCM-199. After 22 hr of the maturation culture, oocytes were washed three times in the maturation medium without hormonal supplements and transferred into 500 ul drops of same medium for additional 22 hr of culture. Then oocytes were matured for total 44 h.

In Vitro Fertilization and Culture

After the completion of maturation culture, cumulus cells were removed with 3 mg/ml hyaluronidase in NCSU 23. Denuded oocytes were washed three times with IVF medium, and sets of 20 oocytes were placed in 50 ul drops of the same medium covered with warm paraffin oil. Oocytes were fertilized with Percoll-treated freshly ejaculated sperm (1×10^5 cells/ml) in mTBM with 0.3% BSA and 2 mM caffeine for 5 hr at 37℃ in a humidified atmosphere of 5% CO_2 in air. At 5 hr after insemination, the embryos were transferred into 50 ul of NCSU-23 medium supplemented with 0.4% BSA.

Analysis of Meiotic Status

After IVM culture (nuclear maturation), oocytes were mounted on slide, fixed for 48 hr in acetic acid: methanol (1:3, v/v) at room temperature. They then were stained with 1% (w/v) orcein in 45% (v/v) acetic acid for 5 min and examined under a phase-contrast microscope at a magnification of X400. Nuclear stages were classified as germinal vesicle (GV), GV breakdown (GVBD), prophase-metaphase I (PI-MI), prophase-metaphase II (PII-MII) (Wang et al., 1994).

Assessment of Pronuclear Formation

At 6 hr of the culture period, the embryos were fixed in 3.7% formaldehyde in PBS for 48 hr at 4℃. Then, the embryos were permeabilized in 0.5% Triton X-100 for 1 hr at room temperature. The embryos were then stained with 10 g/ml propidium iodide for 30 min at 37℃ in the dark. After washing and mounting the embryos with Vectashield mounting medium (Vector Lab, Burlingame, CA) onto a cover slip, the slides were sealed with nail polish and viewed using a fluorescence microscope (Emission filter: 620 mm, excitation: 488 mm) (Nicon Microscopy, UK, Ltd). PN status was classified as no or one PN (unfertilized), 2 PN (normal fertilized) and ≥ 3 PN (polyspermy).

Statistical Analysis

Four replicate trials for examination of oocyte nuclear maturation and pronuclear formation were conducted. All percentage data were subjected to arcsine transformation

before statistical analysis. Comparisons among maturation media containing different concentrations of EGF were analyzed by one-way ANOVA. A probability of $P<0.05$ was considered to be statistically significant.

RESULTS

Effect of EGF on Nuclear Maturation

In the presence of EGF, most oocytes (85%) showed a complete expansion of cumulus layers after 44 hr maturation. Meiotic status of porcine metaphase II oocyte matured for 44 hr is presented in Fig. 1(A). As shown in Table 1, the rates of oocytes that had reached to PII-MII were significantly ($P<0.05$) higher in all groups with EGF than that of non-treated group (67%), but it did not differ among the all EGF added groups (86%, 85%, 79% and 81%, in 5, 10, 20 and 40 ng/ml EGF, respectively).

Effect of EGF on Pronuclear Formation

PN status was classified as no or one PN (unfertilized), 2 PN (normal fertilized) and ≥ 3 PN (polyspermy). Fig. 1(B) shows pronuclei of fertilized egg with 2 PN. No differences on the incidence of 2PN were observed in all treated groups (25%, 30%, 33%, 29% and 29%, in 0, 5, 10, 20 and 40 ng/ml EGF, respectively; Table 2). However, in non-treated group, polyspermy tended to be increased to those of EGF supplemented groups (66% vs. 58%, 54%, 52% and 55%, 0 vs.5, 10, 20, 40 ng/ml EGF, respectively).

Table 1. Meiotic status of oocytes cultured in the presence of 0~40 ng/ml EGF

EGF conc. (ng/ml)	No oocytes examined	No.(%) of oocytes			
		GV	GVBD	P I -M I	P II-M II
0	152	15(10)	12(8)	23(15)	102(67)[a]
5	148	6(4)	3(2)	12(8)	127(86)[b]
10	165	5(3)	3(2)	17(10)	140(85)[b]
20	157	11(7)	4(3)	17(11)	125(79)[b]
40	143	10(7)	4(3)	13(9)	125(81)[b]

GV, germinal vesicle; GVBD, germinal vesicle breakdown; PI, prophase I; MI, metaphase IX
[a,b] : $P < 0.05$, 3 replicates.

Table 2. Pronuclear formation of oocytes cultured inXthe presence of 0~40 ng/ml EGF

EGF conc. (ng/ml)	No. oocytes examined	No.(%) of oocytes		
		No or 1 PN	2 PN	≥ 3 PN
0	71	7(9)	17(25)	47(66)
5	68	8(12)	20(30)	40(58)
10	76	10(13)	25(33)	41(54)
20	75	14(19)	22(29)	39(52)
40	70	11(16)	20(29)	39(55)

*No or 1 PN, unfertilized; 2 PN, normal fertilized; ≥ 3 PN, fertilized with more than 2 sperms 3 replicates.

DISCUSSION

The present study evaluated the effect of EGF supplementation during IVM on meiotic maturation and pronuclear formation of pig oocytes. Oocyte maturation is an important process involving regulation of nuclear required to achieve developmental potency. Cumulus cells surrounding the oocyte have a critical role in oocyte maturation. Interestingly, in our study, compared to no addition group, the presence of EGF in maturation media resulted in many oocytes showing cumulus expansion. It has been shown in mice (Downs et al., 1998), cattle (Lorenzo et al., 1994), and pig (Coskun and Lin, 1995) that exogenous EGF induces oocyte maturation by generating a positive signal in the cumulus cells. According to Wang and Niwa (1995), addition of EGF during IVM did not stimulate further cumulus expansion in porcine COCs in the presence of gonadotropins and serum. However, similar to our results, these authors found that EGF did stimulate cumulus expansion under serum-free culture conditions. The above findings suggest that the presence of gonadotropins and/or serum tend to mask the stimulatory effects of EGF on cumulus expansion. Since pig oocytes could exhibit cumulus expansion in the absence of serum, it has been suggested that cumulus cells may be capable of producing inter-α-trypsin inhibitor, which binds with hyaluronic acid (Abeydeera et al., 2000). Furthermore, it seems that EGF could stimulate the synthesis of this factor as evidenced by better cumulus expansion. It is also possible that, some unknown factor(s) produced by the oocyte or cumulus or both cells that binds with hyaluronic

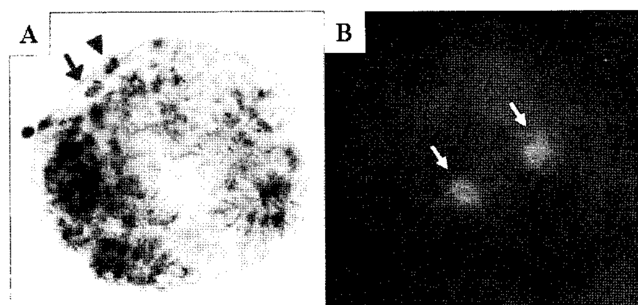

Fig. 1. (A). Meiotic status of porcine metaphase II oocyte matured for 44 hr (x 400) Arrow, MII plate; Arrow head, polar body.X(B). Pronuclei of fertilized egg with 2 PN (x 400).XArrow, Pronucleus.

acid to stabilize the extracellular matrix may result in cumulus expansion (Abeydeera et al., 2000). In addition, the presence of intracellular contacts via gap-junctions plays an important role the metabolic cooperation between oocyte and cumulus cells during the growth phase and final maturation of the oocytes.

The present study showed that EGF at different concentrations during IVM for 44 hr enhanced the nuclear maturation to the metaphase II stage. The addition of EGF to IVM medium supplemented with gonadotropins significantly stimulated the nuclear maturation of pig oocytes (Reed et al., 1993). However, no stimulatory effect of EGF supplementation was observed in the presence of gonadotropins and dialized porcine follicular fluid (Abeydeera et al., 1998a). Similarly, no stimulatory effect of EGF on nuclear or cytoplasmic maturation was observed by Li et al. (2002). The different results on various growth factors from different researchers may due to different culture media used.

It has been reported that EGF might have a physiological role in the regulation of cytoplasmic maturation of oocytes (Singh et al., 1997). It seems that supplementation of IVM medium with EGF has been shown to improve cytoplasmic maturation (as evidenced by male pronuclear formation) of pig oocytes (Ding and Foxcroft, 1994b) and developmental competence to the blastocyst stage (Abeydeera et al., 1998b). Incomplete cytoplasmic maturation of pig oocytes matured *in vitro* was reported previously and included failure of the oocytes to form a male pronucleus after fertilization and inability to develop further. In the present study, the proportion of 2PN formation as judged by a normal appearance for successful fertilization was tended to be higher in EGF added groups than in non-supplemented group, although no significant differences were observed. It has been reported that fertilized oocytes containing both male and female pronuclei can cleave irrespective of polyspermy or monospermy (Wang et al., 1999). Further, the same authors demonstrated the development of polyspermic pig oocytes, although the rate of blastocyst formation and cell number in blastocysts were lower in polyspermic embryos than monospermic embryos. In the present study, addition of EGF during IVM did not reduce the polyspermy of pig oocytes.

In conclusion, no difference was observed in nuclear maturation to the metaphase II stage in oocytes matured *in vitro* for 44 hr with different concentrations of EGF. But *in the* absence of EGF, the rate of oocytes that had reached to PII-MII was significantly ($P<0.05$) lower than in all the groups added with EGF. The concentration of 10 ng/ml EGF enhanced the rates of meiotic maturation and 2 PN formation, but it did not differ among treated groups. These results suggest that EGF can be effectively used as an additive for enhancing oocyte maturation and reducing the incidence of polyspermy in pig.

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