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Morphological Interaction of Porcine Oocyte and Cumulus Cells Study on *in vitro* Oocyte Maturation Using Electron Microscopy

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***Abstract*—**Morphological interaction of porcine cumulus-oocyte complexes (pCOCs) was investigated on *in vitro* condition using electron microscope (SEM and TEM). The totals of 1,923 oocytes were round in shape, surrounded by *Zona pellucida* with layer of cumulus cells ranging between 59.29-202.14 μm in size. They were classified into intact-, multi-, partial cumulus cell layer oocyte, and completely denuded oocyte, at the percentage composition of 22.80% 32.70%, 18.60%, and 25.90 % respectively. The pCOCs classified as intact- and multi cumulus cell layer oocytes were further culturing at 37°C with 5% CO_2, 95% air atmosphere and high humidity for 44 h in M199 with Earle's salts supplemented with 10% HTFCS, 2.2 mg/mL $NaHCO_3$, 1 M Hepes, 0.25 mM pyruvate, 15 μg/mL porcine follicle-stimulating hormone, 1 μg/mL LH, 1μg/mL estradiol with ethanol, and 50 μg/mL gentamycin sulfate. On electron microscope study, cumulus cells were found to stick their processes to secrete substance from the sac-shape end into *Zona pellucida* of the oocyte and also communicated with the neighboring cells through their microvilli on the beginning of incubation period. It is believed that the cumulus cells communicate with the oocyte by inserting the microvilli through this gap and embedded in the oocyte cytoplasm before secreting substance, through the sac-shape end of the microvilli, to inhibit primary oocyte development at the prophase I. Morphological changes of the complexes were observed after culturing for 24-44 h. One hundred percentages of the cumulus layers were expanded and cumulus cells were peeling off from the oocyte surface. In addition, the round-shape cumulus cells transformed themselves into either an elongate shape or a columnar shape, and no communication between cumulus neighboring cells. After 44 h of incubation time, diameter of oocytes surrounded by cumulus cells was larger than 0 h incubation. The effect of hormones in culture medium is exerted by their receptors present in porcine oocyte. It is likely that all morphological changes of the complexes after hormone treatment were to allow maturation of the oocyte. This study demonstrated that the association of hormones in M199 could promote porcine follicle activation in 44 h *in vitro* condition. This culture system should be useful for studying the regulation of early follicular growth and development, especially because these follicles represent a large source of oocytes that could be used *in vitro* for cell technology.

***Keywords*—**Cumulus cells, electron microscopy (SEM and TEM), *in vitro*, porcine oocyte.

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I. Introduction

PIG is a valuable economic animal in Nakorn Pathom Province, Thailand, especially of ovary and oviducts are not uses as human food and they are easy to collect and used for animal technology research such as cell cytotoxicology test, *in vitro* oocyte maturation (IVM), *in vitro* fertilization test (IVF) and *in vitro* embryo development (IVC) [1], [2]. For this project will study the morphological interaction of porcine oocyte and cumulus cells on *in vitro* oocyte maturation (IVM) using scanning electron microscope (SEM) and transmission electron microscope (TEM) for future use the oocyte as cell technology applications.

II. Material and Methods

Porcine ovaries of Large White pigs were obtained from local slaughter house. They were removed and transported to the laboratory within 1 h in a thermos (at 30°C-35°C) containing 0.9% normal saline. Porcine cumulus oocyte complexes (pCOCs) collection, selected healthy follicles of 2-8 mm in diameter were aspirated using a 5-10 ml disposable syringe with 18-gauge needle containing 0.9% normal saline and placed in sterile petri dishes. Follicular fluid was observed under a stereomicroscope and pCOCs were collected using a pipette of narrow pore size (200-250 μm). After aspiration, pCOCs were washed 3 times in TALP-HEPES supplemented with 10% heat treated fetal calf serum (HTFCS) and 50 mg/ml gentamycin. Then, intact- and multi-cumulus cell layers oocytes were cultured in culture medium supplemented with hormones (15 μg/mL pFSH, 1 μg/mL LH, 1 μg/ml estradiol in ethanol) at 37°C with 5% CO_2, 95% air atmosphere, and high humidity for 24-44 h to study ultrastructure under using scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

SEM preparation: Samples were washed 3 times with 0.1 M phosphate-buffer (pH 7.2-7.4) and pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffer for 1-2 h and post-fixed in 1% osmium tetroxide in the same buffer for 24 h. They were then dehydrated in a graded series of ethanol (30, 50, 70 80, 90 and 100%) and dried in a critical point dryer machine. Dry samples were mounted on stubs with conductive

carbon tape, coated with gold particle at 20 nm thick in an ion sputtering, observed and examined under SEM (CamScan Analytical, Maxim 2000S) operating at 10 kV.

TEM preparation: Samples were washed 3 times with 0.1 M phosphate-buffer (pH 7.2-7.4) and pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffer for 1-2 h and post-fixed in 1% osmium tetroxide in the same buffer for overnight. After fixation time they were dehydrated in a graded series of ethanol (30, 50, 70, 80, 90 and 100%). Then, the samples were embedded in spur resin, cut into 1-2 µm thickness, and stained with toluidine blue before examining under a light microscope to identify interested areas. The selected area tissues were ultrathin sectioned to 60-90 nm thickness, stained with 0.5% uranyl acetate and lead citrate prior to examine under TEM (JEOL 1230) operating at 80 kV.

III. Results

A total of 1,923 oocytes were collected from healthy follicles of 2-8 mm in diameter. All oocyte were round in shape and surrounded by *Zona pellucida* with layers of cumulus cells (CCs). They were intact-cumulus cell layer oocyte, (22.80%) multi-cumulus cell layer oocyte (32.70%), partial cumulus cell layer oocyte (18.60%), and completely denuded oocyte (25.90%), depend on their CCs surrounding an oocyte (Table I). The intact-cumulus cell layers surrounding oocyte is the oocyte contained compacted CCs with more than 5 layers. The multi-cumulus cell layers surrounding oocyte is the oocytes were surrounded with 2-4 incomplete layers of CCs. The partial cumulus cell layers surrounding oocyte is the oocytes were partially covered with some CCs layers. The completely denuded oocyte is the oocytes without cumulus cells (Figs. 1, 2). From SEM study of pCOCs showed teardrop-like structure of cumulus cells attached to the oocytes surface cumulus cells explanation (Fig. 3). TEM morphological observation revealed that normal organelle in cytoplasm of incubation oocyte (Fig. 4). Meanwhile, CCs were changing their character from round shape to tear drop (or columnar shape) after 44 h of incubation period (Fig. 5).

TABLE I
Classification of pCOCs

Types	No. of COCs	(%)
intact-cumulus cell layers	439	22.80
multi-cumulus cell layers	629	32.70
partial cumulus cell layers	357	18.60
completely denuded oocyte	498	25.90
total	1,923	100.00

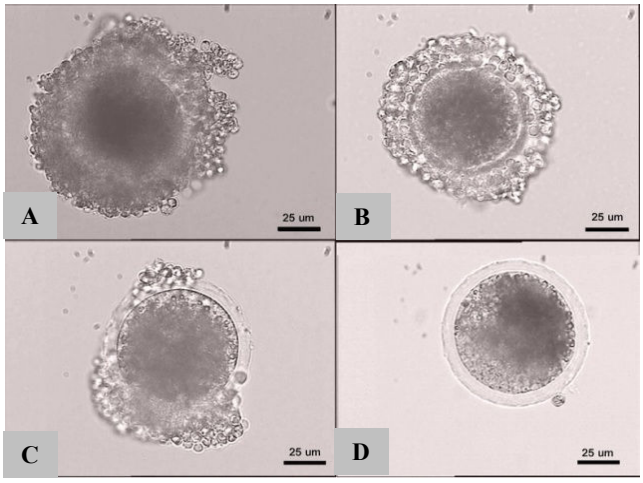

Fig. 1 Inverted microscope micrographs of pCOCs (A) intact-cumulus cell layers, (B) multi-cumulus cell layers, (C) partial cumulus cell layers, (D) completely denuded oocyte

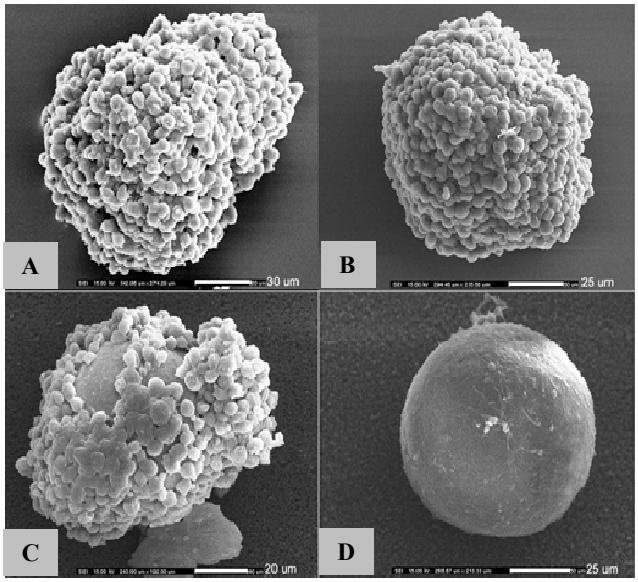

Fig. 2 SEM micrographs of pCOCs (A) intact-cumulus cell layers, (B) multi-cumulus cell layers, (C) partial cumulus cell layers, (D) completely denuded oocyte

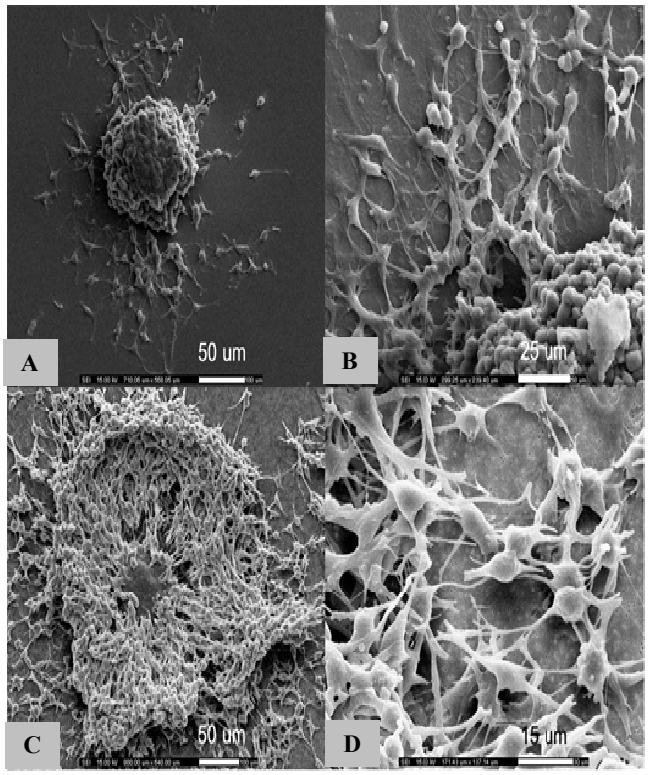

Fig. 3 SEM micrographs of pCOCs (A) showed teardrop-like structure of cumulus cells attached to the oocytes surface (B)-(D) cumulus cells explanation at high magnification

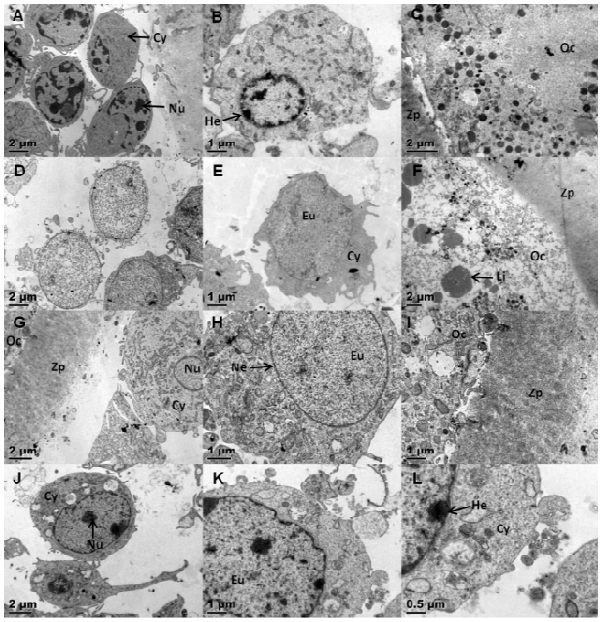

Fig. 4 TEM micrographs showed (A-B) communication of CCs micro villi with the surrounding oocyte at 0 h, (D-E) separate communication of CCs at 3h and (G-H) at 24 h, (J-L) the elongation of CCs at 44 h after culture. C, F and I showed normal ultrastructure of oocyte with several organelles. (Cy, cytoplasm; Eu, euchromatin; He, heterochromatin; Li, lipid droplet; Ne, nuclear enverlope; Nu, nucleoli; Oc, Oocyte; Zp, *Zona pellucida*)

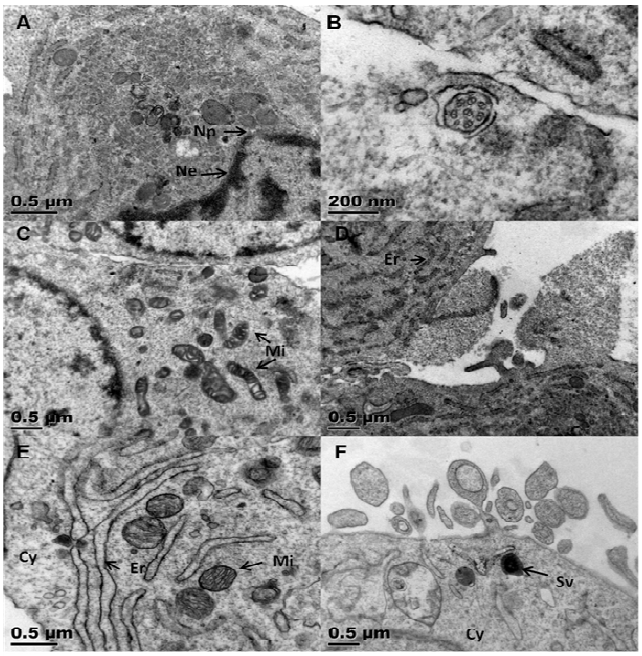

Fig. 5 TEM micrographs showed (A-F) normal ultrastructure of oocyte with several organelles at 44 h culture time. Cy, Cytoplasm; Er, Endoplasmic reticulum; Ne, nuclear envelope; Np, Nuclear pores; Mi, mitochondria; Sv, secretory vesicles

IV. Discussion and Conclusions

Porcine mature oocytes and CCs used in this study were collected from 2-8 mm of antral follicles. One thousand nine hundred and twenty three pCOCs can be classified into 4 types on the basis of oocyte surrounding CCs and their diameter ranging from 59.29-202.14 µm. From the results demonstrated that intact- and multi-cumulus cell layers at the percentage of 55.50% have high potential to develop into matured oocytes in maturation medium condition on 44 h after culture. This is in agreement with the finding of [3] which reported that 2 cell types can be developed into matured oocytes by culture in the medium supplemented with FSH and LH. Results from TEM showed normal organelle in cytoplasm of incubation oocyte. The interaction among CCs and between CCs and the oocyte in the pCOCs at 0 h before culture showed the process of CCs, where at the end of the process it contained secretion, penetrating into *Zona pellucida* of the oocyte and many microvilli of CCs also processed to communicate with neighboring CCs as can be seen. Researcher believed that secretion substance is the oocyte maturation inhibition substance or OMI, via through the sac-shape end of the microvilli, to inhibit primary oocyte development at the prophase I of the oogenesis [4], [5]. We found that intact and multi-cumulus cell layer type had higher potential to become matured oocytes. Two cell types of COCs were successfully cultured in the artificial medium supplemented with follicular stimulating hormone (FSH) and luteinizing hormone (LH) using cell samples from bovine and swamp buffalo [6], [7]. Conclusively, reproductive hormones can be used *in vitro* to

induce immature intact-and multi-cumulus cell layers of pig to further develop until maturation. This finding is merit for used porcine culture oocyte and their secretions on cell technology in term of *in vitro* cytotoxicology, IVM, IVF, IVC [1], [2], supplement in medium for acrosome reaction on frozen bovine spermatozoa [8] and will be used for supplement in cosmetic product [9].

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