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Impact of porcine follicular fluid during folliculogenesis as a supplement on the primary cell culture of oviductal epithelial cells

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ABSTRACT

The impact of varying concentrations of porcine follicular fluid (pFF) derived from three ovarian follicle types and produced during folliculogenesis on porcine oviductal epithelial cell (pOEC) culture was studied for 24 h using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Cells treated with pFF at a protein concentration of 500 µg/ mL (from small- and large-sized ovarian follicles) and 600 µg/mL (from medium-sized ovarian follicles) showed the highest viability, which was significantly different from that observed in the control group (p<0. 05) and not significantly higher than that observed in the positive control group.This study demonstrated that the impact of pFF on pOECs can be used as a model for biotechnological studies. Further, the study showed that instead of costly fetal calf serum, pFF produced during folliculogenesis can be used as a supplement in culture media to promote porcine oviductal epithelium cell growth and development.

Keywords: porcine oviductal epithelial cells; porcine follicular fluid; folliculogenesis

1. INTRODUCTION

Porcine anatomy and physiology are similar to human systems, including their reproductive system (Chen et al., 2013). Thus, many researchers have used pigs as a model to study the reproductive system and basic sciences (Areekijseree and Veerapraditsin, 2008; Areekijseree and Vejaratpimol, 2006; Chen et al., 2013; Sanmanee and Areekijseree, 2009; Sanmanee and Areekijseree, 2010; Pongsawat and Youngsabanant, 2019; Youngsabanant and Mettasart, 2020). Porcine oviduct is an organ that is neglected in slaughterhouses; however, it can be easily collected and used as a model in biotechnological research (Chen et al., 2013; Way, 2006; Youngsabanant and

Mettasart, 2020). In the present study, porcine oviductal epithelial cells (pOECs) were primarily cultured for using them as a model for *in vitro* study because their physiology is similar under *in vivo* conditions (Chen et al., 2013; Miessen et al., 2011). The morphology of pOECs is simple columnar and classified into two types: nonciliated cells that can secrete a substance that stimulates acrosome reaction in spermatozoa, fertilization, and embryo development and ciliated cells that can transport oocytes and spermatozoa in different ways as well as transport the embryo to the uterus (Areekijseree and Chuen-Im, 2012; Areekijseree and Veerapraditsin, 2008; Kim et al., 1996); ciliated cells are present in the lumen of the oviduct (Chen et al., 2013; Miessen et al., 2011).

Porcine follicular fluid (pFF) is a product of the secretory activity of theca and granulosa cells that surround oocytes and follicles. pFF is easy to collect (Bianchi et al., 2007; Revelli et al., 2009; Youngsabanant et al., 2019; Youngsabanant and Rabiab, 2020) from ovarian follicles, which are categorized into three groups as per size (small-sized ovarian follicle: 1-3 mm in diameter, medium-sized ovarian follicle: 4-6 mm in diameter, and large-sized ovarian follicle: >7 mm in diameter) (Orisaka et al., 2009). Follicular fluid plays an important role in oocyte development and sperm activity (Ducolomb et al., 2013; Funahashi and Day, 1993; Revelli et al., 2009). The fluid is rich in reproductive hormones and proteins that stimulate oocyte development and cell growth (Gosden et al., 1988; Ito et al., 2008; Kor, 2014). Previous reports used follicular fluid as a supplement in the culture medium for *in vitro* bovine oocyte maturation and *in vitro* bovine fertilization (Algriany et al., 2004; Oberlender et al., 2013; Vatzias and Hagen, 1999; Youngsabanant and Rabiab, 2020).

The aimed of this research was to study the impact of pFF derived from differently sized ovarian follicles on the *in vitro* morphology and growth of pOECs. Cell viability was studied using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, a colorimetric assay. The results could add value to waste products generated in local slaughterhouses, and pFF derived from pOECs can be used in biotechnological studies as a supplement in the culture medium instead of fetal calf serum to promote cell viability and growth.

2. MATERIALS AND METHODS

2.1 Culture medium

M199 with Earle's salt medium supplemented with 10% heat treat fetal calf serum (HTFCS), 2.2 mg/mL NaHCO3, 0.25 mM pyruvate, and 50 µg/mL gentamycin sulfate was prepared and equilibrated in an incubator at 37°C, 5% CO2, and 95% air atmosphere with high humidity for 12-24 h before use.

2.2 pFF and pOEC collection and isolation

Porcine ovary and oviduct samples were collected from a local slaughterhouse. They were collected 30 min after slaughter and transported to our laboratory in a thermos container at a temperature of 30-35°C using the sterile technique. pFF was collected from small, medium and large-sized ovarian follicles using a syringe connected to an 18-gauge needle in a conical tube and centrifuged for 5 min at 1,500 × *g* to remove oocytes and cells. The fluid was stored at −80°C until use. The protein concentration of pFF derived from the ovarian follicles of all sizes was quantified using the Lowry method. The oviducts were washed three times with 0.9% normal saline supplemented with 250 µg/mL amphotericin B, 100 µg/mL streptomycin, and 100 IU/mL penicillin. pOECs were obtained by scraping the end of the isthmus to the ampulla of the oviduct using a sterile glass slide. The cells and fluid were transferred to sterile test tubes before washing seven times with 0.9% normal saline supplemented with antibiotics. The cells were seeded in the medium at the concentration of 1 × 105 cells/mL and cultured at 37°C, 5% CO2, and 95% air atmosphere with high humidity for 96 h.

2.3 MTT assay and morphological study

The primary oviductal cells were cultured in a 96-well plate for 96 h before testing with pFF derived from the three ovarian follicle types (at the protein concentrations of 2, 4, 20, 40, 200, 400, 500, and 600 µg/mL) for 24 h. Their cell viability was studied using the MTT assay. This method measures the mitochondrial activity of viable cells and is commonly used for studying cell viability because it is economical and has fewer disadvantages (Boncler et al., 2014; Van Meerloo et al., 2011). The cells were cultured with a tetrazolium salt for 4 h and the developed formazan dye was detected using a spectrophotometer at 570 nm. Cell morphology was observed under an inverted microscope during culture and after treatment with pFF derived from the three follicle types.

3. RESULTS AND DISCUSSION

Porcine oviducts and ovaries are considered waste organs in slaughterhouses and were used in this study. The pOECs were cultured in M199 medium supplemented with 10% heat treat fetal calf serum for 24-48 h. pOECs contained two types of cells: moving columnar ciliated cells and round-shaped nonciliated cells (Figure 1). Approximately 50%-60% cells were attached to the substrate after culture in M199 medium for 72 h (Figure 2). After 96 h, approximately 70%-80% cells were attached to the substrate (Figure 3) and showed two cell morphology types: epithelial morphology (regular shape) and fibroblast-like morphology (elongated shape observed after long-term culture for 4 weeks) (Figure 4). The advantages of this culture method included it being a simple and economical method and the ability to culture cells over long term to produce cell line.

After culture for 96 h, pOECs were treated with different pFF concentrations for 24 h. The cells treated with pFF at a protein concentration of 500 µg/mL (from small- and large-sized ovarian follicles) and 600 µg/mL (from medium-sized ovarian follicles) showed the highest cell viability. The viability was significantly different from that observed in the control group (p<0.05) and higher than that observed in the positive control group (p<0.05), except that observed for pFF derived from small-sized ovarian follicles was not significantly from the positive control. The viability of the cells treated with pFF from small-, medium-, and large-sized ovarian follicles was higher, with more control and positive control groups (Figure 5).

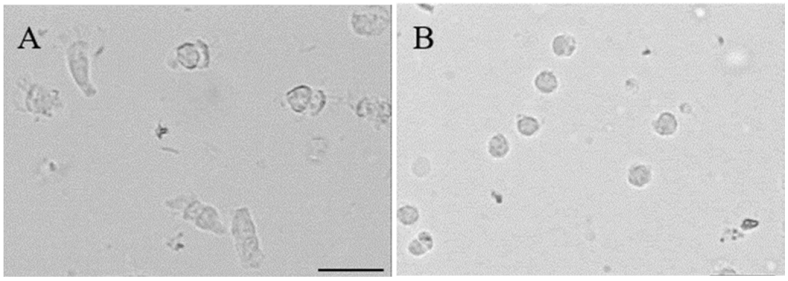

Figure 1. Primary porcine oviductal epithelial cells cultured in M199 medium for 48 h showing (A) moving isolated columnar ciliated cells and (B) round-shaped cells, scale bar = 10 μm

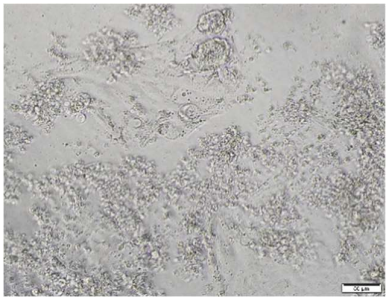

Figure 2. Porcine oviductal epithelial cells attachment to the substrate (approximately 50%-60%) after culture for 72 h in M199 medium (magnification, 200×)

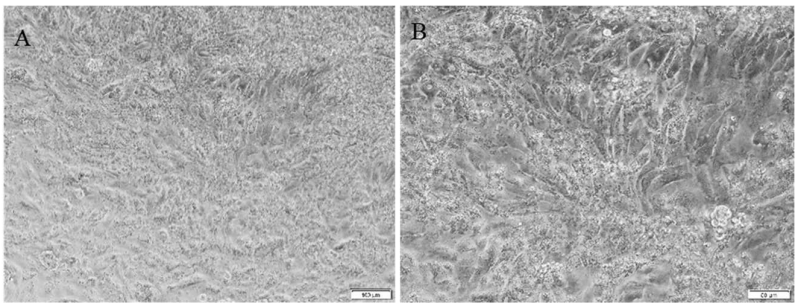

Figure 3. Porcine oviductal epithelial cells attachment to the substrate (approximately 70%-80%) after culture for 96 h in M199 medium (A: magnification, 100× and B: magnification, 200×)

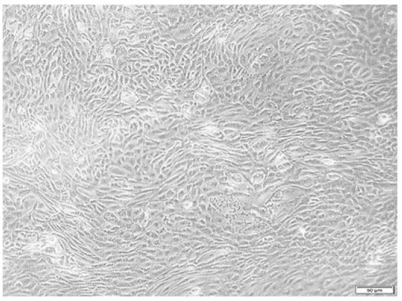

Figure 4. Porcine oviductal epithelial cells showing two cell morphology types: epithelial morphology (regular shape) and fibroblast-like morphology (elongated shape after 4 weeks of culture) (magnification, 200×)

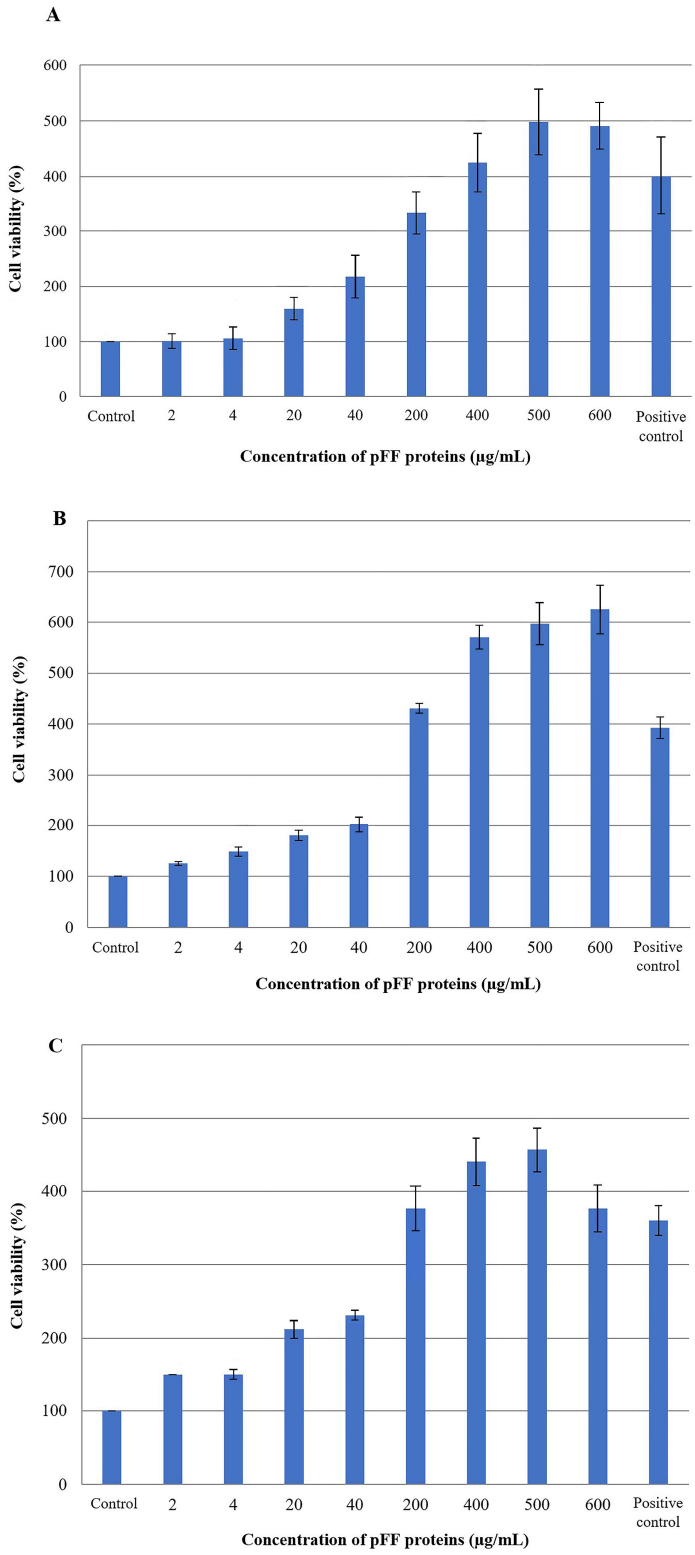

Figure 5. Effects of pFF on pOEC viability; porcine follicular fluid derived from (A) small-, (B) medium-, and (C) large-sized ovarian follicles at the protein concentrations of 2, 4, 20, 40, 200, 400, 500, and 600 µg/mL (mean ± SE)
Note: Control group, medium M199; positive control group, medium M199 supplemented with 10% HTECS

The percentage of cell growth in the test groups with pFF derived from small- and large-sized ovarian follicles was the highest at a protein concentration of 500 µg/mL. The test group with pFF derived from medium-sized ovarian follicles showed the highest percentage of cell growth at a concentration of 600 µg/mL (Figure 5). Moreover, in the test group with pFF derived from small- and large-sized ovarian follicles at a concentration of 500 µg/mL and in the test group with pFF derived from medium-sized ovarian follicles at a concentration of 600 µg/mL, the cell morphology was elongated and the number of viable cells was two-fold higher than that in the control and positive control groups. These findings were observed because the protein components in pFF derived from small-, medium- and large-sized ovarian follicles could stimulate cell growth and viability. The protein components were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed using mass spectrometry. The protein components of pFF from small-sized ovarian follicles contained a 100-kDa protein, which was identified as a truncated form of the epidermal growth factor receptor. It promotes cell growth and differentiation. Meanwhile, another protein with a molecular weight of 92 kDa was found; this protein was identified as a heat shock protein that promotes cell growth (Ducolomb et al., 2013; Youngsabanant-Areekijseree et al., 2019).

In this study, pFF derived from small, medium and large-sized ovarian follicles was used. Porcine follicular fluid contains growth factors, hormones, electrolytes, glucose, uric acid, lipids, cyclic AMP, and proteins (Chang et al., 1976; Spitzer et al., 1996). Brachova et al. (2017) studied human oviductal epithelial cells in a culture medium supplemented with human follicular fluid (hFF) and found that hFF can promote the growth of epithelial cells more than the control group. The present study demonstrated that cells treated with pFF showed more growth than cells in the control group, which is similar to that reported by Brachova et al. (2017). Somigliana et al. (2001) studied the effect of hFF on the endometrial cells of the uterus cultured in Ham's F-10; the results showed increased growth in cells cultured in follicular fluid than in cells in the control group. In addition, pFF from medium- and large-sized ovarian follicles could promote *in vitro* oocyte maturation (IVM). Oberlender et al. (2013) reported that oocytes grown in a culture medium supplemented with follicular fluid derived from large-sized ovarian follicles (6-10 mm in diameter) showed a higher IVM percentage than oocytes grown in the medium supplemented with follicular fluid derived from small-sized ovarian follicles (2-5 mm in diameter). However, the percentage of IVM was not significantly different. Likewise, Ito et al. (2008) found that oocytes cultured in a medium supplemented with follicular fluid derived from large-sized ovarian follicles (5-6 mm in diameter) matured significantly faster than oocytes cultured in the medium supplemented with follicular fluid derived from small-sized ovarian follicles (3-4 mm in diameter). In addition, cumulus cells cultured in the medium supplemented with follicular fluid showed increased cell viability. However, pFF derived from small-sized ovarian follicles contained proteins (the porcine inhibitor of carbonic anhydrase and coagulation factors) that inhibit oocyte maturation. PFF derived from small-sized ovarian follicles affects metaphase I of oocyte development (Ducolomb et al., 2013). Another protein, the oocyte maturation inhibitor, inhibits oocyte development (Tsafriri et al., 1976; Van de Wiel et al., 1983). According to the results of the present study, the cell viability of pOECs treated with pFF derived from small-sized ovarian follicles was lower than that of pOECs treated with pFF derived from medium- and large-sized ovarian follicles. The colorimetric detection method in the MTT assay was used to detect mitochondrial activity in viable cells. This method is common and economical and has fewer disadvantages (Boncler et al., 2014; Van Meerloo et al., 2011).

4. CONCLUSION

pOECs treated with pFF at the concentration of 500 µg/mL from small- and large-sized ovarian follicles and 600 µg/mL from medium-sized ovarian follicles, which were used to supplement the culture medium, showed the highest cell viability. This cell viability was significantly different from the percentage of cell viability observed in the control group and the positive control group. This result implies that pFF from porcine ovaries can be used as a supplement instead of fetal calf serum in the medium to promote the growth of primary porcine oviductal cells.

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