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Histological examination of porcine oviduct, ovary, cumulus-oocyte complexes, and follicular fluid secretion

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ABSTRACT

The porcine reproductive system, while inedible, is a valuable source of hormone and growth factors for cell maturation. The present study investigated the morphological features of the oviduct, ovary, and cumulus-oocyte complexes of Large White pigs and protein patterns of the follicular fluid in the ovaries. Pig ovaries (n=71) were collected from local slaughterhouses in Nakhon Pathom Province, Thailand. A total of 3,510 oocytes were obtained and categorized based on follicle size as small (1-3 mm in diameter; n=2,910), medium (4-6 mm in diameter; n=530), and large (7-8 mm in diameter; n=70). The examination of oocytes revealed that they were intact cumulus cell layer oocytes, multi cumulus cell layer oocytes, partial cumulus cell layer oocytes, completely denuded oocytes, and degenerated oocytes. The oviduct comprised three anatomical regions: the isthmus, ampulla, and infundibulum. The infundibulum had the largest diameter, whereas the isthmus had the smallest diameter. The protein patterns of the follicular fluid were analyzed via a molecular weight-based approach using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The results showed that the fluid in both small and medium follicles contained proteins of molecular weights of 23, 50, 100, 225, and >225 kDa. For large follicles, proteins of molecular weights of 12, 16, 23, 50, 100, 225, and >225 kDa were detected in the follicular fluid. These findings would be useful for biotechnological studies in the future.

Keywords: cumulus-oocyte complexes; follicular fluid protein pattern; pig; ovary; oviduct

1. INTRODUCTION

The porcine ovary, a part of the reproductive tract, plays important gametogenesis and sex hormone production roles. Several studies have described mammalian reproductive cells (Areekijseree and Chuen-Im, 2012; Youngsabanant-Areekijseree et al., 2019; Youngsabanant and Mettasart, 2020). Based on the number of cumulus cell (CC) layers

surrounding the oocyte, cumulus-oocyte complexes (pCOCs) can be classified into four types: type I, intact CC layer; type II, multi CC layer; type III, partial CC layer; and type IV, completely denuded oocyte (Areekijseree and Vejaratpimol, 2006). Ultrastructural observation indicated that the oviduct contained both columnar ciliated cells and spherical nonciliated cells. The identification of the components of the follicular fluid revealed several proteins, including immunoglobulin fragments, cytokeratin, transferrin, and plasminogen precursors, that support *in vitro* oocyte maturation and *in vitro* embryo fertilization of porcine oocytes. Hence, follicular fluid has been used in laboratories and research in various fields, including the application of porcine oocytes in organophosphate insecticide concentration analysis (Ducolomb et al., 2009). Reed et al. (1996) demonstrated that the *in vitro* early embryo development of pig could be further enhanced by coculturing the embryo with the epithelial cells of pig's oviduct. The present study aimed to investigate the morphological features of Large White pCOCs as well as their ovaries and oviducts, including the protein contents in small, medium, and large follicles. The preliminary data from this study may be applicable in the biotechnology research, including *in vitro* oocyte maturation and *in vitro* fertilization, in the future.

2. MATERIALS AND METHODS

2.1 Porcine oviduct and ovary collection

The ovaries and oviducts from Large White female pigs (age, approximately 210-250 days) were collected from local slaughterhouses in Nakhon Pathom Province, Thailand, and transferred to the laboratory in 1 h in a container with 0.9% normal saline kept in water at 30°C-35°C (0.9% w/v normal saline, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 250 mg/mL amphotericin B). Upon arrival at the laboratory, the ovaries and oviducts were rinsed three times using 0.9% normal saline mixed with antibiotic solution. All oviducts were classified as described by Youngsabanant-Areekijseree et al. (2019). The follicular fluid of healthy follicles that were small (1-3 mm in diameter), medium (4-6 mm in diameter), and large (7-8 mm in diameter) in size was collected using a sterile 10-mL disposable syringe with an 18-gauge needle containing saline solution and placed in sterile petri dishes. Then, the color of the follicular fluid of each follicle type was observed. After cell sedimentation, oocytes and follicular contents were observed under a stereomicroscope, and oocytes were collected using a narrow pore-sized pipette (250 µm). Oocytes were washed three times with the washing medium and then classified as described by Areekijseree and Vejaratpimol (2006).

2.2 Histological assessment of porcine oviducts

The morphological features of paraffin-embedded oviducts, including blood vessels, diameter, length, and weight, were observed using histology and light microscopy. For the porcine oviductal epithelial cell (pOEC) study, the oviducts were gently scraped in a sterile petri dish using a sterile glass slide from the end of the isthmus to the infundibulum to extract pOECs from the lumen of the oviduct. Then, the cells were washed and cultured in a culture medium, and their morphological features were observed under a light microscope.

2.3 Histological assessment of pCOCs and follicular fluid protein pattern

Using histology, the morphological features of paraffin-embedded ovaries were observed under a light microscope. The follicular fluids from small, medium, and large follicles were aspirated using an 18-gauge needle with a 10-mL sterile syringe with 0.9% normal saline. The morphological features of pCOCs were inspected under stereomicroscopy, and the diameters of pCOCs were measured using Dino-Eye microscope eye-piece camera.

Then, the follicular fluids were analyzed for total protein concentration using the Lowry method (Lowry et al., 1951), in which bovine serum albumin was used as a standard protein. To characterize protein secretions, 15 µL of the sample was loaded onto 4%-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and run at 80 volts for 1:30 h, and the protein bands were visualized using Coomassie brilliant blue staining (Youngsabanant and Mettasart, 2020).

3. RESULTS

3.1 Morphological features of porcine ovaries and oviducts

The observation of ovaries revealed that small follicles comprise fewer blood vessels than medium and large follicles (Figure 1). Table 1 shows morphological features of porcine oviducts in the follicular phase. Based on the sizes of follicles present in ovaries, they were classified into three types:

Type I (porcine oviduct with the ovaries contained almost all small follicles

Type II (porcine oviduct with the ovaries contained almost all medium follicles

Type III (porcine oviduct with the ovaries contained almost all large follicles

In this study, most oviducts were type I (n=17, 41.46%). All three oviduct types comprised three anatomical regions: the isthmus, ampulla, and infundibulum. The infundibulum had the largest diameter, whereas the isthmus had the smallest diameter.

The observation of porcine oviducts under a light microscope revealed the different characters of the oviductal lumen. The isthmus section of the oviduct contained thick muscular layers, a thin mucus membrane, and lamina propria connected with ciliated cells in narrow lumens (Figure 2). In medium lumens, the ampulla section of the oviduct comprised thin serous membranes, thick muscular layers, mucous membranes, and lamina propria lined with ciliated cells (Figure 3). The ampulla section of the oviduct showed thin serous membranes, mucous membranes, thin muscular layers, and lamina propria lined with ciliated cells in large lumens (Figure 4).

The oviduct epithelial cells of pig from the lumens of oviducts appeared as clusters and free columnar ciliated cells and spherical nonciliated cells. The diameters of columnar ciliated cells and spherical nonciliated cells were 5-7 and 5 µm, respectively (Figure 5).

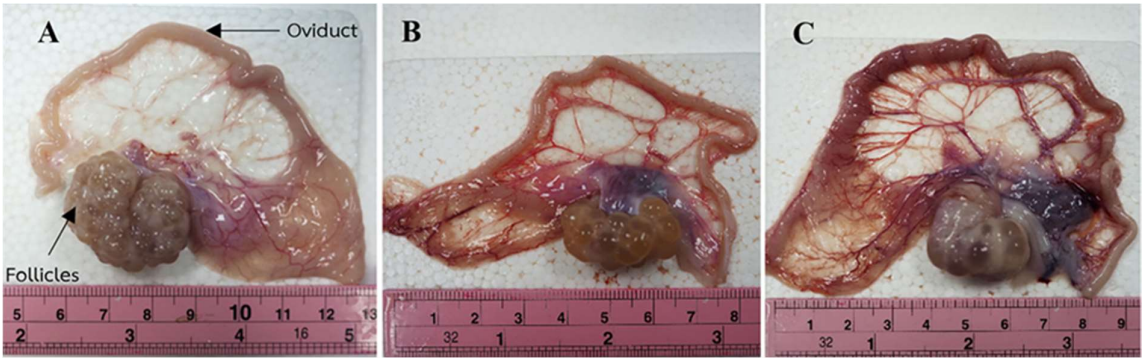

Figure 1. Micrographs show blood vessel supply in an ovary and oviduct with numerous small (A), medium (B), and large follicles (C)

Table 1. Means ± standard deviations of the diameter, length, and weight of porcine oviduct types in the follicular phase

Oviduct type	Diameter (mm)			Length (cm)	Weight (g)
	Isthmus	Ampulla	Infundibulum		
Type I: Porcine oviduct with the ovaries contained almost all small follicles (n = 17)	2.49 ± 0.34	2.94 ± 0.57	3.23 ± 0.45	19.76 ± 2.59	1.39 ± 0.36
Type II: Porcine oviduct with the ovaries contained almost all medium follicles (n = 13)	2.61 ± 0.46	3.26 ± 0.71	3.63 ± 0.48	23.04 ± 2.87	1.93 ± 0.65
Type III: Porcine oviduct with the ovaries contained almost all large follicles (n = 11)	2.93 ± 0.79	3.21 ± 0.75	4.18 ± 0.65	27.04 ± 2.78	2.38 ± 0.53

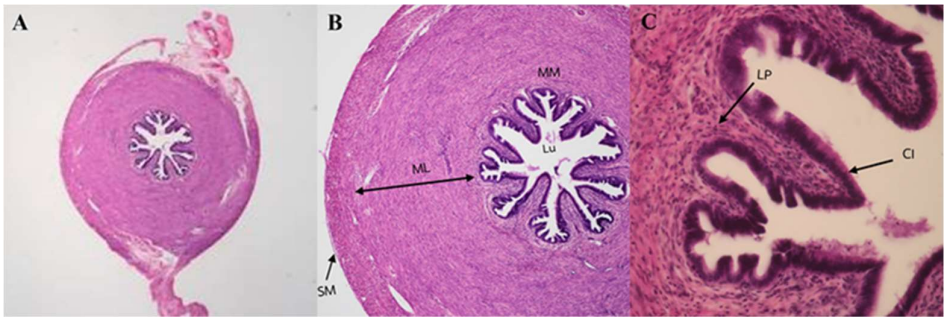

Figure 2. Photographs showing the isthmus section of the oviduct with serous membranes (SM), muscular layers (ML), mucous membranes (MM), lamina propria (LP), ciliated cells (CI), and lumen (LU)

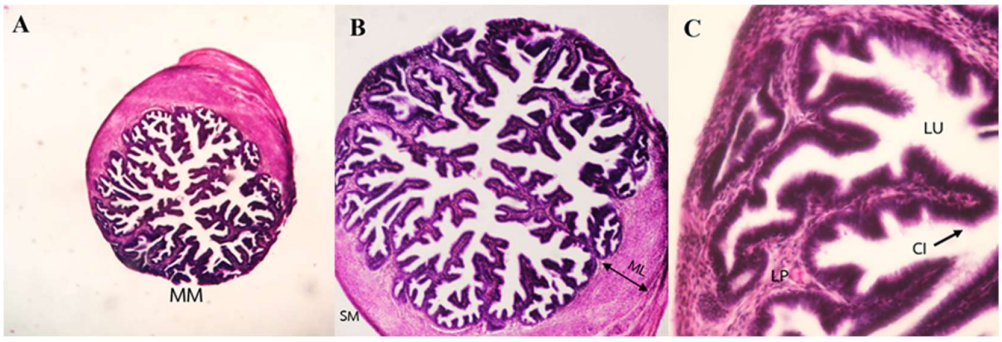

Figure 3. Photographs showing the ampulla section of the oviduct with serous membranes (SM), muscular layers (ML), mucous membranes (MM), lamina propria (LP), ciliated cells (CI), and lumens (LU)

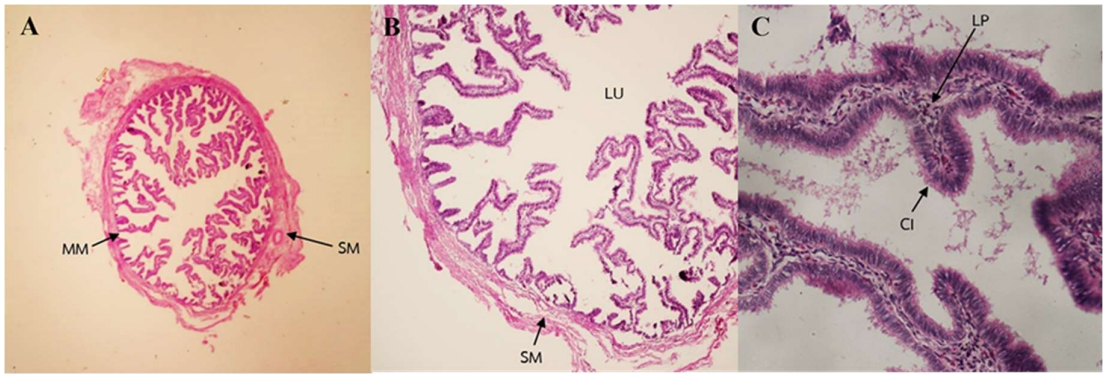

Figure 4. Photographs showing the infundibulum section of the oviduct with serous membranes (SM), mucous membranes (MM), lamina propria (LP), ciliated cells (CI) and lumens (LU)

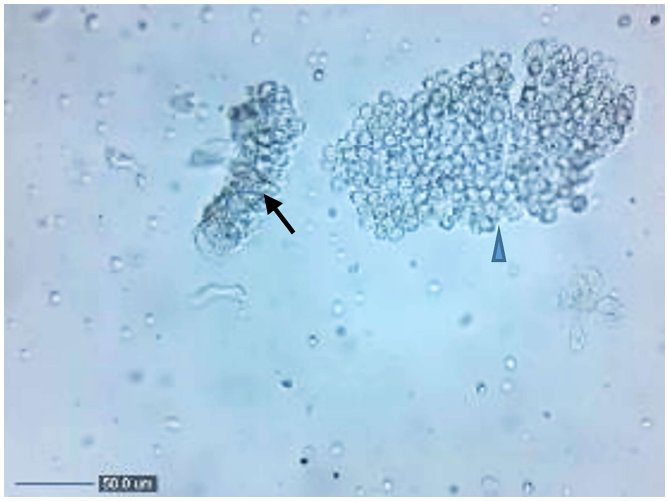

Figure 5. Photographs of pOECs cultured in M199 medium showing clustered columnar ciliated cells (black arrow) and clustered round-shaped nonciliated cells (arrowhead)

3.2 Morphology features of pCOCs

From a total of 64 porcine ovaries, the average length and width of ovaries were 2.66±0.30 and 1.96±0.28 cm, respectively, and their average weight was 3.62±0.99 g. Thus, follicles from all 64 ovaries could be classified into three types based on the follicle size: small, medium, and large. The comparisons of follicular fluid color among the three follicle types revealed that the fluid from small follicles was yellow but darker than that from medium and large follicles (Figure 6).

Porcine oocytes were characterized by collecting 71 ovary samples that yielded 3,510 oocytes (an average of 49 oocytes per ovary) and classified as small, medium, and large follicles. The oocytes were round in shape and surrounded by a zona pellucida (ZP) and several CC layers. Based on the number of oocyte-surrounding CC layers, pCOCs obtained from all ovary types could be classified into five types: type 1 intact CC layer oocyte, the oocytes contained more than five layers of compacted CCs; type 2 multi CC layer oocyte, the oocytes were surrounded with three to five layers of CCs; type 3 partial CC layer oocyte, the oocytes were partially covered with some CCs; type 4 completely denuded oocyte, the oocytes were completely denuded of CCs; and type 5, degenerated oocyte (Areekijseree and Vejaratpimol, 2006). Table 2 shows the amounts and percentages of each type of pCOC. The small, medium, and large follicles contained types 1 and 2 pCOCs at high percentages of 54.68%, 69.06%, and 68.57%, respectively.

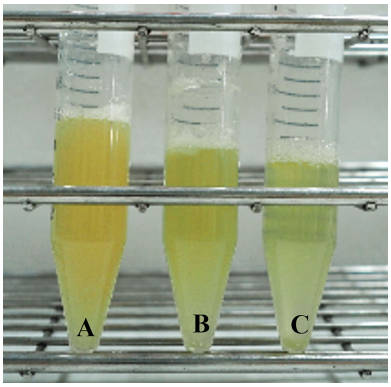

Figure 6. Photographs showing the follicular fluid of (A) small, (B) medium, and (C) large follicles

Table 2. Percentages of each type of COCs classified from small, medium, and large follicles

Type of pCOCs	Follicle sizes					
	Small		Medium		Large	
	No. of COCs	Percentage (%)	No. of COCs	Percentage (%)	No. of COCs	Percentage (%)
1	453	15.57	160	30.19	21	30.00
2	1138	39.11	206	38.87	27	38.57
3	491	16.88	71	13.39	10	14.29
4	580	19.93	70	13.21	6	8.57
5	248	8.52	23	4.34	6	8.57
Total	2910	100.00	530	100.00	70	100.00

A total of 14 pigs could be classified as either in the follicular phase (n=8) or luteal phase (n=6). The obtained follicles (n=92) were categorized as small (n=76), medium (n=14) and large (n=2) follicles. The morphological features of the intact CC layer oocytes from all follicle types differed in the amount and distribution of CC layers. Intact CC layer oocytes isolated from large follicles have more CC layers and more distribution of CC layers than those isolated from medium and small follicles (Figure 7). The mean diameters of type 1 oocytes from small, medium, and large follicles were 217.47±20.40 µm, 260.57±26.49 µm, and 309.71±66.76 µm, respectively (Figure 8 A). The mean diameters of type 2 oocytes from small, medium, and large follicles were 171.78±12.22 µm, 207.43±21.96 µm, and 215.91±24.65 µm, respectively (Figure 8 B). The mean diameter of type 3, 4, and 5 oocytes was 158±12.71 µm, 154.17±7.61 µm, and 148.55±6.39 µm, respectively (Figure. 9).

The histological study of the porcine ovary revealed that the free surface of the ovary was covered by germinal epithelium, comprising simple cuboidal epithelium. The ovary had tunica albuginea (dense irregular connective tissues). The cortex contained ovarian follicles and the corpus luteum. The medulla contained large blood vessels, lymphatics, and nerves. Primordial follicles contained a primary oocyte with simple squamous epithelium (Figure 10 A). Primary follicles contained a primary oocyte surrounded by a single layer of follicle cells (unilinear) and multiple layers of follicle cells (multilaminar) (Figure 10 B). Secondary follicles had more follicular cells, which contained fluid in intracellular spaces (follicular fluid) (Figure 10 C). Graafian follicles contained a secondary oocyte. The follicular fluid filling the single space was called the antrum (Figure 10 D). In the corpus luteum, the granulosa cells were enlarged and vesicular (Figure 11 A). The corpus albicans was pale; it continued to shrink, eventually forming a small scar on the side of the ovary (Figure 11 B).

3.3 Investigation of protein pattern in the follicular fluid

The protein pattern in the follicular fluid was investigated using SDS-PAGE. Follicular fluids from all follicle types contained proteins of molecular weight of 23, 50, 100, 150, 225, and >225 kDa. In addition, proteins of molecular weight of 12 and 16 kDa were observed only in the large follicles. The intensity of protein bands increased from small to large follicles (Figure 12).

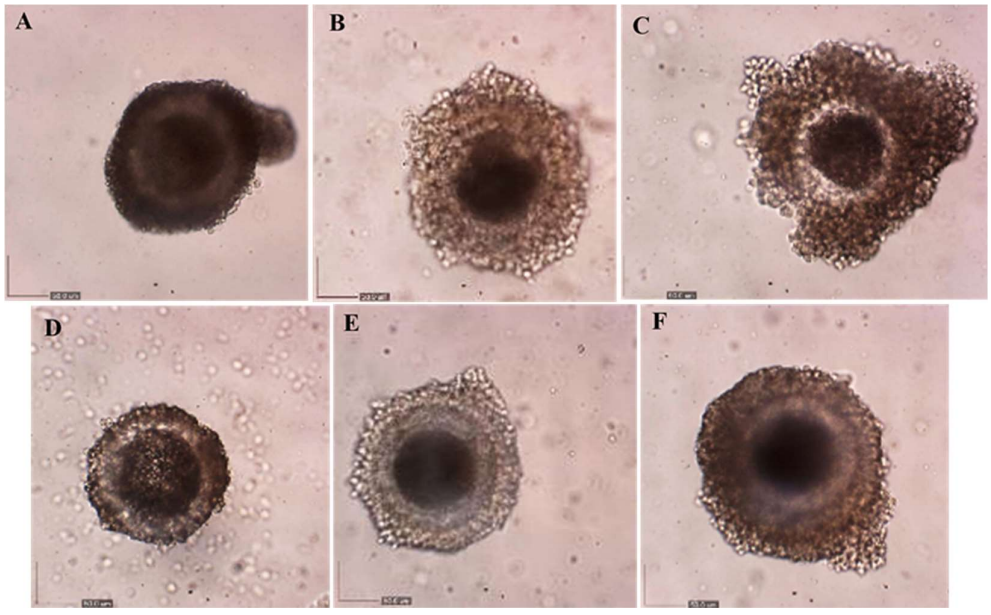

Figure 7. Photographs showing the morphological features of type I COCs from (A) small, (B) medium, and (C) large follicles, and of type II COCs from (D) small, (E) medium, and (F) large follicles

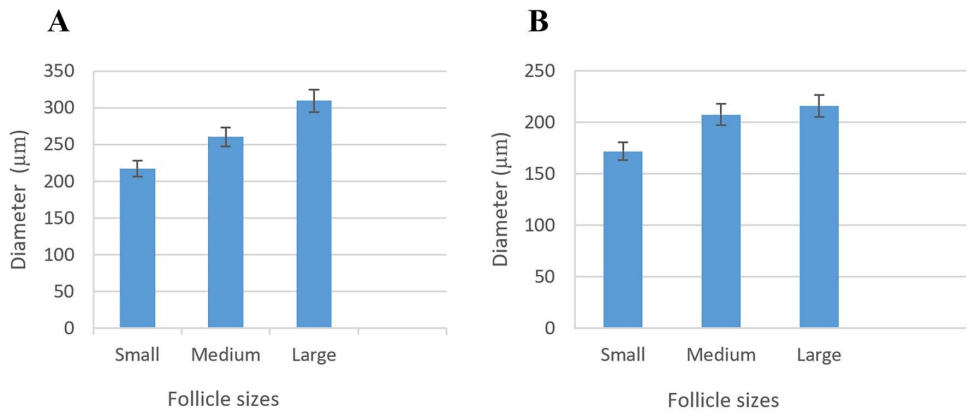

Figure 8. Mean diameters of (A) intact cumulus cell layer oocytes isolated from small, medium, and large follicles and (B) multi cumulus cell layer oocytes isolated from small, medium, and large follicles

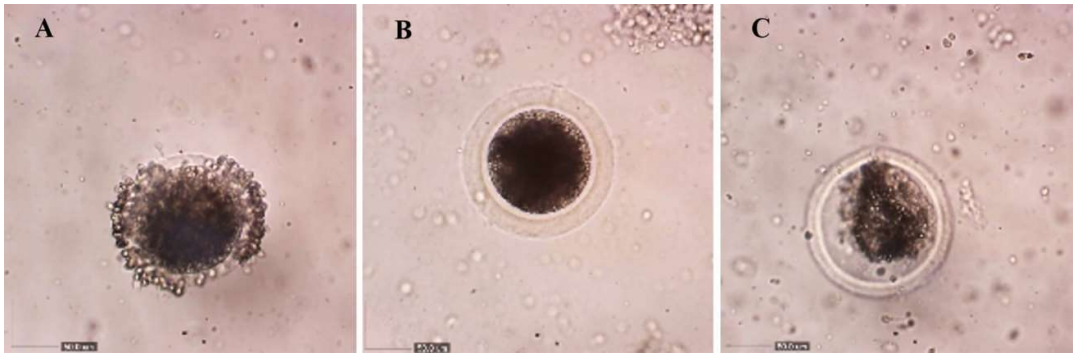

Figure 9. Photographs showing the morphological features of (A) partial cumulus cell layer oocytes, (B) completely denuded oocytes, and (C) degenerated oocytes

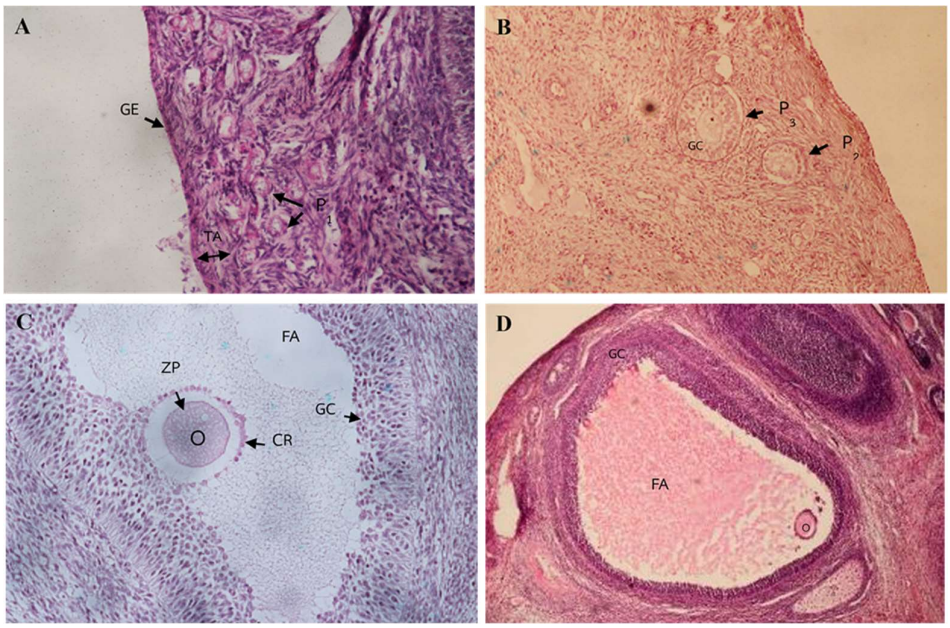

Figure 10. Photographs showing (A) an ovary with primordial follicles (P_1), the germinal epithelium (GE), and tunica albuginea (TA); (B) a primary follicle with unilinear primary oocytes (P_2), multilaminar primary oocytes (P_3), and granulosa cells (GC); (C) a secondary follicle with oocytes (O), GC, corona radiata (CR), zona pellucida (ZP), and follicular antrum (FA); and (D) a Graafian follicle with oocytes (O), GC, and FA

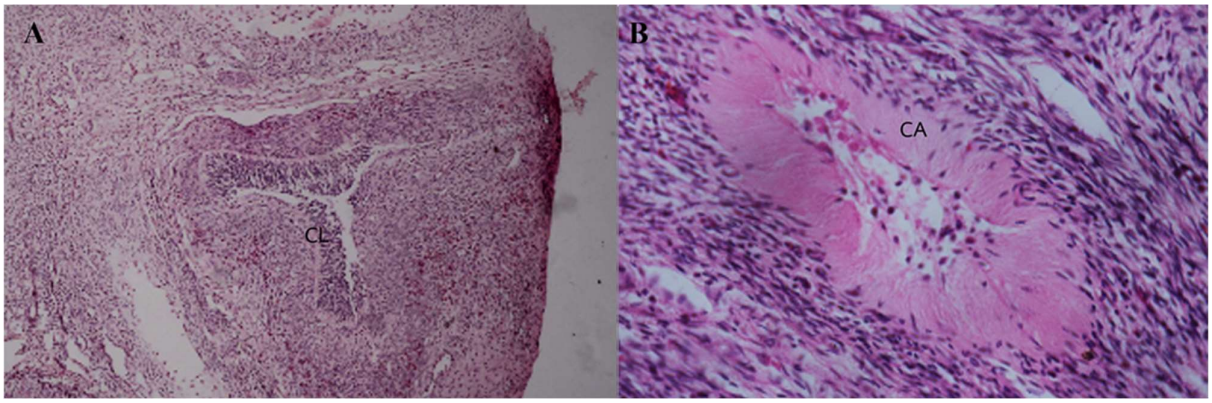

Figure 11. Photographs showing (A) corpus luteum (CL) and (B) corpus albicans (CA)

4. DISCUSSION

The results from the histological investigation revealed that the ovary and oviduct were supplied with arterial blood both from the ovarian and oviduct arteries and their branches. Comparisons among the three follicle types showed that the ovary with small ovarian follicles had fewer mesosalpinx blood vessels than that with medium and large follicles. Ovarian blood vessels supply oxygenated blood, hormones, and nutrients, which are important for oocyte maturation in the ovaries. The number of maturing oocytes in ovaries containing large follicles was higher than those containing medium and small follicles. Accordingly, large follicles contained a larger blood supply, which was necessary for oocyte maturation.

The follicular fluid color of small follicles was darker than that of medium and large follicles. This may be because the follicular fluid was derived from blood flowing through the theca capillaries. Fewer capillaries were observed in the region of the ovarian cortex containing small follicles than that containing large follicles (Rodgers and Irving-Roders, 2010). Subsequently, small follicles contained lesser fluid than medium and large follicles, resulting in a darker color fluid.

The morphological observation of pCOCs of the small, medium, and large follicles revealed that the overall recovery rate was 49 oocytes per ovary. Based on the number of the surrounding CC layers, COCs were characterized into five types: intact CC layer, multi CC layer, partial CC layer, completely denuded oocyte, and degenerated oocyte. The results from the present study revealed that the number of COCs containing small follicles was higher than that containing medium and large follicles. Thus, follicular atresia affected all developmental follicles; however, the proportion of follicles that became atretic decreased as the follicle size increased (Gosden and Spears, 1997).

The type 1 intact CC layer oocytes contained compacted CCs with more than seven layers and uniform cytoplasm. Large follicle-isolated type 1 intact cumulus layer oocytes were covered with more CCs than type 1 oocyte isolated from medium and small follicles. The average diameter of type 1 oocyte isolated from large, medium, and small follicles was significantly different (p<0.05). Compared with type 1, type 2 multi CC layer oocytes were surrounded by three to five incomplete layers of CCs. There was no significant difference in diameter between type 2 oocytes isolated from large and medium follicles (p<0.05). However, there was significant difference in diameter between type 2 oocytes isolated from large and medium follicles and small follicles (p<0.05). During folliculogenesis, CC layers increased. The metabolic coupling of CCs to oocyte occurred via gap junctions by transferring several metabolites into the ooplasm, including regulatory signals. In mammals, a close connection between CCs and oocytes occurs during the maturation period. In addition, gonadotropins, steroids, and other factors from follicular cells interact with oocytes, providing essential support for oocyte maturation. Oocyte maturation support by CCs continues until the metaphase II stage, where cytoplasmic maturation is greatly promoted (Tatemoto et al., 2000). Leroy et al. (2004) determined the biochemical compositions of dairy cattle follicular fluid harvested from three classes of non-atretic follicles, which were <4 mm, 6-8 mm, and >10 mm in diameter. The results showed that increased glucose, β-OHB, and cholesterol levels were observed from small to large follicles, whereas potassium, chloride, lactate, urea, and triglycerides levels were decreased. Their findings suggested that the growth and maturation of oocyte and granulosa cells correlate with the biochemical environments in the follicles. A study on gene expression in oocytes obtained from large (≥8 mm) and medium (3-7 mm) follicles was conducted by Kwak et al. (2012). They found that CCs derived from large follicles showed higher mRNA expression of proliferating cell nuclear antigen (PCNA) than the derived CCs from small follicles. The function of PCNA is related to the initiation of follicle growth (Oktay et al., 1995). This might explain the fact that intact and multi CC layer oocytes isolated from large follicles had significantly larger diameters than those isolated from medium and small follicles.

The observation of type 4 partial CC layer oocytes revealed that these oocytes were partially covered with CCs and that the CCs were loosely connected to ZP. The cytoplasm was initially faint in color. They subsequently became completely denuded oocytes, where the cells had clear cytoplasm. Finally, these completely denude oocytes turned into degenerated oocytes with cytoplasmic shrinkage (Areekijseree and Chuen-Im, 2012; Youngsabanant-Areekijseree et al., 2019).

From the total 14 pigs, high percentages of pCOC type 1 and type 2 were obtained from small (54.68%), medium (69.06%), and large follicles (68.57%). This is comparable with Areekijseree and Chuen-Im (2012) study, in which the total amount of pCOC type 1 and type 2 was 68.18%.

The types 1 and 2 pCOCs, containing intact CC layers and multi CC layers, respectively, can be cultured in medium supplemented with 10% HTFCS, 2.2 mg/mL $NaHCO_3$ in 1 M HEPES, 0.25 mM pyruvate, 15 µg/mL pFSH, 1 µg/mL LH, 1 µg/mL estradiol with ethanol, and 50 µg/mL gentamycin sulfate. These two cell types demonstrated high potential in developing into matured oocytes (Pongsawat and Youngsabanant, 2019). Davachiet et al. (2012) investigated the effects of the number of CC layers on the *in vitro* oocyte maturation, cytoplasm quality, and cumulus expansion of ovine oocytes. They categorized oocytes into four classes (Classes I-IV with more than five, three to four, one to two, and no CC layers, respectively). The cells were separately cultured in TCM 199 medium for 24 h. The highest percentages of cumulus expansion for Classes I-III oocytes were 53.0%±1.0%, 36.3%±2.2%, and 16.3%±1. 8%, respectively. The meiotic resumption rates of Class I-IV oocytes were 77.0%±2.7%, 77.2%±1.9%, 53.0%±2.1%, and 2.7%±1.1%, respectively. The proportions of oocytes with a cytoplasm quality (a dark cytoplasm, cytoplasmic granularity, cytoplasmic vacuoles, refractile bodies in the cytoplasm, smooth endoplasmic reticulum in the cytoplasm) in Class I-IV oocytes were 62.8%±1.5%, 59.4%±1.2%, 36.4%±2.1%, and 0.5%±1.1%, respectively. COCs with more than three CC layers were appropriate for *in vitro* maturation for application in the biotechnology field but not partial COCs and denuded COCs. Consequently, the oocytes for using *in vitro* maturation in the biotechnology field can be obtained from types 1 and 2 pCOCs, categorized from morphological features and diameter

pOECs were found as clusters and free columnar ciliated cells and spherical nonciliated cells. This is in agreement with Areekijseree and Vejaratpimol (2006). They discovered similar results in an *in vivo* study after a 24-h culture. pOECs that were found in clusters contained two different cell types, including columnar ciliated and spherical nonciliated cells. After culture for 48 h, columnar ciliated cells were isolated, whereas spherical nonciliated cells were clearly visible. This is consistent with our previous report (Areekijseree et al., 2005), in which changes in the pOEC morphological features and population of cell types occurred during the estrous cycle. Compared with those in the luteal phase, pOEC in the follicular phase appeared in greater numbers of long ciliated cells. However, pOEC in the luteal phase was covered with many spherical nonciliated cells; the cells contained short microvilli on the apical surface. *In vitro* coculture of pOEC with oocytes can support oocyte maturation and enhance normal fertilization, sperm capacitation, and early embryonic development. Reed et al. (1996) investigated pig embryos cocultured with pOECs, porcine fetal fibroblast monolayer using a combined pOEC, and a PEF coculture system (PEF-pOEC). However, the embryo development rate did not differ (p<0.05) between pOEC and PEF-pOEC. These data indicated that the primary culture of POECs can be used in the enhancement of the *in vitro* development of an early cleavage stage pig embryo.

The results of protein separation on SDS-PAGE demonstrated proteins of molecular weights of 23, 50, 100, 225, and >225 kDa in the follicular fluids from small, medium, and large follicles. Moreover, 12- and 16-kDa proteins were found only in large follicles. In terms of band intensity, the 225-kDA protein bands were found at the same intensity in medium and large follicles. This 225-kDa protein is probably tenascin, which plays a role in vasculogenesis (Jones and Jones, 2000). Therefore, they should be found more in medium and large follicles than in small follicles. The highest band intensities of 50- and 23-kDa proteins were found in large follicles, followed by medium and small follicles. The 50-kDa protein is probably keratin and was the highest amount of protein in follicular fluid. This protein has an important function in folliculogenesis (Ducolomb et al., 2013). The 23-kDa protein band might be prolactin, which is involved in angiogenesis during follicular development (Castilla et al., 2010). A large amount of this protein was present in large follicles; such follicles contained a high number of mesosalpinx blood vessels between oviducts and ovaries. The 16- and 12-kDa protein bands appeared only in large follicles. The 16-kDa protein band is probably the fibroblast growth factor, an important factor for follicular development and luteinization (Buratini and Price, 2010). Therefore, it is expected only in large follicles with a tendency to ovulate. The 12-kDa protein band is probably a proteolytic enzyme, a domain of insulin-like growth factor binding protein-2. Inconsistent with our observations, other studies reported that large follicles contained more proteolytic activity than medium and small follicles (Monget et al., 2002).

5. CONCLUSION

The morphological features of porcine oviducts could be classified into three types. Type I, II, and III oviducts contained almost all small, medium, and large follicles, respectively. The present study found that most oviducts were of type I. All three oviduct types comprised three regions: the isthmus, ampulla, and infundibulum. The infundibulum had the largest diameter, whereas the isthmus had the smallest diameter. The morphology features of the porcine ovary and pCOCs were studied; the results showed that the oocytes were round-shaped with ZP and several layers of CCs. Based on the number of oocyte-surrounding CC layers, pCOCs obtained from all ovary types could be classified into five types. Small, medium, and large follicles contained types 1 and 2 pCOCs, which possessed high potential to develop into mature oocytes. The fluid in all follicles contained proteins of molecular weights of 23, 50, 100, 225, and >225 kDa. Proteins of molecular weights of 12 and 16 were detected in the fluid only in large follicles.

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