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Original Article

Changes in secretory protein of porcine ampulla and isthmus parts of oviduct on follicular and luteal phases

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Abstract

This study aimed to describe the morphology on oviductal epithelial cells (pOEC) and protein secretions in follicular and luteal phases. Result of proteins band were not different from control group. The fresh secretory proteins of ampulla and isthmus parts pOEC in the follicular phase were 45, 90, 105 kDa, while the fresh secretory fluids from ampulla and isthmus pOEC secretion in luteal phase were 38, 45, 90, 105, 220, >220 kDa. Protein of size 220 and >220 kDa was probably trypsin or protease functioning to stimulate ovulation. Protein sized 105 kDa is glycogen phosphorylase and protein kinase or oviductal glycoprotein is the enzyme for glycogenolysis plays an important role in regulation of glucose levels in the blood vessels. Protein of size 90 KDa was found to be heat shock proteins for inducing cell growth and in vitro fertilization development of spermatozoa. Protein sized 45 kDa is immunoglobulin gamma-chain function on oocyte maturation.

Keywords: porcine oviductal epithelial cells (pOEC), follicular and luteal phases, secretion proteins, SDS-PAGE, LC/MS/MS

1. Introduction

The unused organs of porcine reproductive system from slaughterhouse such as oviduct, ovary, and their secretions, have been effectively used as a model for the biological study of mammalian reproductive system and cell technology (Areekijseree & Chuen-Im, 2012; Sanmanee & Areekijseree, 2009; Sanmanee & Areekijseree, 2010). They could be cultured and mature well in the culture conditions for *in vitro* oocyte maturation and sperm preparation (Areekij seree, Thongpan, & Vejaratpimol, 2005; Areekijseree & Vee rapraditsin, 2008; Areekijseree & Vejaratpimol, 2006). The porcine oviduct epithelial cell of reproductive female pig is composed of 2 cell types: columnar ciliated cells and round non-ciliated cells. The oviduct is the environment of ferti-lization, it is found to contain more synthetic secretions (Murray, 1992) and a high number of columnar ciliated cells corresponding to the transportation of ovulated oocytes. Hole and Koos (1994) reported that numerous round non-ciliated cells with short microvilli located in the apical surface also corresponded to the presence of secretory substance for nutritional support of embryonic development. Similar results were reported by Areekijseree *et al*. (2005) and Areekijseree and Vejaratpimol (2006), showing that oviductal epithelial cells of Large White pig underwent changes in both the morphological features and the population of cell types during the estrous cycle. At the follicular phase, porcine oviduct epithelium contained a greater number of long ciliated cells than at the luteal phase. The luteal phase, however, the porcine oviduct epithelium was filled up with numerous round shaped non-ciliated cells having short microvilli on the apical surface.

Even though there are many studies of porcine reproductive cells in vitro, but there is still lack of reports of porcine oviduct samples and their protein secretions during folliculogenesis. Therefore, this study was designed to describe the morphology of oviductal epithelial cells and protein secretions during the follicular and luteal phases using inverted microscope and scanning electron microscope. The results could add value to waste product (porcine oviduct) from local slaughterhouse for the further use of their fresh protein secretion and condition medium (CM) from each part

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of oviduct in the estrous cycle for biotechnology research. The protein secretion could be supplemented to culture medium to promote cell viability and growth (such as in vitro fertilization and embryonic development).

2. Materials and Methods

2.1 Oviductal cell collection

Oviducts of Large White reproductive female pigs (at the age between 210-250 days) were obtained from local slaughterhouses in Nakorn Pathom Province, Thailand, by method of Areekijseree (Areekijseree *et al*., 2005). The organs were removed within 30 min after slaughter and transported for study at the laboratory in a thermos containing a saline solution using sterile technique (0.9% NaCl (w/v), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 250 µg/mL amphotericin B) at temperature between 30-35ºC.

2.2 Oviductal epithelial cells preparation

Porcine oviducts were classified into 2 phases of the estrous cycle (follicular and luteal phases) based on the ovarian morphology and blood supply of oviducts. The follicular phase found growing follicles (2-5 mm diameter), corpus albicans from previous cycle in ovary, and less or no vascularization blood supply in oviduct. The luteal phase found recent corpora hemorrhagica or corpora lutea which was very vascularized in the peripheral and purple stigma, small growing follicles and presence or absence of corpus albicans in ovary, and numerous reddish blood supplies in oviduct. The oviduct were trimmed free from fat and connective tissues and rinsed several times in 0.9% NaCl with 50 µg/mL gentamycin. They were cut in small size pieces (2-3 mm) and prepared for light microscope and scanning electron microscope determination.

2.3 Preparation for light microscope and scanning electron microscopy study

The small pieces of all parts of the oviducts were separated for light microscope and for scanning electron microscopy (SEM) study. For light microscope observation, samples were fixed in Bouin's solution, dehydrated, embedded in paraffin, sectioned and stained with haematoxylin and eosin (Codón & Casanave, 2009). Meanwhile, for SEM study the tissue was pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffer (pH 7.2-7.4) for 24 h. Then, they were dehydrated in a graded series of ethanol (50%, 70% 80%, 90%, 95% and absolute ethanol) and critical point dried in a critical-point dryer machine. All samples were mounted on stubs with conductive carbon tape, coated with gold particles at 20 nm thick in gold-coating machine, and examined under scanning electron microscope operating at 15-20 kV (Areekijseree, Thongpan, & Vejaratpimol, 2005).

2.4 Oviductal fluid preparation

The ampulla and isthmatic parts of follicular and luteal phases of oviducts were prepared for secretory protein study. They were cut and placed in sterile petri dishes. Each part was gently scraped with a sterile glass slide. The extrudates were transferred to 10 mL sterile conical centrifuge tube containing 8 mL of washing medium. They were washed 5-7 times and rewashed in culture medium at a ratio of 1:50. Fresh protein secretions were separated while the other protein secretion was prepared for condition medium (CM). Ten milliliters of culture medium was placed in a 60x15 mm falcon culture dish and cultured at 37ºC with 5% CO_2, 95% air atmosphere and high humidity for 48, 96 and 144 h.

2.5 Culture medium for oviductal condition medium (CM)

M199 with Earle's salts (Sigma Chemical Co., St. Louis MO, USA) supplemental with 10% heat-treated fetal calf serum (HTFCS), 2.2 mg/mL $NaHCO_3$, 1 M HEPES (Sigma Chemical Co., St. Louis MO, USA), 0.25 mM pyruvate, 15 µg/mL and 50 µg/mL gentamycin sulfate was used as medium in this study. The medium was equilibrated at 37 ºC, 5% CO_2, 95% air atmosphere with high humidity for 24 h before use in the experiment.

2.6 Study on protein secretions of oviduct by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and LC/MS/MS technique

Fresh protein secretions and condition medium (CM) of ampulla and isthmatic parts in follicular and luteal phases were determined for the total protein concentration by the Lowry method (1951). The absorbence of the protein solution was measured at a wavelength of 750 nm and the protein concentration estimated by comparing with the standard protein of bovine serum albumin. To separate proteins molecular weight, 50 µL of each sample was loaded into into 12% SDS-PAGE at 200 volts for 50 min. The gel was stained in Coomassie blue stain and destained in destaining solution. After that, the selected protein bands were identified by liquid chromatography/mass spectrometer/mass spectrometer (LC/MS/MS) technique. The selected protein bands were digested by in-gel trypsin digestion procedure. Then, LC/MS/MS machine was used to determine the kinds of a protein. The LC/MS/MS system comprised a liquid chromatography part with added combination electrospray ionization. The LC separation was executed on a reversed phase column chromatography. Mobile phase was water/ formic acid. Mass spectral data from 300-500 m/s were collected in the positive ionization mode. After analysis from LC/MS/MS technique, the individual proteins were identified by the Mascot program with 95 confidence intervals ($p< 0.05$).

3. Results

3.1 Porcine oviductal epithelial cell observation

Observation under light microscope (LM) revealed that the oviductal lumen, the thickness of mucosal wall and connective tissue during the follicular phase (around 2.87 mm, 0.29 mm) was lower than in the luteal phase (around 3.19 mm, 0.35 mm). Moreover, the follicular phase also showed less epithelial folding in lumen than the luteal phase (Figure 1-2).

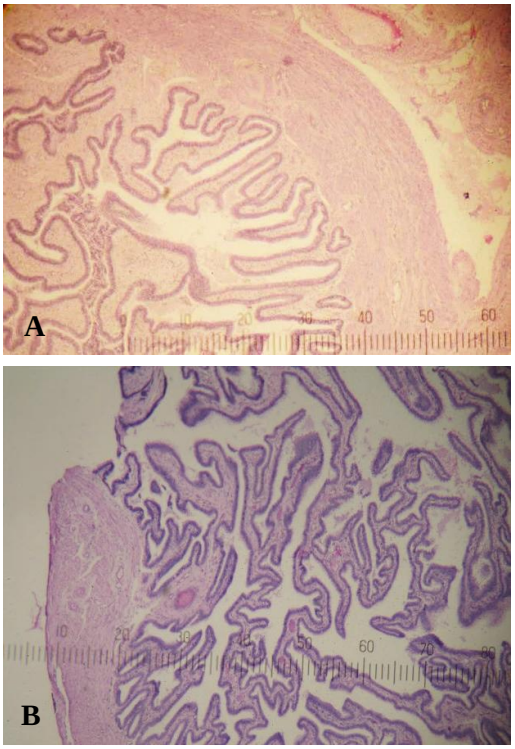

Figure 1. Micrographs showed the porcine ampullar part of oviduct had fewer folds in follicular phase (A) than in the luteal phase (B).

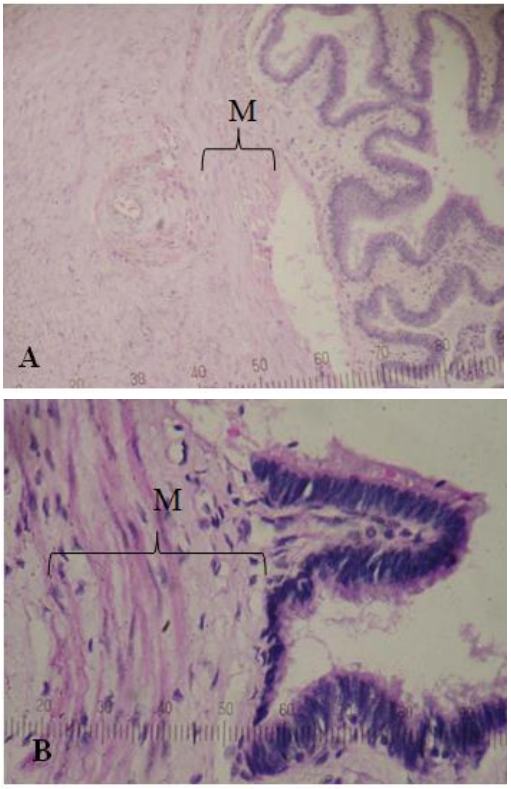

Figure 2. Micrographs showed porcine ampullar part of oviduct was less in the follicular phase (A) than in the luteal phase (B).

The morphological and population changes were studied using SEM. The morphology of oviductal epithelium cells of Large White reproductive female pigs contained 2 different cell types, columnar ciliated cells (7-10 μm in diameter) and round shaped non-ciliated cells (secretory cells) (4-5 μm in diameter) with short microvilli on the apical surface. During the follicular phase, porcine ampullary oviductal epithelial cells contained a greater number of high columnar ciliated cells and a lower number of round shaped non-ciliated cells, while the epithelium during the luteal phase consisted of 50% columnar ciliated cells and 50% round-shaped non-ciliated cells (Figure 3-4). The change of cell morphology and population of the porcine ampullary oviduct during folliculogenesis seems to suit their functions in the reproductive process. From this study, the ciliated cells appeared either in groups or distributed among the non-ciliated cells after culture in culture medium for 24 h. Non-ciliated cells were clearly seen as round-shaped cells. (Figure 5).

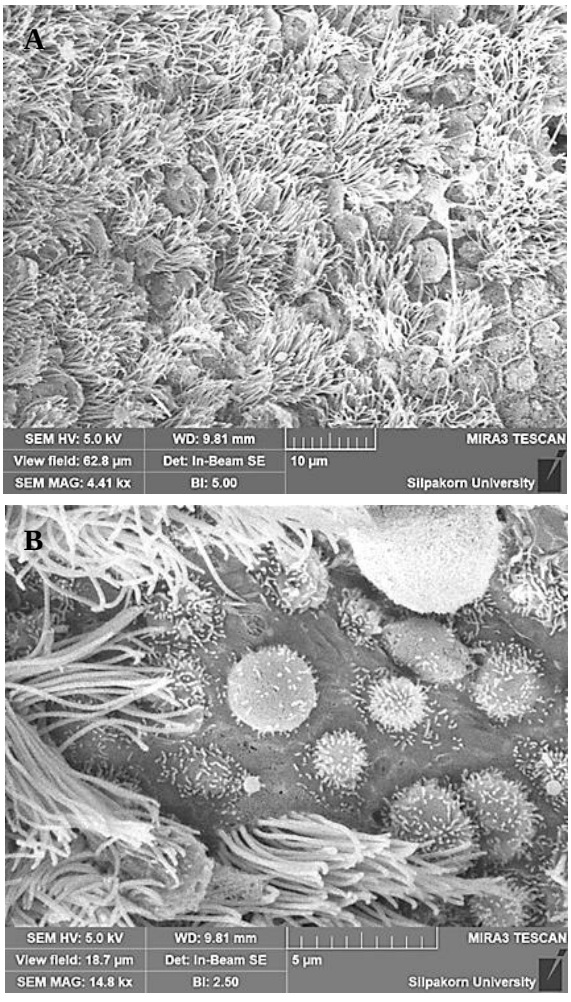

Figure 3. Sanning electron micrographs of in vivo porcine ampullar part of oviduct in the follicular phase (A) showed numerous ciliated cells (CC) more than non-ciliated cells (NC) or secretory cells. At high magnification showing (C) ciliated cells and tiny non-ciliated cells.

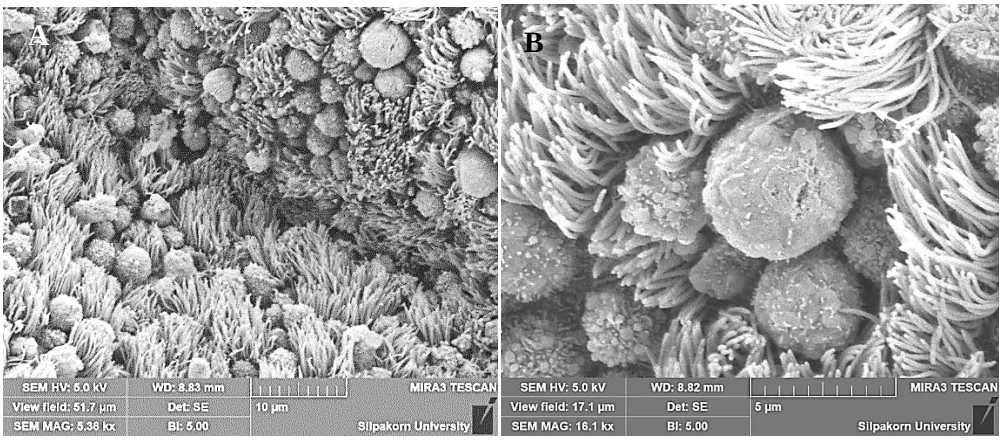

Figure 4. Scanning electron micrographs of in vivo porcine ampullar part of oviduct in the luteal phase (A) showed 50 percent of ciliated cells (CC) and 50 percent of non-ciliated cells (NC) or secretory cells. At high magnification showing (C) ciliated cells and big round-shaped non-ciliated cells.

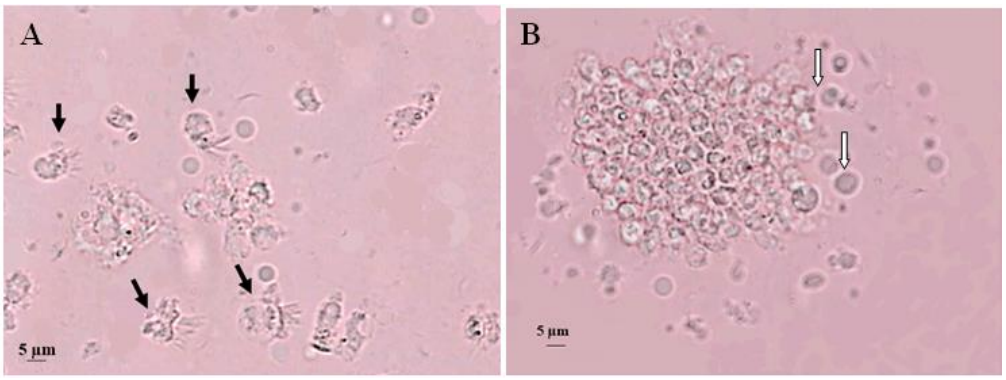

Figure 5. Inverted microscope micrographs showed in vitro porcine oviductal epithelial cells (pOEC). (A) Columnar ciliated cells (black arrows) and (B) round shape non-ciliated cells or secretory cells (white arrows), after 24 h in culture medium.

3.2 Study of the secretory proteins from oviduct and follicle by SDS-PAGE and LC/MS/MS technique

Comparing the proteins secreted into the condition medium (CM) of cultured pOEC in follicular phase and luteal phase after cultured for 48, 96 and 144 hours by SDS-PAGE technique, condition medium proteins bands were not different from the control medium group. It means that pOEC did not secret protein in medium. The results from comparing secretions from pOECs in follicular phase from ampulla and isthmus revealed that proteins in secretion from 2 parts are similar as shown in Figure 5. The molecular weight size of proteins is 45, 95 kDa and 105 kDa in column 5 and this protein column is apparent in column 1. The results from comparing secretions from porcine oviductal epithelial cells in luteal phase from ampulla and isthmus revealed that proteins in secretion from 2 parts are similar as shown in Figure 6. The fresh secretory proteins of ampulla and isthmus parts of pOEC secretion in luteal phase, found 38, 45, 90, 105, 220, >220 kDa. (Figure 7). However, the proteins in column 1 are more obvious than those in column 7. In the upper part of the gel, there are proteins that have molecular weight size 220 and >220 kDa and can be found in column 1 and 7. Therefore, the interesting proteins in column 1 that have molecular weight size 95 and 105 kDa, which are proteins from the fresh secretion of ampulla and isthmus pOEC in luteal phase, were selected to compare with standard protein markers which have molecular weight size between 10 to 220 kDa. Utilization of LC/MS/MS technique for identifying selected proteins of fresh secretion of ampulla and isthmus pOEC secretion revealed that the Protein size 220 and >220 kDa was probably trypsin or protease which functions to stimulate oocyte ovulation. The protein sized 105 kDa is glycogen phosphorylase and protein kinase or oviductal glycoprotein which is the enzyme for glycogenolysis. This plays an important role in regulation of glucose levels in the blood. Protein size 90 KDa was found to be heat-shock proteins inducing cell growth and development of spermatozoa in vitro fertilization. Meanwhile the protein sized 45 kDa is immunoglobulin gamma-chain and functions to stimulate nuclear membrane lysis for oocyte maturation.

4. Discussion

The study of oviductal secretory glycoprotein of pig indicated that the biochemistry and morphology of oviduct change during the reproductive cycle in order to support fertilization and embryo development. These changes are naturally controlled by hormone system from hypothalamus into anterior pituitary gland to the target organ. Hundreds

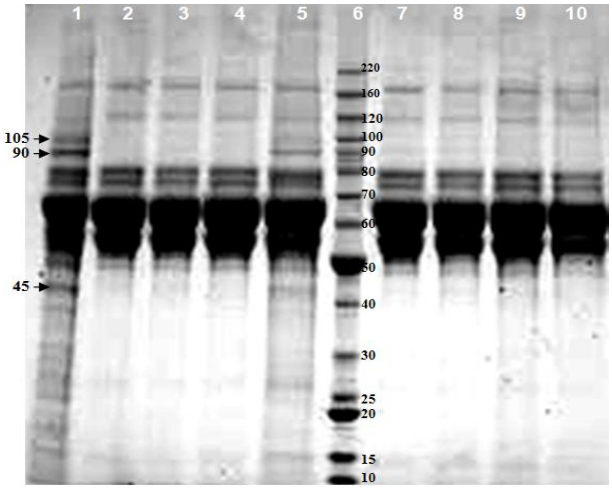

Figure 6. The pattern of fresh protein bands and condition medium (CM) of ampulla and isthmus part pOEC in follicular phase (cultured for 48, 96 and 144 h).
Note: Column 1: Fresh ampulla pOEC secretion
Column 2: CM of ampulla pOEC culturing for 48 h
Column 3: CM of ampulla pOEC culturing for 96 h
Column 4: CM of ampulla pOEC culturing for 144h
Column 5: Fresh isthmus pOEC secretion
Column 6: STD = Standard protein marker
Column 7: Control = M 199 + 10 % fetal calf serum
Column 8: CM of isthmus pOEC culturing for 48 h
Column 9: CM of isthmus pOEC culturing for 96 h
Column 10: CM of isthmus pOEC culturing for 144 h

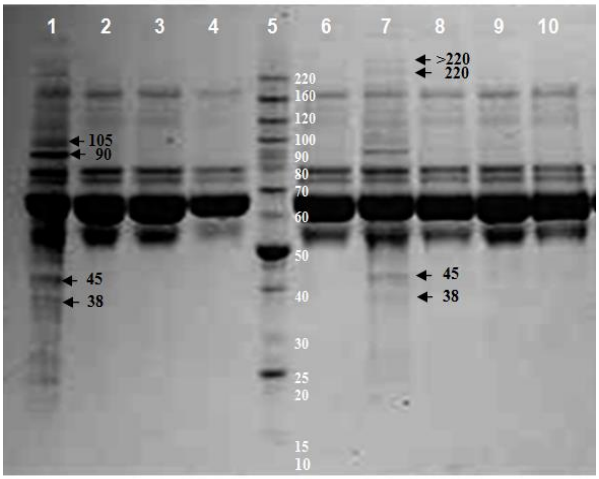

Figure 7. The pattern of fresh protein bands and the condition medium (CM) of ampulla and isthmus part pOEC on luteal phase (cultured for 48, 96 and 144 h).
Note: Column 1: Fresh ampulla pOEC secretion
Column 2: CM of ampulla pOEC culturing for 48 h
Column 3: CM of ampulla pOEC culturing for 96 h
Column 4: CM of ampulla pOEC culturing for 144h
Column 5: Standard protein marker
Column 6: Control =M 199 + 10 % fetal calf serum
Column 7: Fresh isthmus pOEC secretion
Column 8: CM of isthmus pOEC culturing for 48 h
Column 9: CM of isthmus pOEC culturing for 96 h
Column 10: CM of isthmus pOEC culturing for 144 h

macromolecules which are produced and secreted in oviductal lumen have been reported such as estrogen-dependent oviductal secretory glycoprotein (OSP), which helps spermatozoa movement through oviduct to the oocyte. From in-vitro experiments, it was found that several functional proteins such as protease inhibitors, growth factors, and cytokine binding proteins, enzymes and immunoglobulin are also produced and secreted from the oviduct of sheep. The sizes of these proteins range from 14 to 26 kDa (Woldesenbet & Newton, 2003). It is believed that the proteins are essential for fertilization and embryo development during the estrous cycle and early pregnancy, respectively. Secretion proteins in the fluid of the oviduct have different compositions and changes of the composition in the fluid will increase following increasing level of estrogen. Mechanism and function of these proteins need to be further elucidated. It has been reported that glycoprotein present in fluid of porcine oviduct maybe involved in embryo development. Normally, porcine oviduct synthesizes and secretes up to 14 proteins such as porcine oviduct-specific secretory glycoproteins (pOSP), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), and 76 kDa protein (Russinova *et al*.., 2001). Similarly, in this study, we found obvious protein in ampulla part of oviduct in the luteal phase (45, 90, 105, 220, >220 kDa), which is essential for oocyte maturation, ovulation, fertilization and embryo development.

Areekijseree and Veerapraditsin (2008) reported that there were 3 bands observed in both condition medium from pOEC but not the medium control, namely bands sized more than 220, 22, and 17 kDa, and one band observed in only pOEC samples, which is sized about 100 kDa. Although proteins sized about 17 kDa were observed in the condition medium from both cell types, the intensity of the protein presented in condition medium from fresh pOEC is much stronger than that observed in the condition medium from CC+GC, implying that the protein was produced by the CC+GC more than the pOEC. For the 22 -kDa protein, the band intensity was equal in condition medium from pOEC and CC+GC but could not be observed in the control medium. The mechanism of these proteins in supporting fertilization and embryo development is unknown yet; however, identification of all proteins and their functions are currently being investigated.

The morphological study of pOEC of reproductive female pig in estrous cycle in follicular phase and luteal phase revealed that there are 2 types of porcine oviductal epithelial cells, which are ciliated columnar epithelial cells and non-ciliated round shape epithelial cells with short microvilli located on the apical surface (Codón & Casanave, 2009; Dekel & Phillips, 1979; Kamaci, Suludere, Irmak, Can, & Bayhan, 1999; Kress & Morson, 2007; Steffl, Schweiger, & Amselgruber, 2004). The study conducted by Areekijseree *et al*. (2005) and Areekijseree and Vejaratpimol (2006) on morphological differences and structure of porcine oviductal epithelial cells using an inverted microscope and scanning electron microscope reported that there are a greater number of ciliated columnar epithelial in the follicular phase than in the luteal phase. The structure of porcine oviductal epithelial cells at ampulla and isthmus when the oocyte moves in and waits for fertilization process after ovulation is vital in reproductive processes. The ampulla part is the area for fertilization and early development of the embryo (Desantis,

Ventriglia, Zubani, & Corriero, 2009; Leese, 1988). Therefore, there are a greater number of ciliated cells which facilitate movement of mature oocyte at ampulla in the follicular phase than in the luteal phase (Yaniz, Lopez-Gatius, & Hunter, 2006). In contrast, non-ciliated cells are increased in the luteal phase (Abe & Oikawa, 1992; Areekijseree & Vejaratpimol, 2006), corresponding to production and secretion of glycoprotein from secretory granules (DeSouza & Murray, 1995; Murray, 1992). Similarly, in this study we found round-shape (or secretory) cells are increased number in ampulla part of luteal phase corresponding to secretion of essential protein to the lumen of the oviduct. The similar results were reported by Verhage *et al*. (1997), who found that non-ciliated oviductal epithelial cells of baboon secrete more glycoprotein in the stage of rising estrogen level. For the morphological features of oviductal epithelial cells of the isthmus in the estrous cycle, there are a large number of ciliated cells and non-ciliated cell with short microvilli located on the apical surface or secretory cells.

This study focused on the secretion into the condition medium from porcine oviductal epithelial cells which are cultured for 48, 96 and 144 h via SDS-PAGE analysis and on the particular protein which cleaves with trypsin and analysis of types of protein using the LC/MS/MS method with Mascot program at the 95% confidence level ($p< 0.05$). However there were no protein band in condition medium (CM) in all study groups that were different from medium control group. This means that pOEC did not secrete protein into the condition medium. For the fresh pOEC from ampulla part, the particular protein bands in the luteal phase with molecular weight size equal to as exceeding 220 kDa were selected for further investigation. Similar to a study by Areekijseree and Veerapraditsin (2008), which investigated secretion from pOEC and secretion from cumulus cells and granulosa cells that secrete into in to the cultured over 48, 96 and 144 h using SDS-PAGE analysis revealed that there are proteins that have molecular weight size more than 220 kDa in every protein band and the 2 bands with molecular weight size ≥220 kDa have the similar analytical results, that is, they are trypsin precursor and protease.

Particular protein bands from the secretion of ampulla and isthmus pOEC in follicular and luteal phase with molecular weight size are 95 and 105 kDa were selected for study. The 90 kDa protein was heat-shock protein, which is an intracellular protein that activates other synthesized protein (Zhao *et al*., 2002) were selected to observe. King, Anderson, and Killian (1994) reported that 85-95 kDa proteins which are secreted from the bovine oviductal epithelial cells in estrous cycle induce development of sperm for fertilization process in vitro compared with oviductal fluid protein. Staros and Killian (1998) investigated the relation of six different molecular weight size proteins with bovine zona pellucida in estrous cycle. Similary Gerena and Killian (1990) observed bovine oviductal fluid in estrous cycle and found 47 kDa proteins along the cycle and 80-95 kDa proteins three to four days before ovulation. The study also revealed that 105 kDa molecular weight size proteins are glycogen phosphorylase, protein kinase, and oviductal glycoprotein. Verhage *et al*. (1997) reported that 110-130 kDa proteins secreted from baboon oviductal epithelial cells are glycoprotein, which facilitates spermatozoa for fertilization, glycogen phosphorylase, which is the enzyme for glycogenolysis, and protein kinase, which has an important role in glycogenolysis via calcium ion.

The study results revealed that there are a great number of different molecular weight size proteins in the oviduct. Therefore, further study should investigate the functions and benefits of these proteins for the future application. Using non-livestock animals oviduct for scientific research could be benefit for animal reproductive processes including preparation of spermatozoa for fertilization, fertilization facilitation and promotion of the early development of the embryo. (Areekijseree & Veerapraditsin, 2008; Buhi, O' brien, Alvarez, Erdos, & Dubois, 1993; Imam, Ansari, Ahmed, & Kumaresan, 2008; King *et al.*, 1994; Lloyd, Elliott, Fazeli, Watson, & Holt, 2009; Woldesenbet & Newton, 2003).

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