GROWTH CHARACTERISTICS OF LLC-MK2 CELLS DIFFERED FROM VERO AND HELA CELLS WHEN CULTURED IN PORCINE FOLLICULAR FLUID SUPPLEMENTATION USING INVERTED MICROSCOPY

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ABSTRACT
Porcine follicular fluid (pFF) contains biochemical compositions that are essential for follicle growth process. It is known that components and concentrations in pFF are different among small, medium, and large follicles, resulting in different microenvironments. Previously, we reported that pFF from all size follicles can promote growth of Vero and HeLa cells *in vitro*. In this study, impacts of small, medium, and large pFFs on LLC-MK2 cell viability and morphology were studied at different total protein concentrations using MTT assay and inverted microscopy. It was found that cell viability and morphology of LLC-MK2 differed from those of Vero and HeLa cells, in supplementation with pFFs from both different size follicles and different total protein concentrations. This implied that different microenvironments of pFFs from different size follicles and protein concentrations have different growth impacts on each cell line.

Keywords: porcine follicular fluid; LLC-MK2 cells; Vero cells; HeLa cells; cell viability; morphology

INTRODUCTION

Follicular fluid (FF) contains hormones, transforming growth factor-beta (TGF-beta) superfamily, other growth factors and interleukins, reactive oxygen species (ROS), anti-apoptotic factors, proteins&peptides and amino acids, enzymes, polysaccharides, and fatty acids [1]. These components play major roles in biochemical processes [2,3]. Several studies have reported different growth and development of ovarian follicular cells when cultured in a supplement with porcine follicular fluid (pFF) from different size follicles [4,5,6,7]. In consistent with this, characterization of biochemical compositions in porcine, ovine, and caprine FFs revealed metabolite, ion, and enzyme concentrations relating to follicular sizes [8,9,10]. This indicated that different microenvironments of FF from different size follicles have different influences on cell growth and development.

African green monkey kidney Vero, rhesus monkey kidney LLC-MK2, and immortalized cervical carcinoma HeLa cells are widely used as models for study of viral infection and propagation for decades [11,12,13,14,15]. All cell lines were epithelial cell type. HeLa cells were human cervical cell line whereas Vero and LLC-MK2 cells were originated from monkey kidney tissue. Several publications reported that both Vero and LLC-MK2 cells exhibited highly susceptibility to various virus types but displaying different properties to viral infection and replication. There were publications reported that LLC-MK2 cells were more susceptible to infection of mumps

virus and Alkhumra hemorrhagic fever virus than Vero cells [11,16,17]. Conversely, infection efficiency of human metapneumovirus in Vero cells was higher than that to LLC-MK2 cells [18]. Moreover, different characteristics, e.g. cytopathic effects, were also observed between both cell lines after infection with human parainfluenza virus types 1 and 3, and SAR-CoV-2 [13,15,19]. These differences may result from specific nature of each cell line. Previously, efficacy of small, medium, and large pFF supplementations in Vero and HeLa cell cultures has been investigated in comparison with commercial fetal bovine serum [20,21]. Similar to what observed in ovarian follicular cells [6,7], cell viabilities were related to follicle sizes and total protein concentrations. However, for HeLa cells, non-significant difference of viability and abnormal morphology were noted in small pFF supplementation [21]. In this study, impacts of pFF from small-, medium-, and large-sized follicles on growth characteristics and viability of LLC-MK2 cells were investigated using inverted microscopy and MTT assay. It was found that viability and morphology of LLC-MK2 cells with various follicle-sized pFF differed from those of Vero and HeLa cells. The differences of growth characteristics between LLC-MK2, Vero, and HeLa cells have been discussed.

MATERIALS AND METHODS

Porcine follicular fluid (pFF) sampling and preparation

Collection and classification of porcine follicle samples as small, medium, and large sizes were carried out as described by Youngsabanant-Areekijseree et al [6]. Pubertal fresh female large white pig ovaries in the estrous cycle (at the age between 210-250 days old) collected from local slaughterhouses were washed 2-3 times with 0.9% NaCl (w/v) containing 100 IU/mL pennicillin, 100 µg/mL streptomycin, and 250 µg/mL Amphotericin B. After that, pFF was collected using a 5 mL syringe with a 18 gauge needle. Cumulus oocyte complexes (COCs) and other cells, e.g. granulosa cells, were removed by centrifugation at 1,500 x g for 5 min before storing follicular fluids at -80°C until required. Total concentration of protein contents were analyzed using Lowry method [22].

LLC-MK2 cell culture under supplementation of pFFs

LLC-MK2 cells were cultured in DMEM (Gibco BRL Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL Life Technologies, USA) and incubated at 37°C, 5% CO_2, high humidity, and 95% air atmosphere. When reached exponential phase, cells were prepared at a concentration of 10^5 cells/mL and used for cell growth investigation under culture conditions of DMEM supplemented with small, medium, large pFFs at protein concentrations of 2, 4, 20, 40, 200, 400, 500 and 600 µg/mL. Two culture conditions were used as 1) control: cells cultured in medium without supplementation and 2) positive control: cells cultured in medium supplemented with 10% FBS. Cells were incubated at 37°C, 5% CO_2, high humidity, and 95% air atmosphere for 24 h before subjecting to test cell viability by MTT assay. Experiments were carried out in triplicate independently.

MTT cell viability assay

The medium was removed and cells were washed twice with PBS prior to adding MTT solution (Sigma Aldrich). The tested cells were incubated at 37 °C for 4 h. Subsequently, MTT reagent was discarded, and then added 150 µL of DMSO solution to dissolve purple formazan crystal. The samples were then measured absorbance at wavelength of 570 nm. The percentage of cell viability was estimated as follows:

$$\%\text{viability} = \frac{\text{Test} - \text{Blank}}{\text{Reference} - \text{Blank}} \text{ x } 100$$

Where Blank is the absorbance in the non-cultured cells, Reference is the absorbance of the cells cultured without supplementation (control). Test is the absorbance of the

cells cultured with follicular fluid at various size follicles and protein concentrations. Cell growth under cultured condition of pFF supplement and positive control was estimated in relative to the control group. Occurrence of promoting cell development recorded when the estimated cell viability over 100%.

Cell morphological investigation by a scanning electron microscope (SEM)

To prepare samples for scanning electron microscopy, cells were maintained with 10% formalin buffer for 2 h before incubating in 5% formalin buffer for 24 h. Dehydration of the samples was carried out in an ascending alcohol series up to 100 %. The samples were then dried at critical point prior to placing on carbon tape. Subsequently, the samples were coated with gold powder and studying cell morphology using SEM (CamScan Analytical, Maxim 2000S) operating at 10 kV.

Statistical analysis

Statistical analysis of LLC-MK2 cell viability was compared between cells cultured in pFF from small, medium, and large follicles at protein concentrations of 2, 4, 20, 40, 200, 400, 500 and 600 µg/mL using one-way ANOVA and post-hoc Duncan. Significant differences were considered when $p< 0.05$.

RESULTS AND DISCUSSION

Morphology of LLC-MK2 cells when cultured in small, medium, and large pFF supplementations

Initially, LLC-MK2 cells were prepared by culturing in supplementation with 10% FBS and observed morphology using inverted and scanning electron microscopy. At 0 h, the cells were floating in culture medium with oval shape (Fig. 1A and B). Adhesion of cells was seen when cultured for 24 h, appearing in fusiform and polyhedral shapes with about 5 µm diameter (Fig. 1C and D). At 48 h of culture, almost cells were adhered to the culture plate, where they expanded extensively and flattened with approximately 8 µm diameter (Fig. 1E and F). The morphology of LLC-MK2 cells was similar to what reported by Hull et al. [23]. To investigate effects of pFF supplementation on LLC-MK2 cell morphology, cells were cultured in supplementation with small, medium, large pFFs at concentrations of 2, 4, 20, 40, 200, 400, 500 and 600 µg protein/mL for 24 h. For small pFF, cells from all culture conditions including control and positive control appeared in polygonal shape (Fig.2), a conserved property of proliferating epithelial cells and a normal shape of LLC-MK2 cells [24,25]. LLC-MK2 cells cultured in pFF supplementation were more flattened that those of control and positive control groups. At low total protein concentrations (2, 4, and 20 µg protein/mL), some piles of cell were observed (Fig.2C-E).

A number of granules in the cytoplasm as well as multinucleated and giant cells were seen at high protein concentration FFs (>40 µg/mL; Fig. 2F-J). This is in consistent with the report of Cook et al. (1974), where rosettes and giant cells were occasionally observed when cells were maintained for few weeks. Cells with mitotic activity were evidence at >200 µg protein/mL supplementations (big arrows, Fig.2G-J).

In case of medium pFF, similar to control and positive control groups, cells supplemented with low total protein concentrations (2, 4, and 20 µg/mL) were mostly in polygonal shape with few fusiform-shaped cells (Fig. 3A-E). When cultured in $\geq$40 µg protein/mL pFF supplements, cells appeared abnormal, flattened and round. The cytoplasm was expanded and contained a number of vacuoles where association of vacuole amounts with pFF protein concentrations was observed (Fig. 2F-J). At 600 µg protein/mL pFF, cells exhibited clearly abnormal appearance. Large number of granules and various nuclear sizes were observed throughout the

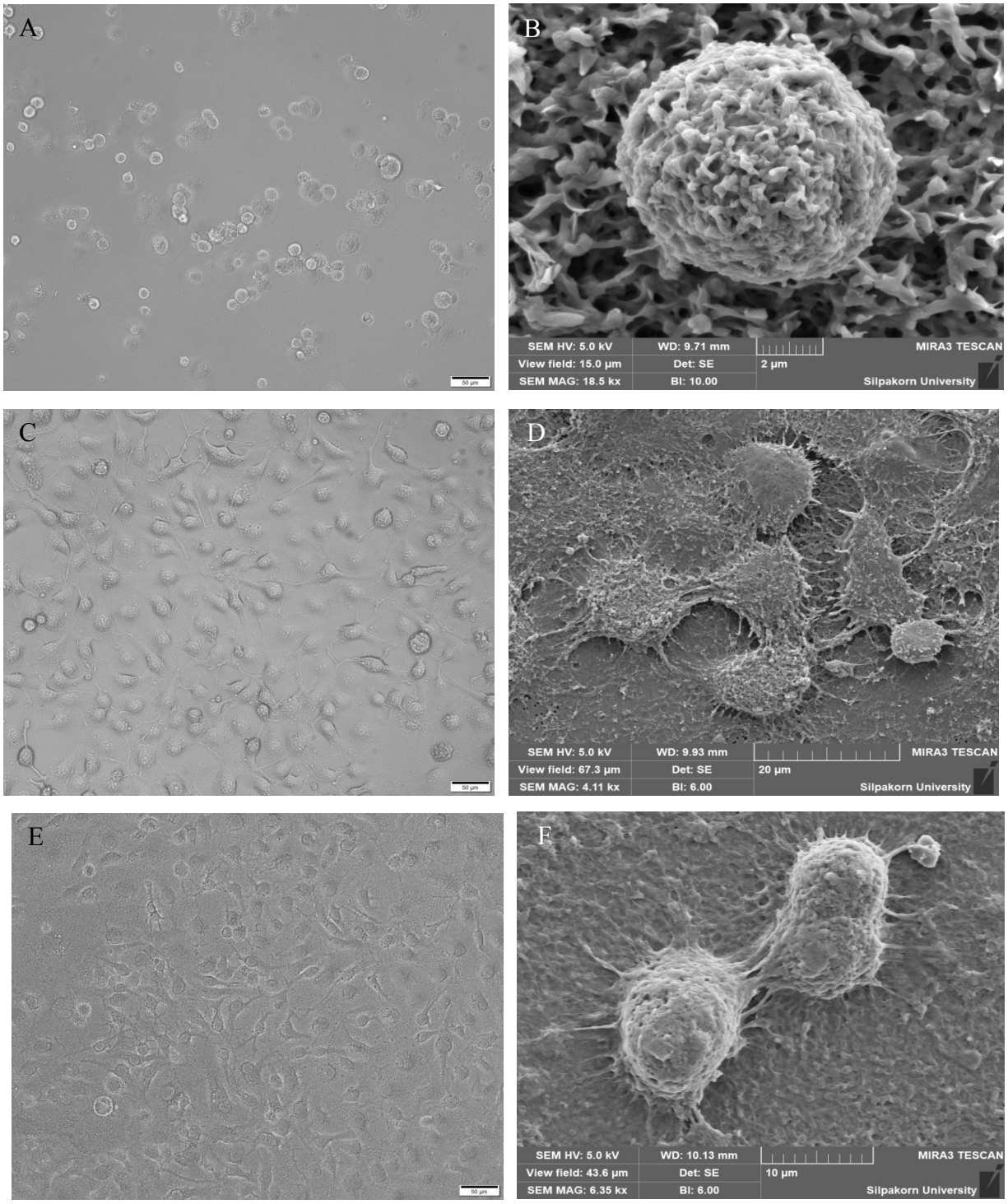
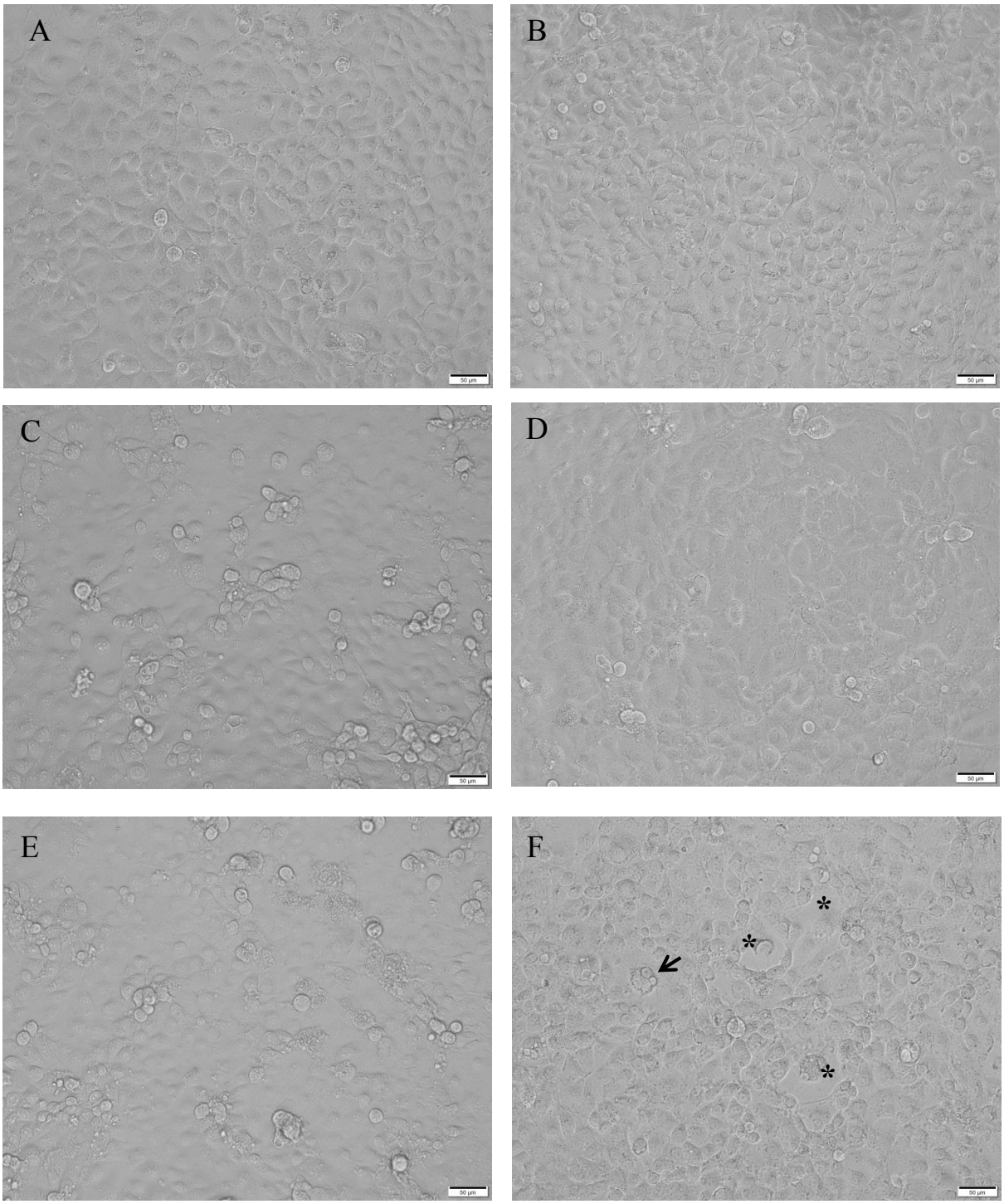


Figure 1 Morphology of LLC-MK2 cells under an inverted microscope at (A) 0 h, (C) 24 h, and (E) 48 h; magnification at 200x); and scanning electron microscope at (B) 0 h, (D) 24 h, and (F) 48 h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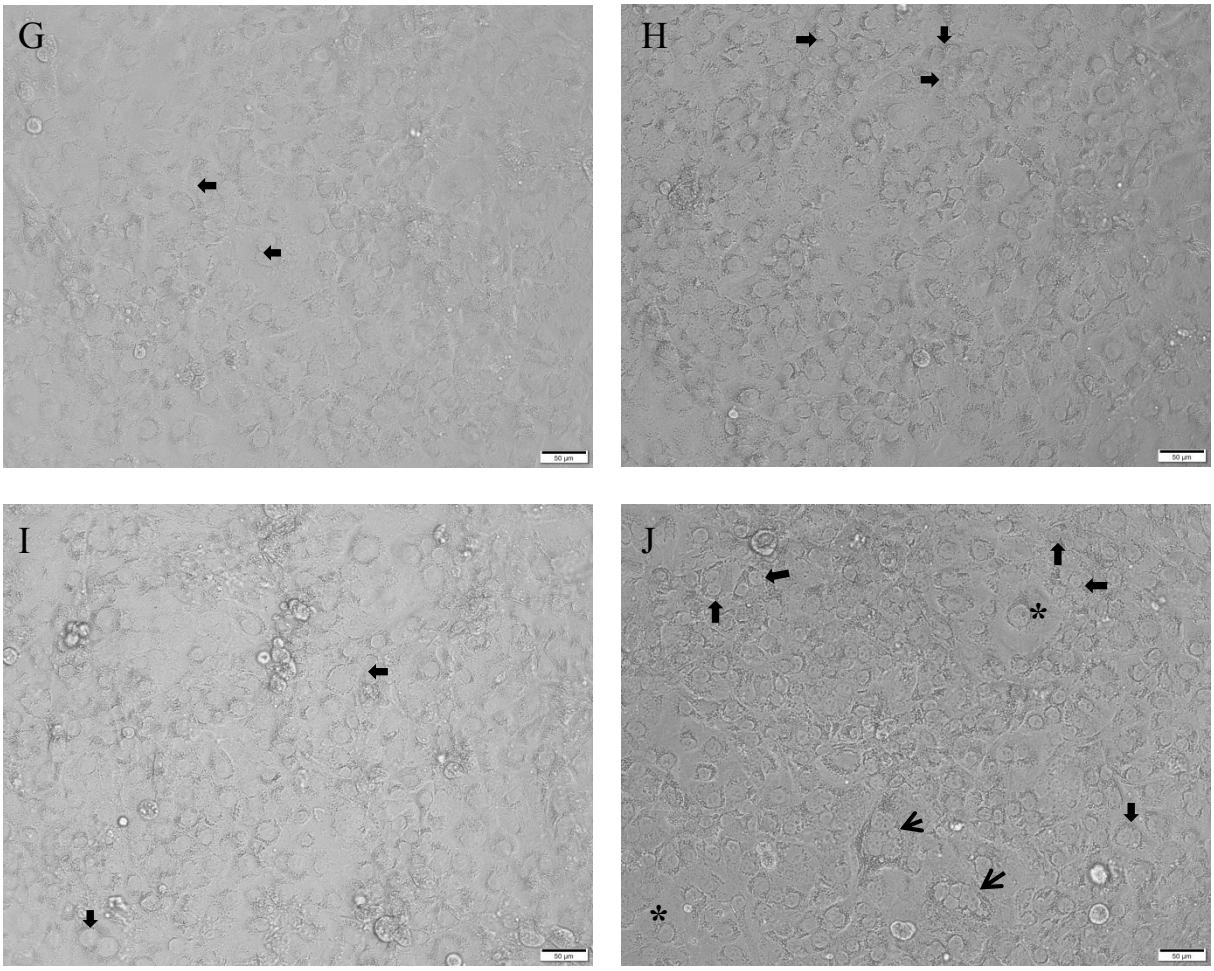
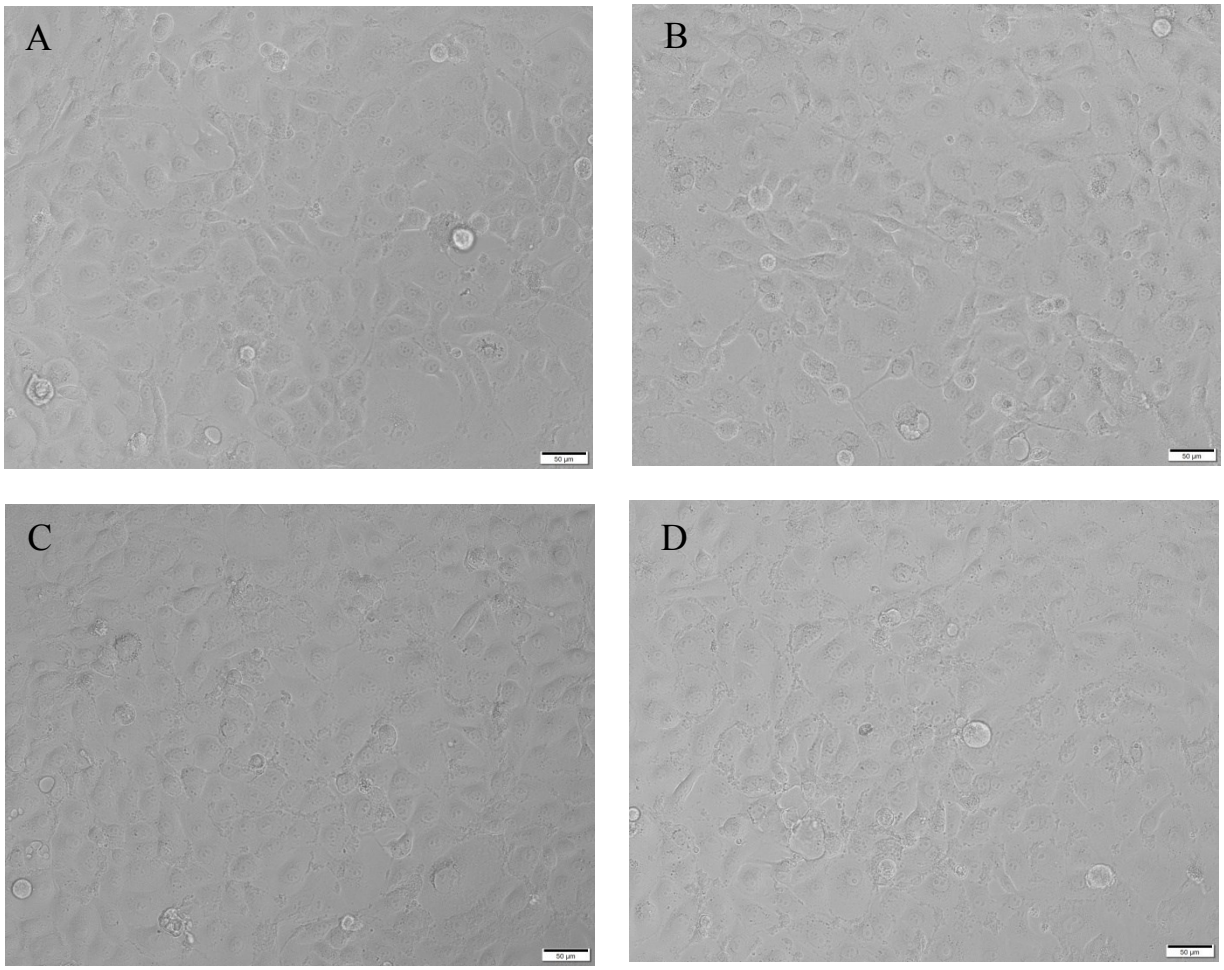


Figure 2 Morphology of LLC-MK2 cells cultured in supplemented with small pFF at 24 h (A) control, (B) positive control, and (C) - (J) cultured in 2, 4, 20, 40, 200, 400, 500, and 600 μg protein/mL pFFs, respectively; magnification at 200x; small arrow indicate multinucleated cell, big arrow indicate cell undergoing mitosis, and star indicate giant cell.

culture plate. The smallest size was less than 1/3 of the main nucleus size (Fig.2J). Results from MTT assay indicated that these cells were metabolically active when culturing for 24 h , and eventually died after 48 h.

The abnormal appearance of LLC-MK2 cells, when cultured in supplementation with 600 μg protein/mL medium pFF, was observed. Morphologically, it is likely that large micronuclei, small DNA structures that separated from cell nucleus, occurred in these cells [26]. Micronuclei are usually found in cytoplasm, round to oval shape, sizes about 1/16 to 1/3 that of main nucleus, containing round smooth membrane, and absence of overlap with or connect to the main nucleus [27]. Presence of micronuclei is an indicator of genetoxic stress and genetic instability, which might contribute carcinogenesis [28,29,30]. Micronuclei formation has been reported in various cell types including lymphocyte, carcinoma cells, oocytes undergoing embryo development, and adenocarcinoma cell line [27,30]. It has been documented that occurrence of micronuclei is associated with various

factors including deficiencies in vitamins (e.g. folate and vitamin B12), ionizing radiation, chemical induction [30]. Additionally, a component of mammal follicular fluid, meiosis activating sterol has been demonstrated to associate with chromosomal abnormality and unequal sizes of human blastomeres [31]. The authors reported that abnormality rates were increased when increased sterol concentration. In addition to micronuclei, cell stress due to pFF treatment might be an explanation for abnormal morphology. This is because one report that investigated influences of imidacloprid- and acetamiprid-based insectides on human neuroblast and found that the chemical substances gave neurocytotoxicity to SH-SY5Y neuroblastoma cells with oxidative stress, leading to cell morphology changes, i.e. neurite branch retraction [32]. However, further experiments, e.g. oxidative stress assessment, to evaluate generation of reactive oxygen species (ROS) are required for this conclusion [32,33].



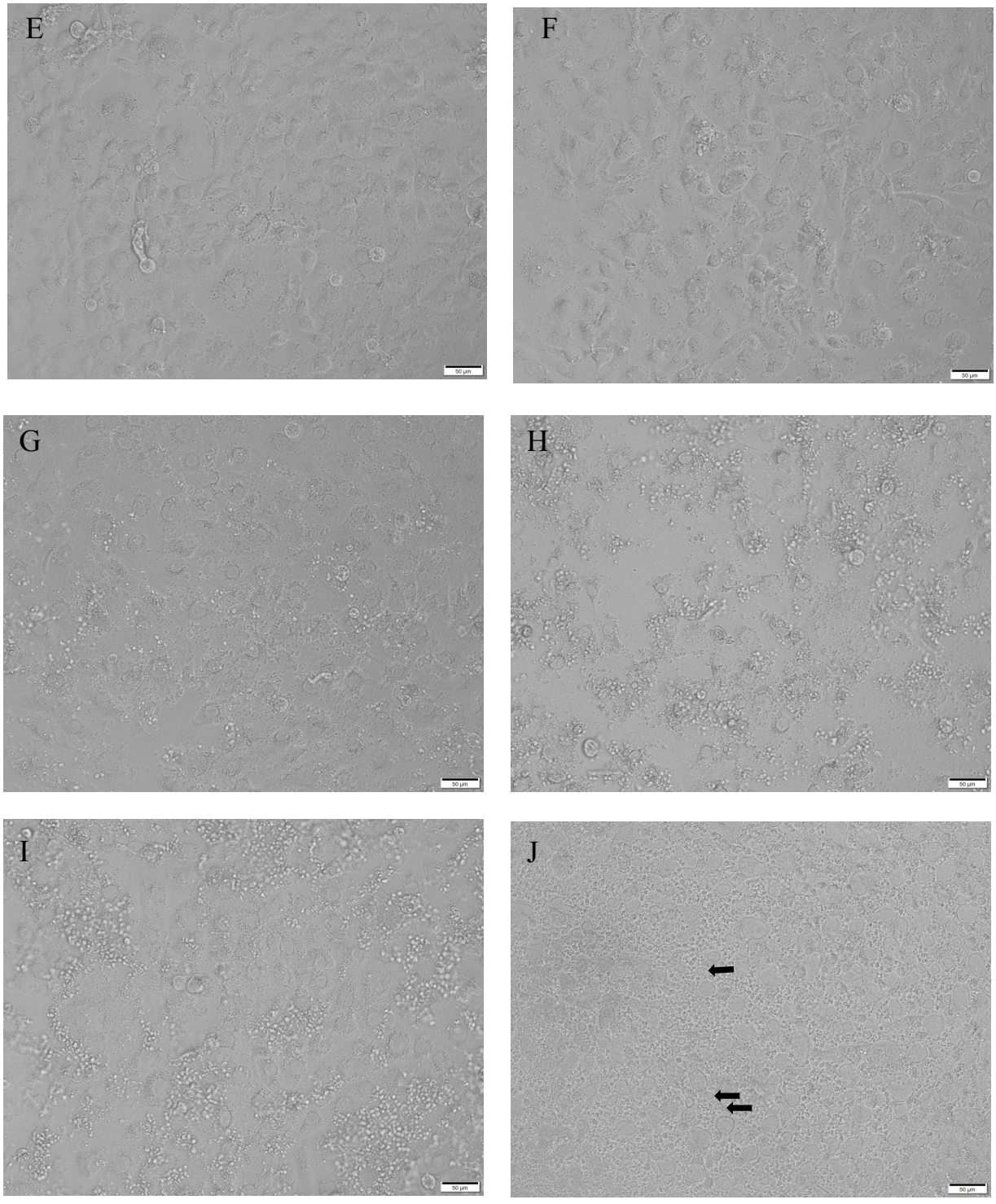
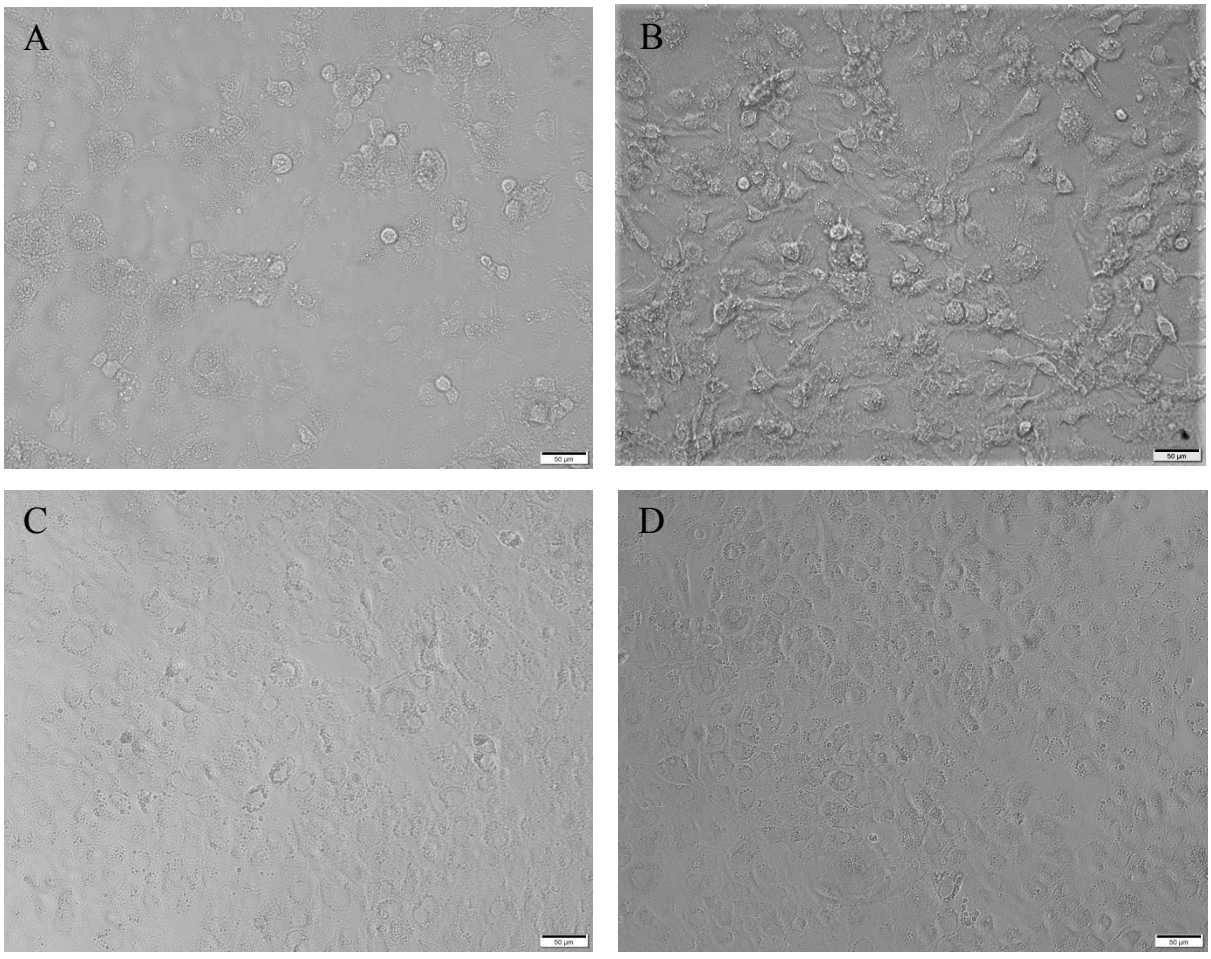


Figure 3 Morphology of LLC-MK2 cells cultured in medium pFF supplement at 24 h (A) control, (B) positive control, and (C) - (J) cultured in 2, 4, 20, 40, 200, 400, 500, and 600 μg protein/mL pFF supplementation, respectively; magnification at 200x; big arrow indicated micronuclei containing cell.

Impacts of large pFF supplementation on LLC-MK2 cell morphology was studied using inverted microscopy. Cells in all tested concentrations were in a polygonal shape (Fig. 4A-J). Compared with control and positive control groups, cells cultured in pFFs were more expanded and flattened, and more confluent. Similar to what seen in small and medium pFFs, a number of granules occurred in the cytoplasm at high protein concentration (≥200 μg/mL) treatments but in lesser degree to those of medium pFF (Fig.4G-J). Mitosis of cells with normal appearance was evidence in 200 and 600 μg protein/mL supplementations (arrows in Fig.4G and J).

Effects of pFF from small-, medium- and large-sized follicles on LLC-MK2 cell viability

Observation of cell morphology using inverted microscopy revealed that small, medium, and large pFFs have different influences on LLC-MK2 cell growth and development. Next, effects of pFF from different-sized follicles on cell viability were determined. After culturing for 24 h in pFF supplementation, cells were determined



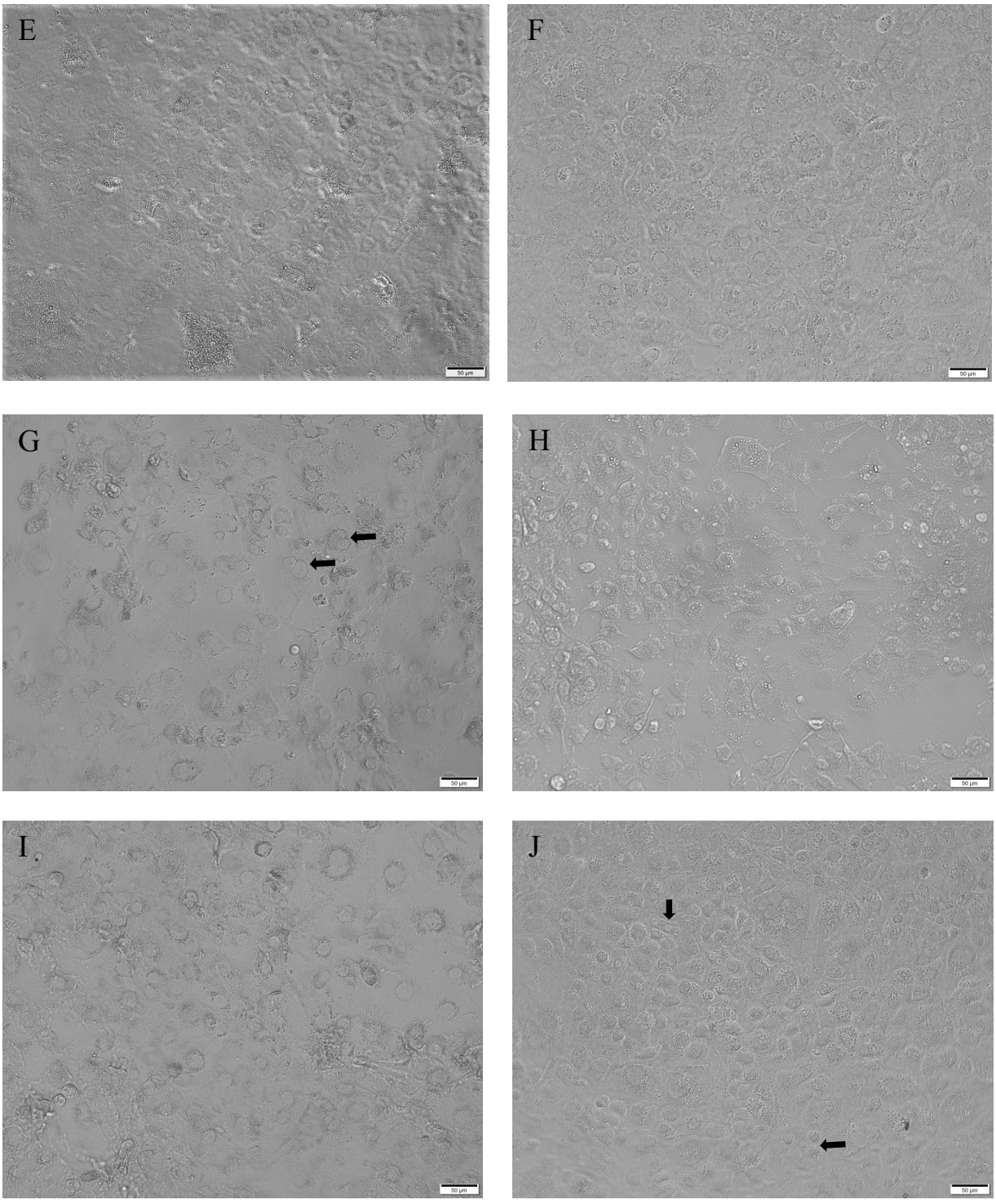


Figure 4 Morphology of LLC-MK2 cells cultured in large pFF supplementation at 24 h (A) control, (B) positive control, and (C) – (J) cultured in 2, 4, 20, 40, 200, 400, 500, and 600 μg protein/mL pFFs, respectively; magnification at 200x; big arrow indicated cell undergoing mitosis.

viability using MTT assay (Fig.5A). Cell viability of each condition was calculated in relative to that of control (100%). For small pFF, LLC-MK2 cells cultured in all protein concentrations had cell viability rate higher than that of control group but lower than the positive control (134.64±1.87%). The highest and the lowest viability percentages obtained from cells cultured in 600 µg protein/mL (101.16±6.08%) and 2 µg protein/mL (130.817±6.28%), respectively. Results from statistical analysis indicated that viability percentage of cells cultured in 2, 4 and 20 µg protein/mL pFF were significantly lower than the positive control ($p < 0.05$) whereas percentage of cells cultured at 40, 200, 400, 500 and 600 µg protein/mL were not significantly different.

In case of medium pFF, viability of cells from all concentrations was higher than control group but lower than positive control (134.39±2.11%) (Fig.5A). The highest and the lowest viability percentages were from cells cultured at 500 µg protein/mL (121.75±1.14%) and 4 µg protein/mL (100.04±1.16%), respectively. Statistical analysis revealed that viability percentage of cells cultured at 2, 4, 20, 40, and 600 µg protein/mL pFF were significantly lower than that of positive control. Similarly, for large pFF, viability of all cells was higher than control group but lower than positive control. The highest and the lowest percentages obtained from 40 µg protein/mL (119.66±4.22) and 600 µg protein/mL pFF (91.07±11.98), respectively. Viability percentage of every pFF protein concentration except at 40 µg protein/mL was significantly lower than that of positive control.

Comparisons of LLC-MK2 cell viability between small, medium, and large pFF supplementation revealed that viability percentages from small pFF were higher than those of medium and large pFFs (Fig.5). For small pFF, cell viability percentage was increased gradually when protein concentrations of fluids increased except for 400 µg/mL. On the other hand, decreased viability percentage was seen in cells supplemented with large pFF at 200 and 600 µg protein/mL. The differences of cell growth may be resulted from biochemical differences between large and small pFF [8,10,34]. Presently, very little information documenting on inhibitory component(s) in FF has been documented. One publication reported unknown component in large bovine FF negatively influencing transcription process of Yoshida ascites cells by interfering the elongation of RNA synthesis [35]. However, it is still no conclusion that whether or not there is inhibitory component in large pFF, where it may have negatively role on cell growth.

We next compared impacts of small, medium, and large pFFs on growth and development of LLC-MK2 (this study), Vero [20], and HeLa cells [21]. Compared with LLC-MK2 cells, viability of Vero cells from medium pFF supplementation was higher than those of small and large pFF treatments (Fig.5B). Percentages of cell viability obtained from small and large pFF were comparable, except at 500 µg/mL pFF. Cell viability trended to be increased when increased pFF concentrations from 4 to 500 µg/mL. Similar to Vero cells, viability of HeLa cells from medium pFF was higher than those of small and large pFFs, where large pFF gave higher viability percentages than that of small pFF (Fig.5C). Also, cell viability trended to be increased when concentration of all size pFFs was increased. Noteworthy, at 600 µg/mL of small pFF, viability of all cell lines was increased whilst decreased cell viability was seen in medium and large pFFs. Furthermore, differences of morphology were noted among three cell lines. Abnormal appearance of LLC-MK2 cells was observed in supplementation with medium pFF at high concentrations (Fig.3G-J). HeLa cells also showed abnormal appearance at high concentrations but in small pFF and lesser degree [21] and no abnormal morphology reported for Vero cells [20].

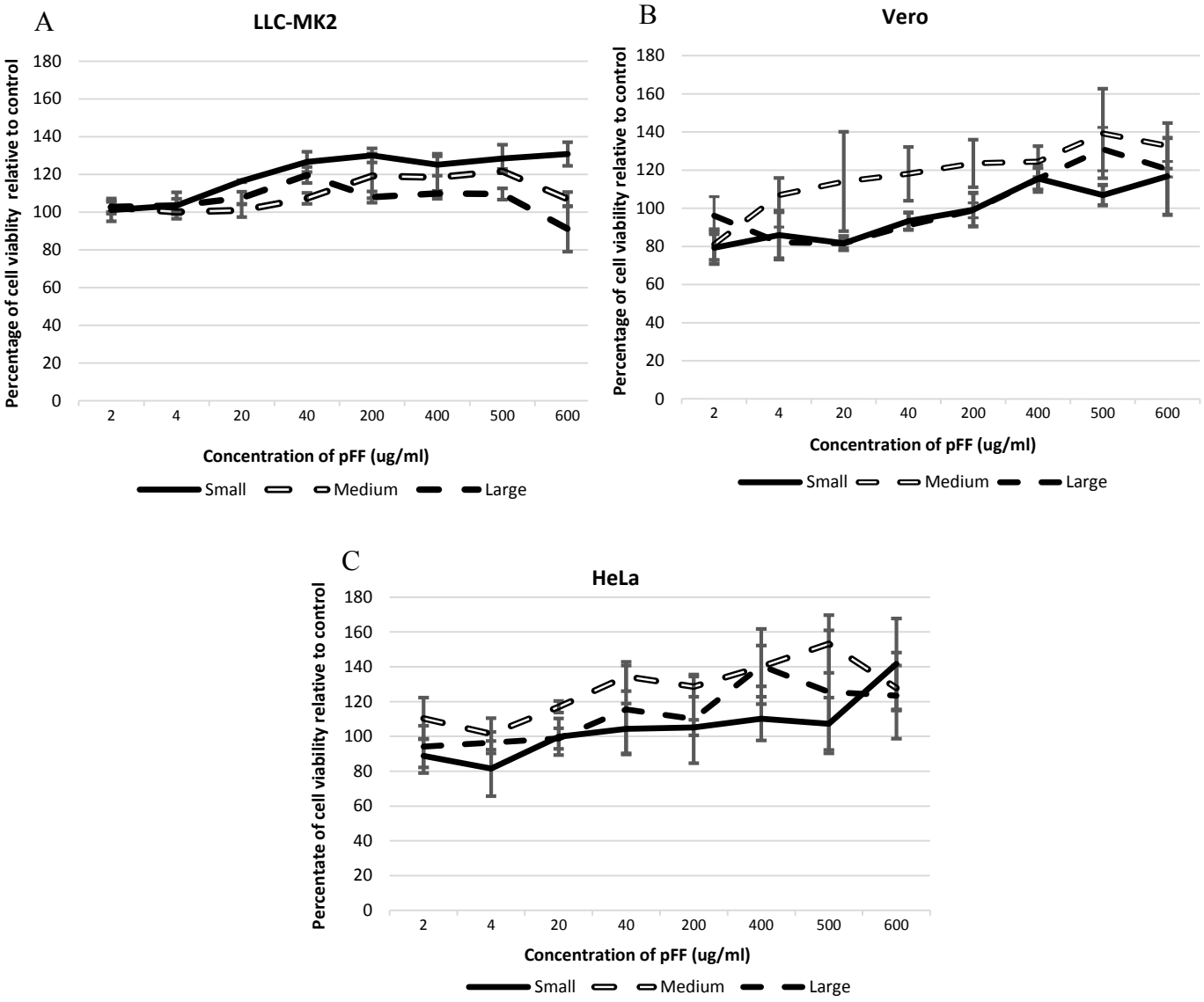


Figure 5 Cell viability of (A) LLC-MK2, (B) Vero, and (C) HeLa cells when cultured in small, medium, and large pFFs for 24 h

As mentioned before that Vero and LLC-MK2 cells were epithelial cells derived from monkey kidney tissues. However, both cells showed different properties and cytopathic effects to viral infection and replication [11,13,15,16,17,18,19]. LLC-MK2 cells were derived from kidney of healthy rhesus monkey, containing little sensitive toxic factors and other adverse factors [23]. Chromosomal counts of LLC-MK2 demonstrated ranging from 60-65 pairs but no nodule formation at high passage level and no tumor occurrence in inoculated animals [23]. Vero cells, an African green monkey kidney derived cell line, have pseudo-diploid karyotypes with chromosome number between 52 and 62 [36]. Similar to LLC-MK2 cells, they were immortalized non-tumorigenic cells.

Results from molecular characterization revealed approximately 9 Mb deletion of Vero cell chromosome 12 causing loss of type I interferon gene cluster and cyclin-dependent kinase inhibitor genes, resulting to high susceptibility to various viruses [36]. It has been noted elsewhere that LLC-MK2 cell appearance was more pleomorphic and mitoses than that in Vero cells [37]. These characteristics might be explanation on presence of abnormal appearance, i.e. micronuclei formation, in LLC-MK2 cells when cultured in pFF supplementations but normal in Vero cells. Clearly, pFFs from different size follicles contain different microenvironments, resulting in different impacts on growth and development between LLC-MK2 and Vero cells.

CONCLUSION

This study investigated impacts of pFF from small, medium, and large follicles on growth and development of LLC-MK2 cells using inverted microscopy and MTT assay. Small pFF can promote growth and development of LLC-MK2 cells, which was comparable with FBS supplement, whereas cell viability from large pFF treatment was significantly lower than that of the positive control group (FBS supplement). LLC-MK2 cells appeared abnormal, i.e. vacuolization, micronuclei formation, when cultured in medium pFF at high concentrations ($\geq$ 200 μg protein/mL). Cell growth and development of LLC-MK2 cells was different to Vero and HeLa cells. That is, medium pFF can efficiently promote growth of both Vero and HeLa cells, where no abnormal appearance reported, and *vice versa* for LLC-MK2. This may be because different microenvironments (biochemical components) between small, medium, and large pFFs differently influencing on growth and development of each cell line. Impacts of pFF on cell growth could be either negative or positive, depending on specific nature of cells.

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