An establishment of long-term culture of porcine granulosa cells and comparison of DMEM and M199 for cell propagation

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Received: June 16, 2021; Accepted: September 4, 2021, 2021

Abstract

The result disclosed that in the initiation of the granular cell culture, the cells were round-shaped, then extended and adhered to the surface of the falcon dish. As cultured for 1-2 weeks, they expanded more, and for 12 weeks, turned reticulated. After 12 weeks of culture time in M199, granulosa cells were sub-culture for compared long-term culture based on 2 formulas. The sub-cultured cells were cultured in M199 and DMEM to investigate the morphological characteristics and cell viability of granulosa cells. The result of culturing porcine granulosa cells using M199 compared to those using DMEM, exhibited that, in the beginning, both were round-shaped, and then turned sharp-headed and sharp-bottomed and stretched out. Therein, granulosa cells culture in M199 shows 39.80$\pm$4.71% of cell viability, while granulosa cells culture in DMEM shows 116.67$\pm$ 8.20% of cell viability. Based on T-test, it is revealed that cell cultures with 2 formulas were statistically significantly different ($p<0.05$). The advantages of this research are that it enables us to culture porcine granulosa cells efficiently by using both primary cell culture and long-term culture methods in DMEM; that we can study the morphological features, as well as the variances of the cell shapes in the laboratory; and that the cells could be utilized practically in the biotechnological field, thus assisting to economize experiment costs and eliminate animal experiments in accordance with moral norms.

Keywords: *porcine granulosa cells; long term culture; MTT assay*

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1. Introduction

Pigs are not only for our consumption purpose, but also for scientific purposes, particularly being animal models, in scientific research, whether in medical or livestock terms (Pond *et al*. 1987) since pigs, as mammals, are considered physically, genetically, and physiologically- in neurological, circulatory, digestive, endocrine, dermatological, pancreatic, and reproductive systems -considerably close to human beings (Hafez, 1992; Swindle *et al*. 2012). Besides, they can be utilized variously in studies concerning relevant biomedical diseases, pathogeneses of viruses, bacteria, as well as parasites, protein-level organ transplantation, neurology (Bassols *et al*. 2014), and especially reproduction. Thanks to the pig's organ functions like the human's, we constantly culture porcine cells in lab as human-stimulating models in the inspection experiment on certain substances in terms of toxicity, as well as embryogenesis (Chen *et al.* 2013; Lossi *et al*. 2016; Swindle *et al.* 2012). There being a study of culturing granulosa cells (Verbraak E.J.C. *et al.* (2011) collected from the cow's 3-6 mm ovaries in the supplement with FSH and without hormones and then extracting tightly lining cumulus cells, results apparently disclosed that the supplement with FSH alone increased metabolism and progesterone production in granulosa cells on the contrary to that with both FSH and tightly lining cumulus cells. Afterwards, analyzing mRNA using microarray made clear that the new-targeting proteins of FSH inhibited DNA binding protein 2 and 3 (ID2 and ID3). The investigation of the expression quantity of both genes using quantitative PCR revealed that the quantity dropped down in cells with FSH alone, whiles augmenting in those with both. Research brought to conclusion that different hormone genres could affect the growth of in-lab cultured follicles and cumulus cells (Areekijseree M. and Vejaratpimol R. (2006). However, the objective of our study was interested in culturing fresh porcine granulosa cells from slaughterhouses using the primary culture method until they developed into long term culture compared based on 2 medium culture formulas. The sub-cultured cells were cultured in M199 and DMEM to investigate the morphological characteristics and cell viability of granulosa cells (M199 is appropriate for culturing endothelium cells, whereas DMEM is modified practically for nurturing both epithelium cells and endothelium cells, cell lines, fibroblast cells). Replaceable cells were experimented in research and studies especially based on in-lab primary culture methods (Kumar and Mallick, 2016). The primary culture method in laboratory is to use fresh cells from recently dead animal in the culture, which have been proved the same cellular characteristics as those alive (Miessen *et al*. 2011). The merit of this study was use cell culture for in vitro test of experimented in fundamental science studies and reproduction (Chen *et al*. 2013). In-lab cultures assumed so significant a role in lessening the use of animal experiments in biological studies (Chen *et al.* 2013; Miessen *et al*. 2011), and experiments can be designed and controlled as desired.

2. Methods

2.1 Culture medium

M199 with Earle's salt supplemented with 10% heat treated fetal bovine serum (HTFBS) , 2.2 mg/mL, $NaHCO_3$, 0.25 mM pyruvate, 50 µg/mL gentamycin sulfate was prepared and equilibrated in incubator at 37°C, 5% CO_2, 95% air atmosphere with high humidity for 12 h before used for primary and long-term cell culture. While DMEM supplemented with 10% HTFBS was prepared and cultured for 12 h before use for long-term cell culture, at 37°C and 5% CO_2 in 95% humidity air atmosphere.

2.2 Collect an ovary sample and take granulosa cells (GC)

Using the method by Areekijseree and Vejaratpimol (2006) as following Porcine ovary was collected from local slaughterhouses**.** Use a pair of scissors to cut the recently dead corpse's ovary, collect it, and rinse 2-3 times using antibiotic-mixed 0.9% normal saline (100 International unit/mL penicillin G, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin-B) . Afterwards, contain the ovary in the sterile sampling bag, keep it in the thermos flask which contains distilled water with controlled temperature 30-35 °C, bring back to the lab within 1 h, and then prepare and culture the cells in the supplements. Rinse the ovary with antibiotic-mixed 0.9% normal saline and use shape scissors to remove the connective tissue. Re-rinse the ovary with antibiotic-mixed 0.9% normal saline and wipe off using a sterile filter cloth. Pierce the follicles from the porcine ovary with an 18-gauge needle connected to the disposable a 5-10 mL syringe. Inside the ovary, there is some secretion, containing follicular cells, cumulus cells (CC) and granulosa cells (GC). Then, pour granulosa cells in a 12 mL cap tube, add 10 mL hepes buffered tyrodes medium in, rinse the cells by gentle shaking for 1-2 minutes, and incubate them in the 37 °C incubator with 5% CO_2 and high humidity for 10 minutes for the cells to fall to the bottom of the tube, and then eliminate the remaining medium water by suctioning, leaving only the cells on the bottom. Re-rinse them 5-7 times. Culture granulosa cells using M199 with Earle's salts (Sigma Chemical Co., St. Louis MO, USA), supplemented with 10% heat-treated fetal calf serum (HTFCS), 2.2 mg/mL $NaHCO_3$, 1M HEPES (Sigma Chemical Co., St. Louis MO, USA), 0.25 mM pyruvate, 15 mg/mL (Areekijseree *et. al*. 2006), at $2x10^5$ cells/mL concentration. Culture the cells in a 60 mL falcon dish, incubate the cells in the 37 °C incubator, enhanced with 5% CO_2 in high humidity, 50 percentage of medium were renewed every 2 days of long-term culture experiment, to study the percentage of cell viability, as well as long-term cell cultures. Granulosa cells were culture for 12 weeks. They were subculture (by using trypsin/EDTA exposed to the cells for long enough to detach cells and transfer $2x10^5$ cells/mL concentration to a new flask containing pre-warmed medium) for use in compare long-term culture based on 2 culture medium-M199 and DMEM (supplemented with 10% HTFCS) on this protocol.

2.3 Examination of the growth of the sample cells by detecting the viability of granulosa cells using MTT assay (Youngsabanant M. and Rabiab S. 2020)

Prepare a 12 mM MTT stock solution (kept in 4 ^{o}C and dark) by adding 1 mL sterile PBS in 5 mg MTT (substance A) and mix substances by shaking until it melts (some may not melt but can be infiltrated). Therein, 5 mg MTT can be employed in up to 100 samples, which need 10 µL MTT per well. Add 10 mL 0.01M HCl in 1 g SDS (substance B), mix it softly by shaking until it melts. Do this immediately as finishing preparing. Add 10 units/mL mixed substances following 1 in each well including control groups. Incubate in 37 ^{o}C for 4 h with the cell density over 1,000 cells/well, or incubate for a short period of time, i.e., 2 h. Add 100 µL SDS-HCl following 2 in each well and incubate in the 37 ^{o}C incubator with high humidity for 4 h. When the incubation finishes, measure the absorbance rate at 570 nm and study the morphological features of the cell samples using an inverted microscope.

2.4 Statistical analysis

Statistical analysis was conducted using SPSS software for Windows, and data was analyzed and compared using ANOVA Analysis.

3. Results and Discussions.

3.1 Study of the morphological features of granulosa cells from medium-sized porcine follicles

The study of the morphological features of granulosa cells in a long period using 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) in the incubator at 37 ^{o}C plus 5% CO_2 and 95% humidity at $2x10^5$ cells/mL concentration under an inverted microscope unveiled that granulosa cells (Figure 1), 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 petri dish. As cultured for 24 h, we found that granulosa cells 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, granulosa cells, both separate and individual, approximately 30-40%, were found adhering to the surface, more stretching and spreading all over the dish, whereas round cells roughly 60% did not, found only floating in the culture. As cultured for 72 h, granulosa cells 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, 80% of granulosa cells 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. As cultured for 1 week, 100% of granulosa cells

attached to the surface, extended more, and appeared fibroblast-like, shape-headed-and-bottomed, and reticulated over the entire dish. As cultured for 2 weeks, the cells tapered, extended more, and appeared fibroblastic and reticulated; some were found floating. As cultured for 4 weeks, the cells appeared tapering, long-stretching, fibroblast-like and reticulated; 90% adhered to the surface; 10% floated in the culture medium; some attached to the interface with over one layer. As cultured up to 6 weeks, the cells appeared tapering, longer-stretching, fibroblast-like and reticulated; 85-90% attached to the surface; 10-15% floated in the culture medium; whereas the adhering cells stretched and substituted for the floating ones; some attached to the interface with more than one layer.

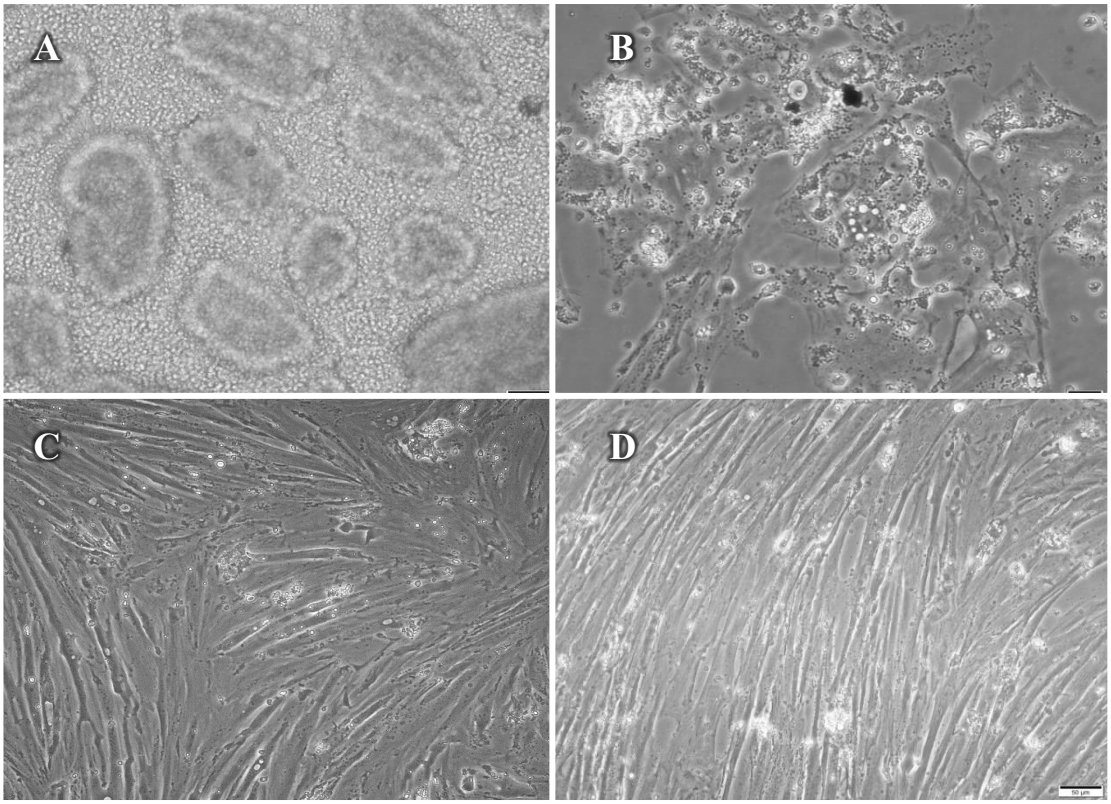

Figure 1 Inverted microscope showed porcine granulosa cells cultured in M199. At concentration $2x10^5$ cells/mL, the cells are round, floating in the medium without the surface attachment. A showed granulosa cells cultured for control (0 h) at 40x magnification. B shows granulosa cells cultured for 72 h. C shows the culture after 1 week; 100% of granulosa cells adhere to the falcon dish surface and appear sharp-pointed from head to bottom, fibroblastic and reticulated over the entire dish. D shows the culture after 2 weeks; granulosa cells adhere to the petri dish surface and appear sharp-pointed from head to bottom and fibroblastic.

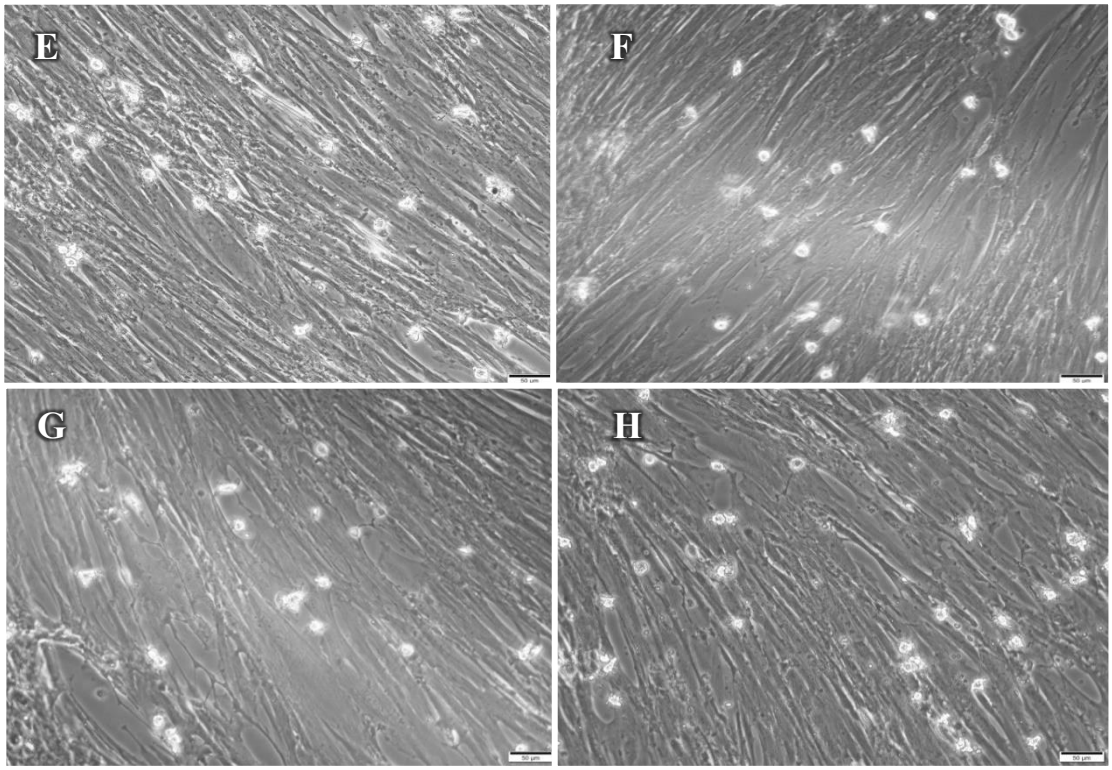

Figure 1 (Continued) Inverted microscope showed porcine granulosa cells cultured in M199. At concentration $2x10^5$ cells/mL, the cells are round, floating in the medium without the surface attachment. E shows the culture after 4 weeks; granulosa cells appear sharp-pointed from head to bottom and fibroblastic; and some cells adhere to the overlap with over one layer. F shows the culture after 6 weeks; granulosa cells appear sharp-pointed from head to bottom and fibroblastic; and some cells adhere to the overlap with over1 layer. G shows the culture after 8 weeks; granulosa cells appear sharp-pointed from head to bottom and fibroblastic; and some cells adhere to the overlap with over one layer. H shows the culture after 12 weeks; granulosa cells appear sharp-pointed from head to bottom and fibroblastic; and some cells adhere to the overlap with over one layer.

having conducted the culture in the longer period, that the number of fibroblast cells seemed to decrease. Up to 8 weeks, the granulosa cells for 80% appeared fastigiated, long stretching, and fibroblastic, and reticulated, adhering to the surface, and expanding in place for 20% which freely floated in the culture; some adhered to the overlapping areas with over one layer. Cultured up to 10 weeks, the number of cells which remained the same lowered down to 70-75%, and the substituting and freely floating cells increased up to a quarter; some still adhered to the overlapping areas. Lasting for 12 weeks, the cells remained the same, decreasing to 65-70% and the substituting and freely floating cells went up to approximately one-third; some

remained in the overlapping areas as shown in Figure 1. As to the study of the 12 weeks long term culture of granulosa cells as in Figure 2, it is discovered that granulosa cells developed step by step and reached the maximum growth rate in roughly 7-14 days. Afterwards, the rate went down, yet could still develop well for 65-70% as cultured for 12 weeks (84 days).

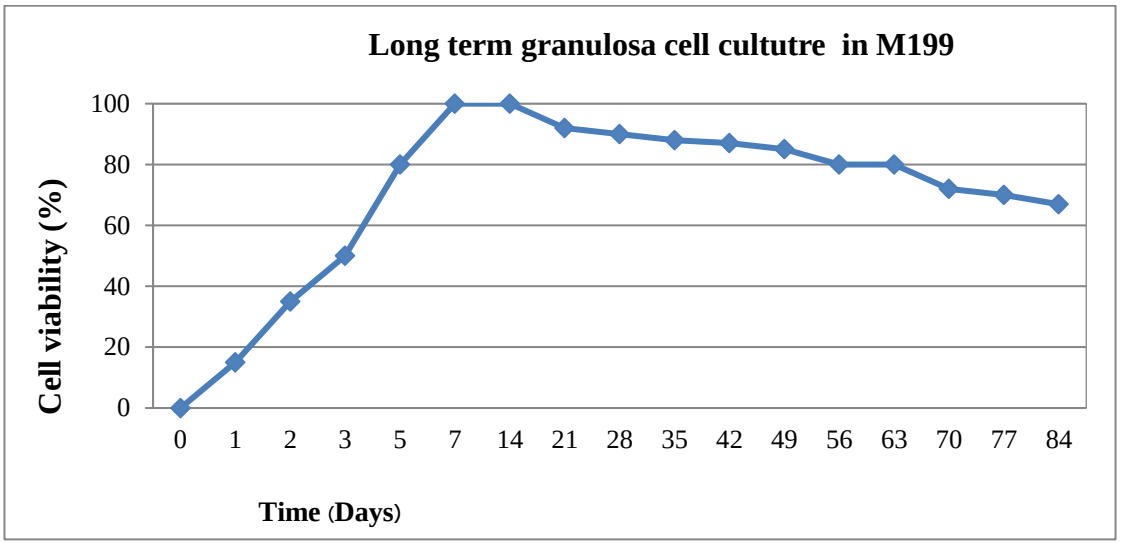

Figure 2 Graph shows the area percentage of the falcon dish occupied by granulosa cells following the 12-weeks (84 days) long-term culture in M199.

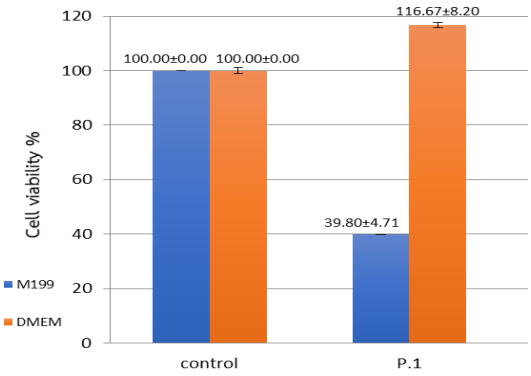

Figure 3 Graph exhibiting the cultures of granulosa cells with $2x10^5$ cells/mL concentration in M199 and DMEM in control group and P1 (experiment group). The percentages of cell growth in P1 compared to in both formulas showed statistically significant differences ($p<0.05$).

3.2 Study of the growth and viability of granulosa cells in M199 and DMEM

The result of culturing granulosa cells from medium-sized porcine follicular cells in 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, and in DMEM supplemented with 10% HTFBS in the 37 °C incubator plus 5% CO_2 and high humidity for 120 h. The cell viability percentage was investigated using MTT assay. Therein, granulosa cells culture in M199 shows 39.80±4.71% of cell

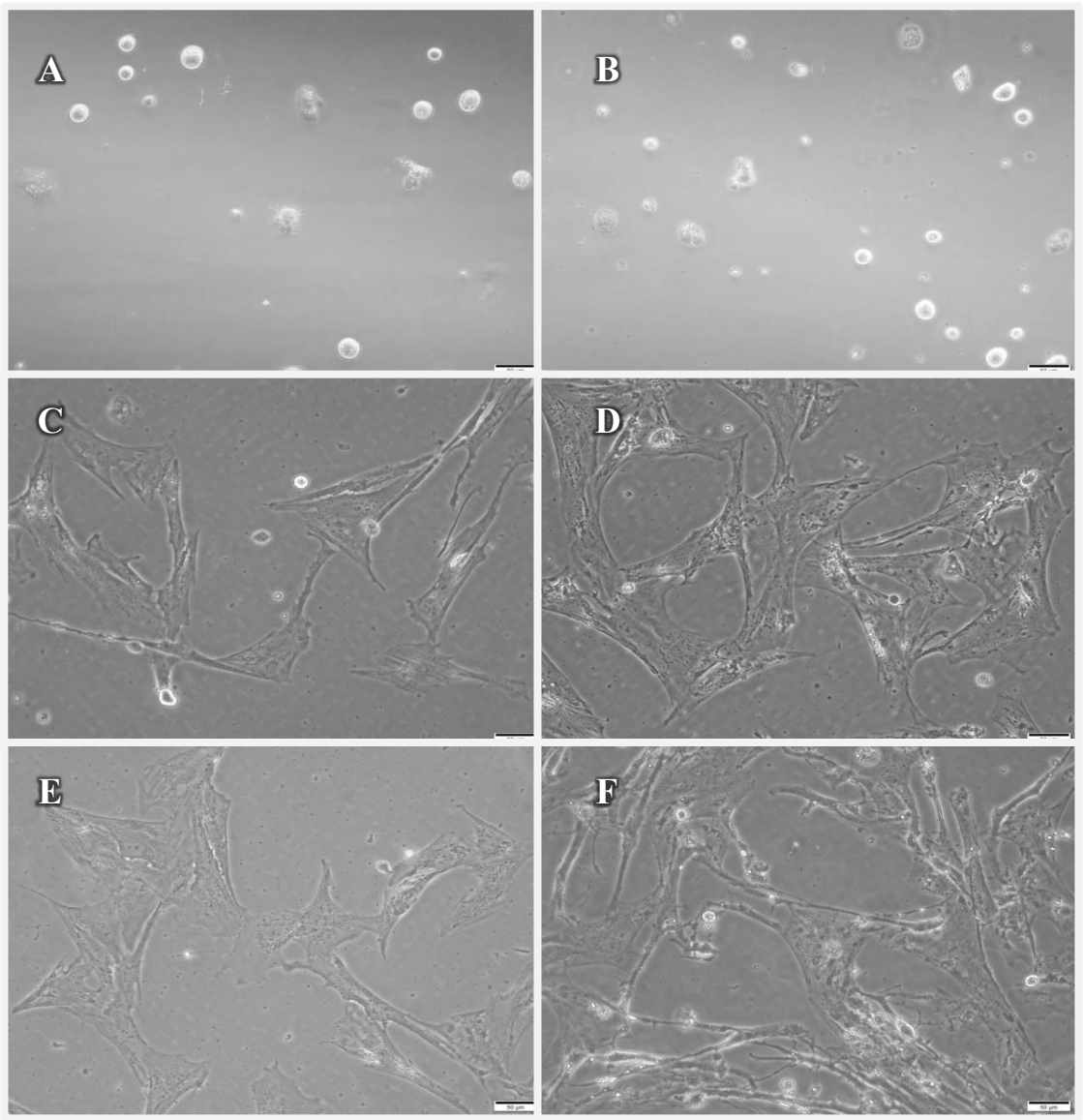

Figure 4 Inverted microscope showed porcine granulosa cells cultured in M199 (left hand side) and DMEM (right hand side) at concentration $2x10^5$cells/mL. A-B showed granulosa cells cultured for 0 h in M199 and DMEM; the cells are round, floating in the medium without the surface attachment. C-D showed granulosa cells cultured for 24 h in M199 and DMEM. E-F showed granulosa cells cultured for 48 h in M199 and DMEM.

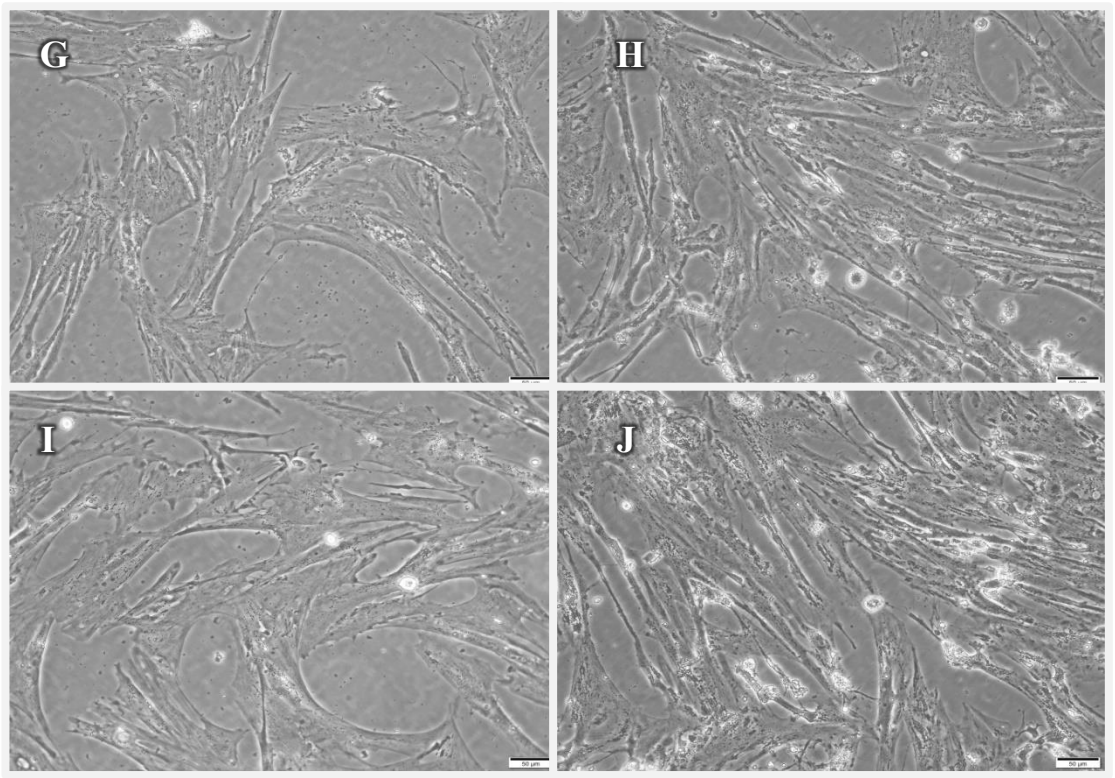

Figure 4 (Continued) Inverted microscope showed porcine granulosa cells cultured in M199 (left hand side) and DMEM (right hand side) at concentration $2x10^5$cells/mL. G-H showed granulosa cells cultured for 72 h in M199 and DMEM. I-J showed granulosa cells cultured for 120 h in M199 and DMEM (40x magnification)

viability, while granulosa cells culture in DMEM shows 116.67± 8.20% of cell viability. Based on T-test, it is revealed that cell cultures with 2 formulas were statistically significantly different ($p<0.05$) (Figure 3). As cultured in M199 and DMEM, cells appeared fastigiated, sharp-headed-and-bottomed, and reticulated all over the falcon dish, having the different density in the entire area. Granulosa cells culture in DMEM was higher density and growth than culture in M199 in all culture time. The morphological of granulosa cell in 2 formulas medium were shows in Figure 4.

3.3 Discussion

The morphology of granulosa cells is round shape which primary cell type in the ovary (in vivo) that provides the physical support and microenvironment required for the developing oocyte. Our study focusing on granulosa cells in culture medium (in vitro) on the long-term culture made obvious that granulosa cells cultured for control were round shape, forming in group; the minority was an individual cell, floating in the culture medium and not adhering to the dish surface. After in vitro culture, granulosa cells were as monolayers on an artificial substrate.

They also showed fibroblastic (or fibroblast-like) cells are bipolar or multipolar and have elongated shapes and grow attached to a substrate. After the 24 h culture, cells were found adhering to the surface and stretching out; some were round-shaped, floating in the medium without adhesion. Cultured for 48 h, the cells 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 granulosa cells cultured for 48 h. The cells were star-like and fastigiated; some were reticulated. Cultured up to 72 h, the cells were found attaching to the surface approximately a half of the entire area, sharp-headed-and-bottomed and multi-star-pointed. Cultured for 120 h, 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. Cultured over 1 week, the cells had an attachment to the surface for 100% of the entire area; the cells stretched out longer to be fibroblast-like, sharp-headed-and-bottomed and reticulated. Similarly, Yoshinao *et al.* (2012) studying the morphological features of porcine granulosa cells and observing using the microscope for 7 days discovered that initially the cells were round in shape; as cultured for 24 h, granulosa cells started to attach tightly to the surface of the falcon dish and stretched longer, then turning fibroblastic as cultured for 48 h; Cultured up to 96 h, the cells became fibroblast-like and extended more until connecting all over the falcon dish when cultured in the following 7 days.

In this study, we found that granulosa cells in long-term can be alive for 12 weeks. This type of culture can be applied in the study of the structures, mechanisms, or even variations of the cell in long term, and can be utilized in the investigation of cell toxicity (Chen *et al*. 2013). According to the result of culturing granulosa cells in long-term, it's found that the cells were fastigiated, sharp-headed-and-bottomed, fibroblast-like and reticulated; some cells adhered to the interface with over one layer in a similar way as a study of Cornelia (1970) focusing on the morphological features of the different sizes of porcine follicular cells and eventually finding that as cultured for 2-14 days, granulosa cells stretched out and proliferated their number. Furthermore, we concluded that granulosa cells developed better gradually. The growth rate was at the highest by day 7-14; afterwards, it initiated to go down, but still maintained at around 65-70% as cultured for 84 days in conformity with a study of Winston *et al*. (2001) leading to conclusion that the cells were able to grow day by day until fully by day 8 of the culture process and proved to be viable longer for 12 weeks; as no research was ever performed as his before.

Such the long-term culture shows the cell viability, as well as good cell structures. The cells cultured in long term period can possibly be further developed as cell lines. For example, Lin (2004) who developed porcine granulosa

cells to be granulosa cell lines by culturing them in M199 for 11 weeks and conducting the subculture. The sub-cultured cells were then cultured in M199 and DMEM to investigate the morphological characteristics of granulosa cells. Therein, cells in control (0 h), as primarily cultured, were found that their features cultured in both M199 and DMEM had no significant difference; the cells were round, forming in group floating in the medium without surface attachment. When culturing from 24 h, it is revealed the cells in both mediums appeared sharp-headed-and-bottomed and developed attachments to the surface, extended out, formed in group, and turned reticulated. It correlated to a study of Lin (2004) culturing them using the same method and discovering the cells were fibroblast-like; and correlated to a study of Sadowska *et al*. (2015) culturing granulosa cell lines in DMEM found, as cultured up to 48 h, sharp-headed-and-bottomed and fastigiated like fibroblast, and developed as monolayer cells, spreading out all over the dish. Moreover, the outcome of the culture of granulosa cells in both formulas disclosed that the cells cultured in DMEM attached to the surface and extended out rather than the ones cultured in M199. The results showed that, granulosa cells culture in DMEM was higher density and growth than culture in M199 in all culture time. So, DMEM could used for long term culture of granulosa cells. These disparities were consequences of both formulas which consisted of different ingredient components and ought to be utilized for different purposes. That is, M199 is appropriate for culturing endothelium cells, whereas DMEM is modified practically for nurturing both epithelium cells and endothelium cells, and is a popular medium formula used in culturing cell lines, fibroblast cells rather than the primary cells. That is why granulosa cells from the subculture, as cultured in DMEM, had a better evolution than the ones cultured in M199. If continually furthered for the subculture, they are prone to grow successfully to be cell lines.

In this study, we detected the development outcome of the cell viability based on MTT assay. MTT assay could measure the cell viability -the process of reduction of the mitochondria in living cells by changing tetrazolium salt color to formazan crystal color (yellow to purple) (Alzbeta *et al,* 2018). The light absorbance rate was then evaluated. According to the long-term culture of granulosa cells focused on the cell viability using MTT assay, we discovered that the time which the cell culture was performed over 2 weeks showed the highest percentage of cell development which had a statistically significant contrast to that of the control group ($p<0.05$). We additionally developed granulosa cells into cell lines by sub-culturing cells in two medium formulas - M199 and DMEM - then inspecting the cell viability using MTT assay; and finally found that the results from both formulas were statistically significantly distinct ($p<0.05$). Likewise, Tiemann *et al*. (2007) reported the utilization of MTT assay in the investigation of the cell viability of granulosa cells cultured based on the monolayer culture. The 570 nm light absorbance was then

tested using a microplate. Alzbeta *et al.* (2018) also cultured granulosa cells for studying the relevant effects on cellular mechanisms, detecting the cell viability using MTT assay, and measuring the 570 nm light absorbance rate based on the microplate as well as this study.

4. Conclusion

This study could culture porcine granulosa cells on primary and 12 weeks long-term culture in M199 (added with 10% heat treated fetal bovine serum 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). After 12 weeks of culture time in M199, granulosa cells were sub-culture for compared long-term culture based on 2 formulas. So, the sub-cultured cells were then cultured in M199 and DMEM to investigate the morphological characteristics of granulosa cells. The results showed that, granulosa cells culture in DMEM was higher density and growth than culture in M199 in all culture time. Sub-culture cells and long-term culture can possibly be further developed as granulosa cell lines.

5. Acknowledgement

The project was funded by a grant from Silpakorn University, Program Management Unit (PMU-B), Thailand, and Faculty of Science, Silpakorn University, Thailand.

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