

Original Article

Determination of porcine oocyte and follicular fluid proteins from small, medium, and large follicles for cell biotechnology research

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Abstract

Porcine oocytes from small, medium, and large follicles can be classified into five types. Type I (intact-cumulus oocytes) and type II oocytes (multi-cumulus cell layers surrounding oocytes) have high potential to be developed into *in vitro*-matured oocytes. Determination of porcine follicular fluid (pFF) proteins from 3 follicle sizes by SDS-PAGE and LC/MS/MS, 6 protein bands sizes 52, 65, 79, 90, 160, and >220 kDa were identified as immunoglobulin gamma chain, keratin, porcine inhibitor of carbonic anhydrase, heat shock protein or plasminogen precursor or both, transthyretin, and protease. All proteins were reported for their important roles in promotion and regulation on growth and development of reproductive cells except for the protein band at 79 and 160 kDa which had diminished function in the first metaphase of oocyte. This study provides knowledge on the oocyte and pFF proteins as biological models and *in vitro* cell maturation supplements in biotechnology research.

Keywords: follicular fluid protein, porcine oocyte, LC/MS/MS, SDS-PAGE

1. Introduction

The porcine reproductive system that includes the ovaries, oviducts, and their secretions are unused organs at a slaughterhouse. These organs have been used effectively as models for cell biotechnology research (Areekjseree & Chuen-Im, 2012; Areekjseree & Vejaratpimol, 2006; Areekjseree & Veerapraditsin, 2008; Sanmanee & Areekjseree, 2009; Sanmanee & Areekjseree, 2010). Previously, oocytes, oviductal cells, and cumulus cells were shown to mature well under culture conditions (Areekjseree & Chuen-Im, 2012; Areekjseree, Thongpan, & Vejaratpimol, 2005; Areekjseree & Vejaratpimol, 2006; Areekjseree & Veerapraditsin, 2008). The porcine ovary is composed of numerous ovarian follicles.

There primordial follicles develop into primary follicles (small follicle: 1-2 mm in diameter), secondary follicle (medium follicle: 3-6 mm in diameter), and tertiary follicle or graafian follicle (large follicle: 7-10 mm in diameter). All follicles contain one oocyte surrounded by the zona pellucida and a layer of cumulus cells (or cluster granulosa cells). The primary oocyte in the primary follicle and secondary follicle complete meiosis I and turn into a secondary oocyte in the tertiary follicle. They undergo ovulation in the estrous cycle. The number of cumulus cells surrounding the oocyte increase directly in response to heightened levels of circulating gonadotropin-releasing hormone (GnRH) in the estrous cycle. So, the number of cumulus cells surrounding the oocyte in the estrous cycle was used to classify the oocytes in each follicular size (Areekjseree & Chuen-Im, 2012). Classification of oocytes after the oocytes isolate from different sized follicles is essential for improvement in the quality of *in vitro* oocyte maturation (Areekjseree & Chuen-Im, 2012; Kwak *et*

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al., 2014). The granulosa cells around the follicle produce follicular fluid proteins involved in ovarian hormone synthesis regulation. The follicular fluid proteins in oocyte follicles are an essential environment that is composed of molecules that are useful for *in vivo* oocyte maturation, ovulation, and fertilization. Therefore, various components of follicular fluid proteins were found that depended on the size of the follicle. It was reported that oocytes from small and medium follicles showed differences in *in vivo* maturation that were associated with follicular fluid content (Kwak *et al.*, 2014). So, identification of the protein contents in the follicular fluid is useful to determine the proteins in each follicle size to use in *in vitro*-matured oocytes. (Ducolomb *et al.*, 2013). Several studies on porcine reproductive cells have been published, but none of these studies reported on the secretion proteins during the porcine estrous cycle. For this reason, the study was designed to identify the porcine follicular fluid protein bands of small, medium, and large follicles using the SDS-PAGE and LC/MS/MS techniques. The results provide knowledge for selection of the best follicular fluid in each follicle size to use as supplement to promote embryo maturation, *in vitro*-mature oocytes, and other cell biotechnologies.

2. Materials and Methods

Ovaries of Large White reproductive female pigs were obtained from local slaughterhouses in Nakorn Pathom Province, Thailand. They were removed within 30 min after the pigs were slaughtered and transported to the laboratory within 1 h in a thermos of saline solution (0.9% NaCl (w/v), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 250 µg/mL amphotericin B) at 30-35 °C. The morphologies of the blood vessels of the ovaries were observed. The ovaries were trimmed free from fat and connective tissues, and rinsed 3 times with a washing medium (TALP-HEPES with 10% HT FCS and 50 µg/mL gentamycin).

The follicular fluid of healthy follicles that were small (1-2 mm in diameter), medium class I and II (3-4 and 5-6 mm in diameters), and large (7-8 mm and 10 mm in diameters) were collected by sterile technique using a sterile 10 mL disposable syringe with an 18-gauge needle containing saline solution, and placed in sterile petri dishes. Then, the follicular fluid color of each size follicle was observed.

After cell sedimentation, preparation of oocytes and follicular contents were observed under a stereomicroscope and oocytes were collected using a pipette of a narrow pore size (200-250 µm). Oocytes were washed 3 times with the washing medium and then classified as described by Areekijserree and Vejaratpimol (2006).

Secretion follicular fluid protein pattern bands of small, medium, and large follicles were investigated using SDS-PAGE. To do this, follicular fluid was determined for the total protein concentration according to the Lowry method (Lowry *et al.*, 1951) and the absorbance was measured at a wavelength of 750 nm. Characterization of the protein pattern was carried out by loading 10 µL of each sample into 4-12% SDS-PAGE at 200 volts for 50 min. The gel was stained in Coomassie brilliant blue solution. The selected follicular fluid protein bands were then identified by amino acid sequence by the LC/MS/MS technique.

3. Results and Discussion

Observation of the blood supply vessels revealed that the ovaries that contained many small follicles had fewer blood vessels than those of medium and large follicles. This implied a relationship between the number of blood vessels and the growth of follicles in which the larger size and higher growth rate follicles contained a greater number of vessels which resulted in follicles that were reddish. A study of follicular fluid color and volume of each size found that the fluid from small, medium, and large follicle sizes had the same character (Figure 1).

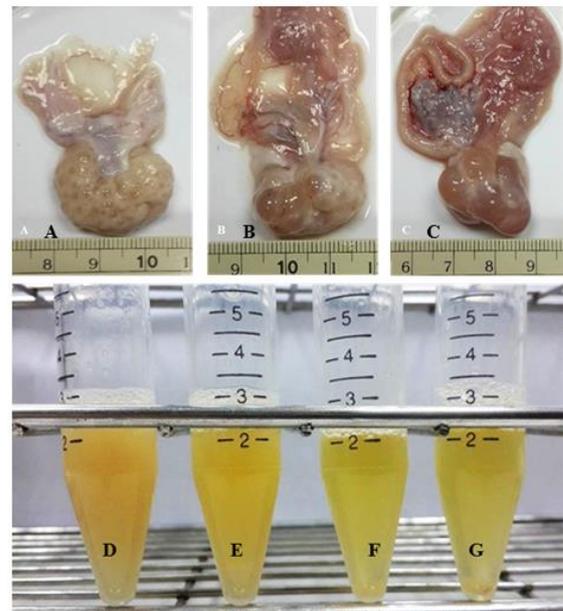


Figure 1. Photographs showed (A) small follicles, (B) medium follicles, (C) large follicles (D) follicular fluid of small follicles, (E-F) follicular fluid of medium follicles, (G) and follicular fluid of large follicles.

The oocytes from small, medium, and large follicles were round in shape and surrounded by the zona pellucida with layers of cumulus cells (CCs). When measured under a stereomicroscope, these oocytes were categorized into 5 types based on the number of cumulus cell layers surrounding the oocyte. Type I was intact-cumulus cell layers surrounding the oocytes and the oocytes were contained in more than 5 compact layers of CCs. Type II was multi-cumulus cell layers surrounding oocytes and the oocytes were surrounded with 2-3 layers of CCs. Type III was partial cumulus cell layers surrounding oocytes and the oocytes were partially covered with one layer or some CCs. Type IV was completely denuded oocytes and the oocytes were completely denuded of CCs. Finally, Type V was degenerated oocytes.

The diameters of Type I oocytes from small, medium, and large follicles were 89.51 µm, 108.02 µm, and 149.47 µm, respectively. We found that Type I oocytes from small, medium, and large follicles were contained in CCs of more than 5, 7, and 10 layers, respectively (Figure 2). The

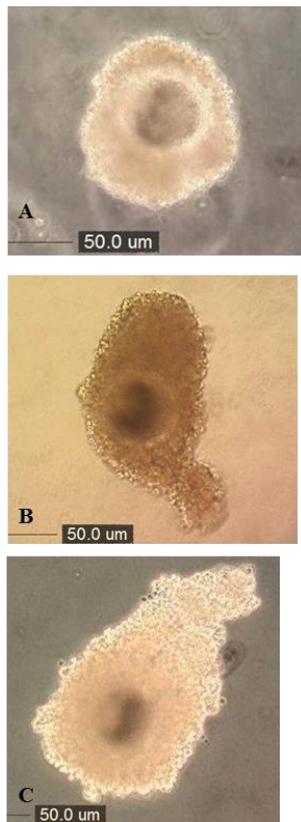


Figure 2. Photographs showed Type I: Intact-cumulus cell layers surrounding oocyte from small follicle with compact cumulus cells more than 5 layers (A), Type I oocyte from medium follicle with compact cumulus cells more than 7 layers (B), and Type I oocyte from large follicle with compact cumulus cells more than 10 layers (C).

diameters of Type II oocytes from small, medium, and large follicles were 69.57 µm, 72.71 µm, and 66.62 µm, respectively. Type II oocytes from small, medium, and large follicles showed the same character of oocytes (Figure 3). The diameters of Type III, IV, and V oocytes ranged from 59 to 60 µm (Figure 4).

The porcine oocytes from small follicles can be further classified into 5 types (intact-, multi-, partial cumulus cell layers, completely denuded, and degenerated oocytes) which were found in percentages of 13.41%, 40.07%, 17.14%, 22.76%, and 6.62%, respectively (Figure 5). The 5 types of oocytes of medium follicles were found in percentages of 33.33%, 23.61%, 15.28%, 18.06%, and 9.72%, respectively (Figure 6). Meanwhile, porcine oocytes from large follicles were found as intact-, multi-, partial cumulus cell layers, completely denuded oocytes, and degenerated oocytes in percentages of 26.09%, 30.43%, 26.09%, 4.34%, and 13.04%, respectively (Figure 7). It should be noted that all oocytes observed in this study were mostly either in Types I or II in small, medium, and large follicles in high percentages of 53.48%, 56.94%, and 56.52%, respectively.

Interestingly, the diameters of Type I oocytes from medium (4-6 mm) and large follicles (7-10 mm) were 108.02 µm and 149.47 µm. They contained compact cumulus cells

ranging from 7 to 10 layers (Figures B and C) more than Type I oocytes from small follicles (Figure A). The indication was that Type I oocytes of medium and large follicles would be the best oocytes to have high potential to develop into mature oocytes *in vitro*. According to the report of Areekijserree and Chuen-Im (2012), porcine cumulus oocyte complexes Type I (from follicles with diameter of about 4-6 mm) have high potential to develop into mature oocytes when cultured in medium containing 10% HTFCS, 2.2 mg/mL NaHCO₃ 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. This is in agreement with the report by Kwak *et al.* (2014) which reported that oocytes obtained from large follicles (more than 8 mm) can develop into mature oocytes by culturing in medium supplemented with FSH and LH. Meanwhile, it was reported that Type II oocytes of medium and large follicles could develop into mature oocytes *in vitro*. Similarly, Mori, Amano, and Shimizu (2000), Chen *et al.* (2007), and McElroy *et al.* (2008) reported that Types I and II porcine cumulus oocyte complexes could develop into mature oocytes by culturing in medium supplemented with follicle stimulating hormone (FSH) and luteinizing hormone (LH). Moreover, they also used the oocytes as a model for cell cytotoxicity assay (Pongsawat, 2014).

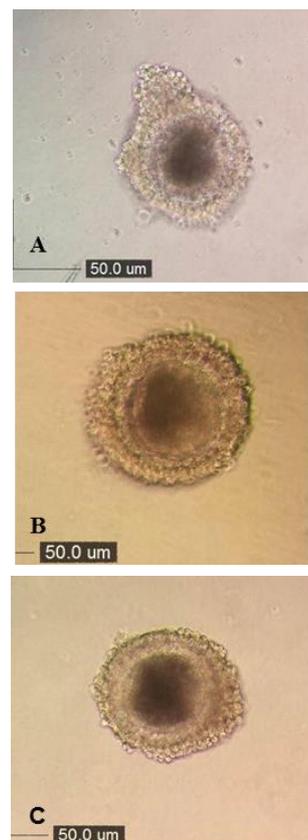


Figure 3. Photographs showed the same character of Type II: Multi-cumulus cell layers surrounding oocyte from small follicle (A), Type II oocyte from medium follicle (B), and Type II oocyte from large follicle (C).

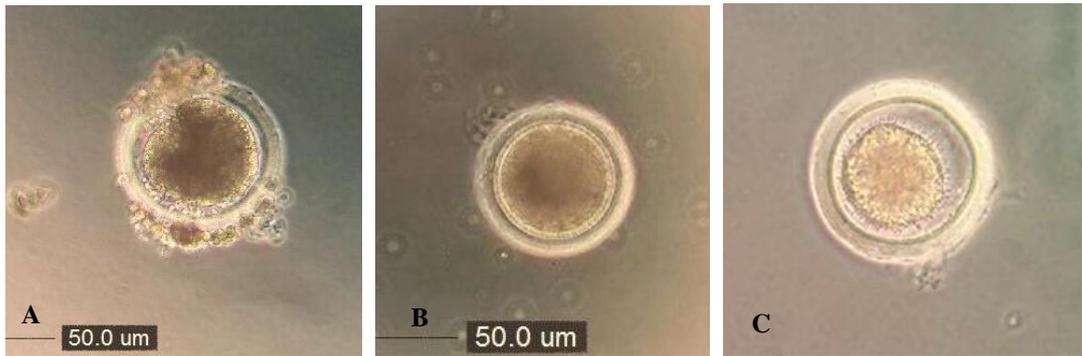


Figure 4. Photographs show Type III: Partial cumulus cell layers surrounding oocyte (A), Type IV: Oocyte that is completely denuded of cumulus cells (B) (Two types will be degenerated oocytes), and Type V: Degenerated oocyte (C).

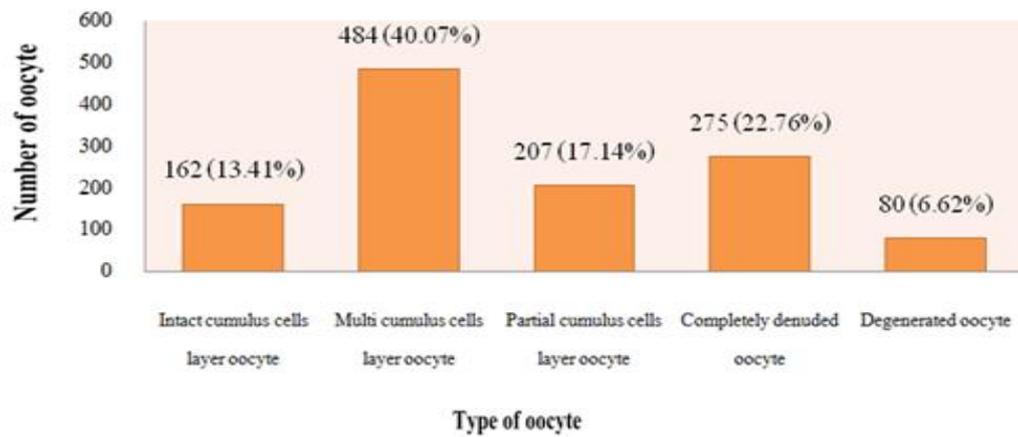


Figure 5. Percentages of 5 types of porcine oocytes that were aspirated by puncturing oocyte from small follicles.

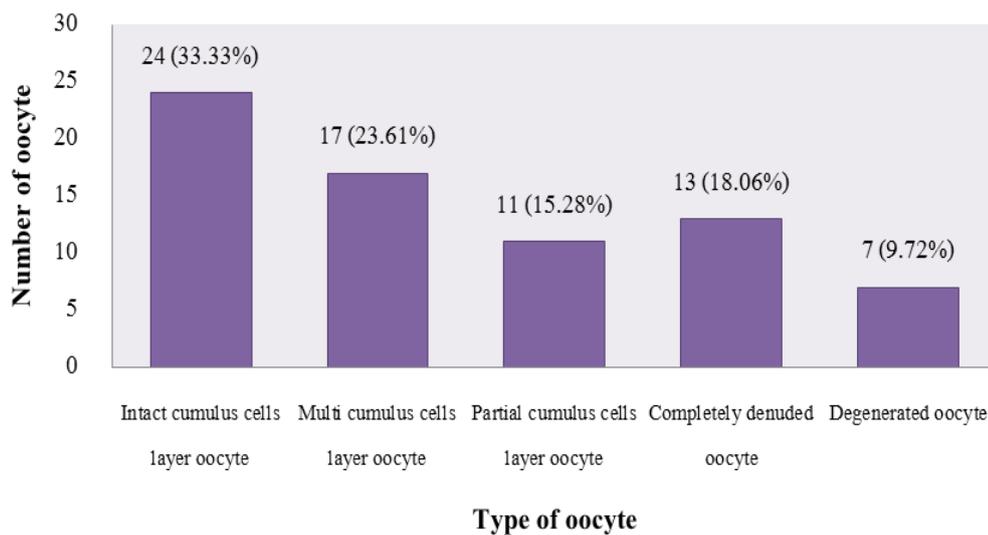


Figure 6. Percentages of 5 types of porcine oocytes that were aspirated by puncturing oocyte from medium follicles.

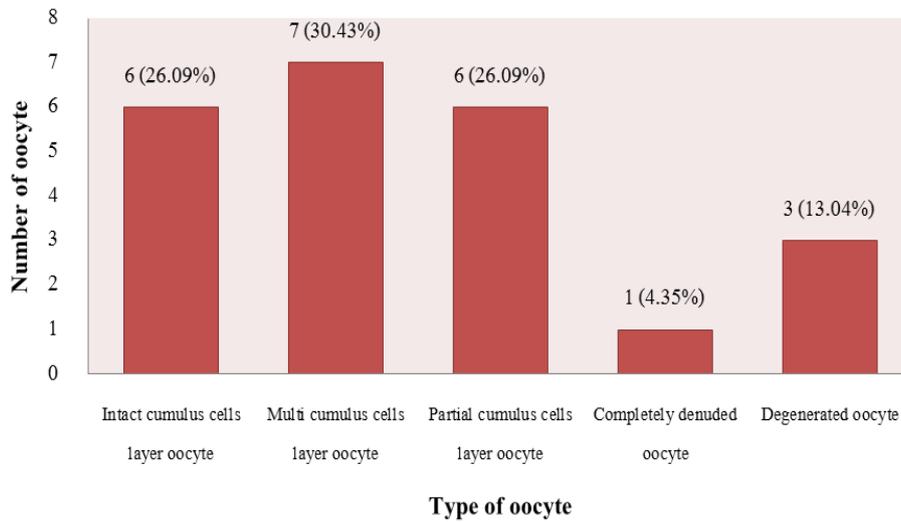


Figure 7. Percentages of 5 types of porcine oocytes that were aspirated by puncturing oocyte from large follicles.

Next, porcine follicular fluid (pFF) proteins of small, medium, and large follicles were investigated as to whether or not some of these proteins play important roles in promotion and regulation on growth, and development of the oocytes. The protein patterns determined by SDS-PAGE illustrated that the protein bands were in sizes between >220 kDa and <24 kDa. The results of pFF from small and medium follicles class I showed protein bands that had sizes of 24, 60-65, 79, 110, 140, 160, and >220 kDa. The protein band sizes at 24, 110, and >220 kDa in pFF of medium follicles appeared in stronger color than those of small follicles (Figure 8). This implied that these proteins in pFF of medium follicles were in higher concentration compared with the small follicle proteins.

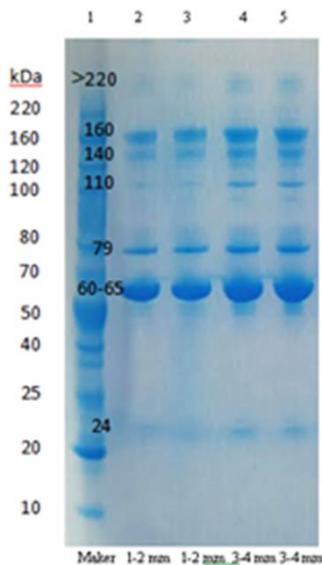


Figure 8. SDS-PAGE showed the patterns of protein bands from small and medium follicles. Lane 1: standard protein marker; lane 2-3: follicular fluid of small follicles (1-2 mm); lane 4-5: follicular fluid of medium follicles class I (3-4 mm).

The results of pFF from medium follicles class II and large follicles revealed 9 bands which had sizes of 52, 65, 79, 90, 110, 120, 160, 190, and >220 kDa. However, protein bands of sizes about 90 and 110 kDa were not observed in porcine follicular fluid of large follicles at 10 mm in diameter. The intensity of the protein bands at 79 and 160 kDa that presented in small (3-4 mm) and medium (5-6 mm) follicles were much stronger than those observed in the large follicles with diameters of 7-8 and 10 mm (Figure 9). These two bands (79 and 160 kDa) had diminished function in the first metaphase (Ducolomb *et al.*, 2013).

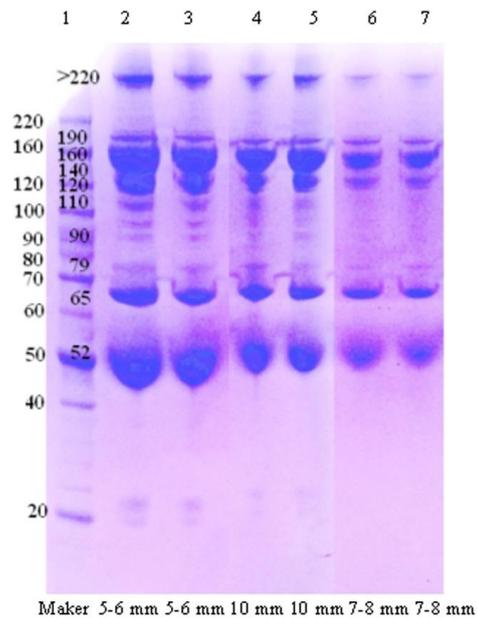


Figure 9. SDS-PAGE shows the pattern of protein bands from medium and large follicles. Lane 1: standard protein marker; lane 2-3: follicular fluid of medium follicle class II (5-6 mm); lane 4-5: follicular fluid of large follicle (10 mm); lane 6-7: follicular fluid of large follicle (7-8 mm).

The results of pFF from small, medium, and large follicles revealed 6 bands which were protein bands at 52, 65, 79, 90, 160, and >220 kDa (Table 1). Identification of the protein band size at 79 kDa from pFF by LC/MS/MS technique found that this protein might be porcine inhibitor of carbonic anhydrase (Ducolomb *et al.*, 2013). Furthermore, there were reports on the identification of several proteins from pFF showing that the 52 kDa protein band was an immunoglobulin gamma chain, the 65 kDa protein was keratin, the 90 kDa protein was heat shock protein or plasminogen precursor or both (Ducolomb *et al.*, 2013), and the large size protein >220 kDa was probably protease (Mettasart, 2009). The functions of all identified proteins function as supplements and promote oocyte maturation, ovulation, and embryo development in *in vivo* and *in vitro* conditions (Mettasart, 2009; Ducolomb *et al.*, 2013) with exception of the protein band at 79 and 160 kDa which has diminished function in the first metaphase in oocyte maturation. The results from this study allow us to better understand the protein functions in pFF. This information is important for the use of pFF proteins as a valuable resource for economical animal culture and farming. For example, it is easy to use as a supplement substance in *in vitro* fertilization and oocyte maturation. Because

Table 1. Identified proteins and function of porcine of small, medium, and large follicles.

Molecular mass (kDa)	Identified proteins	Biological activity	References
50-52	Immunoglobulin gamma chain	Promotion of <i>in vitro</i> maturation (IVM) and <i>in vitro</i> fertilization (IVF)	Ducolomb <i>et al.</i> (2013)
65	Keratin	favored germinal vesicle breakdown and promoted IVM	Ducolomb <i>et al.</i> (2013)
79	Porcine inhibitor of carbonic anhydrase	Decreased in the first metaphase	Ducolomb <i>et al.</i> (2013)
90	Heat shock protein and/or plasminogen precursor	Promotion of IVM and IVF	Mettasart (2009) and Ducolomb <i>et al.</i> (2013)
160	Tranthyretin	Germinal vesical breakdown and Decreased in the first metaphase	Ducolomb <i>et al.</i> (2013)
>220	Protease	Involved in digesting long protein chains into shorter fragments and Promotion of IVM and IVF	Mettasart (2009)

Abbreviations: IVM, *in vitro* maturation; IVF, *in vitro* fertilization; pFF, porcine follicular fluid

of its convenience in use and economy, it might be possible to replace the use of chemicals in the future. Moreover, it is not necessary to prepare fresh follicular fluid for biotechnology work as these follicular fluid proteins can be stored at -80 °C for up to 3 months and still show high induction ability (data not shown).

4. Conclusions

The findings demonstrated that porcine oocytes from small, medium, and large follicles were round in shape and surrounded by the zona pellucida with layers of CCs. Type I and II oocytes of medium and large follicles have high potential to develop into *in vitro*-matured oocytes which could be used for cell biotechnology and models for cell cytotoxic tests. The pFF of medium and large follicles revealed strong protein bands as supplement substances to replace chemicals in *in vitro* oocyte maturation and fertilization. The protein bands at 79 and 160 kDa, which presented in small follicles, were much stronger than those observed in the large follicles. However, those two bands have diminished function in the first metaphase.

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