Research Article *Science, Engineering and Health Studies 2019, 13(3), 143-152*

In vitro characterization and viability of Vero cell lines supplemented with porcine follicular fluid proteins study

Mayuva Youngsabanant[1*], Suphaphorn Rabiab[1]
Nongnuch Gumlungpat[1] and Busaba Panyarachun[2]

[1]*Department of Biology, Faculty of Science, Silpakorn University, Nakhon Pathom 73000, Thailand*
[2]*Department of Anatomy, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110, Thailand*
[*]*Corresponding author: areekijseree_m@su.ac.th*

Received: July 22, 2019; Revised: October 29, 2019; Accepted: October 30, 2019

ABSTRACT

The purpose of this research was to study the morphological features of cells and test the porcine follicular fluid (pFF) supplements in Vero cell lines culture. The pFF in the estrous cycle was collected and kept sterile properly. The fluid was categorized into three types: small size (1-3 mm), medium size (4-6 mm) and large size (>7 mm) follicles. In the control group, Vero cell lines were cultured in dulbecco's modified eagle medium (DMEM). In the positive control group, Vero cell lines were cultured in DMEM and supplemented using 10% heat-treated fetal bovine serum (HTFBS). In the third group, Vero cell lines were cultured in DMEM and supplemented using pFF from small, medium and large sized ovarian follicles at 2, 4, 20, 40, 200, 400, 500 and 600 µg protein/mL concentrations for 24 h, using MTT assay. The results related to pFF from small-, medium- and large-sized follicles indicated that the percentage of cell viability in tested group at 400-600 µg protein/mL showed the percentage of viability came out to be the highest, significantly than that in the control group ($p<0.05$), and not different from that in the positive control group. In summary, according to morphological studies, the pFF effectively helped increase the growth of Vero cell lines closely as compared to the cultured group supplemented by HTFBS in the laboratory.

Keywords: cell viability; porcine follicular fluid; pFF; MTT assay; Vero cell line

1. INTRODUCTION

Nowadays, pigs have become a significant animal for human consumption in Thailand. Each part of their bodies can be transformed into edible products, with the exception of female pig ovaries, which are not typically consumed by humans. Due to the fact that pigs are mammalian, like humans both anatomically and physiologically, relevant studies today enable researchers to apply ovaries practically and variously in biotechnological terms (Youngsabanant-Areekijseree et al., 2019). Blood circulation, skin, sexual reproduction, urinary system, excretory system and immune system in pigs are engaged tremendously in medical research, i.e. studies of viral and bacterial infections, and vaccine developments (Hafez, 1992; Swindle et al., 2012). In Nakhon Pathom province, Thailand, there are a great number of porcine slaughterhouses; therefore, leftover carcasses are disposed of each

ISSN Online 2630-0087

day. More putrid garbage is accumulated because of this large amount of waste. In order to ultimately utilize natural resources, we used the ovaries from female pigs. Within ovaries, there are follicles. Inside the follicles are follicular cells and cell-development-supporting follicular fluid that exist and remain sterile, and are versatile for cell-related technological development. Former research reported that follicular fluid contains the components of cell coat secreted from granulosa cells, comprising of reproductive hormones and proteins that stimulate oocyte development and cell growth. (Ito et al., 2008; Kor, 2014). Previous reports revealed that using follicular fluid as a supplement in culture medium can stimulate IVM/IVF (Algriany et al., 2004; Oberlender et al., 2013; Vatzias & Hagen, 1999). Following the study of Alberto et al. (2009), the components of follicular fluid in humans can be categorized into nine groups: follicle stimulating hormone (FSH), luteinizing hormone, estrogen and progesterone; sugar, hyaluronan; growth factors of the transforming growth factor-beta superfamily; growth factors and interleukins; reactive oxygen species; anti-apoptotic factors; proteins, peptides and amino-acids; anti-apoptotic factors; and prostanoids. Suchanek et al. (1994), illuminated in his research that during the time cumulus cells expand from oocyte-cumulus cell complex in humans, FSH has a crucial duty in increasing hyaluronic acid by stimulating t-granulosa cells in follicular cells and help estrogen boost the growth of cytoplasm, causing complete growth of the follicular cells. Furthermore, Zhao et al. (2002) and Ducolomb et al. (2013) disclosed that the components of proteins in medium- and large-sized porcine ovarian follicular cells contains essential proteins for supporting the decomposition of follicular nucleus membranes, boosting oocyte maturation and stimulating ovulation. Meanwhile, the components of proteins in small sized porcine ovarian follicular cells contain immunoglobulin (Ig), immunizing against five groups of diseases - IgG, IgA, IgM, IgD and IgE - which perform different roles in conformity in a study by Mettasart (2009) and contain keratin which helps decompose nucleus membranes and sustain the growth of follicular cells (Ducolomb et al., 2013).

Hence, the purposes of this research was to study porcine follicular fluid (pFF) from small-, medium- and large-sized follicles in the estrous cycle which contained necessary components in aiding the growth of Vero cell lines using sterile cultures to substitute costly import fetal bovine serum; and to study the morphological features of the Vero cell lines cultured in the laboratory.

2. MATERIALS AND METHODS

2.1 Culture medium

Dulbecco's modified eagle medium (DMEM) with 10% heat treated fetal bovine serum (HTFBS) and cultured for 12 h before use, at 37°C with high humidity and 5% carbon dioxide in 95% air atmosphere.

2.2 pFF collection and isolation

The porcine follicles were classified as healthy and atretic follicles on the basis of morphological criteria while those with opaque, hemorrhagic or milky follicular fluid were excluded. pFF was collected from local slaughterhouses. They were removed within 30 min after the pigs were slaughtered and transported to the laboratory in a sterile thermos container at temperature 30-35°C by sterile technique. They were classified based on three sizes of ovarian follicles (small-, medium- and large-sized) by using a syringe connected with an 18-gauge needle. Then, the fluid was collected in a conical tube and centrifuged for 5 min at 1,500x g to remove oocytes and cells, and stored at temperature of -80°C, until usage. Protein quantification was performed by the Lowry method (Lowry et al., 1951) for calculating the protein concentration of pFF from three sizes of follicles to

supplement with DMEM culture medium.

2.3 MTT assay and cells morphological study

Two-hundred microliters of the Vero cell lines were cultured in 96-well plates for 48 h before being treated with pFF from three sizes of follicles at concentration of 2, 4, 20, 40, 200, 400, 500, and 600 µg protein/mL for 24 h. After incubation, the cell viability was analyzed using MTT assay. The assay method was utilized for cell incubation with tetrazolium salt for 4 h, and the quantification of formazan dye was detected using a spectrophotometer at 570 nm. Meanwhile, cell morphology was observed during the culture period and after being supplemented with pFF by using an inverted microscope.

3. RESULTS AND DISCUSSION

Vero cell lines showed normal morphological characteristics under the inverted microscope observation. In the beginning of the culture, Vero cell lines were oval-shaped (Figures 1A). After being cultured for 24 h, Vero cell lines transformed into flat-shaped cells and spread over the surface (Figures 1B). After the 48 h cultures, they transformed into spindle-shaped cells, and increasingly extended (Figures 1C).

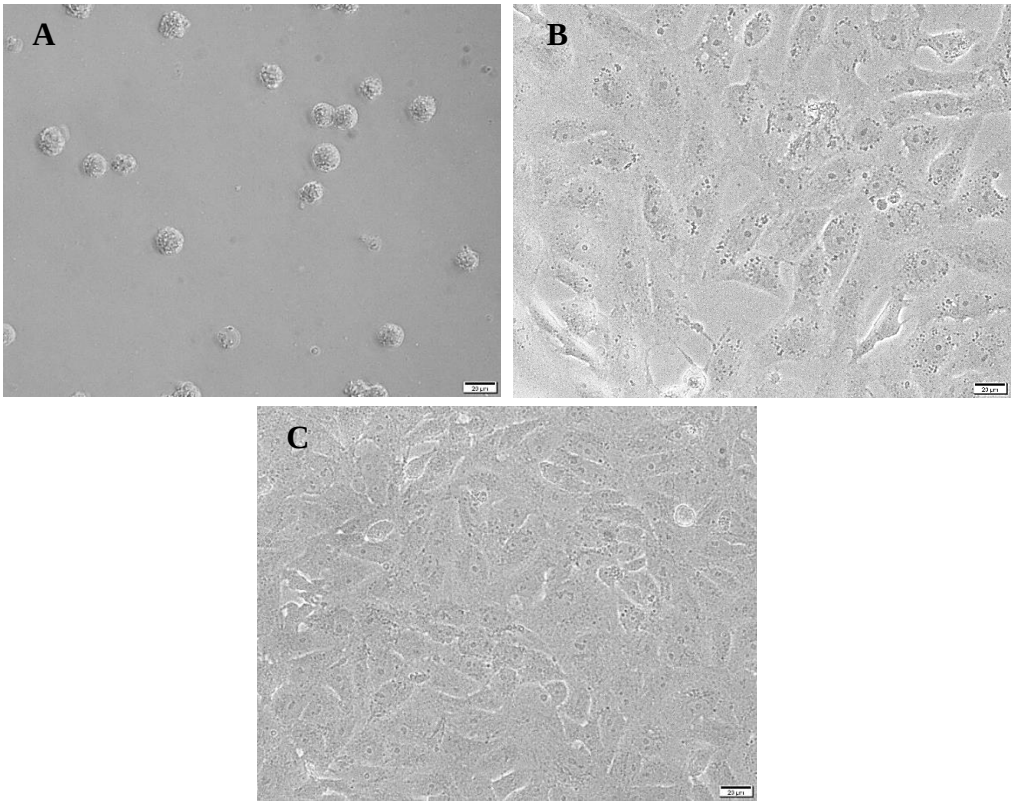

Figure 1 Images via an inverted microscope, exhibiting the Vero cell line cultured in DMEM supplemented with 10% heat treated bovine calf serum x400; (A) Cultured for 0 h, cells remain spherical; (B) Cultured for 24 h, cells propagate, turning fusiform; (C) Cultured for 48 h, cells increasingly extend.

Moreover, the morphology of Vero cell line was also studied using a scanning electron microscope (SEM). During culture initiation, it was found that Vero cell lines were normally spherical; their surface

had tuberculation with numerous microvilli (Figure 2 A-B). For 24 h, it showed that the cells attached themselves to the surface and propagated up to 3 times (Figure 2 C-D). Cultured for 48 h, the cells become spindle-shaped attached themselves to the surface and extended more (Figure 2 E-F).

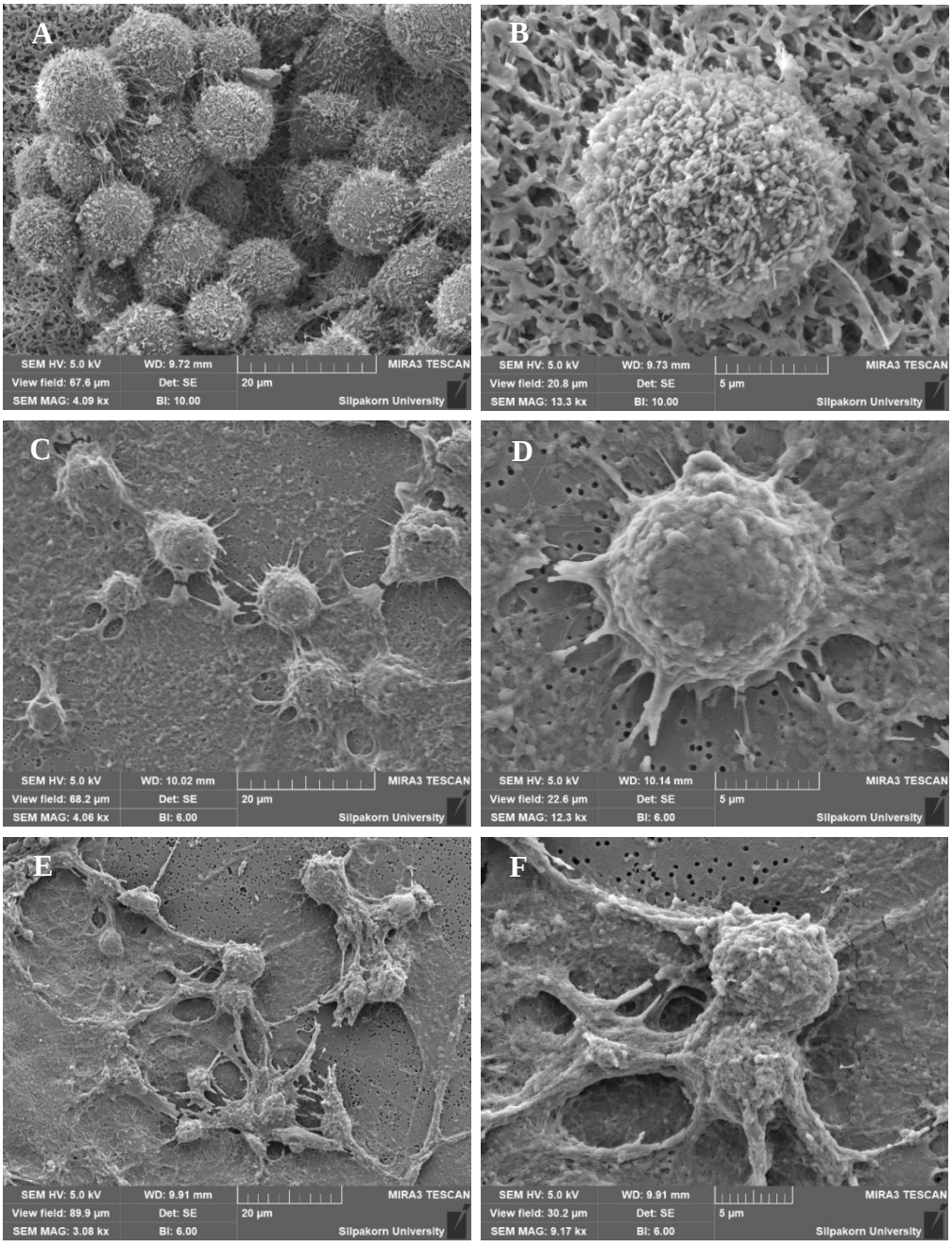

Figure 2 Scanning electron micrographs, exposing normal morphological features of Vero cell lines cultured based on different times; (A-B) for 0 h, the cells are spherical; their surface appears tuberculation (C-D); for 24 h, the cells propagate, attaching to the surface; (E-F) for 48 h, the cells get attached and extend.

3.1 Efficacy examination of pFF from small, medium and large-sized ovarian follicles on Vero cell lines

According to the efficacy examination of pFF from small-, medium-, and large-sized follicles on Vero cell lines, it exposed the percentage of cell viability of Vero cell lines examined using pFF. The horizontal axis showed 2, 4, 20, 40, 200, 400, 500, and 600 µg protein/mL concentrations of pFF, which were utilized in order to test Vero cell lines for 24 h. Two comparison groups were conducted: the control group, where Vero cell lines were cultured solely in DMEM; and the positive control group, where Vero cell lines were cultured in DMEM as well as supplemented by 10% HTFBS. The examination of the cell viability percentage was based on MTT assay and statistically analyzed using one-way ANOVA as well as post-hoc Duncan. The differences amongst each group were identified as a, b, c, d...

3.2 Efficacy study of pFF from small-sized ovarian follicles on the growth of Vero cell lines

MTT assay results showed that the Vero cell lines' viability after 24 h treated with pFF at 2, 4, 20, 40 and 200 µg protein/mL were 79.27±8.35, 86.00 ±12.70, 81.64±2.62, 93.27±4.48, and 99.27±8.82 respectively. Their viability was less than control group and had no statistically significant difference. The Vero cell lines' viability of 400, 500 and 600 µg protein/mL was 115.64±6.95, 106.91±5.27, 116.73 ±20.15, respectively, while the viability of Vero cell lines of the positive control group was 124.73±15.81. At 600 µg protein/mL concentration, viability appeared the highest but statistically lower than that of the positive control group ($p<0.05$), as illustrated in Figure 3. Vero cell lines were fibroblastic and elongated after small-sized ovarian follicles were treated with pFF.

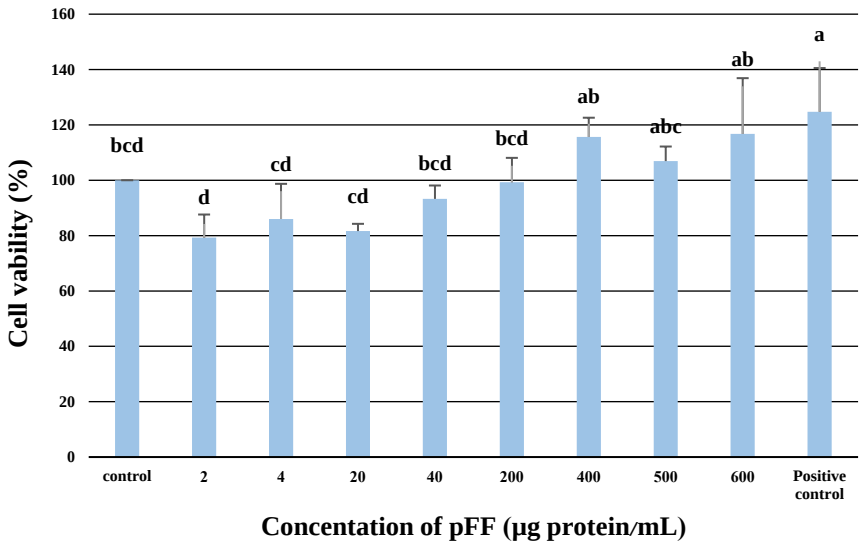

Figure 3 Effect of pFF from small-sized ovarian follicle groups at concentrations of 2, 4, 20, 40, 200, 400, 500 and 600 µg protein/mL supplemented in DMEM for 24 h on percentage of Vero cell lines viability, compared to those of the control group (DMEM) and the positive control group (DMEM supplemented with 10% HTFBS).

3.3 Efficacy study of pFF from medium-sized ovarian follicles on the growth of Vero cell lines

In accordance with the results comparing the control group and the group tested using medium-sized pFF, it made clear that the percentage of cell viability of Vero cell lines at concentrations of 200, 400, 500 and 600 µg protein/mL turned out to be 123.54±12.46, 124.44±8.15, 139.24±23.46 and 132.74±11.93, respectively. As compared with the group based on pFF from medium-sized follicles at 400, 500 and 600 µg protein/mL concentrations with the positive control group with cell viability of 154.26±15.08, it uncovered that the group based on medium-sized pFF possessed a lower viability percentage than the positive control group, and there was no statistically significant difference ($p>0.05$) (Figure 4).

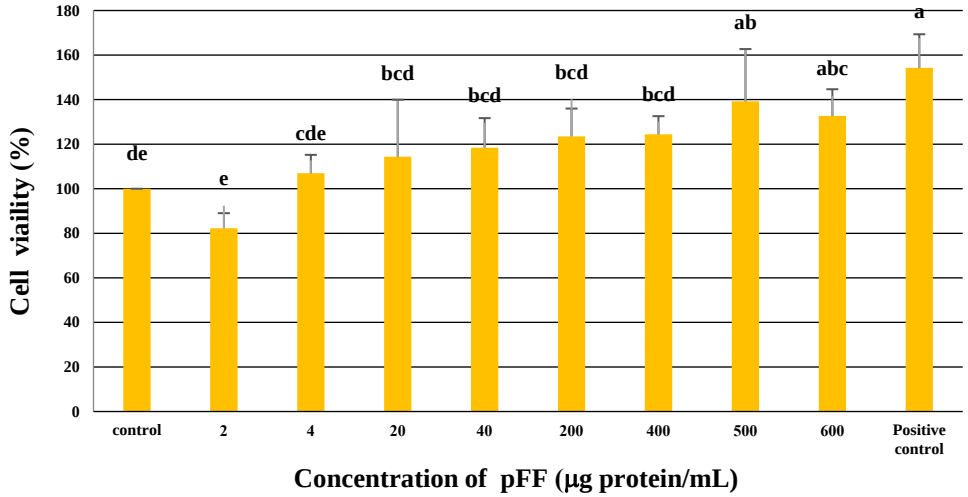

Figure 4 Percentage of cell viability of Vero cell lines inspected using pFF from medium-sized follicles (n=3) for 24 h. The percentage of Vero cell lines' viability is compared with those of the control group (DMEM) and the positive control group (DMEM supplemented with 10% HTFBS).

3.4 Efficacy study of pFF from large-sized pFF on the growth of Vero cell lines

Due to the result of the comparison study between the control group and pFF from large-sized follicle, it shed light on the fact that the percentage of the cell viability of Vero cell lines at concentrations of 400, 500 and 600 µg protein/mL came out to be 115.43±5.41, 131.04±11.37 and 120.45±4.09, respectively. As compared to the control group with cell viability of 100.00±0.00, there was no statistically significant difference ($p>0.05$). As comparing the group based on pFF from large-size follicle at concentrations of 500 and 600 µg protein/mL with the positive control group with cell viability of 136.25±8.24, it unveiled that the group based on pFF from large-sized follicle at concentration of 500 µg protein/mL held a lower viability percentage than the positive control group, while statistically significant difference was not found ($p>0.05$) (Figure 5).

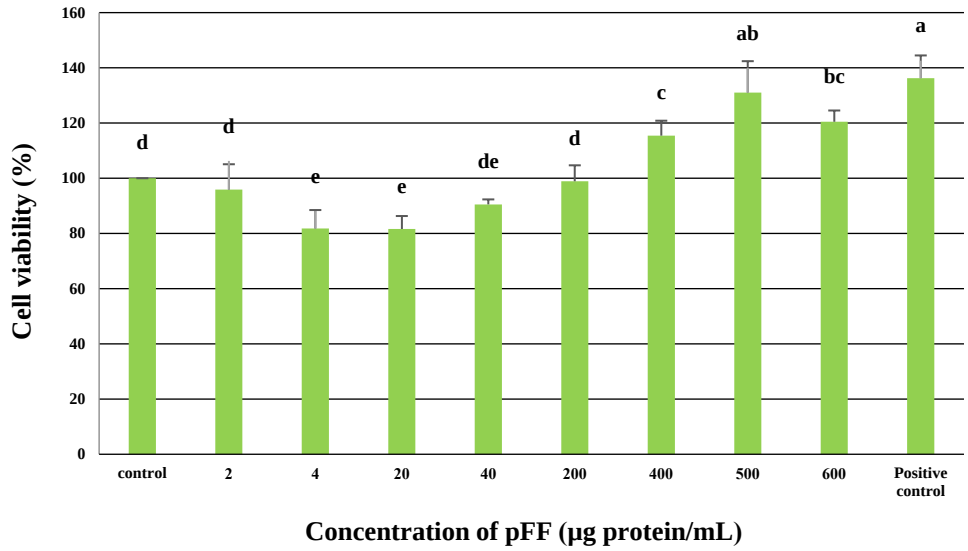

Figure 5 Percentage of cell viability of Vero cell lines, inspected using pFF from large-sized follicles (n=3) for 24 h on percentage of Vero cell lines viability, compared with the control group (DMEM) and the positive control group (DMEM supplemented with 10% HTFBS).

The morphological study of Vero cell lines cultured in the laboratory using an inverted microscope unveiled that Vero cell lines propagated entirely in a well-plate and turned fusiform in conformity with a research of Ammerman et al. (2008). Based on the growth of Vero cell lines, it is found that the cells developed to be monolayer-fibroblast-like (Genari et al., 1995). Cultured on culture papers instead of on plates, the morphological study of the cells via SEM enlightened that the Vero cell lines cultured on papers appeared spherical, not fusiform, yet extended less than those cultured on plates. After attempting to culture Vero cell lines on unconventional surfaces for 24 h, the cells turned spherical and their microvilli were found growing from the cell surface.

Vero cell lines were cultured using DMEM with 10% FBS added, i.e., hormones, vitamins, minerals, as well as growth factors, which are essential for cell viability (Brunner et al., 2010). When compared with the control group, there was a statistically significant difference ($p<0.05$). The efficacy study of Vero cell lines supplemented by pFF from medium- and large-sized follicles in the culture medium in place of HTFBS was found to cause a statistically significant percentage increase of cell viability ($p<0.05$), compared to that of the control group cultured in DMEM alone. Meanwhile, it was found lower than that of the positive control group with no statistically significant difference ($p>0.05$). In harmony with a study conducted by Ayoub et al. (1993), it has been discovered that ovarian follicular cells examined by pFF from small-, medium-, and large-sized follicle performed a lower development ratio than the group given fetal calf serum (FCS), whereas ovarian follicular growth and development ratio significantly augmented, as compared to the control group with no ovarian follicular fluid added (Romero et al., 1994). Within ovarian follicular cells, certain proteins were found assuming certain vital duties in biochemical and physiological processes, for instance, ovarian follicular growth and development, follicular production processes, ovulation, as well as reproduction-related processes (Edwards, 1974; Ali et al., 2004). Additionally, the cells both provided nourishment to

both growth and development in ovarian follicular cells and maintained proper conditions to ovarian follicular growth (Ali et al., 2008). According to Ito et al. (2008), focusing on the efficacy study of pFF from small-sized follicle (3-4 mm) and large-sized follicle (5-6 mm), it uncovered that the supplementation in culture medium using large-sized follicular fluid can result in nucleus production in the cell, promoting a reproduction ratio and better development in ovarian follicular cells. The supplement examination in the culture medium based on pFF from small-sized follicle, like in the Oberlender et al. (2013) study, identifying the efficacy study of small-sized pFF (2-5 mm) and large-sized pFF (6-10 mm), showed the higher growth ration of ovarian follicular cells cultured in laboratory culture medium with large-sized pFF added than ovarian follicular cells cultured in culture medium with small-sized pFF added, and large-sized pFF resulted in higher cell viability. Furthermore, a study showed that certain proteins in large-size pFF with molecular weights ranging from 30-100 kDa took part in ovarian follicular growth and development effectively via the production cycle of cell nuclei and cumulus cells (Ito et al., 2008). Ducolomb et al. (2013) found that by emphasizing proteins in medium-sized pFF (3-6 mm), the proteins, as components in ovarian follicular fluid, nurtured ovarian follicular growth, such as immunoglobulin fragments, cytokeratin, transferrin, plasminogen precursors, serum albumin as well as keratin. A type of protein, the so-called heat shock protein, weighing 90 kDa, was found to stimulate cell growth (Mettasart, 2009). This study also found that the viability percentage of Vero cell lines inspected based on small-sized pFF was lower than those of medium- and large-sized follicles. Moreover, a study of Ledwitz-Rigby et al. (1977) disclosed that small-sized pFF (1-2 mm) can inhibit the ovulatory cycle of ovarian follicles prior to ovulation in tubes, discovering that the supplementation using 50% small-sized pFF in the culture medium performed a better duty in inhibiting ovulation than that of using 20% small-sized pFF, and that using large-sized follicles (6-12 mm) had no capacity to inhibit ovulation.

This result implied that pFF of small-, medium-, and large-sized ovarian follicles at concentrations of 400-600 µg protein/mL can be optimized for using as a supplement in the Vero cell line culture medium to promote cell viability instead of growth hormone from fetal bovine serum. This merit can be applied in relevant cell biotechnology research.

4. CONCLUSION

The normal morphological features of Vero cell lines cultured in DMEM and supplemented by 10% HTFBS, were found spherical as cultured in the beginning (0 h), gradually attaching to the surface as cultured for 24 h, and spindle-shaped and extended as cultured for 48 h, respectively. The Vero cell line examination using pFF from small-, medium- and large-sized follicles at concentrations of 2, 4, 20, 40, 200, 400, 500 and 600 µg protein/mL revealed higher percentage of cell viability than using the control group at a concentrations of 400-600 µg protein/mL. For small-sized follicles, the highest percentage of cell viability at concentration of 600 µg protein/mL was 116.73±20.15. For pFF of medium-sized follicles, the highest percentage of cell viability at a concentration of 500 µg protein/mL was 139.24±23.46, analogous to that of the positive control group (154.26±15.08). For large-sized follicles, the highest percentage of cell viability at a concentration of 500 µg protein/mL was 131.04±11.37, showing no significant difference with that of the positive control group (136.25±8.24). We were able to add pFF in the culture medium in place of fetal bovine serum since the study made it clear that pFF can promote cell growth and development in the laboratory, thereby helping economize because it costs nothing, whereas fetal bovine serum was costly. As a result, pFF can be applied instead of fetal bovine

serum, yet the responses to specific cells still vary and need to be further researched. We hope that this research can help in other relevant cell cultures in the laboratory in the future.

ACKNOWLEDGEMENTS

The project 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 Korea) for their kind to support cell lines for this work. We are also grateful to Assistant Professor Dr. Thongchai Taechowisan (Department of Microbiology, Faculty of Science Silpakorn University, Thailand) for his kindness to render his cell line culture technique. We are also thanks Miss Suphaphorn Rabiab (Department of Biology, Faculty of Science Silpakorn University, Thailand) for her assistance in cell line culture and MTT assay.

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