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Effect of EGF and AREG Treatment During Porcine *in vitro* Maturation on *in vitro* Developmental Potential of Preimplantation Embryos

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Abstract: Porcine *In Vitro* Maturation (IVM) and *In Vitro* Fertilization (IVF) technique has been used and improved. However, polyspermic penetration, low rate of Blastocyst (BL) formation and poor quality of BLs are induced by imperfect nuclear and cytoplasmic maturation. It has been reported that Epidermal Growth Factor (EGF) is beneficial for oocyte maturation to improve the IVM system and Amphiregulin (AREG) is a growth factor containing an EGF-like domain. Consequently, the present study was performed to investigate whether during porcine IVM system EGF and/or AREG supplementation is profitable for improving oocyte maturation and embryo development. In Experiment 1, oocytes were maturated with EGF (15 ng mL^{-1}) or AREG (15 ng mL^{-1}) or combination of both and after 44 h, oocytes were stained with Hoechst and metaphase II and development rate were evaluated. Without any growth factor served as a control. In Experiment 2, maturated oocytes from the previous groups were fertilized with proven sperm and penetration rate after 10 h of post-insemination was evaluated. In Experiment 3 *in vitro* matured porcine oocytes were stained with fluorescein isothiocyanate-labeled peanut agglutinin and the distribution of Cortical Granules (CGs) was evaluated by laser confocal microscopy. In Experiment 1, the oocytes maturation rate were significantly higher in EGF, AREG or combined group (70.9±15.5, 75.8±9.8 and 70.5±10.8%, respectively) compared to control (51.6±18.3%) but no significant difference among the treatments group. In Experiment 2, there was no significance of sperm penetration rate among the groups however, polyspermy and sperm number per oocytes were decreased significantly ($p<0.05$) in combined group (26.7±12.0 and 1.35±0.2%, respectively) compared with control (43.5±13.1 and 1.63±0.3%, respectively). In Experiment 3, BL formation rate were significantly ($p<0.05$) higher in combined group (17.1±5.0%) compared with control and EGF group (9.4±3.2 and 13.1±4.6%, respectively) but the TE cell number and total cell number were significantly higher in ($p<0.05$) combined group compared to control and other treatment group. In Experiment 3, the CG area was increased significantly ($p<0.05$) in all treatment groups compared with control group (11.1±3.4, 9.7±3.0 and 10.4±2.8 vs. 4.1±1.8%, respectively). These results indicate that the addition of EGF and/or AREG to porcine IVM medium that enhances oocyte maturation and increases the total cell number. Combination of both growth factors also increases development rate and decreases polyspermy. Therefore, it is suggested that AREG can assist immature porcine oocytes to the metaphase II stage, enhance developmental potential in *in vitro* system and that there is due to synergistic effect of EGF and AREG during oocytes and embryonic development.

Key words: IVM, IVF, EGF, amphiregulin, cortical granule, polyspermy

INTRODUCTION

In mammal, preovulatory oocytes are maintained in a diplotene stage of meiotic prophase by an oocyte maturation inhibitor secreted from follicles (Tsafriri and Pomerantz, 1984). However, the preovulatory Luteinizing Hormone (LH) surge induces reentry into the first meiotic division of oocytes (Hsieh *et al*., 2007). The oocyte maturation represents that female gamete changes from a developmentally incapable cell to one with ability to fertilization and embryonic development. *In vivo*, the oocyte is exposed to Follicle-Stimulating Hormone (FSH), LH, steroid, maturation-promoting factor, growth factors and other factors and these may interact to regulate the maturation of oocyte.

In vitro, the suitable medium and supplements can mature oocyte to be fertilizable. However, imperfect nuclear and cytoplasmic maturation can cause

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polyspermic fertilization and formation of poor quality Blastocysts (BLs) (Han *et al*., 1999) and many studies have experimented to decrease polyspermic fertilization and improve embryo development (Abeydeera and Day, 1997a, b). In pigs, porcine Follicular Fluid (pFF) (Naito *et al*., 1988), cysteine (Yoshida *et al*., 1992), insulin (Singh and Armstrong, 1997) and Epidermal Growth Factor (EGF) (Grupen *et al*., 1997; Abeydeera *et al*., 2000; Hong *et al*., 2004) can contribute to nuclear and cytoplasm maturation. Among the factors, EGF is reported beneficial on oocyte to improve *In Vitro* Maturation (IVM) system (Ding and Foxcroft, 1994; Grupen *et al*., 1997; Abeydeera *et al*., 1998). However, Tissue Culture Media (TCM)-199 supplemented with EGF is used on porcine oocyte maturation in many studies. The main positive effect of EGF is to stimulate the synthesis of intracellular Glutathione (GSH) (Abeydeera *et al*., 2000) and followed high concentration of intracellular GSH effects on embryo development, acts on the protein synthesis and amino acid transport and promotes male pronuclear formation (Whitaker and Knight, 2004).

It has been known that Amphiregulin (AREG) which is a member of EGF family and expresses in the luminal epithelium of mouse uterus (Das *et al*., 1995) and in ovarian epithelial cell that could affects cumulus expansion and oocyte maturation in human (Shoyab *et al*., 1989). However, it is believed that it could be involved to improve oocyte quality during oocyte maturation (Lee *et al*., 2009) but the role of AREG on oocytes yet to be clear. Consequently, the present study was performed to investigate whether TCM-199 supplemented with EGF and/or AREG is profitable for improving the oocyte maturation and development of porcine *In Vitro* Fertilization (IVF) embryos.

MATERIALS AND METHODS

Sampling of ovaries, aspiration and selection of oocytes: Slaughterhouse-derived porcine (mixed type-Yorkshire, Landrace and Duroc about 6 months old gilt) ovaries were carried to the laboratory in antibiotics (amoxicillin 20 mg mL^{-1} and clavulanic acid 4 mg mL^{-1}) added 0.9% NaCl within 2 h. Collected ovaries were washed (2 times) with 0.9% NaCl and maintained at 39°C. Oocytes were aspirated from 3-6 mm follicular size with 18 gauge needle attached to a 10 mL syringe and collected in a 15 mL conical centrifuge tubes for sedimentation. After 10 min, supernatant fluid was removed and the conical tube was filled with tyrode lactate HEPES-Polyvinylalcohol (TLH-PVA) up to 5-6 mL. Thereafter, the contents were poured in petri dish and oocytes which had homogenous ooplasm and intact culumus oophurus were selected for IVM.

***In Vitro* Maturation (IVM):** Forty to fifty selected cumulus oocytes complexes were maturated in 500 μL modified Tissue Culture Medium-199 (TCM-199; Invitrogen Corporation, Carlsbad, CA, USA) supplemented with EGF and AREG according to experimental design. First 20-22 h they were cultured with eCG 4 IU mL^{-1} (Intevet, Folligon) and hCG 4IU mL^{-1} (Intervet, Chorulon) at 39.5°C, 5% CO_2. After 22 h, oocytes were transferred to M199 without eCG/hCG at 39.5°C, 5% CO_2.

***In Vitro* Fertilization (IVF):** Maturated oocytes were denuded by 0.1% hyaluronidase (Sigma, H-3506) and washed 3 times with TLH-PVA. Thereafter, 15 oocytes were transferred to 40 mL modified Tris-Buffered Medium (mTBM) drop covered with mineral oil. A straw of frozen boar semen was thawed at 39°C for 2 min in a water bath and washed in 10 mL Phosphate Buffered Saline (PBS) by centrifuging twice at 2000 rpm for 2 min. The sperm pellet was resuspended in mTBM that was pre-equilibrated for 18 h at 39°C under 5% CO_2. After appropriate dilution, 5 μL of sperm suspension was added to a 40 mL drop of fertilization medium (mTBM) to give a final sperm concentration of 2×10^6 sperm mL^{-1}. Gametes were co-cultured for 6 h at 39°C in a humidified atmosphere of 5% CO_2 and 95% air.

***In Vitro* Culture (IVC):** After 6 h of fertilization, gametes were removed from the mTBM drops, washed 3 times in TLH-PVA and cultured in 30 μL microdrops (10 gametes per drop) of North Carolina State University (NCSU-23) medium covered with mineral oil and incubated under 5% O_2, 5% CO_2 and 90% N_2 at 39.8°C for 168 h. The cleavage rate and BL formation were evaluated under a stereomicroscope at 48 and 168 h after insemination, respectively.

Maturation rate check: After 40-42 h of IVM, denuded oocytes were washed TLH-PVA and fixed with 4% paraformaldehyde in PBS for 10 min and stained with Hoechst for 5 min. Thereafter, oocytes were mounted and assessed by epifluorescence microscopy (Leica, DMI 4000B). Polar body and MII plate were considered as matured oocytes.

Penentration rate (polyspermy) check: About 10 h after fertilization the zygotes were washed with TLH-PVA and zona pellucida was removed by 0.5% protease by gentle pipetting. Thereafter, oocytes without zona pellucid were washed in TLH-PVA medium containing a solution of 0.1% formaldehyde and 0.01% PVA in PBS for 1 min and fixed in 1% formaldehyde and 0.01% PVA in PBS for 10 min at room temperature. Fixed embryos were placed in a drop of mounting medium on slide and covered with a

cover slide. The mounting medium consisted with 25% glycerol in PBS containing 2.5 mg mL^{-1} sodium azide and 2.5 μg mL^{-1} Hoechst. After 5 min, numbers of sperm head penetrating the zona pellucid, polyspermy were assessed by epifluorescence microscopy.

Assessment of the number of cells in BL: The qualities of BLs were assessed by differential staining of Inner Cell Mass (ICM) and the Trophectoderm (TE) cells according to Thouas *et al.* (2001) with little modifications. Briefly, TE cells of BLs at 168 h were stained with 0.1 mg mL^{-1} fluorochrome propidium iodide for 10 sec after treatment with permeabilizing solution containing 2% (v/v) Triton X-100 ionic detergent. BLs was then incubated in a second solution containing 3 μg mL^{-1} Hoechst in 100% ethanol at 4°C for 90 min. After Hoechst stain, BLs were transferred to third solution containing 100% ethanol for 80 min. Fixed and stained whole Bls were mounted and assessed for cell number using epifluorescence microscopy.

Cortical Granule (CG) stain and assessment: Methods of CG visualization were based on those described by Wang *et al.* (1998). After IVM, denuded oocytes were fixed by 3.7% paraformaldehyde in PBS. After 30 min, oocytes were washed 3 times in PBS. After being treated for 5 min in PBS containing 0.5% Triton X-100, oocytes were washed 2 times. Oocytes were then cultured in 100 μg mL^{-1} fluorescein isothiocyanate-labeled peanut agglutinin (FITC-PNA, Sigma) in PBS for 30 min in a dark. After staining, the oocytes were washed in PBS. Confocal microscopy was used for assessment of CGs. Area of CGs was calculated by EZ-C1 (Gold Version 3.20 build 610) and total area of oocyte were divided to the area except CG of oocyte (Fig. 1).

Experimental designs: In Experiment 1, effects of AREG (15 ng mL^{-1}) and/or EGF (15 ng mL^{-1}) on *in vitro* maturation of porcine oocytes were evaluated after 42-44 h of culture. Following IVM, oocytes were stained and with Hoechst and assessed by epifluorescence microscopy. In Experiment 2, sperm penetrations were checked in regards to different groups after 10 h of insemination. In Experiment 3 and 4, the *in vitro* developmental potential of porcine IVF embryos and areas of cortical granules were assessed after matured with AREG and/or EGF during IVM system.

Statistical analysis: All percentage data and data sets obtained from this study were expressed as mean±SD. Differences in developmental rate and cell numbers of the embryos between experimental groups were analyzed by

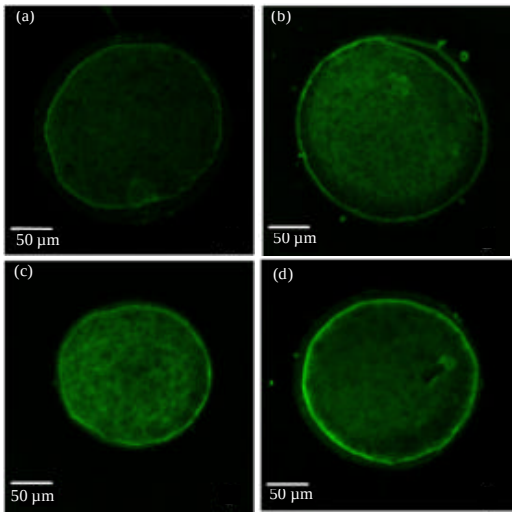

Fig. 1: Confocal microscopic images of CGs distribution in pig oocytes stained with FITC-PNA. A monolayer CG distribution under plasma membrane was observed in an oocyte after 44 h of culture. Green images represent CG staining; a) control; b) EGF 15 ng mL^{-1}; c) AREG 15 ng mL^{-1} and d) EGF 15 ng mL^{-1}+AREG 15 ng mL^{-1}

Duncan multiple range test using SPSS Version 17.0 for Windows (SPSS, Inc., Chicago, IL). Differences were considered to be significant if $p<0.05$.

RESULTS

Experiment 1 (effect of EGF and/or AREG on *in vitro* maturation of oocytes): Metaphase II stage was significantly increased in EGF, AREG and EGF+AREG (70.9±15.5, 75.8±9.8 and 70.5±10.8%, respectively) compare to control (51.6±18.3%) and it was also observed that metaphase I stage was significant decreased in groups of EGF, AREG and EGF+AREG (24.3±9.9, 20.0±8.6 and 24.5±8.7%) than the control group (40.4±13.1%). But there was no difference in the rates of GV and GVBD stage among the groups (Table 1).

Experiment 2 (effect of EGF and/or AREG on sperm penetration): Polyspermic penetration was significantly decreased in EGF+AREG group compare to control (26.6±12.0 vs. 43.5±13.1%, respectively). But there was no any significant difference in penetration rate among the treatment groups (Table 2). However, number of sperm penetrate per oocytes was significantly ($p<0.05$) lower in EGF+AREG combined group (1.35±0.2) compared to control (1.63±0.3).

Table 1: Effect of EGF/AREG treatment during porcine *in vitro* maturation on nuclear maturation

Groups	No. of oocytes examined	No. (mean±SD%) GV	GVBD	MI	MII
Control	188	4 (2.1±2.7)	11 (5.9±6.6)	76 (40.4±13.1)[a]	97 (51.6±18.3)[a]
EGF 15 ng mL^{-1}	189	0 (0.0±0.0)	9 (4.8±6.5)	46 (24.3±9.90)[b]	134 (70.9±15.5)[b]
AREG 15 ng mL^{-1}	190	2 (1.1±1.6)	6 (3.2±2.0)	38 (20.0±8.60)[b]	144 (75.8±9.80)[b]
EGF 15 ng mL^{-1}+AREG 15 ng mL^{-1}	200	5 (2.5±2.9)	5 (2.5±5.0)	49 (24.5±8.70)[b]	141 (70.5±10.8)[b]

Within the same column, values with different superscripts letters (a and b) were different ($p<0.05$). GV: Germinal Vesicle; GVBD: Germinal Vesicle Breakdown; MI: Metaphase I; MII: Metaphase II

Table 2: Penetration and polyspermic fertilization rates of porcine oocytes cultured in TCM-199 supplemented with absence or presence of EGF and/or AREG

Groups	No. of oocytes examined	No. (mean±SD%) of penetrated	No. (mean±SD%) of polyspermy	No. (mean±SD%) of Pns 2PN	3PN	4PN	No. (mean±SD%) of spermatozoa in penetrated oocyte
Control	232	161 (69.4±7.30)	70 (43.5±13.1)[a]	91 (56.5±13.1)[a]	39 (24.2±9.60)	31 (19.3±14.1)[a]	1.63±0.3[a]
EGF 15 ng mL^{-1}	242	167 (69.0±10.3)	54 (32.3±12.1)[a, b]	113 (67.7±12.1)[a, b]	42 (25.2±15.6)	12 (7.2±5.70)[b]	1.40±0.1[a, b]
AREG 15 ng mL^{-1}	248	162 (65.3±8.50)	48 (29.6±12.1)[a, b]	114 (70.4±12.1)[a, b]	30 (18.5±11.3)	18 (11.1±6.80)[a, b]	1.41±0.2[a, b]
EGF 15 ng mL^{-1}+ AREG 15 ng mL^{-1}	231	146 (63.2±8.60)	39 (26.7±12.0)[b]	107 (73.3±12.0)[b]	27 (18.5±9.60)	12 (8.2±6.00)[b]	1.35±0.2[b]

Within the same column, values with different superscripts letters (a and b) were different ($p<0.05$). PN: Pronucleus

Table 3: Effects of EGF/AREG treatment during porcine IVM on preimplantation embryonic development after *in vitro* fertilization

Groups	No. of oocytes examined	Embryo development: No. (mean±SD%)* of ≥2 cells	No. (mean±SD%)* of blastocysts	No. of blastocysts evaluated	Cell number (mean±SD): ICM	TE	Total cells
Control	701	394 (56.2±12.6)	66 (9.4±3.2)[a]	18	11.2±2.2	45.2±24.6[a]	56.3±25.5[a]
EGF 15 ng mL^{-1}	709	397 (56.0±14.9)	93 (13.1±4.6)[a, b]	19	11.8±2.7	69.6±31.0[b]	81.4±32.0[b]
AREG 15 ng mL^{-1}	711	393 (55.3±11.4)	98 (13.8±6.0)[b, c]	16	11.7±3.4	68.0±27.4[b]	79.7±27.3[b]
EGF 15 ng mL^{-1}+ AREG 15 ng mL^{-1}	708	402 (56.8±11.5)	121 (17.1±5.0)[c]	19	11.6±3.8	68.7±25.6[b]	80.3±28.6[b]

Within the same column, values with different superscripts letters (a-c) were different ($p<0.05$). ICM: Inner Cell Mass; TE: Trophectoderm; *Percentage of the number of oocytes cultured

Table 4: Area of cortical granule in equator of oocytes maturated in medium for 44 h

Groups	Area fraction of cortical granules (mean+SD%)
Control	4.1±1.8[a]
EGF 15 ng mL^{-1}	11.1±3.4[b]
AREG 15 ng mL^{-1}	9.7±3.0[b]
EGF 15 ng mL^{-1}+AREG 15 ng mL^{-1}	10.4±2.8[b]

Within the same column, values with different superscripts letters (a and b) were different ($p<0.05$)

Experiment 3 (effects of EGF and/or AREG on *in vitro* developmental potential of porcine *in vitro* fertilized embryos): There was no significant difference of cleavage rate among the groups.

However, BL developmental rates in the EGF+AREG group (17.1±5.0%) were significantly increased compared with control, AREG and EGF group (9.4±3.2 and 13.1±4.6%). Total cell number as well as TE cell number was significantly increased in all treatment groups compared to control (Table 3).

Experiment 4 (effects of EGF and/or AREG on area of CG distribution): Area of cortical granule distribution was significantly increased in all treatment groups compared to control but no any significant difference among the treatment groups (Table 4).

DISCUSSION

AREG is a member of the EGF family and its role can be inferred from the study of EGF. Previously it was reported that ampherulin could influence trophoectoderm cell number of porcine *in vitro* IVF and parthenogenetic embryos and also this protein was detected in cytoplasm (Lee *et al*., 2009). The exact role of AREG was not elucidated but it could be involved to improve oocyte quality. It was reported that EGFs promote emission of first polar bodies (Abeydeera, 2002) and the results have shown a similar result of porcine IVF embryos. An influence to nuclear maturity with the help of exogenous AREG which is EGF-like peptide and AREG may operate like EGF. Conti *et al.* (2006) are appearing about AREG roles. However, AREG receptor existed but still separately operate to that place to EGF receptor where they operated and the effect should have been born, reveals and support after combined operating which is not but the process which happens is thought being similar.

Cytoplasmic maturity is important for embryonic developmental during *in vitro*. Cytoplasmic organelle are redistributed and migrated during meiosis towards periphery and the CG density and cortical localization in

matured oocytes are important markers for cytoplasmic maturation (Cran and Esper, 1990; Liu *et al.* 2005). CGs are a specialized group of membrane-bound secretory granules composed of specialized enzymes and glycoproteins that are exquisitely localized near their site of release at non-penetrated oocytes (Gulyas, 1980; Dandekar and Talbot, 1992) and close to their site of action such as the zona pellucida. Thousands of CGs are situated within 2 μm of the plasma membrane of the mature egg and few are detected in subcortical regions (Ducibella *et al.*, 1990). In mammals, the release of CGs by exocytosis after gamete membrane fusion appears to block polyspermy by changing the properties of the ZP and forming a new envelope like structure in the perivitelline space (Long *et al.*, 1994). In order to evaluate the ooplasmic maturity, the CG areas were stained with FITC-PNA and observed with confocal microscopy. In matured oocyte the CG are dispersed uniformly to periphery of the cytoplasm (Li *et al.*, 2003). To evaluate CG area, researchers used the method to divide the bulk of the ovum whole with CG area and the fact that CG area is not governed in compliance with an oocyte size. Researchers observed CG area was relatively increased in EGF/or AREG or in both treated oocytes than controls. In the result showed that sperm penetration was not any significant different among the groups, monospermic penetration was significantly higher in EGF and AREG group compared to controls and also the polyspermic penetration was significantly lower in EGF and AREG group compared to control. This result indicated that addition of AREG combination with EGF have some beneficial effects during oocytes maturation. During sperm penetration CG prevents polyspermy by cortical reaction and CG exocytosis (Miyazaki *et al.*, 1993). Cortical reaction is thought to play a major rule to secrete materials which makes zona hardening that reduced polyspermy during the conventional IVF system (Miyazaki *et al.*, 1993). In the study indicated that addition of AREG along with EGF prevents sperm penetration per oocyte significantly compared to controls.

In vitro blastocyst formation is dependent on oocytes cytoplasmic quality during oocyte maturation. During *in vitro* maturation oocyte-synthesized materials are involved to prepare oocyte cytoplasm for proper fertilization. Normal fertilization would help to proper embryos development. In the study showed that addition of AREG along with EGF during *in vitro* maturation of porcine oocytes significantly increased embryonic developmental potential and the total cell number per blastocyst of *in vitro* fertilized embryos compared with the control group and this is could be due to synergy effect of EGF and AREG to the embryos during oocyte maturation. During *in vitro* maturation proper CG distributions also important for proper fertilization and subsequent embryo development. Improper CG distribution could lead to abnormal sperm penetration and reduced embryos development and sometime lethal to the oocytes (Hunter, 1991). The oocytes which were treated with EGF or AREG alone during IVM that contribute to increase BL formation and as well the trophectoderm cell number and those may result of nuclear and cytoplasmic maturation. Furthermore, the increased trophectoderm could help to placentation and researchers hypothesized that AREG could affect the SCNT embryo implantation.

CONCLUSION

In this study, the addition of exogenous AREG during IVM period could help nuclear and cytoplasmic maturation of porcine oocytes. Moreover, increased BL formation and cell number were observed in IVF with AREG treated oocytes.

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