Research Article *Science, Engineering and Health Studies 2020, 14(2), 141-151*

Potential effect of porcine follicular fluid (pFF) from small-, medium-, and large-sized ovarian follicles on HeLa cell line viability

Mayuva Youngsabanant* and Suphaphorn Rabiab

Department of Biology, Faculty of Science, Silpakorn University, Nakhon Pathom 73000, Thailand
**Corresponding author: areekijseree_m@su.ac.th*

Received: December 16, 2019; Revised: May 6, 2020; Accepted: May 13, 2020

ABSTRACT

The experiments reported herein show the effect of porcine follicular fluid (pFF) at concentrations of 2, 4, 20, 40, 200, 400, 500, and 600 µg protein/mL on HeLa cell line viability. We found that 600, 500, and 400 µg proteins/mL of pFF from small-, medium-, and large-sized ovarian follicles showed the highest viability (141.65±26.07, 153.14±16.58, and 140.52±11.71%), which was significantly different from the control group ($p<0.05$), but lower than the positive control. The cell morphology of those cultured in pFF from the medium- and large-sized follicles remained normal. Cell morphology tended to be abnormal at high concentrations of pFF from small-sized ovarian follicles. Thus, we suggest the applicability of pFF from medium- and large-sized ovarian follicles at concentrations of 40, 200, 400, 500, and 600 µg protein/mL as a substitute for heat-treated fetal bovine serum (HTFBS), which is more expensive. However, it should be noted that not only did pFF extracted from small-sized ovarian follicles have no significant effect on the viability of HeLa cell lines; cell morphology tended to be abnormal at high concentrations of pFF. This study contributes to biotechnology research by discovering applications of pFF extracted from ovarian follicles of different sizes as supplements in culture media.

***Keywords*:** HeLa cell lines; porcine follicular fluid (pFF); porcine ovarian follicles; MTT assay

1. INTRODUCTION

This research focused on using porcine follicular fluid (pFF) in an extensive study on the effect of pFF on cell viability and growth. An example of an application of pFF is its use as a supplement in cell culture medium for cancer and other cell lines in laboratory settings. Our protein analysis indicated that pFF is composed of several substances generated by granulosa cells. Particularly, we used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to analyze the molecular weights of proteins found in ovarian follicles of three sizes: small (1-3 mm in diameter), medium (4-6 mm in diameter), and large (7-10 mm in diameter). The molecular weights of proteins from small-sized ovarian follicles are 50, 65, 75, 90, 95, 110, 120, 160, 190, and greater than 220 kDa. Proteins from medium-sized ovarian follicles have molecular weights of 50, 65, 75, 90, 110, 120, 160, 180, and greater than 220 kDa. In addition, proteins with molecular weights of 50, 65, 90, 110, 120, 160, 180, and greater than 220 kDa in large-sized ovarian follicles were reported (Youngsabanant et al., 2019). Previous studies suggested that the protein component of pFF plays an important role in nuclear envelope breakdown of the oocyte, which compliments the processes of oocyte maturation and ovulation (Ducolomb et al., 2013; Youngsabanant-Areekijseree et

al., 2019; Youngsabanant et al., 2019). An immunological study suggested that the 27 kDa proteins are immunoglobulin (Ig), which function in immunity, and can be categorized into five groups: IgG, IgA, IgM, IgD, and IgE, each of which functions differently (Mettasart, 2009). Another study suggested that keratin, the 62-65 kDa protein, has effects on nuclear envelope breakdown and oocyte maturation. They also suggested that the 70 kDa protein is a coagulation factor which could potentially delay the transition of the oocyte to metaphase I. The 80-kDa proteins are porcine inhibitors of carbonic anhydrase, which also delay the transition of the oocyte to metaphase I (Ducolomb et al., 2013). Moreover, follicular fluid is composed of hyaluronic acid (HA), a complex compound of polysaccharides and glycosaminoglycans (GAGs). The water absorption capacity of HA gives cells their gel-like texture and an abundance of fibrous proteins such as keratin, collagen, albumin, immunoglobulin, and ceruloplasmin. Along the same lines, the compounds of human follicular fluid are categorized into nine groups: 1. follicle stimulating hormone (FSH), luteinizing hormone (LH), estrogen, and progesterone hormones; 2. sugars and hyaluronan; 3. growth factors of the transforming growth factor-beta (TGF-beta) superfamily; 4. growth factors and interleukins; 5. reactive oxygen species (ROS); 6. anti-apoptotic factors; 7. proteins, peptides, and amino acids; 8. anti-apoptotic factors; and 9. prostanoids. In another study, the cumulus cell expansion phase of the oocyte-cumulus cell complex (COCs) occurred, and FSH was found to be essential for the development of HA. Particularly, FSH stimulates granular cells and supports estrogen in the developing cytoplasm during the oocyte maturation process (Suchanek et al., 1994). Kimura et al., (2002) studied the development of HA, a component of the extracellular matrix of the cumulus-oocyte complex. They studied the roles of the mRNA of hyaluronan synthase 2 (has2), hyaluronan synthase 3 (has3), and CD44 during the mammalian preovulatory phase. The study showed that HA is related to electrocardiogram (eCG) and pFF of the genes in COCs, oocytectomized (OXCs). They also found that cumulus cells expressed the mRNA of has2 and CD44. In addition, oocytes expressed the mRNA of has3. Together, these studies suggested that pFF is composed of hyaluronic acid, collagen, hormones, and growth factors, all of which play an important role in cell development. This study focused on the effect of pFF from small-, medium-, and large-sized ovarian follicles on HeLa cell line viability, growth, and cell morphology. The results could provide knowledge useful for selecting the optimal concentration of pFF from antral follicles of different sizes for future use in cell culture.

2. MATERIALS AND METHODS

2.1 Materials

Pubertal porcine ovaries from pigs (aged between 210 and 250 days) were collected from a local slaughterhouse using the method reported previously (Areekijseree et al., 2005; Pongsawat and Youngsabanant., 2019). HeLa cell lines were obtained from The Korean Cell Line Bank (Seoul, South Korea).

2.2 Porcine follicular fluid (pFF) collection

Porcine ovaries were removed within 30 minutes after being slaughtered and then transported to a laboratory in a thermos container at a temperature of 30-35°C. pFF was collected from pubertal pig ovarian follicles of three sizes: small (1-3 mm in diameter), medium (4-6 mm in diameter), and large (more than 7 mm in diameter) by using a syringe connected to an 18-gauge needle. Then, fluid was collected in a conical tube and centrifuged for 5 minutes at 1500 × g to remove oocytes and cells. pFF was stored at −80°C until use. Protein quantification was carried out using the Lowry method (Lowry et al., 1951) to calculate the protein concentrations of pFF from three sizes of ovarian follicles to supplement the culture medium.

2.3 HeLa cell line culture

HeLa cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% heat-treated fetal bovine serum (HTFBS), at 37°C, in high humidity, in a 5% carbon dioxide and 95% air atmosphere, at 37°C before experiments were performed. The separated cells were seeded in culture medium at 5 × 10^5 cells/mL at 37°C, in a 5% carbon dioxide and 95% air atmosphere, in high humidity, for 48 hours.

2.4 Experimental setup

pFF from small-, medium-, and large-sized ovarian follicles were cultured *in vitro* with HeLa cell lines in experiments consisting of three groups. Group 1: control group consisted of HeLa cell lines cultured in DMEM. Group 2: positive control group comprised HeLa cell lines cultured in DMEM and 10% HTBFS. Group 3: treatment group consisted of 2, 4, 20, 40, 200, 400, 500, and 600 µg protein/mL of pFF extracted from small-, medium-, and large-sized ovarian follicles, all of which were *in vitro* cultured in HeLa cell lines for 24 hours before using the MTT assay to determine the number of viable cells present. For the statistical analyses, one-way ANOVA and Duncan's *post hoc* analysis were used to examine the similarities and differences between the three groups.

2.5 MTT assay and cell morphological study

HeLa cell lines were seeded in a 96-well plate at 20,000 cells/well for 48 hours before treatment with pFF from three sizes of ovarian follicles at concentrations of 2, 4, 20, 40, 200, 400, 500, and 600 µg proteins/mL for 24 hours. After incubation, they were incubated with tetrazolium salt for 4 hours, and cell viability was quantified by detecting formazan dye crystal formation with a spectrophotometer at 570 nm. Meanwhile, cell morphology was observed during the culture period and after treatment with pFF under an inverted microscope.

3. RESULTS AND DISCUSSION

3.1 Morphological study

The morphological characteristics of HeLa cell lines were examined by using an inverted microscope and a scanning electron microscope. During the first culture, HeLa cell lines were oval shaped with numerous knobs and micro villi (Figures 1A and 1B). After 24 hours in culture medium, they transformed into flattened cells and spread out over the surface of the culture paper (Figures 1C and 1D). After 48 hours in culture medium, they transformed into spindle-shaped cells, numerous spine shapes, and doubled in size at around 80%-90% confluence (Figures 1E and 1F).

3.2 Potential effect of pFF from small-sized ovarian follicles on HeLa cell line viability

The results indicated that HeLa cell line viability after 24 hours in cells treated with pFF at 2, 4, and 20 µg protein/mL were 88.80±9.92, 81.53± 15.83, and 99.80±10.58, respectively. Their viability was less than that of the control group, but the difference was not statistically significant. The HeLa cell line viability under treatments with 40, 200, 400, and 500 µg protein/mL was 104.32±14.65, 105.12±4.42 , 110.22±12.56, and 107.27±14.98, respectively. The viability of HeLa cell lines in the positive control group was 181.34±21.22, and at a concentration of 600 µg protein/mL, percent viability was 141.65±26.07, which was the highest, but significantly lower than that of the positive control group (p<0.05), as illustrated in Figure 2. HeLa cell lines treated with 200, 400, 500, and 600 µg proteins/mL of pFF from a small-sized ovarian follicle were transformed into abnormal morphology with a vacuole in the cytoplasm, which indicated that cells degenerated, as illustrated in Figure 3.

3.3 Effect of pFF from a medium-sized ovarian follicle on HeLa cell line viability

The viability of groups treated with pFF from a medium-sized ovarian follicle at 2, 4, and 20 µg proteins/mL were 110.34±12.01, 101.42±9.11, and 117.04±3.34, respectively. Their viability were higher than those of the control group, but not statistically significant difference. The HeLa cell line viability of 40, 200, 400, 500, and 600 µg protein/mL were 134.48±8.43, 128.60±5.79, 140.16±21.58, 153.14±16.58, and 127.79±12.90, respectively. The viability of HeLa cell lines under these treatments were higher than those of the control group (p<0.05). The groups treated with 500 µg protein/mL showed the highest cell viability, but the level was statistically lower than that of the positive control group, as illustrated in Figure 4. All cell cultures in the treatment groups showed normal morphology, as illustrated in Figure 5.

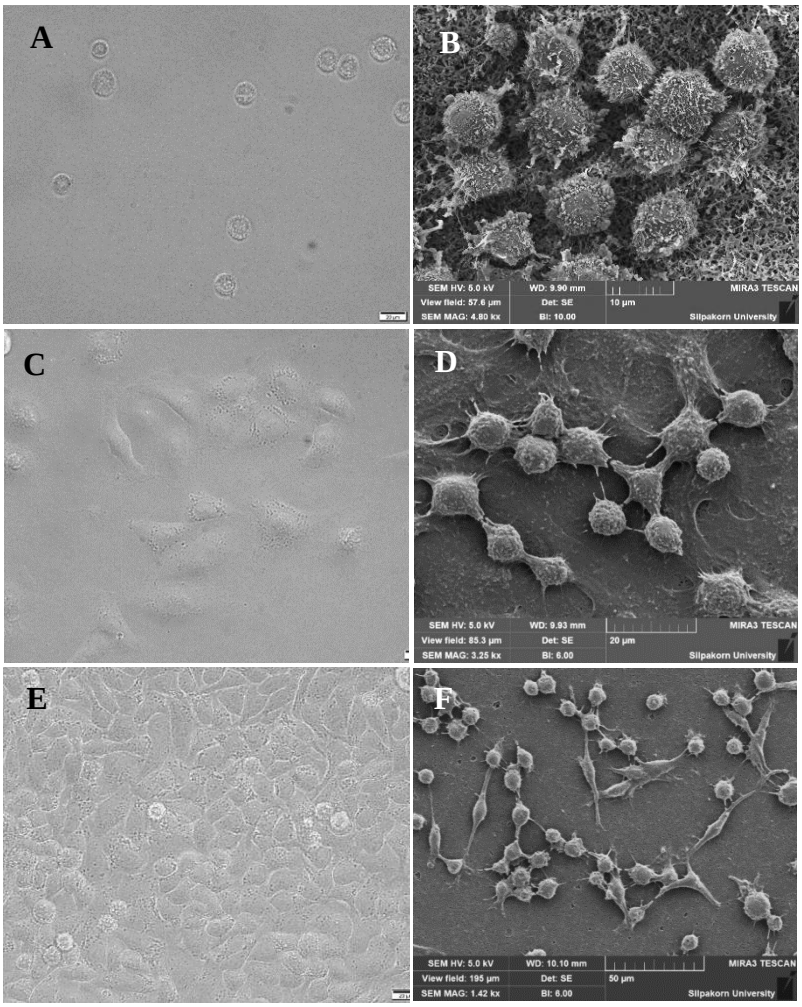

Figure 1 Images from inverted microscope (400 × magnification) (left hand side) and from scanning electron microscope (right hand side) of HeLa cell lines cultured in DMEM with 10% HTFBS showing rounded cells in the first culture period (A-B), attachment to the substrate after culture for 24 hours (C-D), and some took on a fibroblast-like shape and spread out over the surface of the culture paper after culture for 48 hours (E-F)

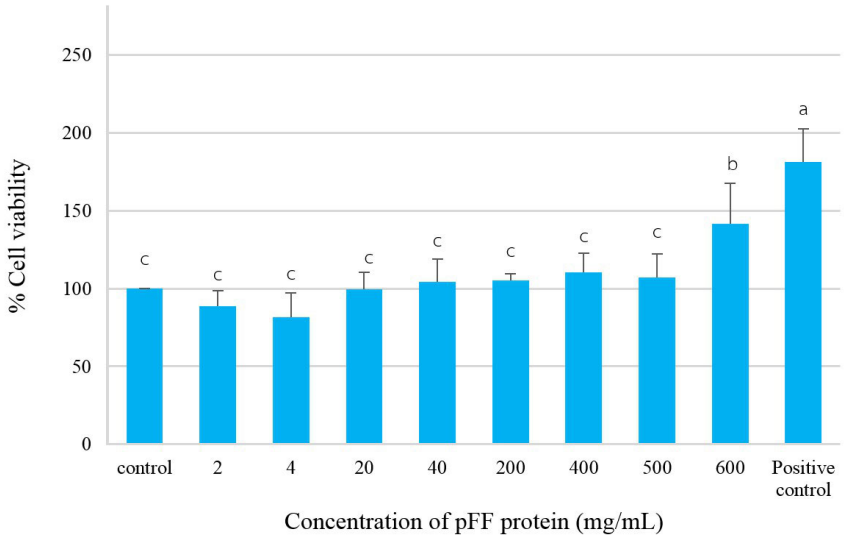

Figure 2 Effect of pFF from small-sized ovarian follicle groups at concentrations of 2, 4, 20, 40, 200, 400, 500, and 600 µg protein/mL in DMEM for 24 hours on the percent viability of HeLa cell lines, compared with control group (DMEM) and positive control group (DMEM supplemented with 10% HTFBS)

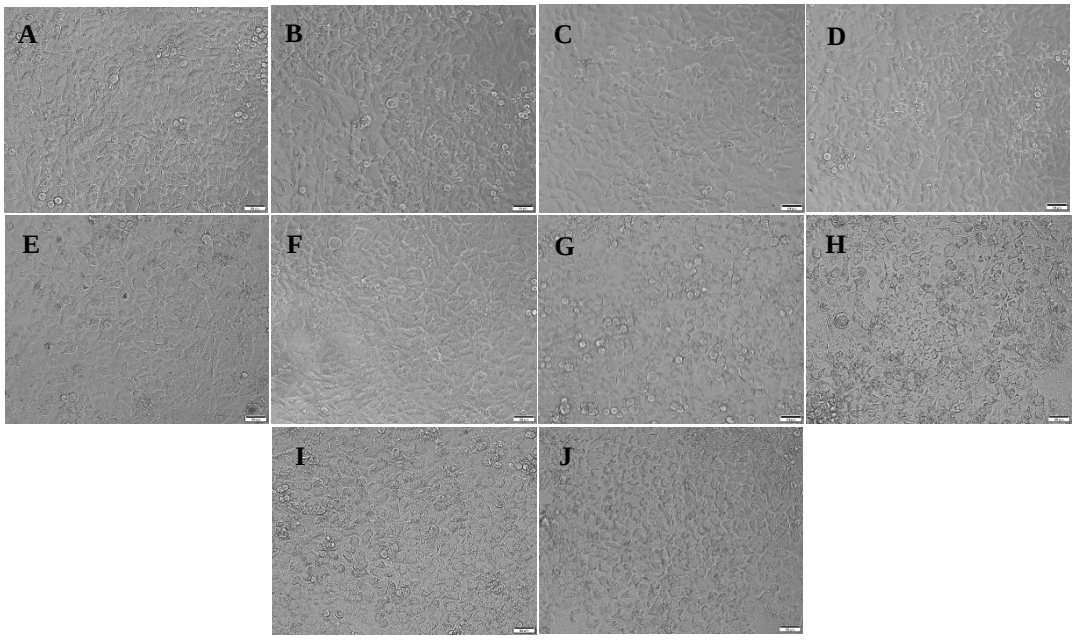

Figure 3 Images from inverted microscope (200 × magnification) showing HeLa cell line morphology; control group (A), positive control (B), the treated groups with porcine follicular fluid from a small-sized ovarian follicle at 2, 4, 20, and 40 µg proteins/mL (C-F), groups treated for 24 hours with pFF from a small-sized ovarian follicle at 200, 400, 500, and 600 µg proteins/mL showed abnormal cell shapes (G-J) (scale bar = 20 µm)

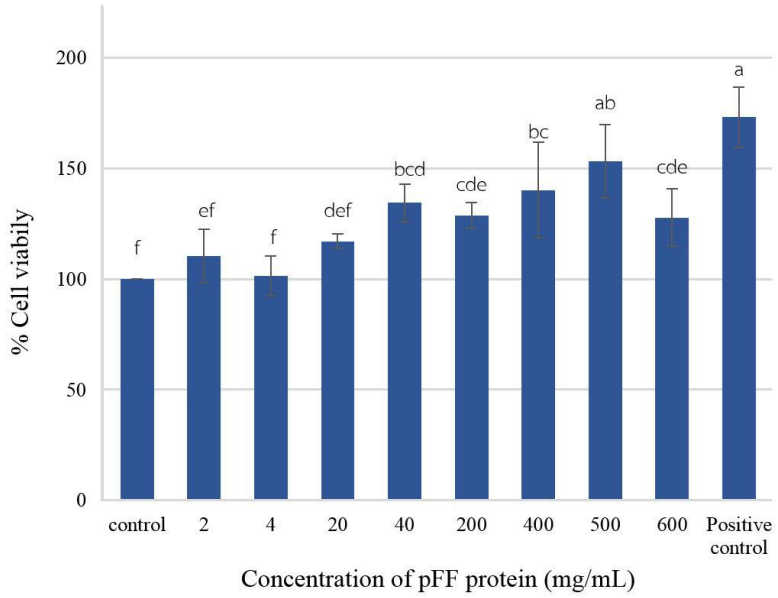

Figure 4 Effect of porcine follicular fluid from medium-sized ovarian follicle groups at concentrations of 2, 4, 20, 40, 200, 400, 500, and 600 µg protein/mL in DMEM for 24 hours on the percent viability of HeLa cell lines, compared with control group (DMEM) and positive control group (DMEM supplemented with 10% HTFBS)

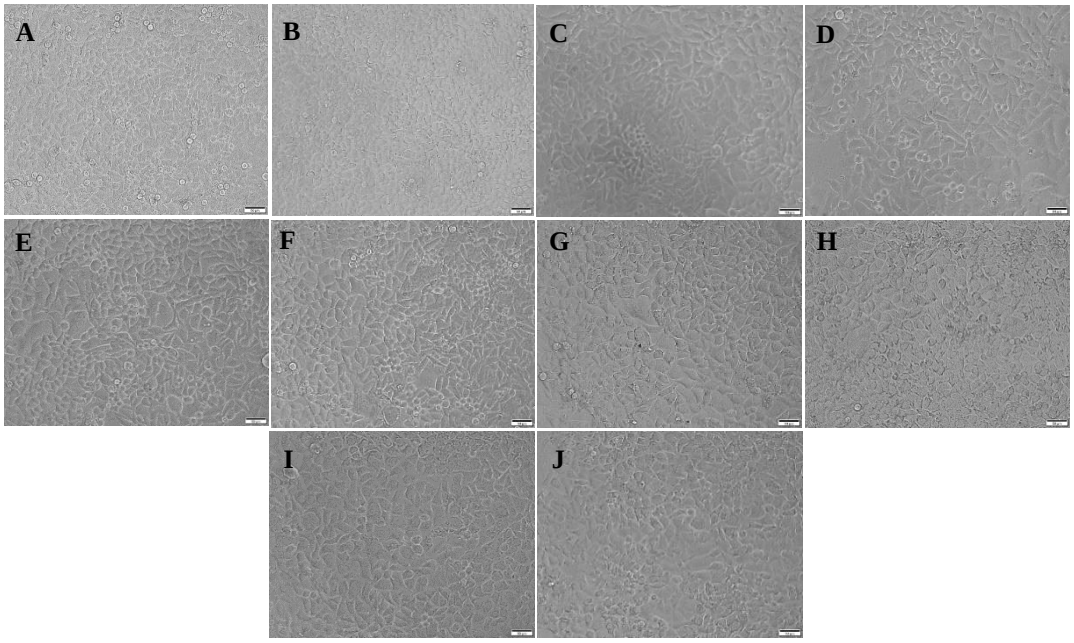

Figure 5 Images from inverted microscope (200 × magnification) showing HeLa cell line morphology; control group (A), positive control (B), and the groups treated for 24 hours with porcine follicular fluid from a medium-sized ovarian follicle at 2, 4, 20, 40, 200, 400, 500, and 600 µg proteins/mL (C-J) (scale bar = 20 µm)

3.4 Potential effect of pFF from a large-sized ovarian follicle on HeLa cell line viability

The effect of pFF from a large-sized follicle on HeLa cell line viability indicated that the viability of groups treated with pFF at 2, 4, and 20 µg proteins/mL were 94.15±12.00, 96.37±6.16, and 98.79±5.95, respectively. Their viability was insignificantly lower than that of the control group. The viability of HeLa cell lines treated with 40 and 200 µg protein/mL was 115.52±25.24 and 110.08±25.50, respectively. The viability of HeLa cell lines under these treatments was also insignificantly higher than those of the control group. The HeLa cell line viability under treatment with 400, 500, and 600 µg protein/mL was 140.52±11.714, 125.60±35.40, and 123.39±24.76, respectively. The tested group at 400 µg protein/mL showed the highest cell viability, but this value was statistically lower than the positive control group, as illustrated in Figure 6. All HeLa cell lines in all treatment groups showed normal morphology, as illustrated in Figure 7

The cell morphology of *in vitro* cultured HeLa cell lines was examined using an inverted microscope. The results suggested that HeLa cell lines spread over a cell culture flask and formed an epithelial-like morphology. These results are similar to the research of Kniss and Summerfield (2014), who indicated a similar transformation of cell morphology and monolayer cultures. The characteristics of cell morphology were examined by using culture paper rather than a culture flask. When using a scanning electron microscope, the morphology of HeLa cell lines differed from those examined by an inverted microscope. Specifically, those cultured with culture paper exhibited an epithelial-like morphology but appeared to have less expansion than those cultured in a culture flask. The results are supported by Majumdar et. al. (2001), who found that, when culturing cells for 48 hours, the cells transform into an epithelial-like morphology with numerous micro villi and also lamellipodia (the organ for cell movement). Their results indicated that HeLa cell lines cultured with a cell culture flask are more expanded (around 40 µg in length) than when cultured on culture paper (around 30 µg in length).

DMEM was supplemented with 10% HTFBS, which contained several vital components for cells, such as hormones, vitamins, minerals, and growth factors (Brunner et. al., 2010; Youngsabanant et al., 2019). The experiments on the effect of pFF extracted from small-, medium-, and large-sized ovarian follicles on HeLa cell lines showed that using pFF as a substitution on HTFBS yielded significantly higher viability and growth than the control group (only DMEM) ($p<0.05$). The viability was lower than that of the positive control group, although the difference was not statistically significant. These results were similar to those of Ayoub and Hunter (1993) and Romero-Arredondo and Seidel (1994), who showed that the oval cells cultured with pFF from small-, medium-, and large-sized ovarian follicles had higher viability and growth than otherwise, but still lower than those cultured with HTFBS. pFF is composed of proteins essential for biochemical and physiological processes such as follicular development, oocyte development, and ovulation (Ali et al., 2004; Edwards, 1974). In addition to their nutrients that are essential for cell viability, pFF also provides and maintains the conditions necessary for cell development. Ali et al. (2008), and Ito et al. (2008) studied the effect of pFF collected from small (3-4 mm in diameter) and large (5-6 mm in diameter) sized ovarian follicles. They found that pFF from large-sized ovarian follicles has effects on nuclear maturation and increased the oocyte maturation and fertilization rates over those collected from small-sized ovarian follicles. Similarly, Oberlender et al. (2013) studied the effect of pFF collected from small- and large-sized ovarian follicles and found that the development of cells cultured in pFF from the large-sized ovarian follicles is more advanced than that of cells cultured in small-sized ovarian follicles.

The study also indicated that the 30-100 kDa proteins found in pFF play an important role in cell development through nucleus and cumulus maturation (Ito et al., 2008).

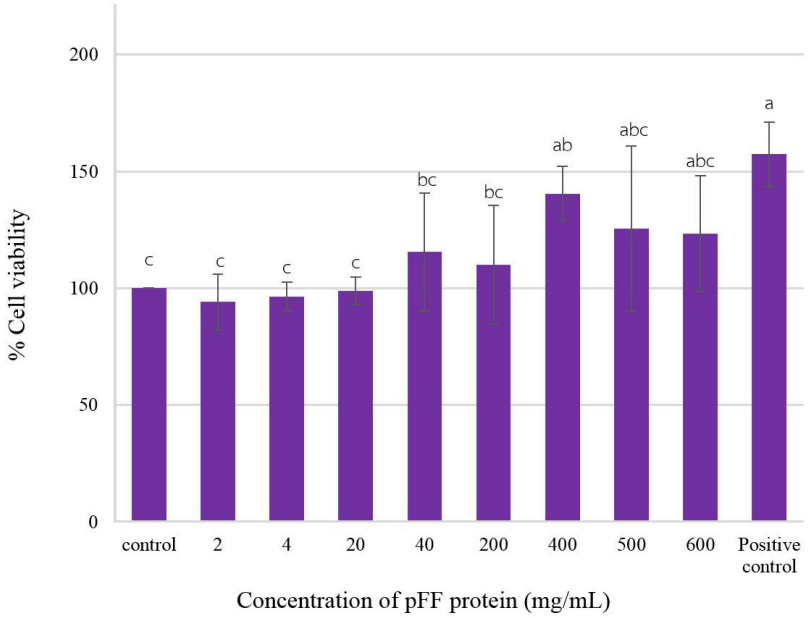

Figure 6 Effect of porcine follicular fluid from large-sized ovarian follicle groups at concentrations of 2, 4, 20, 40, 200, 400, 500, and 600 µg protein/mL in DMEM for 24 hours on the percent viability of HeLa cell lines, compared with control group (DMEM) and positive control group (DMEM supplemented with 10% HTFBS)

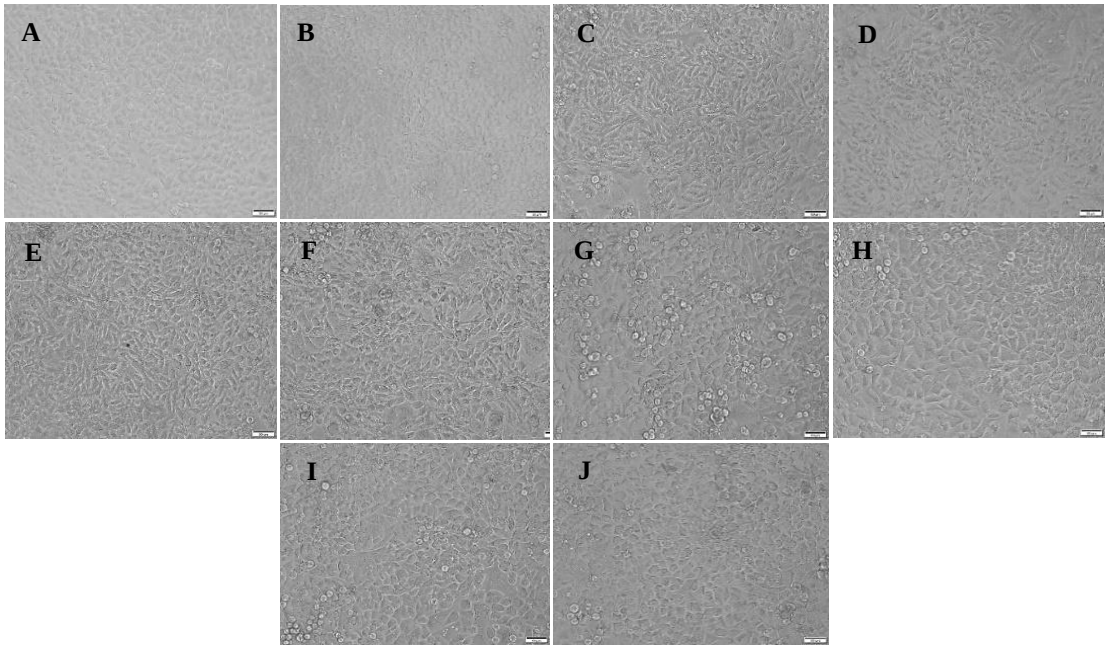

Figure 7 Images from inverted microscope (200 × magnification) showing HeLa cell line morphology; control group (A), positive control (B), and group treated for 24 hours with porcine follicular fluid from a large-sized ovarian follicle at 2, 4, 20, 40, 200, 400, 500, and 600 µg proteins/mL (C-J) (scale bar = 20 µm)

In this study, the viability of HeLa cell lines was higher when cultured with pFF from medium-sized ovarian follicles than those cultured with pFF from the small- and large-sized ovarian follicles. Ducolomb et al. (2013) suggested that pFF is composed of some protein compounds essential for oval cell development such as immunoglobulin fragment, cytokeratin, transferrin, plasminogen precursor, serum albumin, and keratin. The 90-kDa proteins, known as heat shock proteins, were also found for stimulating cell development (Mettasart, 2009). The same occurred in this finding on pFF from the small follicles. Ledwitz-Rigby et al. (1977) found that pFF inhibited *in vitro* oocyte maturation. Their results showed that supplementation with 50% pFF inhibits *in vitro* oocyte maturation more than supplementation with 20% and that pFF from large-sized follicles (6-12 mm) has no effect on ovulation.

This study showed that culturing HeLa cell lines with pFF from three sizes of ovarian follicles yielded significantly higher viability than the control group, but lower than the positive control group. The latter difference was not statistically significant. The analysis of cell morphology suggested that the cells cultured with a high concentration of pFF from small-sized ovarian follicles exshibited abnormal morphology. Specifically, they transformed into flat-shaped cells and expanded 1.5 times more than the positive control group. This cell morphology was abnormal and indicated cellular dysfunction, which agreed with the study of Adams et al. (2015), who found that nutrient abundance could lead to a plasmolytic shape. Specifically, it was an abnormal morphology that occurred from shrinkage of protoplasm and numerous vacuoles in the cytoplasm. The abnormal morphology of a dark color on the rim of the cytoplasm of HeLa cell lines was also found. This observation was supported by Adams et al. (2015), who also found plasmolytic-shaped cells. Hasasna et al. (2015) studied the effect of *Rhus coriaria* ethanolic extract (RCE) on breast cancer cells and found that cells transform into flat shapes with vacuolization. RCE has some effects on the destruction of breast cancer cells. It should also be noted that the MTT assay is a measure of succinate dehydrogenase enzyme release from mitochondria. This enzyme in capable of reducing the tetrazolium dye, MTT 3-(4, 5-dimethylthiaol- 2-yl)-2, 2-diphenyltetrazolium bromide, to its insoluble formazan (change in purple color) (Chacon et al., 1997). Thus, the findings of higher viability and growth could also be falsely positive, as the mitochondria of abnormal cells are still functioning.

The experiments also showed that at 40, 200, 400, 500, and 600 µg proteins/mL of pFF extracted from medium- and large-sized ovarian follicles yielded higher viability than the control group. Compared to the control and positive control groups, cell morphology remained normal when cultured with pFF from medium- and large-sized follicles at any concentration. This study, thus, suggested that 40, 200, 400, 500 and 600 µg proteins/mL of pFF extracted from medium- and large-sized ovarian follicles can be used as a substitute for HTFBS.

4. CONCLUSION

HeLa cell lines cultured with DMEM and 10% HTFBS appeared rounded in the first culture period. After 24 hours in culture, they transformed into flattened cells and spread out over the surface of the culture paper. After 48 hours in culture, they transformed into spindle-shaped cells, numerous spine shapes, and doubled in size at around 80%-90% confluence. The 600 µg proteins/mL of pFF from small-sized ovarian follicles yielded the highest viability when compared with the control group, but lower than the positive control group. The 500 µg proteins/mL of pFF extracted from the medium follicles yielded the highest viability compared with the control group. The 400 µg proteins/mL of pFF from the large-sized ovarian follicles yielded the highest viability. When cultured with 400, 500, and 600 µg proteins/mL of pFF extracted from the small follicles, HeLa cell lines

exhibited an abnormal morphology. However, those cultured with pFF from the medium and large follicles exhibited similar morphology to the positive control group. As evidenced by our experiments, pFF has merit as a supplement in cell culture medium. However, we note that different cells might respond to pFF differently, and further studies should be conducted.

ACKNOWLEDGEMENT

This research was funded by a grant from Silpakorn University and Faculty of Science, Silpakorn University, Thailand. The authors are grateful to The Korean Cell Line Bank (Seoul, South Korea) for their kindness in supporting HeLa cell lines research for this project. We are also grateful to Assistant Professor Dr. Thongchai Taechowisan (Department of Microbiology, Faculty of Science, Silpakorn University, Thailand) for his kindness to render cell line culture techniques.

REFERENCES

Adams, T., Anwar, R., Mfarej, M., Rundatz, T., Coyle, M., and Mc Laughlin, J. S. (2015). Nutritional stress of cultured Vero cells causes altered growth and morphology as seen in neoplastic transformation. *American Journal of Undergraduate Research*, 12(3), 63-75.

Ali, A., Coenen, K., Bousquet, D., and Sirard. M. A. (2004). Origin of bovine follicular fluid and its effect during *in vitro* maturation on the developmental competence of bovine oocytes. *Theriogenology*, 62(9), 1596-1606.

Ali, S., Ahmad, N., Akhtar, N., and Zia-Ur-Rahman, Noakes, D. E. (2008). Metabolite contents of blood serum and fluid from small and large sized follicles in dromedary camels during the peak and the low breeding seasons. *Animal Reproduction Science*, 108(3-4), 446-456.

Areekijseree, M., Thongpan, A., and Vejaratpimol, R. (2005). Morphological features of porcine oviductal epithelial cellsand cumulus-oocyte complex. *Kasetsart Journal: Natural Science*, 39(1), 136-144.

Ayoub, M. A., and Hunter, A. G. (1993). Inhibitory effect of bovine follicular fluid on *in vitro* maturation of bovine oocytes. *Journal of Dairy Science*, 76(1), 95-100.

Brunner, D., Frank, J., and Appl, H., Schöffl, H., Pfaller, G., Gstraunthaler, G., (2010). Serum-free cell culture: The serum-free media interactive online database. *ALTEX*, 27(1), 53-62.

Chacon, E., Acosta, D., and Lemasters, J. J. (1997). Primary cultures of cardiac myocytes as *in vitro* models for pharmacological and toxicological assessments. *In Vitro Methods in Pharmaceutical Research*, 209-223.

Ducolomb, Y., González-Márquez, H., Fierro, R., Jiménez, I., Casas, E., Flores, D., Bonilla, E., Salazar, Z., and Betancourt, M. (2013). Effect of porcine follicular fluid proteins and peptides on oocyte maturation and their subsequent effect on *in vitro* fertilization. *Theriogenology*, 79(6), 896-904.

Edwards, R. G. (1974). Follicular fluid. *Journal of Reproduction and Fertility*, 37(1), 189-219.

Hasasna, H. E. l., Athamneh, K., Samri, H. A. l., Karuvantevida, N., Dhaheri, Y. A. l., Hisaindee, S., Ramadan, G., Tamimi, N. A. l., AbuQamar, S., Eid, A., and Iratni, R. (2015). *Rhus coriaria* induces senescence and autophagic cell death in breast cancer cells through a mechanism involving p38 and ERK1/2 activation. *Scientific Reports*, 5, 13013.

Ito, M., Iwata, H., Kitagawa, M., Kon, Y., Kuwayama, T., and Monji, Y. (2008). Effect of follicular fluid collected from various diameter follicles on the progression of nuclear maturation and developmental competence of pig oocytes. *Animal Reproduction Science*, 106(3-4), 421-430.

Kimura, N., Konno, Y., Miyoshi, K., Matsumoto, H., and Sato, E. (2002). Expression of hyaluronan

synthases and CD44 messenger RNAs in porcine cumulus-oocyte complexes during *in vitro* maturation. *Biology of Reproduction*, 66(3), 707-717.

Kniss, D. A., and Summerfield, T. L. (2014). Discovery of HeLa cell contamination in HES cells: Call for cell line authentication in reproductive biology research. *Reproductive Sciences*, 21(8), 1015-1019.

Ledwitz-Rigby, F., Rigby, B. W., Gay, V. L., Stetson, M., Young, J., and Channing, C. P. (1977). Inhibitory action of porcine follicular fluid upon granulosa cell luteinization *in vitro*: assay and influence of follicular maturation. *Journal of Endocrinology*, 74(2), 175-184.

Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, 193(1), 265-275.

Majumdar, S. K., Valdellon, J. A., and Brown, K. A. (2001). *In vitro* investigations on the toxicity and cell death induced by tamoxifen on two non-breast cancer cell types. *Journal of Biomedicine and Biotechnology*, 1(3), 99-107.

Mettasart, W. (2009). *Cell and protein secretion from porcine oviduct and ovary in estrous cycle*. (Master thesis, Silpakorn University).

Oberlender, G., Murgas, L. D. S., Zangeronimo, M. G., da Silva, A. C., de Alcantara Menezes, T., Pontelo, T. P., and Vieira, L. A. (2013). Role of insulin-like growth factor-I and follicular fluid from ovarian follicles with different diameters on porcine oocyte maturation and fertilization *in vitro*. *Theriogenology*, 80(4), 319-327.

Pongsawat, W., and Youngsabanant, M. (2019). Porcine cumulus oocyte complexes (pCOCs) as biological model for determination on *in vitro* cytotoxic of cadmium and copper assessment. *Songklanakarin Journal of Science and Technology*, 41(5),1029-1036.

Romero-Arredondo, A., and Seidel, G. E. J. (1994). Effects of bovine follicular fluid on maturation of bovine oocytes. *Theriogenology*, 41(2), 383-394.

Suchanek, E., Simunic, V., Juretic, D., and Grizelj, V. (1994). Follicular fluid contents of hyaluronic acid, follicle-stimulating hormone and steroids relative to the success of *in vitro* fertilization of human oocytes. *Fertility and Sterility*, 62(2), 347-352.

Youngsabanant-Areekijseree, M., Tungkasen, H., Srinark, C., and Chuen-Im, T. (2019). Determination of porcine oocyte and follicular fluid proteins from small, medium, and large follicles for cell biotechnology research. *Songklanakarin Journal Science Technology*, 41(1), 192-198.

Youngsabanant, M., Gumlungpat, N., and Panyarachun, B., (2019). *In vitro* characterization and viability of Vero cell lines supplemented with porcine follicular fluid proteins study. *Science, Engineering and Health Studies*, 13(3), 143-152.