Biological research on morphological features of in vitro porcine granulosa cells culture

Sukjai Rattanayuvakorn

Department of science and Mathematics, Faculty of Agriculture and Technology, Rajaqmangala University of Technology Isan, Surin Campus

Nongnuch Gumlungpat* and Mayuva Youngsabanat*

Department of Biology, Faculty of Science, Silpakorn University

Maijukkee Areekijseree

Department of Mechanical Engineering, Faculty of Engineering, King Mongkut's University of Technology Thonburi

Napatsorn Meedech

Department of Environmental Technology, Faculty of Applied Science, King Mongkut's Institute of Technology North Bangkok

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Abstract

The purpose of this research was to study the morphology and culturing porcine granulosa cells (GC) collected from large white pig ovaries with sterile technique. The primary cell culture methods were utilized based on M199 formula (supplemented with 10% HTFBS, 15 µg/mL FSH, 1 µg/mL LH, 1 µg/mL estradiol, 2.2 mg/mL NaHCO3, 0.25 mM pyruvate and 50 µg/mL gentamycin sulfate at high humidified atmosphere with 5% CO2 in 95% air atmosphere at 37°C) at 0, 24, 48, 72, 96 and 120 h (short-term culture). The result of culturing GC, using M199 based on cell morphology study, exhibited that, in the beginning, cells were round-shaped and non-ciliated cells. Porcine granulosa cells showed healthy characteristic and changed their morphology from round shape to fibroblast-like morphology, then extending and adhering to the surface of the petri dish. After 24 h, they multiplied and spread 100% all over the culture dish and then turned sharp at both head and bottom and expanded. As cultured for 48, 72, 96, 120 h. they were expanded more, fibroblast-like shape, epithelial-like isolated and cluster group cells under microscopy. The viability of GC culture for 0, 24, 48, 72, 96 and 120 h were 100, 98, 95, 92, 90, and 86%, respectively. The advantages of this research were: that it enabled us to culture GC cells collected from the slaughterhouse efficiently by using short-term culture

**Corresponding author: gumlungpat_n@su.ac.th, areekijseree_m@su.ac.th*

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methods in culture medium; that we were capable of studying the morphological features, as well as the variance of the cell shape in the laboratory; sub-culture cells and long-term culture can possibly be further developed as granulosa cell lines; and that the cells could be utilized practically in innovative biological research field, thus helping economize costs and eliminate the animal experiments correctly in accordance with moral norms.

Keywords: Biological research**;** porcine granulosa cells (GC); porcine oocyte

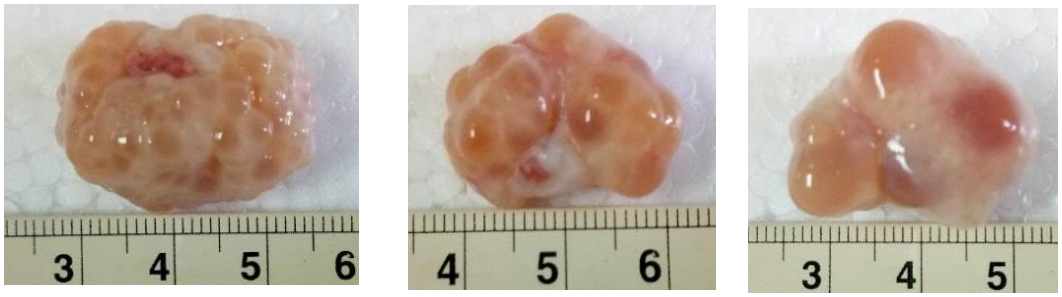
1. INTRODUCTION

Porcine ovary is unused organ form slaughter house which can be collected and used as a model in biology research. In porcine ovary composed of numerous granulosa cells which separated and cultured well in M199. This cell can be co-culture with embryonic cells for IVM, IVF, and IVP research as well as for toxicity testing (Areekijsere et al., 2008, Pongsawat and Youngsabanant, 2019; Youngsabanant and Mettasart, 2020). Short-term cell culture is used as a tool for innovative biological research such as toxicity on reproductive system and basic science (Sanmanee and Areekijsere, 2009, Areekijsere and Veerapraditsin (2008). In this study, we examined the morphological characteristic of primary porcine granulosa cells (GC) on short-term culture on culture hormone condition in place of experiments on animals. The primary granulosa cell culture method in vitro is to use fresh cells from slaughterhouse in the culture, which have been proved to have the same cellular characteristics (Youngsabanant and Rabiab (2020). Porcine granulosa cells can be experimented in innovative biological studies and reproduction. It also in vitro cultures assumed so significant a role in lessening the use of animal experiments in all biological study design and control (Chen et al., 2013, Youngsabanant et al., (2019); Youngsabanant-Areekijseree et al., (2019).

2. METHODS

Porcine ovary was collected and preparation of porcine granulosa cells (GC) for in vitro culture (Areekijseree and Vejaratpimol (2006). Porcine ovary was collected from local slaughterhouses in Nakorn Pathom Province, Thailand. Sample oocytes were collected from female Large White Pig Cross Land Race (aged around 5-7 month and weight around 150-220 kg.) as well as sample cells from different sizes of porcine oocytes during the estrus (small, medium and large size ovarian follicles). The porcine ovary was collected by cutting, and rinse 3 times using antibiotic-mixed 0.9% normal saline (100 international unit/mL penicillin G, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin-B). Then,

the ovary was contained in the sterile sampling bag, keep it in the thermos flask which contains distilled water with controlled temperature 30-35 oC, bring back to the laboratory within 1 h. After that, preparation of GC for in vitro culture by rinse the ovary with antibiotic-mixed 0.9% normal saline and use shape scissors to remove the connective tissue 2 times. Then, follicles were done by suction with an 18-gauge needle connected to the disposable a 5-10 mL syringe. The secretion was containing of oocyte and GC. Then, the oocyte was removed out and pour GC in a 12 mL cap tube, add 10 mL HEPES Tyrodes medium in, rinse the cells by gentle shaking for 2 min, and incubate them in the 37 oC incubator with 5% CO2 and high humidity for 10 min for the cells to fall to the bottom of the culture tube. Next, supernatant medium were removed, leaving only the cells on the bottom and re-rinse cells 7 times with washing medium. Finally, GC were cultured using M199 with Earle's salts (Sigma Chemical Co., St. Louis MO, USA), supplemented with 10% HTFBS, 15 μg/mL FSH, 1 μg/mL LH, 1 μg/mL estradiol, 2.2 mg/mL NaHCO3, 0.25 mM pyruvate and 50 μg/mL gentamycin sulfate and plate in the incubator at 37 oC plus 5% CO2 and high humidity, at 2x105 cells/mL concentration was used to study the percentage of cell viability and cell proliferation for 120 h. The morphological character of cells was also studied. Statistical analysis was performed using SPSS program version 28. One-way Anova was used to calculate the difference of means and SD among group.



A B C

Figure 1 (A) Small size follicle (1-3 mm) (B) medium size follicle (4-6 mm) (C) large size follicle (7-8 mm).

3. RESULTS and DISCUSSION

3.1 Study of porcine ovary

Granulosa cells from 720 porcine ovaries of Large White x Landrace x Duroc (5-7 months -2 years and weight between 150-220 kg) were investigated from local slaughterhouses in Nakhon Pathom

Province, Thailand. The measurement and weight of 30 ovaries were performed. We found that, the average weight of ovaries is 3.18±0.72 g, the average wide of ovaries is 1.65±0.31 cm, and the average length of ovaries is 2.34±0.31 cm. Moreover, the oocytes were varied in sizes and can be classified as small size follicles (1-3 mm in diameters; n=253), medium size follicles (4-6 mm in diameters; n=444), and large size follicles (7-8 mm in diameters; n=23) (Fig 1).

3.2 Morphological study of porcine granulosa cells (GC)

The granulosa cells (GC) from medium-sized porcine follicles were used for this experiment. Because GC from medium-sized porcine follicle were demonstrated at high developing cells (Pongsawat and Youngsabanant, 2019; Panyarachun et al., 2021). The morphological study of GC in M199 (added with 10% HTFBS, 15 µg/mL FSH, 1 µg/mL LH, 1 µg/mL estradiol, 2.2 mg/mL $NaHCO_3$, 0.25 mM pyruvate and 50 µg/mL gentamycin sulfate) and was in incubator at 37 oC plus 5% CO_2 and high humidity at 2x105 cells/mL concentration. The cells were observed under an inverted microscope at 0, 24, 48, 72, 96, 120 h. Under an inverted microscope unveiled that GC, as cultured for control (0 h), was morphologically round-shaped in group; some separated as individuals, floating in the culture medium without adhering to the surface of the falcon dish. As cultured for 24 h, we found that GC started adhering to the surface and stretched out around 10-20%, while round cells which were counted as 80% did not, floating in the culture. As cultured for 48 h, GC both separate and individual, approximately 30-40%, were found adhering to the surface, more stretching and spreading all over the falcon dish, whereas round cells roughly 60% did not, found only floating in the culture. As cultured for 72 h, GC approximately 50% adhered to the surface and turned sharp-headed and -bottomed; some compounded in group and reticulated; the others roughly 50% separated and floated all over the dish without surface adhesion. As cultured for 120 h, GC approximately 80% attached to the surface and turned more shape-headed-and-bottomed; some formed in group and reticulated, whiles the others for 20% separated all over the dish, floating without adhering to the surface (Fig. 2). As cultured in M199 cells appeared fastigiated, sharp-headed-and-bottomed, and reticulated all over the dish, having the different density in the entire area.

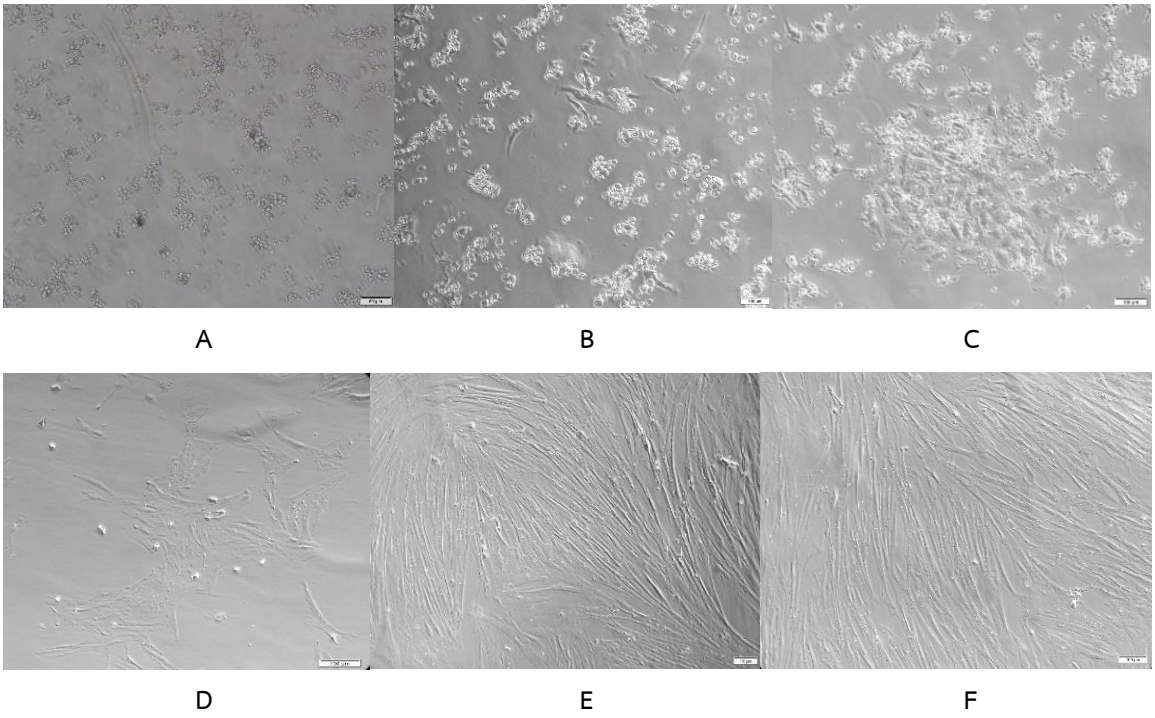


Figure 2 Photograph from inverted microscope showed GC cultured in M199. At concentration $2x10^5$ cells/mL, the cells are round, floating in the medium without the surface attachment. Photograph A showed GC cultured for 0 h; the cells are round, floating without the surface attachment. Photograph B-F showed GC adhering to the petri dish surface and appear sharp-pointed from head to bottom, fibroblastic and reticulated over the entire dish cultured after culture for 24, 48, 72, 96, 120 h.

The prolonged of porcine granulosa cells (GC) culture in M199 on different culture times (24, 48, 72, 96 and 120 h) were 162.22± 5.38, 218.33± 6.89, 327.47± 8.51, 349.76± 6.35, and 382.55± 7.53 micrometer. Statistical analysis Mean and standard deviation of the prolong of GC was calculated. The results from a statistical SPSS program by one-way ANOVA test, post hoc Duncan when compared the prolong growth percentage of each time of culture showed that significant differences (p<0.05). The differences amongst each group were identified as a, b, c, d, and e (Fig. 3).

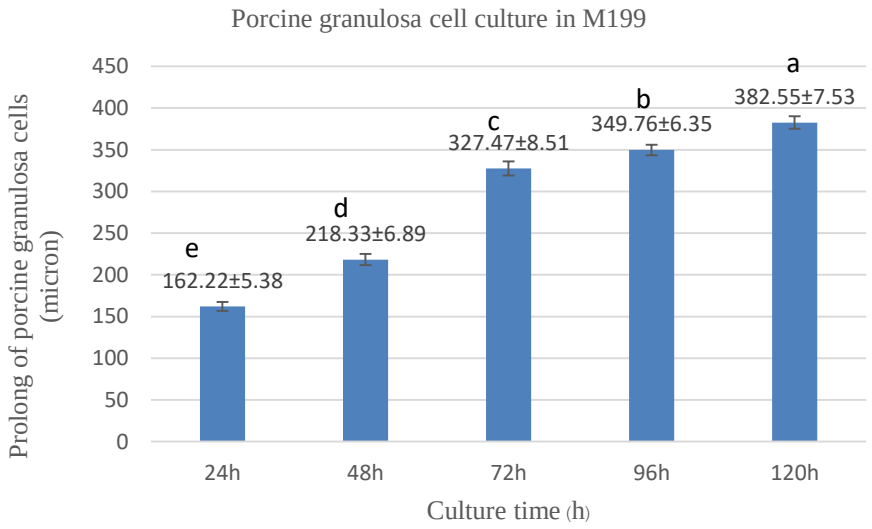


Figure 3 Graph shows prolonged of porcine granulosa cell culture in M199 on different culture time (24, 48, 72, 96 and 120 h). The prolong growth percentage of each time of culture showed significant differences (p<0.05). The differences amongst each group were identified as a, b, c, d, and e.

Table 1 The percentage of porcine granulosa cell viability under investigated inverted microscope shows 100, 98, 95, 92, 90, 86 percent on 0, 24, 48, 72, 96, 120 h of culture time.

Time (h)	Percentage (%)
0	100
24	98
48	95
72	92
96	90
120	86

3.3 Study of the growth and viability of porcine granulosa cells (GC) in *in vitro* short-term culture

The results of percentage cell viability of culturing GC from medium-sized porcine follicular cells in *in vitro* short-term culture (M199 supplemented with 10% HTFBS, 15 µg/mL FSH, 1 µg/mL LH, 1 µg/mL estradiol, 2.2 mg/mL $NaHCO_3$, 0.25 mM pyruvate, as well as 50 µg/mL gentamycin sulfate, plus

5% CO_2 and high humidity) for 0, 24, 48, 72, 96, and 120 h. were 100, 98, 95, 92, 90, 86, respectively as shown on Table 1.

The study of the morphology of granulosa cells based on the shot-term culture made obvious that granulosa cells cultured for 0 h were round without cilia, mostly forming in group and some minority was an individual cell, floating in the culture medium and not adhering to culture dish surface. After the 24 h culture, cells were found adhering to the surface and stretching out; some were round-shaped, floating in the M199 medium without adhesion. Cultured for 48 h, the GC attached to the surface and extended for 30-40% of the entire area; in the meantime, some initiated to turn sharp-headed-and-bottomed and multi-star-pointed in conformity with the study conducted by Xiaowei *et al.,* (2019) reporting regarding the morphological features of rat GC cultured for 48 h. The cells were star-like and fastigiated; some were reticulated. Porcine granulosa cells were found attaching to the surface approximately a half of the entire area, sharp-headed-and-bottomed and multi-star-pointed after 72 h of culture. The cells developed an adhesion to the surface for 80% of the entire area, sharp-headed-and-bottomed and multi-star-pointed; some formed in group and reticulated after culture for 120 h. These characters are the same as the porcine oviductal cell and oocyte culture (Panyarachun *et al.,* 2021; Gumlungpat *et al.,* 2023). Porcine GC could be used for future study as long-term culture by using several sub cultures. The long-term period cells cultured can possibly be further developed into cell lines. For example, Lin (2004) who developed porcine GC to be GC lines by culturing them in M199 for 11 weeks and conducting the subculture. Also, with a study of Youngsabanant *et al.,* (2021) porcine GC as cultured for 1-2 weeks, they expanded more and more and fastigiated like fibroblast, and developed as monolayer cells, spreading out all over the culture dish. We hope that our study can successfully cultivate porcine GC into cell lines and use them for innovative biological research (Chuen-im *et al.,* 2023).

4. CONCLUSION

This study reviewed that porcine granulosa cells could be cultured on primary for 120 h. in laboratory with normal morphology character. We found that, we could use fresh cells from recently dead living things in the culture, which have been proved to have the same cellular characteristics as those alive. Moreover, the porcine granulosa cells culture in M199 could possibly be further sub-culture cells and long-term culture and also developed as granulosa cell lines in the future study.

5. AECKNOWLEDGMENT

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