5. กิตติกรรมประกาศ (Acknowledgements)

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6. เอกสารอ้างอิง (References)

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เรณู เวชรัชต์พิมล 2540 **เทคนิคการเพาะเลี้ยงเซลล์สัตว์** ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศิลปากร นครปฐม หน้า 65-87

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Out put ที่ได้จากโครงการ

1. ผลงานวิจัยที่ตีพิมพ์ในวารสารระดับนานาชาติ

1.1 ตีพิมพ์งานวิจัยเรื่อง *In vivo* and *in vitro* study of porcine oviductal epithelial cells, cumulus oocyte complexes and granulosa cells: a scanning electron microscopy and inverted microscopy" ในวารสาร Micron เป็นวารสารของประเทศสหรัฐอเมริกา **มี impact factor 1.537 (ปี 2002)** (ผลงานวิจัยฉบับเต็มอยู่ในภาคผนวก)

1.2 ตีพิมพ์งานวิจัยเรื่อง "Morphological features of porcine oviductal epithelial cells and cumulus-oocyte complexes" ในวารสาร The Kasetsart Journal (Nat. Sci.) Volume 39 Number 1 (January-March 2005) ซึ่งเป็นวารสารที่เป็น International Journal และ post อยู่ใน CABI Publishing และ Thailand Citation Index Centre (TCI) **มีค่า Impact factor = 0.06 (2004)** (ผลงานวิจัยฉบับเต็มอยู่ในภาคผนวก)

2. กิจกรรมอื่น ๆ ที่เกี่ยวข้อง

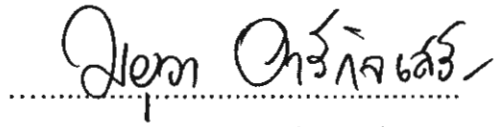
2.1ผู้วิจัยได้นำเสนอผลงานวิจัยรูปแบบโปสเตอร์ในการประชุมวิชาการระดับประเทศเรื่อง "Porcine oviductal epithelial cells and cumulus-oocyte complex observance" ในการประชุม 22th EMST Annual Conference ของ Microscopy Society of Thailand เมื่อวันที่ 2 ถึง 4 กุมภาพันธ์ พ.ศ. 2548 ณ มหาวิทยาลัยบูรพา จังหวัดชลบุรี (ผลงานวิจัยอยู่ในภาคผนวก)

2.2 ผู้วิจัยได้นำเสนอผลงานวิจัยในรูปแบบโปสเตอร์ในการประชุม "นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส" จัดโดยสำนักงานกองทุนสนับสนุนการวิจัยและสำนักงานการอุดมศึกษา ณ.โรงแรมรีเจน ชะอำ จังหวัดเพชรบุรี ระหว่างวันที่ 13-15 ตุลาคม พ.ศ. 2548 (ผลงานวิจัยอยู่ในภาคผนวก)

2.3 ผู้วิจัยได้รับเชิญเป็นวิทยากรในหัวเรื่อง "นักวิทยาศาสตร์พบนักเรียนวิทยาศาสตร์" ณ หอประชุมใหญ่โรงเรียนพรหมานุสรณ์ จังหวัดเพชรบุรี ในวันที่ 18 พฤศจิกายน พ.ศ. 2548 ได้บรรยายถึงเรื่องการเป็นนักวิจัยรุ่นใหม่ของ สกว. และงานวิจัยที่ได้ศึกษาให้นักเรียนมัธยมปลายสายวิทยาศาสตร์รับฟัง

2.4 ผู้วิจัยจะได้นำเสนอผลงานวิจัยในรูปแบบ Oral presentation ในการประชุมที่จะจัดขึ้นโดยสำนักงานกองทุนสนับสนุนการวิจัยและสำนักงานการอุดมศึกษา ต่อไป

ลงนาม



ผศ. ดร. มยุวา อารีกิจเสรี

(หัวหน้าโครงการวิจัยที่รับทุน)

ภาคผนวก

ผลงานวิจัยที่ตีพิมพ์ในวารสารระดับนานาชาติ

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In vivo and in vitro study of porcine oviductal epithelial cells, cumulus oocyte complexes and granulosa cells: A scanning electron microscopy and inverted microscopy study

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Abstract

...phology and structure of porcine oviductal epithelial cells (POEC), cumulus-oocyte complexes (COCs) and granulosa cells (GC) were ...ied in vivo and in vitro conditions using scanning electron microscopy (SEM) and inverted microscopy. The POEC contained columnar ...lls and spherical shaped non-ciliated cells. Both non- and ciliated cells appeared either in groups or distributing among each other. ...the isolation of cells was observed after culture for 48 h. A total of 921 oocytes from 20 ovaries was isolated resulting in an average of 46 ...per ovary. They were round in shape, surrounded by zona pellucida with layers of cumulus cells ranging between 89.16 and 144.68 μm in ...the COCs, they were classified into 4 types; intact-, multi-, partial-cumulus cell layers and completely denuded oocyte. Interestingly, changes ...nology of COCs with intact and multi-cumulus cell layers were observed in the in vitro study. The GCs in the follicular fluid were also round ...and found as clusters. After culturing in in vitro for 48 h, no change in morphology was observed. The GC appeared in smaller clusters or ...most as single cells and their sizes ranged from 6 to 8 μm. The results obtained from this study allow us to have a better understanding of the ...gy and nature of cells under both in vivo and in vitro conditions. This information is also important for the study of their secretions and ...ical compositions, which is of great importance to the use of cells as feeder cells in in vitro fertilization in current studies.

...Cumulus cell complexes; Granulosa cells; Porcine oviductal epithelial cells; Scanning electron microscopy

...oduction

...malian oviductal epithelial cells and granulosa cells ...an be isolated and cultured in culture medium. ...ger cultures of these cells have been widely used for ... co-culture because they have a direct effect and ...on which contribute to the success of fertilization ...ntt et al., 1993, 1995; Vatzias and Hargen, 1999; ...et al., 2001, 2003; Kidson et al., 2003; Lucidi et al., ...In animals that produce several offspring at one time ... pigs or rodents, there are some secretions in the ...ctive tract of the animals and these secretions were ... facilitate better growth and development of embryos of ...cies (Kitiyanant et al., 1993). Since the reproductive ...of pigs are not used as human food, they are readily ...e and can be readily collected from slaughterhouses for research. Kitiyanant et al. (1993) reported on the use of porcine oviductal epithelial cells (POEC) to support in vitro bovine embryo development. Besides, there is also evidence that porcine oviductal cells can support in vitro maturation in sow oocytes and fertilization (Kidson et al., 2003).

Cumulus cells and GC are cells that are found surrounding an oocyte and lining antral follicles. Both cells promote the penetration of spermatozoa into the oocyte by inducing an acrosome reaction via its expansion of the cell mass (Anderson and Killian, 1994; Mattioli et al., 1998; Romar et al., 2001) leading to a higher rate of in vitro fertilization (Romar et al., 2001, 2003). In addition, these cells are very useful in the determination of the toxic effects of the environment which could be accumulative and lead to abnormal development of an embryo. Moreover, it is ethically more acceptable.

To be able to make the best utilization of cumulus cells, POEC and GC, attempts have been made have been made to thoroughly study their characteristics. Unfortunately, basic knowledge on the morphological aspects of these cells both in vivo and in vitro is poorly characterized, including the

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...n an oocyte and its cumulus complex during ... cumulus maturation in vitro. Thus, the ... work were to establish the culture technique ...lus oocyte complexes (COCs) and GC and to ...ological features of in vivo and in vitro POEC, ... by scanning electron microscopy (SEM) and ...copy.

... and methods

...edium

...edium, consisting of M199 with Earle's salts ..., St Louis MO) supplement with 10% heat-... serum (HTFCS), 2.2 mg/mL $NaHCO_3$ (Sigma ... Louis MO), 1 M Hepes (Sigma, St Louis MO), ...ate, 15 μg/mL porcine follicle-stimulating ...mL LH, 1 μg/mL estradiol with ethanol and ...gentamycin sulfate. The culture medium was ...7 °C, 5% CO_2, 95% air atmosphere with high ...h before use.

...COCs and GC collection and preparation

...and ovary collection

...ducts and ovaries of Large White pigs were ... local slaughterhouses. They were removed ... of slaughter and transported to the laboratory ... a thermos containing a saline solution kept ...35 °C. The saline solution consisted of 0.9% ...supplemented with 100 IU/mL penicillin, 100 μg/... and 250 μg/mL amphotericin B).

...collection

...were trimmed free from fat and connective tissues ...times in a washing medium (TALP-HEPES ...with 10% HTFCS and 50 μg/mL gentamycin). ...rated into 2 groups. The first group of oviducts ...small pieces (2–3 mm) and prepared for SEM ... The second group was used for in vitro culture ...ph), and oviducts were placed in sterile Petri ... were gently scraped with a sterile glass slide ...of isthmus to the ampula for extracting porcine ...helium mucosal cells from the lumen of oviduct. ...cales were transferred to a 12 mL sterile conical ...be containing 10 mL of washing medium. They ...7 times and then resuspended in the culture ...ratio of 1:50. Ten milliliters of the suspension ...in a 60 mm Falcon culture dish and cultured at ...% CO_2, 95% air atmosphere and high humidity ... The POEC viability was determined by trypan ... v/v, final concentration) using an inverted ...

...and GC collection

...aries of Large White pigs were trimmed free ...ive tissues and rinsed 3 times in washing medium. The follicular contents of selected healthy follicles of 4–6 mm in diameter were aspirated by a 5 mL disposable syringe with an 18-gauge needle containing saline solution. The follicular contents were pooled in sterile Petri dishes. After sedimentation, COCs were recovered under a stereomicroscope. They were allocated to different groups depending on the number of cumulus cells layers. The diameters of COCs were measured. The COCs were washed 2 times with the washing medium and separated into 2 groups. The first group was studied under inverted microscopy and prepared for SEM (in vivo group). The second group was cultured in small drops of culture medium (100 μL) at 37 °C with 5% CO_2, 95% air atmosphere and high humidity for 48 h. After incubation, COCs were studied under inverted microscopy and prepared for SEM (in vitro group). Meanwhile, GC in the follicular fluid were pooled in 12 mL conical tubes. They were washed 7 times with the washing medium and separated into 2 groups. The first group of GC was studied under inverted microscopy and prepared for SEM (in vivo group). The second group was cultured in the culture medium at a ratio of 1:50. Ten milliliters of GC suspension were placed in a 60 mm Falcon culture dish and cultured at 37 °C with 5% CO_2, 95% air atmosphere and high humidity for 48 h. Granulosa cells viability was determined by trypan blue (0.4%, v/v, final concentration) with an inverted microscope and GCs were prepared for SEM (in vitro group).

2.3. POEC, GC and COCs preparation for SEM

All samples were pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffer at pH 7.2 for 2 h, rinsed in phosphate buffer and post-fixed in 1% osmium tetroxide for 24 h. They were then dehydrated in a graded series of ethanol (30%, 50%, 70% 80%, 90% and absolute ethanol) and dried in a critical point dryer machine. They were then mounted on stubs with conductive carbon tape, coated with gold particle at 20 nm thickness in an ion sputtering, observed and examined under SEM (CamScan Analytical, Maxim 2000S) operating at 10–15 kV.

2.4. Statistical analysis

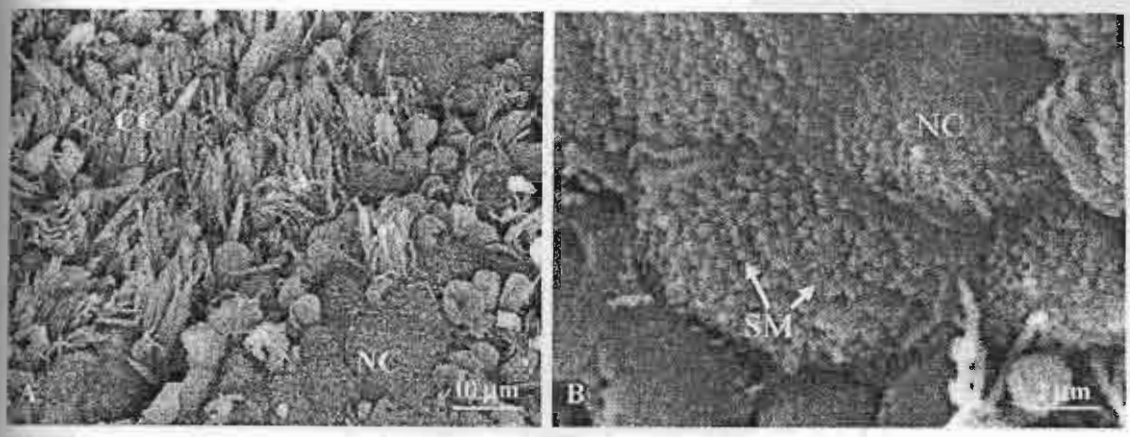
Mean and standard deviation of the means of each diameter of COCs were calculated. Statistical analysis at 95% significance level was determined using analysis of variance (ANOVA), and multiple comparisons were analyzed by Student-Newman-Keuls (SNK).

3. Results

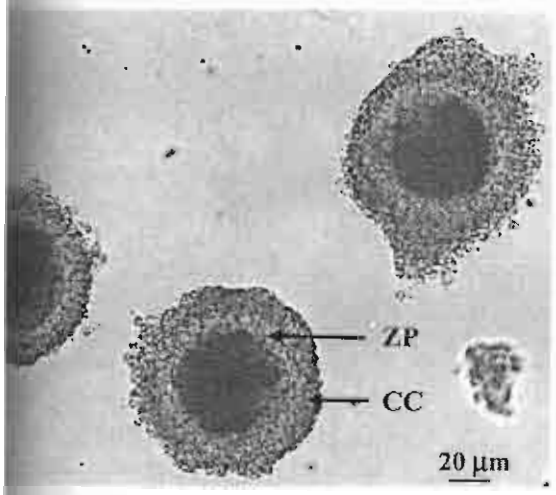
3.1. In vivo study

3.1.1. POEC observation

From SEM observation (Fig. 1A), the surface of porcine oviductal epithelium contained two different cell types, ciliated cells and non-ciliated cells. The ciliated cells appeared either in groups or were found to be distributed among the non-ciliated cells. As could be clearly seen in the micrograph, the cilia of the



micrograph of POEC (A); showing numerous non-ciliated cells (NC) and ciliated cells (CC). At high magnification (B); non-ciliated cells of spherical short microvilli (SM) on the apical surface.



graph of COCs showing round shape of oocytes surrounded with (ZP) and several layers of cumulus cells (CC).

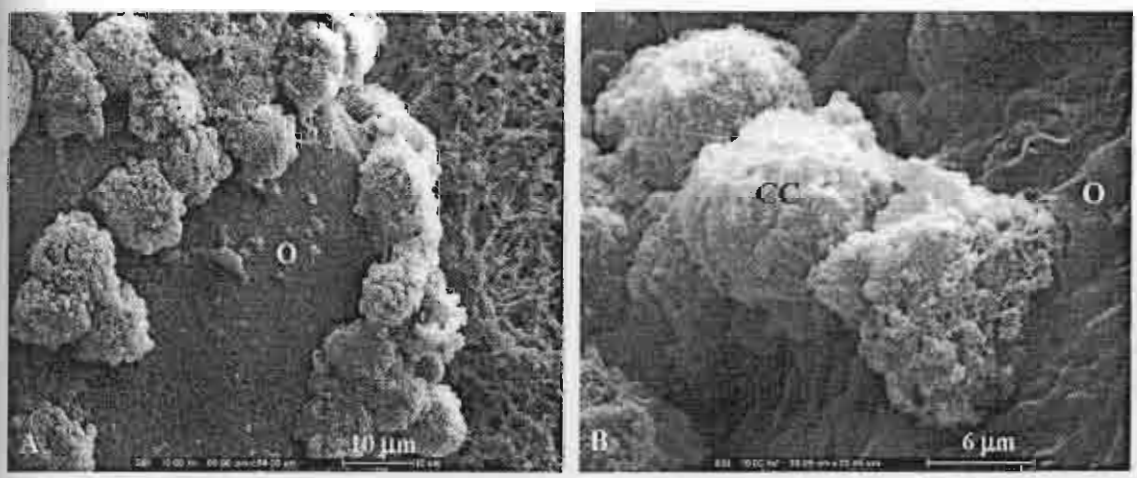
lls consistently project themselves above the apex of liated cells. At high magnification, non-ciliated cells ly seen as spherical shape cells with numerous short (Fig. 1B).

3.1.2. COCs observation

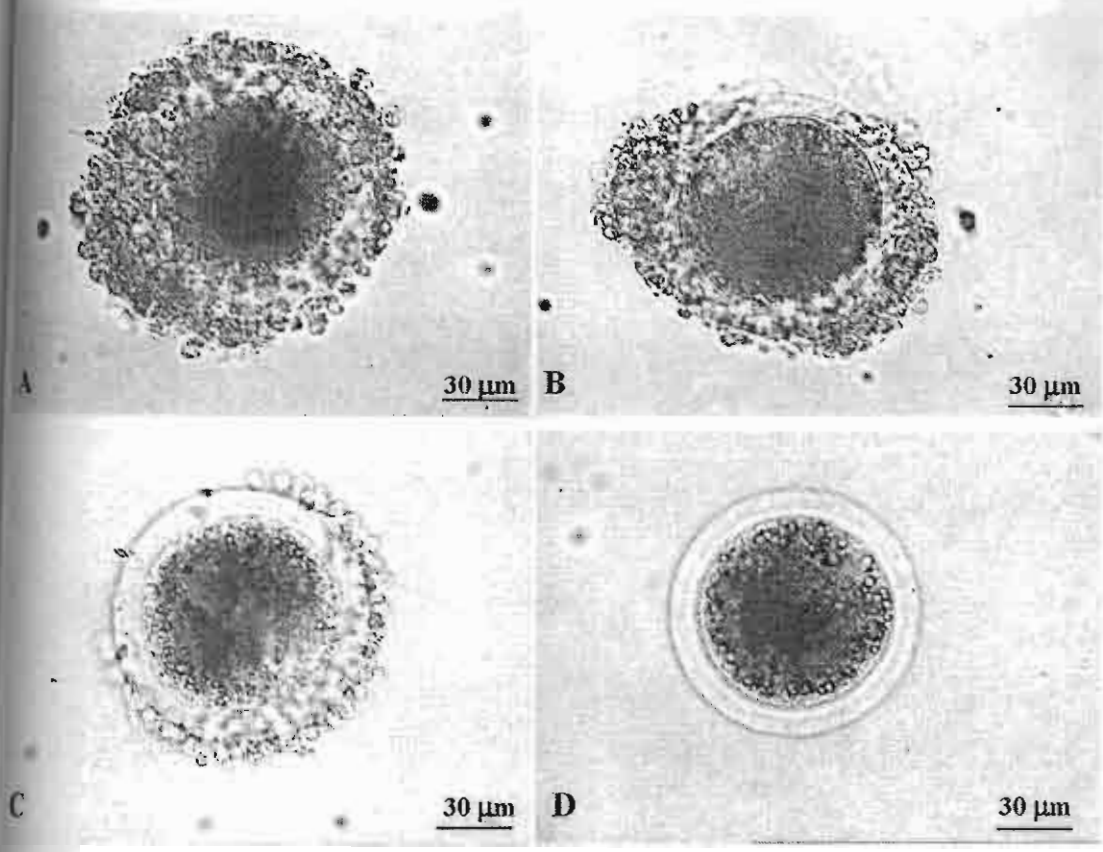
A total of 921 oocytes were isolated from 20 ovaries: an average of 46 oocytes per ovary. Fig. 2 shows observation of the COCs collected from the follicular fluid using inverted microscopy. The oocytes were round in shape and ranged between 89.16 and 144.68 µm in size. They were surrounded by a zona pellucida and several layers of cumulus cells.

By using SEM, the surface appearance of both oocytes and cumulus cells from COCs was observed. As can be seen in Fig. 3A, oocytes and cumulus cells have distinct surface appearance. Similar to what was observed in the oocytes, the cumulus cells were round in shape and they contained no microvilli on the surface membrane (Fig. 3A and B).

Based on the number of cumulus cell layers surrounding the oocyte, these COCs were classified into 4 types as follows (Fig. 4): Type I: Intact cumulus cells layer. The oocytes of this type were at an early development stage. Several compact layers of cumulus cells were seen on the surface of these oocytes and the cytoplasm of the oocytes was homogeneous with a dark zone around the periphery. This complex type was found in secondary follicles (Fig. 4A). Type II: Multi cumulus cell layer. The oocytes contained 2–3 incomplete layers of



micrographs of COCs collected from the follicular fluid (A); the cumulus cells (CC) on surface of the oocyte (O). At high magnification (B; showing of cumulus cells and non-ciliated cells.



of COCs collected from the follicular fluid (A); intact cumulus cell layer; (B); multi cumulus cell layer; (C); partial cumulus cell layer; and (D); oocyte.

(Fig. 4B). Type III: Partial cumulus cell layer. were partially covered with some cumulus cells. cells were loosely attached to the zona pellucida. was faint in color (Fig. 4C). Type IV: denuded oocyte. The oocytes were completely mulus cells. These oocytes were located in the . The cytoplasm was pale in color (Fig. 4D).

that 33.12% of these COCs has completely ytes, 28.88% were of the partial cumulus cell layer ose of multi cumulus cell layer and intact cumulus ypes were found at only 18.13% and 19.87%, Table 1).

diameters of four types of COCs were measured graphs ($n = 30$). It was found that, the mean intact cumulus cell layer from the healthy antral gnificantly different from those of multi cumulus cell layer, partial cumulus cell layer and completely denuded oocyte (144.68 ± 8.79 µm, 122.82 ± µm, 106.20 ± 8.72 µm and 89.16 ± 5.69 µm, respectively) (Fig. 5).

3.1.3. GC observation

Similar to the COCs, GCs were located in the follicular fluid. These cells did not have cilia and were present as either single

nd COCs based on number of cumulus cell layers surrounding the

	No. of COCs	Percentage (%)
cell layer	183	19.87
ell layer	167	18.13
ell layer	266	28.88
ded oocyte	305	33.12
	921	100.00

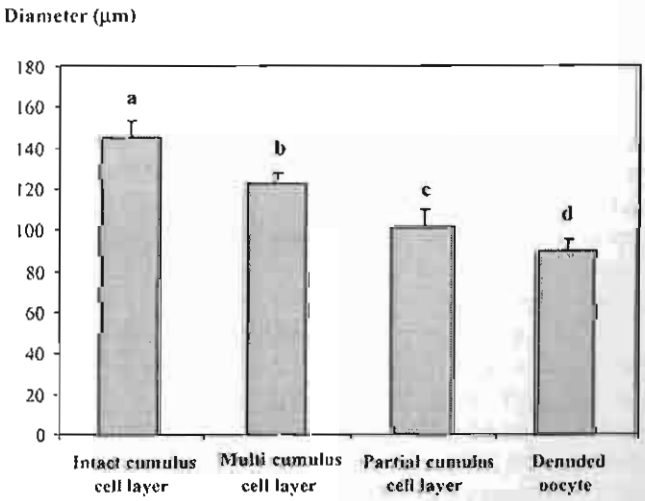
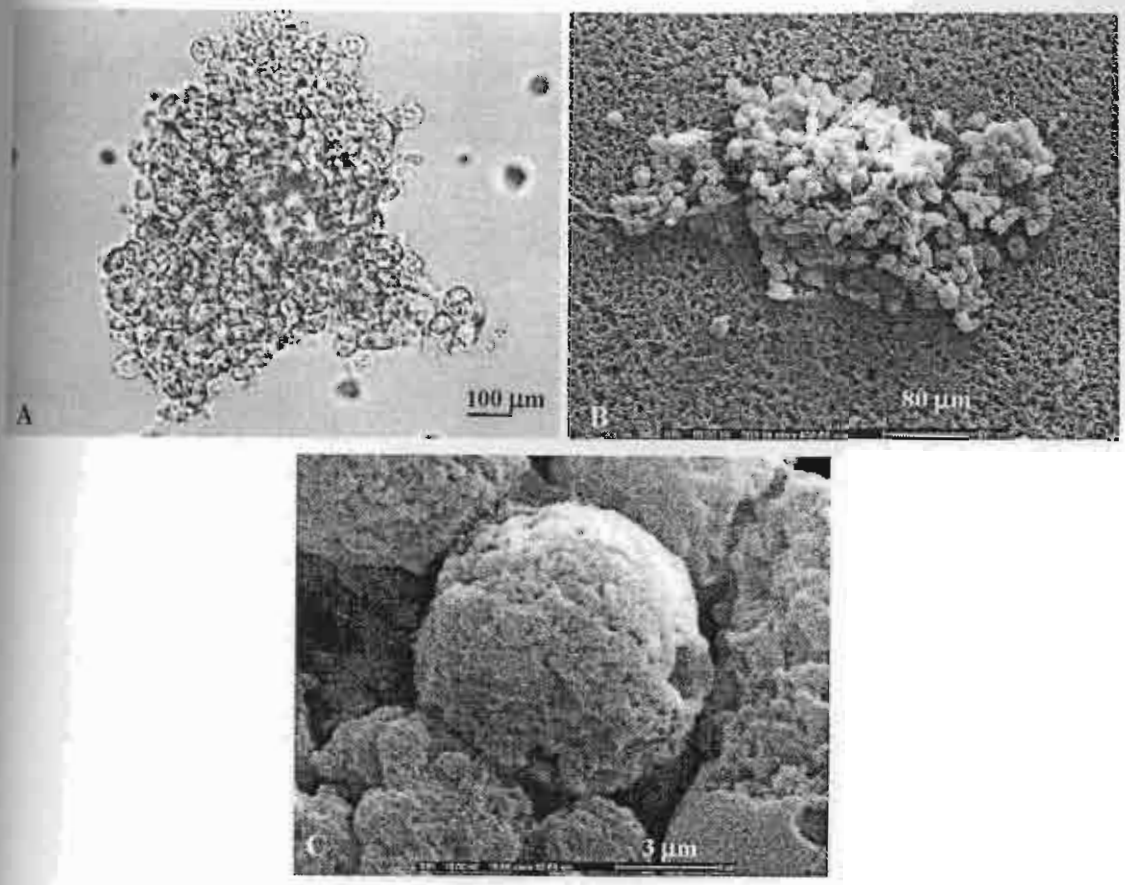


Fig. 5. Mean diameters of the isolated COCs from antral follicles. Different letters above the bar denote statistically significant differences ($P < 0.05$).



...tograph of GC (A); and SEM micrograph of GC (B) showing round shape of the cluster GC. At high magnification (C); showing the round shape of the ...C without microvilli.

...clusters. They were all round in shape and their sizes ...6–8 μm (Fig. 6).

...study of ultrastructures of POEC, COCs and

...inverted microscopic observation of POEC and GC, ...cultured in M199 with Earle's salts supplement with ...FCS, 2.2 mg/mL $NaHCO_3$, 1 M Hepes, 0.25 mM ..., 15 μg/mL porcine follicle-stimulating hormone, ... LH, 1 μg/mL estradiol with ethanol and 50 μg/mL ...cin, at 37 °C with 5% CO_2, 95% air atmosphere and ...idity for 48 h, the viability of these cells was found to ...94–95% (Table 2).

...of POEC and GC viability during culturing in the culture medium ...an ± S.E. (n = 3 replicates; replicates were different cell batches)

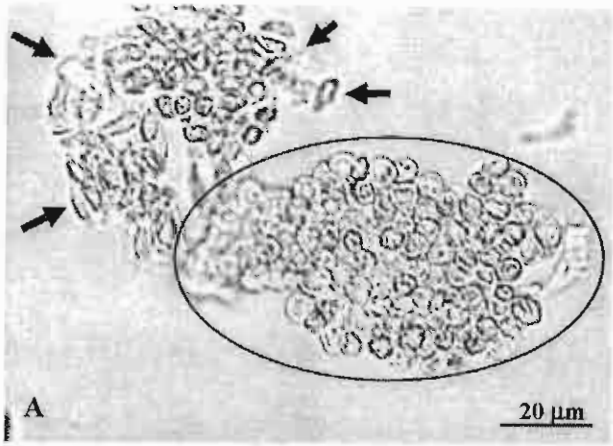
...e (h)	Percentage (%)	
	POEC	GC
	100.00	100.00
	96.33 ± 1.25	96.33 ± 1.70
	95.00 ± 0.82	94.00 ± 0.82

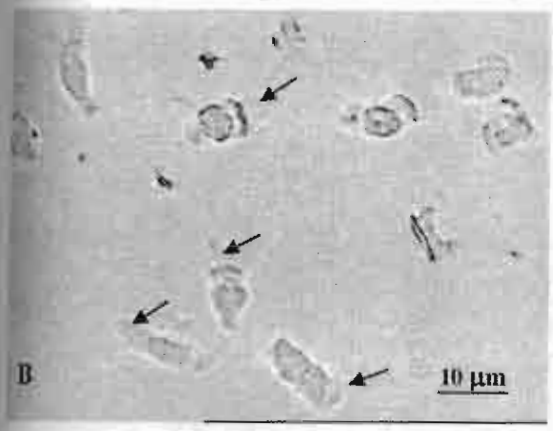
3.2.1. POEC observation

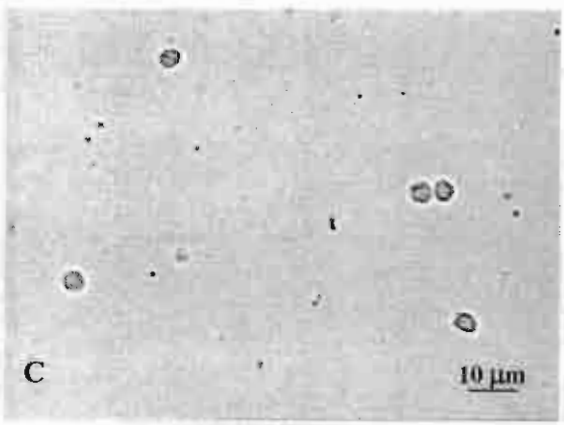
Similar to the observation in the in vivo study, after 24 h in culture, POEC were found in clusters (Fig. 7A) and contained two different cells types. They were columnar ciliated cells (Fig. 7A; arrows) and spherical non-ciliated cells (Fig. 7A; in a circle). Following longer in culture (48 h), the movement of columnar ciliated cells (arrows) in isolation from each other (Fig. 7B) and the isolated spherical non-ciliated cells were clearly apparent (Fig. 7C).

3.2.2. COCs observation

Cumulus cells of COCs classified as Type I (intact cumulus layer) and Type II (multi cumulus layer) were collected and cultured in M199 as described in Section 2. After culturing for 24 h, the cell complex was examined by SEM and compared with the cells from the in vivo condition. The cumulus cells from COCs Type I and Type II before culturing in the culture medium appeared spherical in shape (Fig. 8A and C). Interestingly, changes in the morphology of the complex were observed after culturing for 24 h. In Type I COCs the cumulus cell layer was peeling off from the oocyte (Fig. 8B), but the cell shape was still round. Moreover, it was also found that COCs Type II contained expanded cumulus cell shape, while the cells remained intact on the surface of the oocytes (Fig. 8D).







...graphs of POEC in culture medium for 24 h (A) showing columnar ciliated cells (arrow) and round shape non-ciliated cells (in circle); (B and C) POEC ...dium for 48 h showing moving isolated columnar ciliated cells and spherical cells.

...n additional 24 h culture of COCs in the culture ...it was observed that the round shape cumulus cells, ...ched to the surface of the oocyte before (Fig. 9A), ...d themselves into a teardrop-like shape. They had a ...ical end that clearly stuck into the oocyte (Fig. 9B). ...magnification, Fig. 10A and B illustrate formation of ... cells as a mesh-like structure with numerous ... covering the oocyte. Meanwhile, the elongated ...ls of COCs Type II were detached from the oocyte ...g. 11A and B).

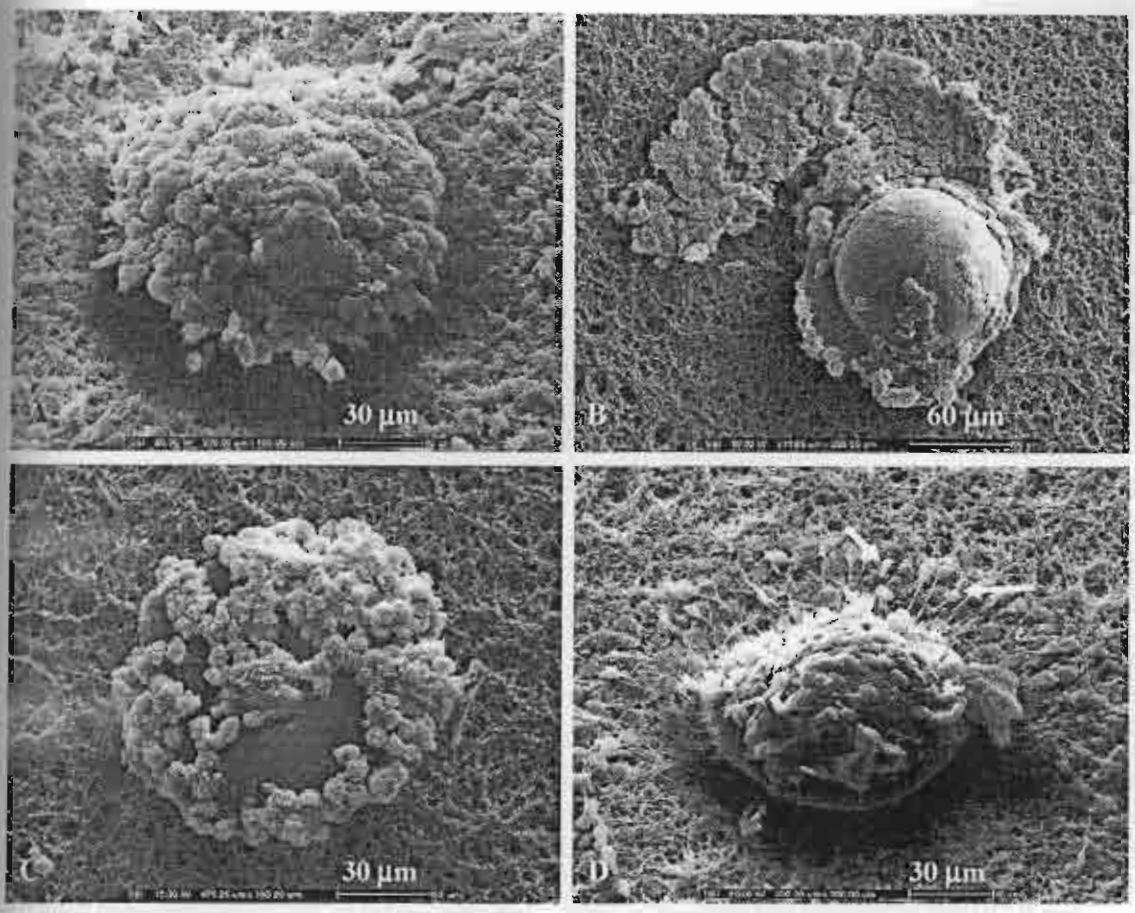
...bservation

...during in the culture medium for 48 h, there was no ...nge in morphology of the GC. Similar to those ...the in vivo condition, the cells were still all round in ...out microvilli but the GC clusters were present as ...gle cells or in smaller clusters (Fig. 12).

...sion

...ference in morphology and structure of POEC, ...d GC under in vivo and in vitro conditions was ...sing SEM and inverted microscopy. It was found ...o condition, POECs were composed of two types of ...epithelial cells, non-ciliated and ciliated cells. The ...y of the porcine epithelial cells was distinctly different from bovine. In bovine, oviductal epithelial cells form a "wormlike" structure (Xu et al., 1992) while those in pig were columnar ciliated cells and round the non-ciliated cells.

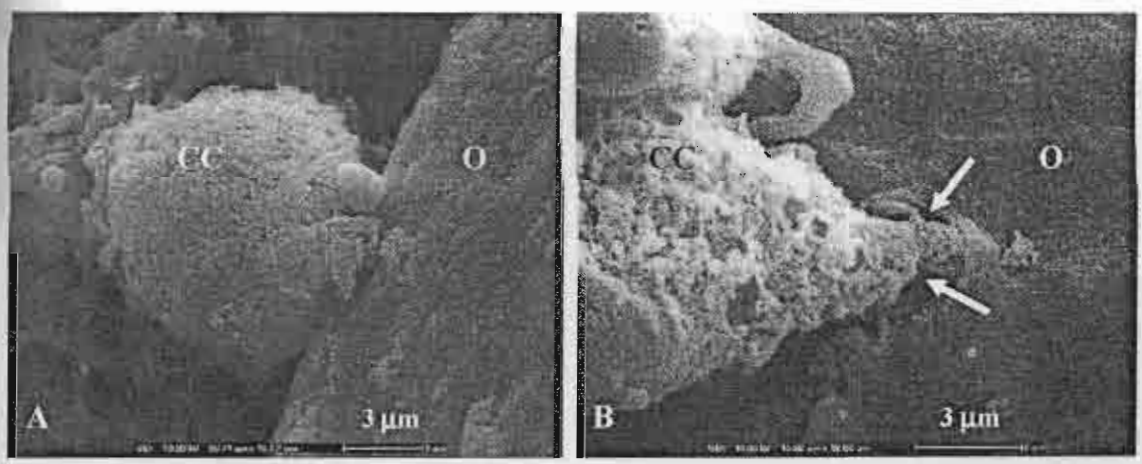
Since oviduct is the site of fertilization, it is found to contain more synthetic secretions (Murray, 1992) and a high number of columnar ciliated cells corresponding to the transportation of ovulated oocytes. Hole and Koos (1994) reported that, numerous round non-ciliated cells with short microvilli located in the apical surface also corresponded to the presence of secretory substance for nutritional support of embryonic development. A similar result was reported by Areekijseree et al. (2005) who found that POEC underwent changes in both the morphological features and the population of cell types during the estrus cycle. At the follicular phase, POEC contained a greater number of long ciliated cells than at the luteal phase. The luteal phase, however, was filled up with numerous round shaped non-ciliated cells having short microvilli on the apical surface. Under in vitro condition, POEC showed moving isolated columnar ciliated cells and non-moving spherical cells in the culture medium. The POEC were used in in vitro co-culture to support oocytes maturation and to increase normal fertilization, sperm capacitation and early embryonic development (Kitiyanant et al., 1993; Park and Sirard, 1996; Vatzias and Hargen, 1999; Romar et al., 2001, 2003). Nagai and Moor (1990) also suggested that glycoproteins secreted from non-



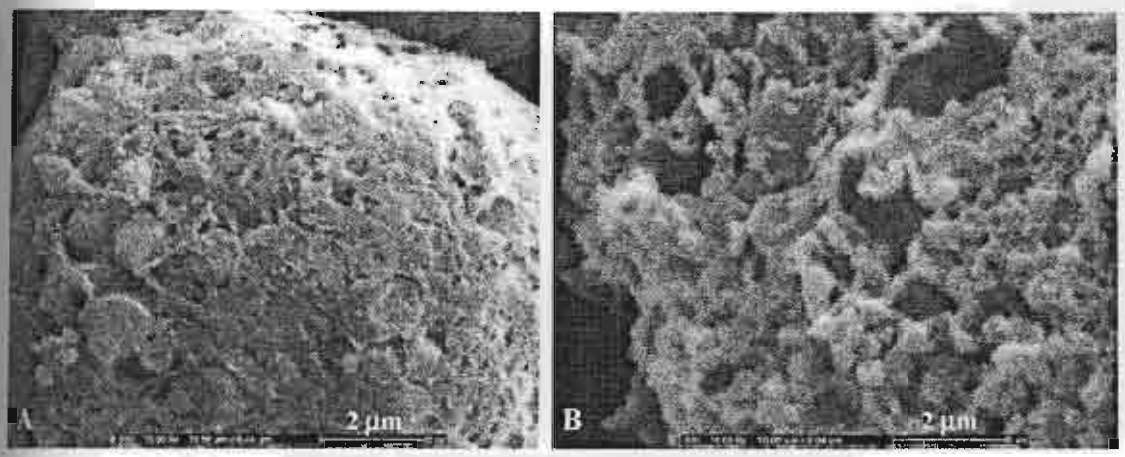
rographs of COCs showing (A and C) Type I and Type II before culture. Cumulus cells were peel off (B); or expanded (D) after culture in culture for 24 h.

oviductal epithelial cells could bind to the porcine and reduce the incidence of polyspermy.
udy exhibited that antral porcine follicular aspiration in the collection of a high number of oocytes with cumulus cells. The overall recovery rate was per ovary. The COCs were characterized into 4 types their accumulation and arrangement of cumulus cells around the oocytes; intact cumulus cell layer, multi cumulus cell layer, partial cumulus cell layer and completely denuded oocyte at the percentage composition of 19.87%, 18.13%, 28.88% and 33.12%, respectively. The diameters of the 4 types of COCs were 89–145 µm.

Mori et al. (2000) found that COCs Type I (intact cumulus cell layer) and Type II (multi-cumulus cell layer) had a higher



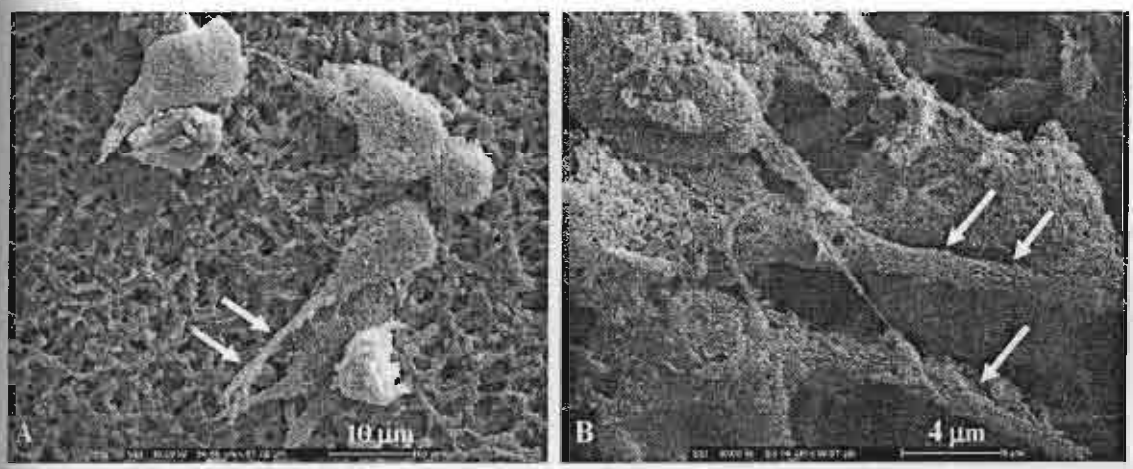
M micrographs (A: cumulus cells of COCs Type I showing round shape attached to the oocyte surface (cumulus cells: CC, oocyte: O). Cumulus cells of e I after culturing for 48 h showing; (B); the conical end (arrows) pointed towards the oocyte surface.



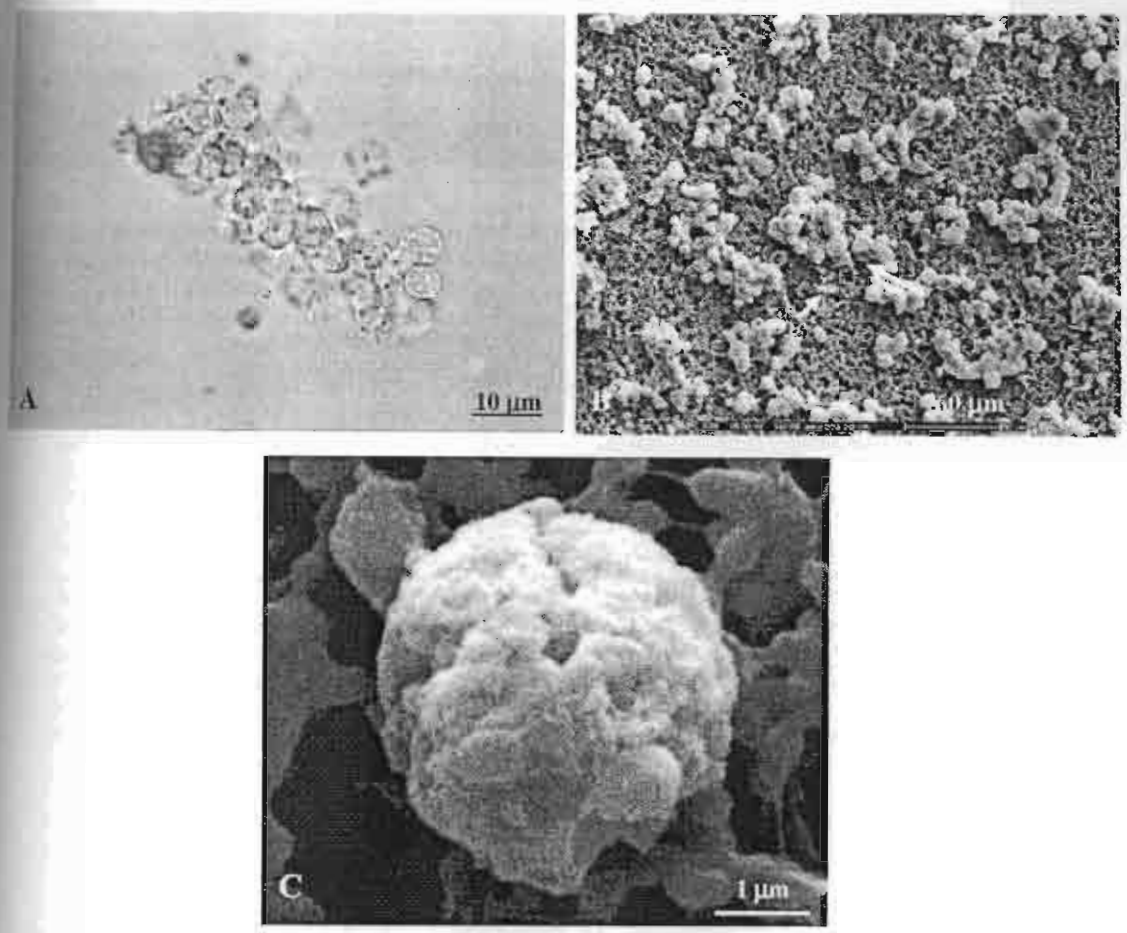
micrographs of cumulus cells (A and B) showing round shape of cumulus cells having mesh-like structure with numerous fenestrations on the surface.

become mature oocytes in vitro. These types of COCs fully cultured in the culture medium supplemented r stimulating hormone (FSH) and leutinizing H) using cell samples from rat (Magnusson, 1980), miller and Moor, 1984), bovine and swamp buffalo et al., 1989, 1995). Similarly, we cultured the COCs ype II in M199 supplemented with 10% HTFCS, $NaHCO_3$, 1 M Hepes (Sigma, St Louis MO), pyruvate, 15 μg/mL porcine follicle-stimulating μg/mL LH, 1 μg/mL estradiol with ethanol and gentamycin sulfate. The SEM study indicated that, of COCs Type I were peeled off and changed from into a tear-drop like structure. Meanwhile, COCs ed signs of expansion such as elongation of cumulus ulture for 24 h. It indicated that, the oocyte became culturing in the culture medium. After longer COCs Type II showed the elongation of cumulus cells detached from the oocyte.

ically, the antral follicles have two types of follicular so-called cumulus cells, which are adjacent to the the GC lining in the antral follicles. Both cells have tions. The GC were reported to secrete estradiol into ar fluid. It is known to function in transmitting low molecular weight substances, i.e., ion nucleotides and amino acids to oocytes in the young non-reproductive females. These substances are named oocyte maturation inhibitor (OMI) or meiosis arresting factor which arrests oocyte development at the diplotene stage of prophase I, leading to preventing the primary oocytes from progressing to the secondary oocytes (Eppig, 1993; Whitaker, 1996; Li et al., 2000). The cumulus cells play an important role in transmitting and/or modulating the inhibitory activity of the GC on oocytes maturation (Richard and Sirard, 1996). When the COCs were isolated from the antral follicles, this may separate the cumulus cells from the GC. Thus the COCs were cultured in the absence of the GC thereby releasing the suppression of maturation oocytes. As a result, peeling off or expansion of the cumulus cells from the oocytes occurred to allow oocyte maturation after culture for 24 h. Hinrichs (1997) also reported a similar observation in equine oocytes. In vitro maturation of horse oocytes showed a high proportion of oocytes with COCs Type I and Type II in the maturation medium. Our investigation demonstrated that the porcine ovary had around 40 percent of COCs Type I and Type II with the diameter ranging from 120 to 145 μm. Consequently, the morphology and diameters of Type I and Type II could be used as criteria for selection of oocytes for in vitro embryo production.



11. SEM micrographs of detachable cumulus cells surface showing (A and B) elongation of the end point of cumulus cells (arrows).



...graphs of GC (A and B); showing small cluster of round shape of GC after culturing for 48 h. At high magnification (C); showing the single round ...without microvilli.

...ee-floating GC in the follicular fluid were round in ...d were found as clusters. After culturing in in vitro ...for 48 h, no change in the morphology was observed. ...e found in smaller clusters or as single cells. However, ...l GC clusters secreted the substance for oocyte ...into the culture medium (Richard and Sirard, 1996). ...cells and GC conditioned medium could be used as ...lls for in vitro fertilization. Their biochemical ...ms and secretion are being further investigated to ...best use of them.

...lusion, SEM and inverted microscopy are excellent ...the determination of morphological changes in the in ...in vitro study of POEC, COCs and GC. This ...is important to the furture study of their secretions ...mical compositions. The results could allow us more ...on the use of these cells for mammalian in vitro ...on.

...dgements

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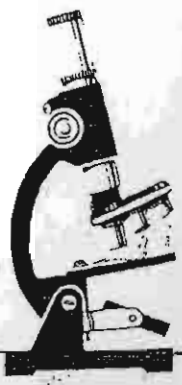

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Morphological Features of Porcine Oviductal Epithelial Cells and Cumulus-Oocyte Complex

Mayuva Areekijseree[1] Amara Thongpan[2] and Renu Vejaratpimol[1]

ABSTRACT

Porcine oviductal epithelial cells (POEC) and cumulus-oocyte complexes (COCs) were observed using inverted microscopy and scanning electron microscopy. At follicular phase, POEC contained a great number of long ciliated cells whereas those at luteal phase consisted mostly of round shaped non-ciliated cells with short microvilli on the apical surface. This change in morphological features of POEC seemed to serve well on their functions as oocyte transporters at follicular phase. As for COCs, they were morphologically classified into 4 types based on the accumulation and arrangement of cumulus cells around the oocytes. These were intact cumulus cell layer, single cumulus cell layer, partial cumulus cell layer and completely denuded oocytes at the percentages of composition of 19.87%, 18.13 %, 28.88 % and 33.12 %, respectively. Cumulus cells attached on the surface of oocytes were teardrop-like shape having the conical ends pointed towards the oocytes membrane surface while free-floating cumulus cells in the follicular fluid and those at the outer layers were round in shape. These POEC and COCs were high potential feeder cells and could be further cultured for *in vitro* fertilization use.

Key words: cumulus-oocyte complexes, morphology, porcine oviductal epithelial cells

INTRODUCTION

Cells in the mammalian female reproductive system, i.e., oviductal epithelial cells and cumulus cells have direct effect and interactions which contribute to the success of fertilization. They are, therefore, high potential cellular materials to be used as cultured feeder-cells for gamete development and *in vitro* fertilization (White *et al.*, 1989; Nagai and Moor, 1990; Kitiyanant *et al.*, 1989, 1993, 1995; Park and Sirard, 1996; Vatzias and Hargen, 1999; Romar *et al.*, 2001, 2003). Since the reproductive organs of pig are not used as human food, they are readily available and can be collected from the slaughter house for research work. Kitiyanat *et al.* (1993) reported the use of porcine oviductal cells to support *in vitro* bovine embryo development. It is also known that there are some materials in the reproductive tract of animals that produce several offspring at one time, i.e., pig, rodents, could better facilitate the growth and development of embryo in other types of animal in culture. These cells not only help the sperm capacitation and acrosome reaction but also assist the penetration of oocytes and hence, gave higher percentage of *in vitro* fertilization (White *et al.*, 1989; Anderson and Killian, 1994; Hyttel *et al.*, 1997; Park and Sirard, 1996; Romar *et al.*, 2001, 2002). In addition, these cells are very useful in determining the toxic effect of environment

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which could be accumulated and lead to the abnormal development of the embryo. The results of these effects could be clearly seen and evaluated than using the experimental animals and therefore, ethically acceptable.

To be able to make the best use of these cells, attempts are made to thoroughly study the characteristics of them. Unfortunately, basic knowledge on their morphological aspects of these cells are still unknown. This work was aimsed to discern the morphological features of porcine oviductal epithelial cells during the estrous cycle and those of cumulus oocytes complex using inverted microscopy and scanning electron microscopy (SEM).

MATERIALS AND METHODS

POEC and COCs collection and preparation

Oviducts and ovaries of Large White pigs were obtained from slaughter house at Nakorn Pathom Province. They were removed within 30 minutes after being slaughtered and transported to the laboratory within 1 hour in a thermos containing 0.9% normal saline.

The oviduct from follicular phases (estrous cycle, day 15) and luteal phases (estrous cycle, day 1-2) were trimmed free from fat and connective tissues and rinsed 3 times in 0.1 M phosphate buffer (pH 7.2). They were cut in small size (2-3 mm) and prepared for SEM observation.

For COCs collection, selected healthy follicles of 2-6 mm in diameter were aspirated using a 5 ml disposable syringe with 18-gauge needle containing 0.9% normal saline and placed in petri dishes. Follicular content was observed under a stereomicroscope and COCs were collected using a pipette of narrow pore size (200 µm). After aspiration, COCs were washed 3 times in TALP-HEPES supplemented with 10% heat treated fetal calf serum (HTFCS) and 50 mg/ml gentamycin, then observed under an inverted microscope and prepared for SEM observation.

Preparation for scanning electron microscopy

Samples were pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffer (pH 7.2) for 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 absolute ethanol) and dried in a critical point dryer machine (CPD). All 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.

RESULTS

Ultrastructures of POEC

SEM of porcine ampullary oviduct (PAO) showed two different cells types, ciliated and non-ciliated cells. The porcine ampullary oviductal epithelium at the follicular phase contained numerous ciliated cells and some non-ciliated cells. The cilia consistently projected themselves above the apex of the non-ciliated cells (Figure 1A, B and C). At high magnification, non-ciliated cells were clearly seen as spherical shape cells with numerous short microvilli (Figure 1D).

At luteal phase, the number of ciliated cells decreased while non-ciliated cells increased (Figure 2A). At high magnification, the apical surfaces of the non-ciliated cells were round in shape with numerous small microvilli (Figure 2B).

Ultrastructures of COCs

From twenty collected ovaries, 921 oocytes were isolated resulting in the average of 46 oocytes per ovary. Inverted microscopic observation of COCs showed the oocytes from follicular fluid were round in shape and 120-145 µm in size. They were surrounded with zona pellucida and several layers of cumulus cells (Figure 3A, C). The SEM gave distinct surface appearances of both oocytes and cumulus cells (Figure 3B, D, F, H). Cumulus

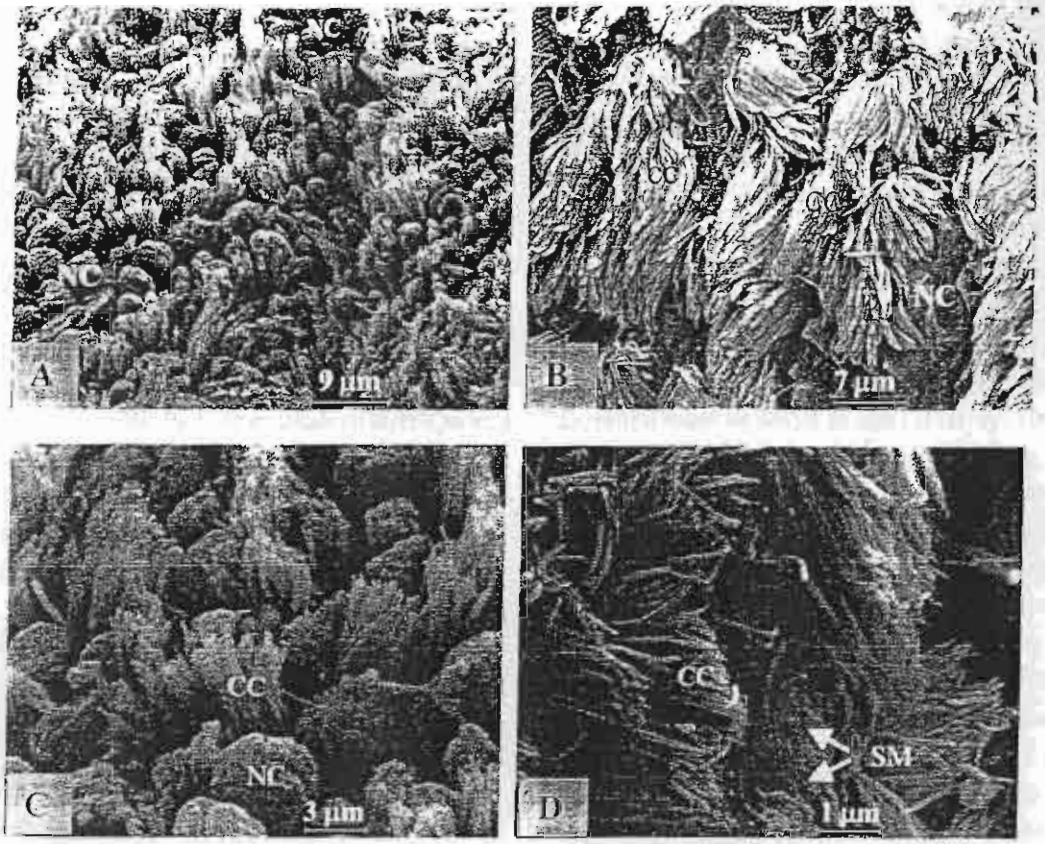

Figure 1 Scanning electron micrographs of POEC at follicular phase. (A, B) showing numerous ciliated cells (CC) and non-ciliated cells (NC). At high magnification (C,D) showing non-ciliated cells (NC) of spherical shape having short microvilli (SM) on the apical surfaces.

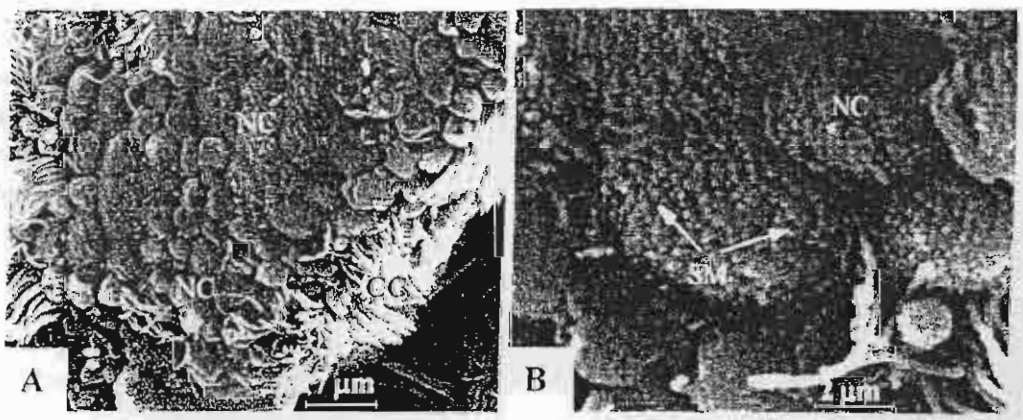

Figure 2 Scanning electron micrographs of POEC at luteal phase showing (A) numerous non-ciliated cells (NC) small number of ciliated cells (CC). At high magnification (B) showing round shape non-ciliated cells (NC) with short microvilli (SM) at the apical surface.

cells were also round in shape and contained no microvilli on the surface membrane. Based on the surrounded cumulus cells of the oocytes, COCs were classified into 4 types as follows:-

Types I- Intact cumulus cells layer- The oocytes were at early development stage having several compact layers of cumulus cell. They were found at secondary follicle part (Figure 3 A,B).

Types II- Single cumulus cell layer- Oocytes were found with one or incomplete two layers of cumulus cell (Figure 3 C,D).

Type III- Partial cumulus cell layer-Oocytes were partially covered with some cumulus cells. The cumulus cells were loosely attached to the zona pellucida. Cytoplasm became fainted in color (Figure 3 E,F).

Type IV- Completely denuded oocyte - Oocytes were completely free from cumulus cells. These oocytes were from follicle atresia. Cytoplasm was pale in color (Figure 3 G,H).

It was found that 33.12% of these COCs was completely denuded oocyte, 28.88% was partial cumulus cell layer type while those of single cumulus cell layer and intact cumulus cell layer types were found at only 19.87% and 18.13 %, respectively (Table 1).

Cumulus cells attached to the oocyte surface were not round in shape like those in the follicular fluid but conformed to a teardrop-like structure having the conical end pointed towards the oocyte surface membrane (Figure 4). The remaining cumulus cells were all round in shape and could be found as a single cell or as a monolayer surrounding the oocyte (Figure 5).

DISCUSSION

Two types of mammalian oviductal epithelial cell, i. e., non-ciliated and ciliated cell, were present in porcine ampullary oviduct (PAO) at both follicular and luteal phases. Ciliated cells in ampulla, however, were found at increased number at the follicular phase than at the luteal phase. This finding agreed with that of bovine oviductal epithelial cells as reported by Songthaveesin (1998). Although the alternation of population of two types of cell in both phases were similar in these two different species, the morphology of the epithelial cells were distinctly different. In bovine, oviductal epithelial cells form a "wormlike" structure (Xu *et al.*, 1992) while those in porcine were small and round in shape.

Since ampullary oviduct was the site of fertilization, it was found to contain more synthetic secretion than the whole oviduct (Murray, 1992). In the ampullary oviduct at follicular phase, the high number of ciliated cells corresponded to the transporting of ovulated oocytes. At luteal phase, numerous non-ciliated cells with short microvilli at the apical surface also corresponded to the secretory substance for nutritional support of embryonic development (Hole and Koos, 1994). It has been suggested that the cycle of long ciliated and non-ciliated cells population as seen in the mammalian oviduct depends on the levels of circulating estrogen and progesterone (Verhange and Jaffe, 1986). Furthermore, POEC are used as co-culture *in vitro* to support oocytes maturation and increase normal fertilization, sperm capacitation and early embryonic development (White *et al.*, 1989; Nagai and Moor, 1990; Kitiyanant *et al.*, 1993; Park and Sirard, 1996; Vatzias and Hargen, 1999; Romar *et al.*, 2001, 2003). Nagai and Moor (1990) also suggested that glycoproteins secreted from non-ciliated oviductal epithelial cells could bind to the porcine spermatozoa and reduce the incidence of polyspermy. Further studies should be carried out to elucidate the characterization of protein synthesis from non-ciliated POEC during luteal phase.

COCs were collectively characterized into 4 types based on their accumulation and arrangement of cumulus cells around the oocytes. The co-existences of these 4 types of COC were found in all follicular fluid samples collected, even from the similar follicle sizes. However, these 4

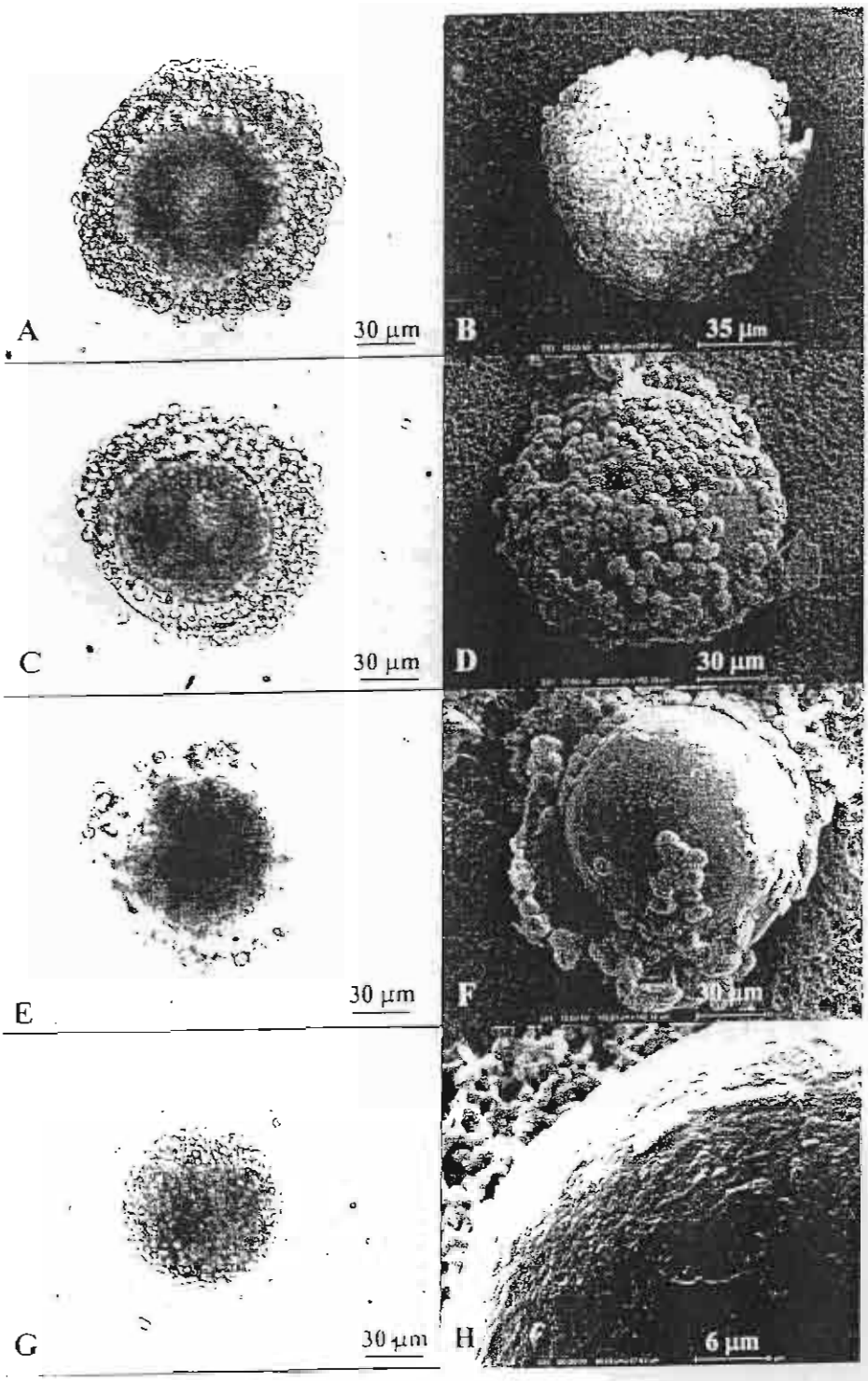

Figure 3 Micrographs and scanning electron micrographs of COCs. (A,B) intact cumulus cell layer. (C, D) single cumulus cell layer. (E,F) partial cumulus cell layer. (G,H) completely denuded oocyte.

Table 1 Classification of COCs based on the types of cumulus cells surrounding oocyte.

Types	No. of COCs	(%)
Intact cumulus cell layer	183	19.87
Single cumulus cell layer	167	18.13
Partial cumulus cell layer	266	28.88
Completely denuded oocyte	305	33.12
Total	921	100.00

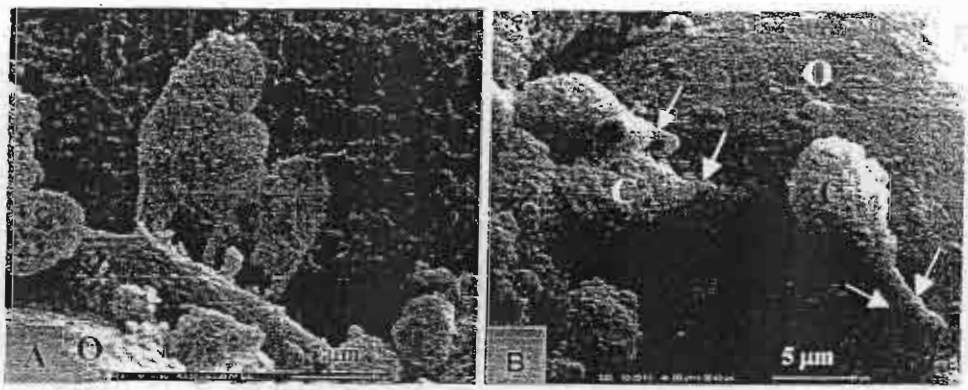

Figure 4 Scanning electron micrographs of COCs, (A) teardrop-like structure of cumulus cells attached to the oocytes surface (cumulus cells: C, zona pellucida: Z, oocytes :O). (B) showing the conical end (arrow) pointed towards the oocyte membrane.

types of COC could be selectively used for different experimental purposes. The partial cumulus cell layer type and the completely denuded oocyte are considered more mature in their natural stage of development and ready for sperm penetration. As for culturing oocyte cells to reach the maturation, Mori *et al.* (2000) found that intact cumulus cell layer type and single cumulus cell layer type had higher potential to become matured oocytes. These types of COC were successfully cultured in the artificial medium supplemented with follicular stimulating hormone (FSH) and leutinizing hormone (LH) using cell samples from rat (Magnusson, 1980), sheep (Staigmiller and Moor, 1984), bovine and swamp buffalo (Kitiyanant *et al.*, 1989, 1995).

Cumulus cells are known to transmit low molecular weight substances, i. e., ion nucleotides and amino acids to oocytes in the young non-reproductive females (Dekel and Beers, 1980). These substances are collectively called oocytes maturation inhibiting factor (or meiosis arresting factor) which arrest the oocyte development at the diplotene stage of prophase I, thereby preventing the primary oocytes from progressing to secondary oocytes (Eppig, 1993). Further study on these molecular secretion from cumulus cells and the oviductal epithelial cells in culture could render us more information on the use of these cells for fertilization control.

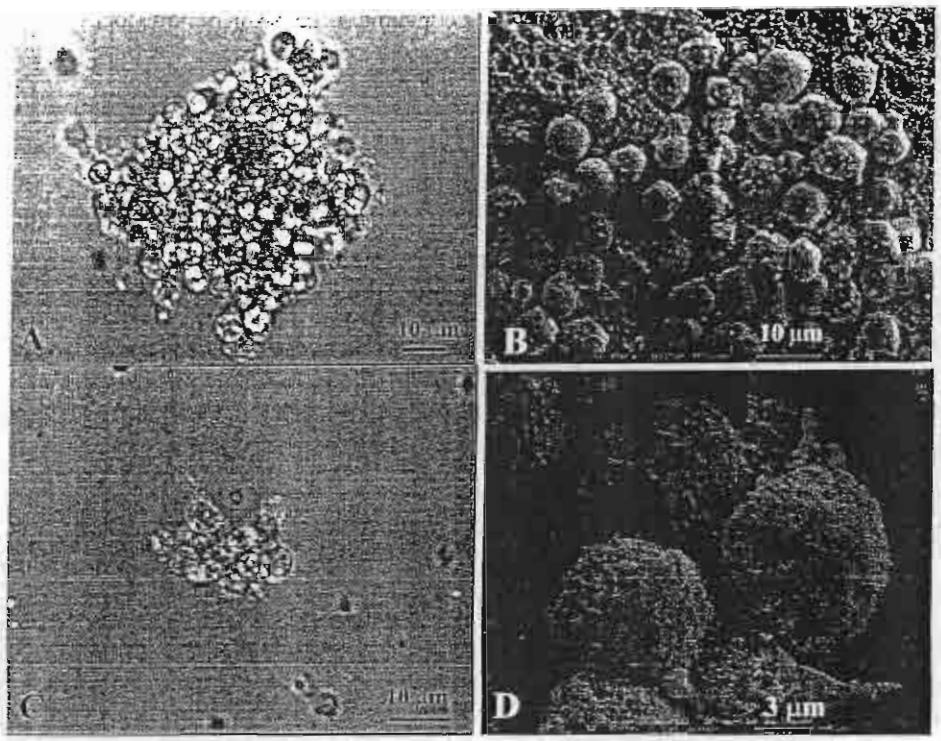

Figure 5 Micrographs (A,C) and scanning electron micrographs (B,D) showing the round shape cumulus cells in follicular fluid (free-floating cumulus).

CONCLUSION

These findings indicated that POEC changed both the morphological features and the population of cell types during the estrus cycle. At follicular phase, POEC contained the greater number of long ciliated cells than at luteal phase. The luteal phase, however, was filled up with numerous round shaped non-ciliated cells having short microvilli on the apical surface.

COCs could be collected from the antral follicle of porcine. They were classified according to the surrounding cumulus cells into 4 types, i. e., intact cumulus cell layer, single cumulus cell layer, partial cumulus cell layer, and completely denuded oocytes at the percentage composition of 19.87, 18.13, 28.88 and 33.12%, respectively. The first two types of COC could be further developed into matured eggs in culture while the last two types were too advanced in their developmental stages and became deteriorated in culture. The first layer of cumulus cells attached to the oocyte membrane were teardrop-like in shape having the conical ends pointed towards the surface membrane while free-floating cumulus cells in the follicular fluid were round in shape and were found both as single cell or forming a monolayer. These cumulus cells could be used as feeder cells for *in vitro* -fertilization. Their biochemical compositions and secretion are being further investigated for the best use of them.

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Porcine oviductal epithelial cells and cumulus-oocyte complex observance

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[a] *Department of Biology, Faculty of Science, Silpakorn University, Nakorn Pathom 73000, Thailand*
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Abstract-Porcine oviductal epithelial cells (POEC) and cumulus-oocyte complexes (COCs) were observed using light microscopy and scanning electron microscopy. At follicular phase, POEC contained a great number of high ciliated cells whereas those at luteal phase consisted mostly of round shaped non-ciliated cells with short microvilli on the apical surface. This change in morphological features of POEC seemed to serve well on their functions as oocyte transporters at follicular phase. As for COCs, they were morphologically classified into four types based on the accumulation and arrangement of cumulus cells around the oocytes. These were intact cumulus cell layer, single cumulus cell layer, partial cumulus cell layer and completely denuded oocytes at the percentage composition of 19.87%, 18.13%, 28.88% and 33.12%, respectively. Cumulus cells attached on the surface of oocytes were teardrop-like shape having the conical ends pointed towards the oocytes membrane surface while free-floating cumulus in the follicular fluid and those at the outer layers were round in shape. These POEC and COCs are high potential feeder cells and could be further cultured for *in vitro* fertilization use.

Keywords-Cumulus-oocyte complexes, Morphology, Porcine oviductal epithelial cells

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Poster Presentation

Porcine Oviductal Epithelial Cells and Cumulus-Oocyte Complexes Observance

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Key words
Cumulus-oocyte complexes, Light microscopy, Morphological, Porcine oviductal epithelial cells, Scanning electron microscopy

Background
Cells in the mammalian female reproductive system, i.e., oviductal epithelial cells and cumulus cells have direct effect and interactions which contribute to the success of fertilization. They are, therefore, high potential cellular materials to be used as cultured feeder-cells for gamete development and *in vitro* fertilization [1-9]. Since the reproductive organs of pig are not used as human food, they are readily available and can be collected from the slaughter house for research work. Kitiyanat *et al.* [4] reported on the use of porcine oviductal cells to support *in vitro* bovine embryo development. It is also known that there are some materials in the reproductive tract of animals that produce several offspring at one time, i.e., pig, rodents, could better facilitate the growth and development of embryo in other types of animal in culture. These cells not only help the sperm capacitation and acrosome reaction but also assist the penetration of oocytes and hence, gave higher percentage of *in vitro* fertilization [1, 6, 8-11]. In addition, these cells are very useful in determining the toxic effect of environment which could be accumulated and lead to the abnormal development of the embryo. The results of these effects could be clearly seen and evaluated than using the experimental animals and therefore, ethically acceptable.

To be able to make the best use of these cells, attempts are made to thoroughly study the characteristics of them. Unfortunately, basic knowledge on their morphological aspects of these cells are still unknown in pigs. This work aims to discern the morphological features of porcine oviductal epithelial cells (POEC) during the estrous cycle and those of cumulus-oocyte complexes (COCs) using light microscopy (LM) and scanning electron microscopy (SEM).

Materials and Methods

POEC and COCs collection and preparation

Twenty oviducts and twenty ovaries of Large white pigs were obtained from slaughterhouse at Nakorn Pathom Province. They were removed within 30 min after being slaughtered and transported to the laboratory within 1 h in a thermos containing 0.9% normal saline.

The ampullar part of oviduct from follicular phases (estrous cycle, day 15) and luteal phases (estrous cycle, day 1-2) were trimmed free from fat and connective tissues and rinsed 3 times in 0.1 M phosphate buffer (pH 7.2). They were cut in small size (2-3 mm) and prepared for SEM observation.

For COCs collection, selected healthy follicles of 2-6 mm in diameter were aspirated using a 5 ml disposable syringe with 18-gauge needle containing 0.9% normal saline and placed in petri dishes. Follicular content was observed under a stereomicroscope and COCs were collected using a pipette of narrow pore size (200 μm). After aspiration, COCs were washed 3 times in TALP-HEPES supplemented with 10% heat treated fetal calf serum and 50 mg/ml gentamycin, then observed under an inverted microscope and prepared for SEM observation.

Preparation for scanning electron microscopy

Samples were pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 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 absolute ethanol) and 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 an ion sputtering and examined under SEM (CamScan Analytical, Maxim 2000S) operating at 10 kV.

Results

Ultrastructures of POEC

SEM observation of the porcine ampullary oviduct showed two different cells types, ciliated and non-ciliated cells. The porcine ampullary oviductal epithelium at the follicular phase contained numerous ciliated cells. The cilia consistently projected themselves above the apex of the non-ciliated cells (Figure 1A-C). At high magnification, non-ciliated cells were clearly seen as spherical shape cells with numerous short microvilli (Figure 1C and D).

At luteal phase, the number of ciliated cells were decreased while non-ciliated cells were increased (Figure 2A). At high magnification, the apical surfaces of the non-ciliated cells were round in shape with numerous small microvilli (Figure 2B).

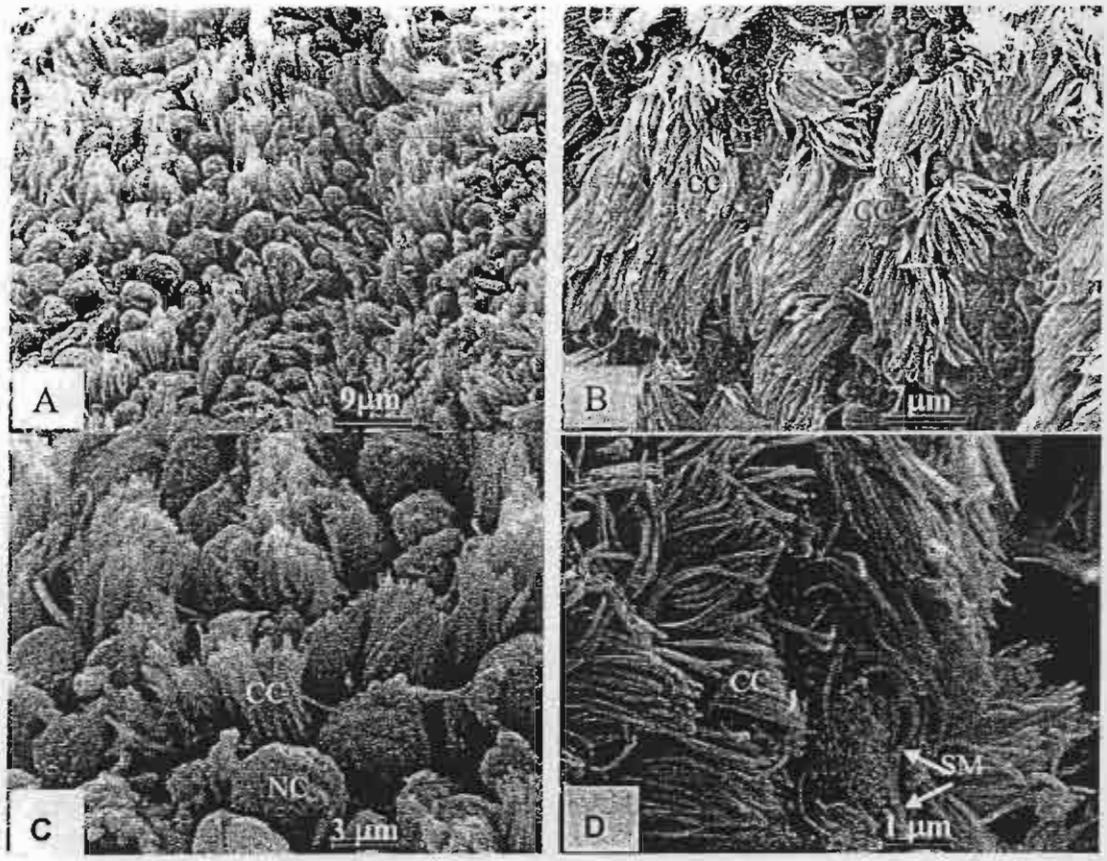

Figure 1. SEM micrographs of the POEC at follicular phase showed (A-B) numerous ciliated cells (CC). An enlargement of (C-D) non-ciliated cells (NC) of spherical shape having short microvilli (SM) on the apical surfaces.

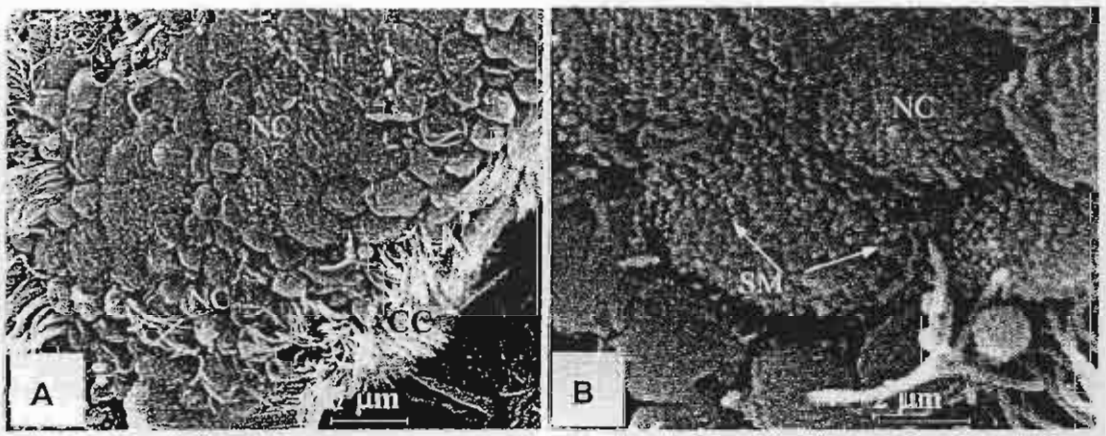

Figure 2. SEM micrographs of the POEC at luteal phase showed (A) numerous non-ciliated cells (NC) and small number of ciliated cells (CC). An enlargement of round shape showed (B) non-ciliated cells (NC) with short microvilli (SM) at the apical surfaces.

Ultrastructures of COCs

From twenty collected ovaries, 921 oocytes were isolated resulting in the average of 46 oocytes per ovary. LM observation of COCs showed the oocytes from follicular fluid were round in shape and 120-130 μm in size. They were surrounded with zona pelucida and several layers of cumulus cells (Figure 3A, C). SEM demonstrated distinct surface appearances of both oocytes and cumulus cells (Figure 3B, D, F, H). Cumulus cells were also round in shape and contained no microvilli on the surface membrane. Based on the surrounded cumulus cells of the oocytes, COCs were classified into four types as follows:-

Type I- Intact cumulus cell layer. The oocytes were at early development stage having several compact layers of cumulus cells. They were found at secondary follicle part (Figure 3A-B).

Type II- Single cumulus cell layer. The oocytes were found with one or incomplete two layers of cumulus cells (Figure 3C-D).

Type III- Partial cumulus cell layer. The oocytes were partially covered with some cumulus cells. The cumulus cells were loosly attached to the zona pelucida. The cytoplasmic appearance of these cells was faint (Figure 3E-F).

Type IV- Completely denuded oocyte. The oocytes were completely free from cumulus cells. These oocytes were from follicle atresia. The cytoplasmic appearance of these cells was pale (Figure 3 G-H).

It was found that 33.12% of these COCs was completely denuded oocyte, 28.88% was partial cumulus cell layer type while those of intact cumulus cell layer types and single cumulus cell layer were found at only 19.87% and 18.13%, respectively (Table 1).

Table 1. Classification of COCs based on the types of cumulus cells surrounding oocyte.

Types	No. of COCs	(%)
Intact cumulus cell layer	183	19.87
Single cumulus cell layer	167	18.13
Partial cumulus cell layer	266	28.88
Completely denuded oocyte	305	33.12
Total	921	100.00

Cumulus cells attached to the oocyte surface were not round in shape like those in the follicular fluid but conformed to a teardrop-like structure having the conical end pointed towards the oocyte surface membrane (Figure 4). The remaining cumulus cells were all round in shape and could be found as a single cell or as a monolayer surrounding the oocyte (Figure 5).

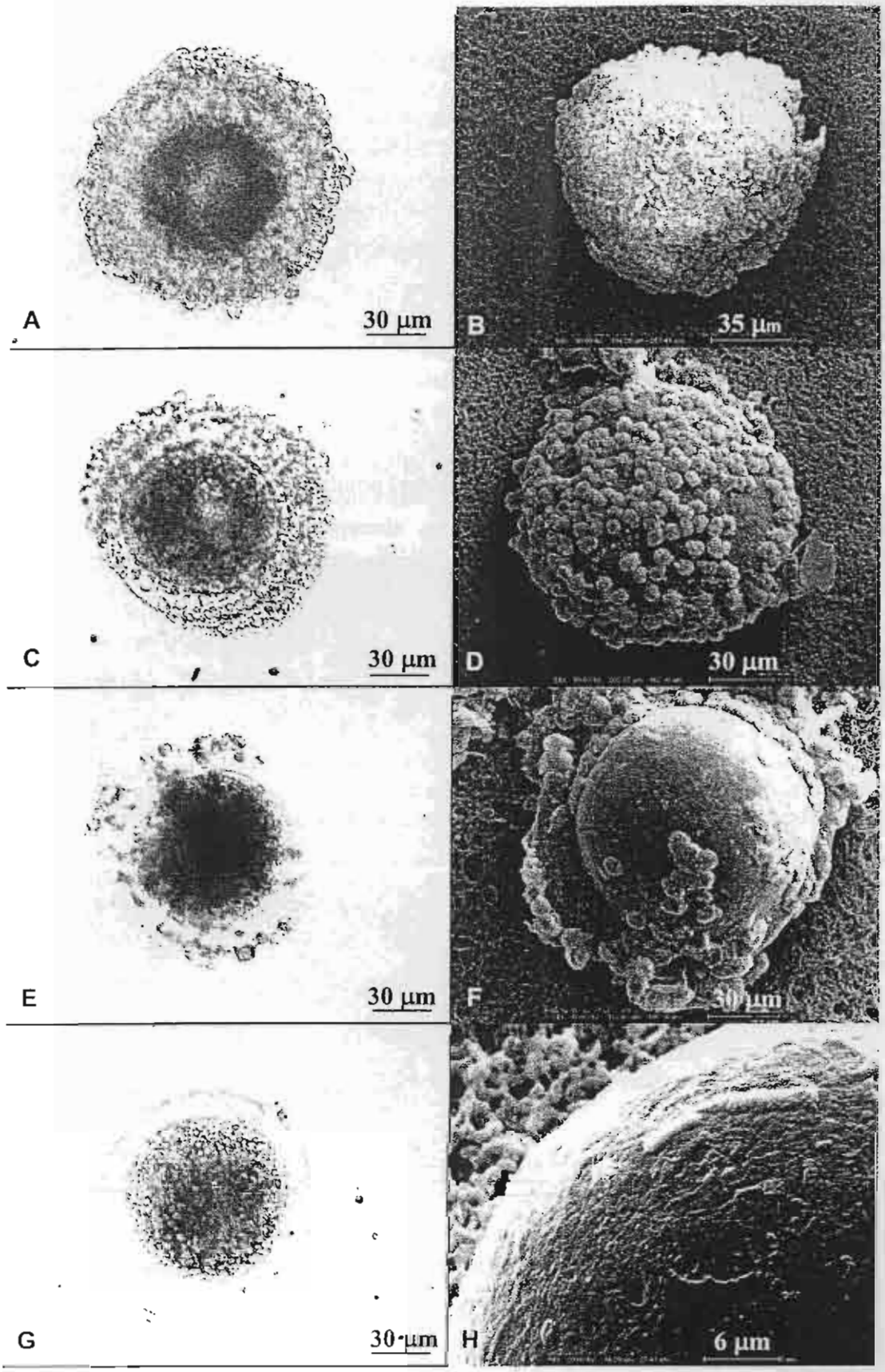

Figure 3. LM and SEM micrographs of COCs showed (A-B) intact cumulus cell layer, (C-D) single cumulus cell layer, (E-F) partial cumulus cell layer, (G-H) completely denuded oocyte.

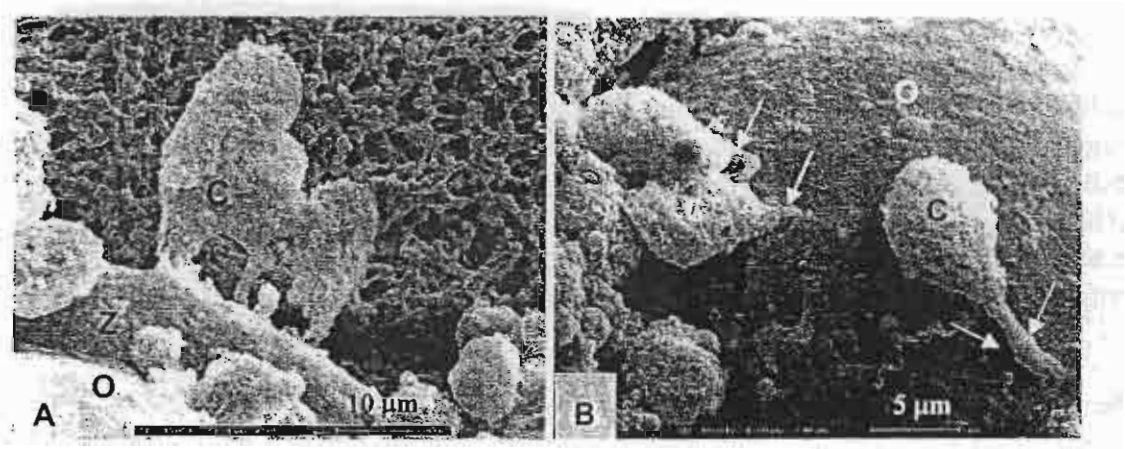

Figure 4. SEM micrographs of COCs showed (A) teardrop-like structure of cumulus cells attached to the oocytes surface (cumulus cells: C, zona pellucida: Z, oocytes :O), (B) the conical end (arrows) pointed towards the oocyte membrane.

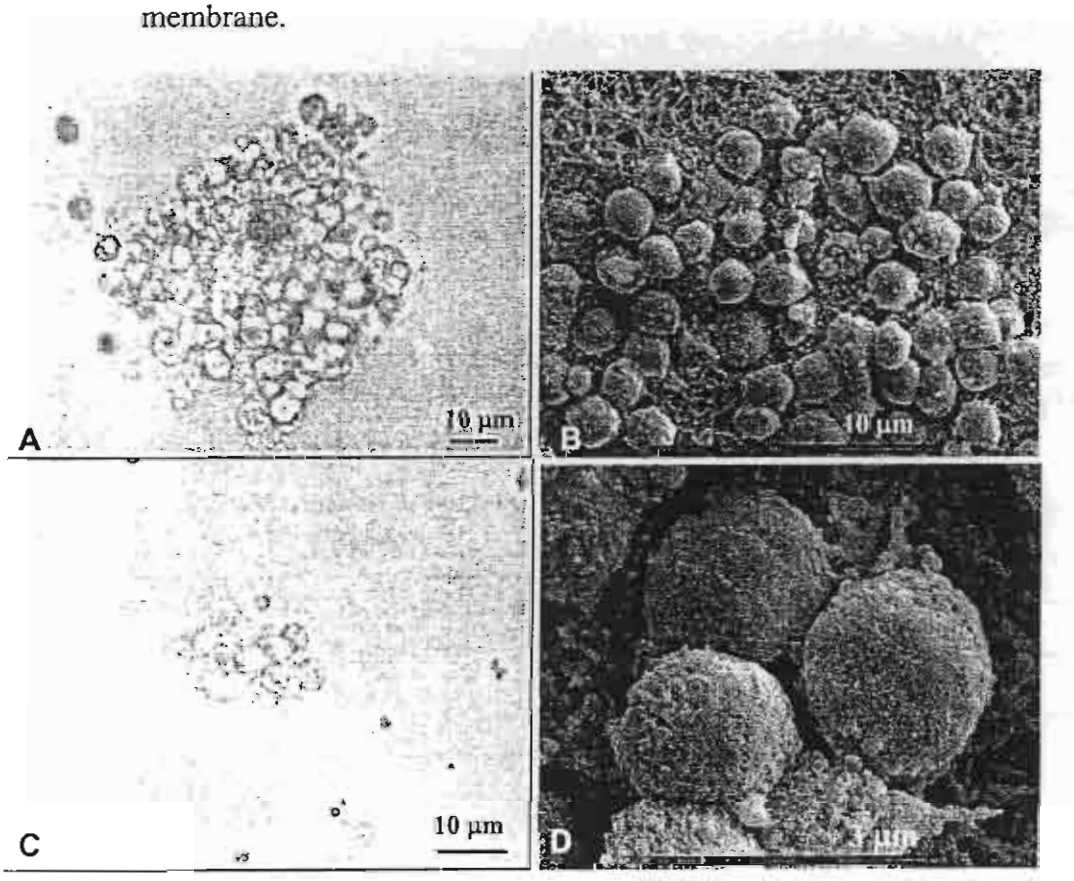

Figure 5. LM (A-C) and SEM micrographs (B-D) showed the round shape cumulus cells in follicular fluid (free-floating cumulus).

Discussion

Two types of mammalian oviductal epithelial cells, i. e., ciliated cell and non-ciliated, were present in porcine ampullary oviduct at both follicular and luteal phases. Ciliated cells in ampulla, however, were found at increased number at the follicular phase than at the luteal phase. This finding agreed with that of bovine oviductal epithelial cells as reported by Songthaveesin [12]. Although the alternation of population of two cell types in both phases were similar in these two different species, the morphology of the epithelial cells were distinctly different. In bovine, oviductal epithelial cells form a "wormlike" structure [13] while those in pig were small and round in shape.

Since ampullary oviduct is the fertilization area, it was found to contain more synthetic secretion than the whole oviduct [14]. In the ampullary oviduct at follicular phase, the high number of ciliated cells corresponded to the transporting of ovulated oocytes. At luteal phase, numerous non-ciliated cells in the small spheres with short microvilli at the apical surfaces corresponded to the secretory substance for nutritional support of embryonic development as also seen by Hole and Koos [15]. It has been suggested that the cycle of high ciliated and non-ciliated cells population as seen in the mammalian oviduct depends on the levels of circulating estrogen and progesterone [16]. Furthermore, POEC were used as co-culture *in vitro* to support oocytes maturation and increase normal fertilization, sperm preparation and early embryonic development [1-9]. Nagai and Moor [2] also suggested that glycoproteins secreted from non-ciliated oviductal epithelial cells could bind to the porcine spermatozoa and reduce the incidence of polyspermy. Further studies should be carried out to elucidate the characterization of protein synthesis from non-ciliated POEC during luteal phase.

COCs were collectively characterized into four types base on their accumulation and arrangement of cumulus cells around the oocytes. The consistent mixing of these four types of COCs were found in all follicle fluid samples collected, even from the similar follicle sizes. However, these four types of COCs could be selectively used for different experimental purposes. The partial cumulus cell layer type and the completely denuded oocyte are considered more mature in their natural stage of development and ready for sperm penetration. As for culturing oocyte cells to reach the maturation, Mori et al. [17] found that intact cumulus cell layer type and single cumulus cell layer type had higher potential to become matured oocytes. These types of COCs were successfully cultured in the artificial medium supplemented with follicular stimulating hormone and leutinizing hormone using cell samples from rat [18], sheep [19], bovine and swamp buffalo [3,5].

Cumullus cells are known to transmit low molecular weight substances, i.e., ion nucleotides and amino acids to oocytes in the young non-reproductive females [19]. These substances are called oocyte maturation inhibiting factor (or meiosis arresting factor) which arrest the oocyte development at the diplotene stage of prophase I, thereby preventing the primary oocytes from progressing to secondary oocytes [20]. Further study on these molecular secretions from cumulus cells and the oviductal epithelial cells in culture could render us more information on the use of these cells for fertilization control.

Conclusions

Our findings indicated that POEC changed both the morphological features and the population of cell types during the estrous cycle. At follicular phase, POEC contained the greater number of high ciliated cells than at luteal phase. The luteal phase, however, was filled up with numerous round shaped non-ciliated cells having short microvilli on the apical surfaces. COCs could be collected from the antral follicle of pig. They were classified according to the surrounding cumulus cells into four types, i.e., intact cumulus cell layer, single cumulus cell layer, partial cumulus cell layer, and completely denuded oocytes at the percentage composition of 19.87%, 18.13%, 28.88%, and 33.12%, respectively. The first two types of COCs could be further developed into mature eggs in culture while the last two types were too advanced in their developmental stages and became deteriorated in culture. The first layer of cumulus cells attached to the oocyte membrane were teardrop-like in shape having the conical ends pointed towards the surface membrane while free-floating cumulus cells in the follicular fluid were round in shape and were found both as single cell or forming a monolayer. These cumulus cells could be used as feeder cells for *in vitro* fertilization.

Acknowledgement

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