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Original Article

Establishment and characterization of female reproductive tract epithelial cell culture

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ABSTRACT

The oviductal and uterine epithelial cells have a crucial role, but are still poorly understood. Numerous studies have tried to isolate the epithelial cells from different organs in various models. The current study aimed to establish and characterize an *in vitro* monolayer culture of the oviduct and uterine horn epithelial cells by using two different techniques. Female reproductive epithelial cells from sows were cultured in follicular phase. Combined protocols to isolate the epithelial cells were performed. The viability and cell number were determined. Monolayers of epithelial cells from each group were cultured in four-well plates and were subjected to immunostaining using a Vector ABC Elite Kit. The immunohistochemical staining step was performed to evaluate the quality of the epithelial cells. Oviductal cells reached confluence faster than uterine horn cells. Cilia were seen in oviduct and uterine horn tissue culture. All the isolated cells reached confluence prior to harvesting. The number of cells was increased over the time of incubation. Monolayer culture using the trypsin/EDTA method took longer than culture with the collagenase method. Immunohistochemistry of epithelial cells showed strong staining for cytokeratin. Oviductal and uterus epithelial cells were cultured and established. Both techniques used in this experiment were useful and showed no significant differences. This cell culture model has the potential to study the secretory interactions of the female reproductive tract with spermatozoa, oocytes or embryos.

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1. Introduction

In vitro cultures of cells from the female reproductive tract of different species, including humans, have been successfully established and characterized [1–5]. Numerous studies have tried to isolate epithelial cells from different

organs in various models such as rats [6], cattle [5], swine [7], and humans [8,9]. Several protocols and techniques have been used to isolate the epithelial cells from different organs and organisms.

Epithelial cells are formed in the early stage of embryo development. Three distinct layers are formed, the ectoderm, mesoderm, and endoderm. The mesoderm lies between the ectoderm and endoderm. The mesoderm generates skeletal and connective tissues, while the ectoderm and endoderm generate the epithelial layers [10]. The function of epithelial cells is to transport essential nutrients, oxygen, fluids, and ions. They also secrete several components and produce certain signals. The generation of these

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signals could be due to other factors or can be generated by itself [11].

In term of physiological processes, the epithelium can be found either internally in organs or exteriorly on their surface. *In vivo*, epithelial cells are the site for malignant transformation due to their regenerative nature. Therefore, the epithelium is considered in the studies of several types of tumors [12]. The malignant tissues which composed of epithelium cells that derived for carcinogenesis tumor in prostate, lung, breast [10]. In histological sections, the stroma cell, basement membrane, is acting to separated the epithelial cell layer from other tissue. The stromal cell is also acting to regulate the different functions of the epithelial cells [13]. Squamous epithelial cells have been also studied in skin cells and determined the physiology and pathology of keratinocytes [14].

In vitro cultures are simpler and easier to establish compared with *in vivo* cultures. However, there are still several shortcomings in using *in vitro* cell culture. Specific features and functions of the oviductal epithelium are lost during *in vitro* culture [2]. Some studies showed that an increased number of oviduct culture passages resulted in decreased expression of several genes [8,15]. In addition, cell morphology alterations have also been reported with continuous cell culture [16,17].

Thibodeaux et al [5] determined a method to isolate and culture endometrial epithelium from adult bovine tissue using a trypsin–EDTA protocol. They maintained good cell viability for up to eight subcultures. In addition, a good level of post-thaw subculture was also determined. Cell separation refers to separation of epithelial cells from fibroblasts. Different components in the culture medium support epithelial cell growth and exclude fibroblasts. A simple vascular monolayer could be used for subculture and is sufficient for production of multilayers [18].

Prichard et al [19] investigated the ability to coculture oviduct and uterine cells from goats. They examined the progress of embryo development in each group with different cells. Eventually, they concluded that coculture of the embryo with oviductal or uterine cells yielded a higher rate of development. However, coculture of the embryo in medium with oviductal and uterine cells together did not improve embryo development.

In this study, we aimed to establish and characterize an *in vitro* monolayer culture of oviduct epithelial cells (OVECs) and uterine horn epithelial cells (UHECs) using two different techniques. Immunohistochemical staining was performed to confirm that the cells were epithelial cells.

2. Materials and methods

2.1. Sample collection and preparation

Reproductive tracts were obtained from sows from the local slaughterhouse (G. Wood and sons, Mansfield, UK) and transferred to the laboratory at ambient temperature. Ovaries that showed dominant follicle growth and absence of corpus luteum were selected as follicular phase. Samples in luteal phase, ovaries with pathological signs, or ambiguous samples were discarded. Samples of 15 oviducts

and uterine horns were prepared at lengths of 20 cm and 15 cm, respectively. The excess tissues were trimmed away. The uterine horns and oviducts were washed three times and flushed with 37 °C phosphate-buffered saline (PBS; Gibco, Invitrogen, Paisley, UK) without magnesium and calcium, supplemented with 100 µg/mL streptomycin + 100 U/mL penicillin + 0.25 µg/mL amphotericin B mix antibiotics (Sigma, Poole, Dorset, UK). Epithelial cells were isolated from the oviducts and uterine horns.

2.2. Epithelial cell isolation from oviducts and uterine horns

Two different protocols for cell isolation were performed. The first protocol was based on an enzymatic method and the other on a mechanical or scraping technique [7]. Two separate methods were performed based on an enzymatic protocol; one with 0.25% trypsin–EDTA, as described by Thibodeaux et al [5] with some modifications. The other enzymatic method used collagenase digestion as described by Freshney [10]. Fifteen samples from each group (oviduct and uterine horn) were used. M199 medium (Invitrogen) was used as the culture medium. M199 was supplemented with 10% fetal bovine serum (Invitrogen) and 100 µg/mL streptomycin + 100 U/mL penicillin + 0.25 µg/mL amphotericin B mix (Sigma).

2.3. The 0.25% trypsin–EDTA method

Samples were kept in warm PBS (no Mg²⁺ or Ca²⁺; Gibco) after flushing with PBS. One end of each sample was sealed tightly with cotton thread. Three to five millimeters of warm 0.25% trypsin–EDTA was injected into the sample using a 10-mL syringe, and the sample was sealed from the other side. The sealed samples were incubated at 39 °C with 5% CO₂ for 90 minutes. The cotton threads were then cut at both ends. Each sample (oviduct and uterine horn) was milked well to collect the fluid. An equal volume of the culture medium was added to the 50-mL centrifuge tube. The tubes were centrifuged at 300g for 10 minutes. The pellet was resuspended in M199 medium and washed with PBS. This step was repeated two times to ensure high quality and clean pellet. Red blood cell lysis buffer was added to remove the red blood cells. The cells were washed and added to 5 mL M199 medium. The viability test was performed by 0.4% trypan blue exclusion (Sigma) to evaluate cellular integrity. Five milliliters of M199 medium was added to the pellet, which was divided into several tissue culture flasks (T-75; Nalge Nunc International, Naperville, IL, USA). The flasks were incubated in air at 39 °C and 5% CO₂. The culture medium was replaced every 12 hours.

2.4. Collagenase digestion

Collagenase Type 1A (Sigma) was prepared with Hank's Balanced Salt Solution (HBSS) without magnesium or calcium (Invitrogen). The prewarmed collagenase was filled inside the uterine horn and oviduct. The oviduct and

uterine horn samples were incubated at 39°C in 5% CO₂ humidified air for 2 hours. The samples were properly squeezed and the fluid was collected in clean tubes. Collagenase helped to release the cells from the attached tissues. A cell pellet was formed by centrifugation at 300 g for 5 minutes. The pellet was suspended in 5 mL M199 medium and allocated into tissue culture flasks (T-75). The flasks were incubated in air at 39°C and 5% CO₂. The culture medium was replaced every 12 hours.

2.5. Scraping technique

The samples from both techniques (trypsin–EDTA and collagenase) for each group (oviduct and uterine horn) were opened longitudinally. Cells were scraped using a clean glass slide (once) and collected in the tube with culture medium. The cells were centrifuged at 300 g for 5 minutes and the supernatant was discarded. The red blood cells were removed using red blood cell lysis buffer. The pellet was later resuspended in M199 medium and allocated into appropriate tissue culture flasks (T-75). The flasks were incubated in air at 39°C and 5% CO₂. The culture medium was replaced every 48 hours.

2.6. Harvesting and cell detachment

Cells reached confluence after continuous feeding with fresh medium. Most of the cells reached 95–100% confluence. The proteolytic enzyme technique was used to release the attached cells. Four to five milliliters of trypsin (0.5 mg/mL) with EDTA (0.2 mg/mL) was used to detach the cells from the surface. The flasks were washed with warm PBS (Mg²⁺/Ca²⁺ free; Invitrogen) and incubated for 3–5 minutes in air at 39°C, in 5% CO₂. Gentle tapping and mechanical shaking by hand were used to increase removal of the cells. Culture medium with fetal calf serum was added to stop trypsinization. The cells were collected in a tube and centrifuged at 300 g for 4 minutes. The supernatants were discarded and the cell pellet was suspended in 5 mL M199 medium.

2.7. Subculture and freezing cells

The 5-mL pellet with M199 medium was allocated into three T-75 flasks. After incubation in air at 39°C, in 5% CO₂ for 48 hours, the cells reached confluence. We used the similar protocol for the detachment of cells by trypsin–EDTA. To perform several passages, 1 mL of the cell suspension was aliquoted into a T-75 flask with 10 mL M199 medium. For cell freezing, the cell pellet was resuspended in freezing medium rather than culture medium. Freezing medium contained 10% glycerol (Sigma) with fetal calf serum. No antibiotics were used in the freezing medium. Three milliliters of the freezing medium was added to the pellet and aliquoted into three different cryovials. The cryovials were chilled at –20°C and transferred to a freezer at –80°C overnight. The cryovials were transferred to a liquid nitrogen container for further analysis.

2.8. Cell growth measurement

The cell count was performed using a Neubauer hemocytometer (Hausser Scientific Company, Horsham, PA). Thirty microliters of the suspended pellet was taken and spread on the counter slide. The number of cells in four large squares (1 mm²) was counted to determine the total cell count. The concentration of the cells from the uterine horn and oviduct were determined.

2.9. Immunohistochemical staining

The cells were stained immunohistochemically to evaluate the quality of the cells. A Vector ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA) was used for this purpose. Epithelial cells contained cytokeratin, which yield a brownish color, whereas nonepithelial cells did not. Cells (10⁵) were cultured overnight in four-well plates (Nunc). The cells reached confluence within 30 hours and then the staining process was initiated. Cells were fixed in 2% formaldehyde buffer in four-well plates (Nunc). The cells were blocked by 300 µL blocking solution at room temperature for 1 hour to remove any endogenous biotin. The blocking solution contained 25% (v/v) avidin and 0.2% (v/v) horse serum. The primary antibodies (dilutents at 1:200 with antibody dilutents media, Sigma) were added. The secondary biotinylated antibody (Vector Laboratories) was added and incubated for 30 minutes. The vector stain (ABC solution) was applied for 30 minutes at room temperature. The working solution (chromagen), 3,3'-diaminobenzidine (DAB) was prepared by mixing DAB stock solution with hydrogen peroxide solution in distilled water, and applied for 10 minutes. DAB yields a brown stain and the binding was observed with brown color. Finally, the plate was washed gently with PBS to remove excess moisture. The control wells were incubated without primary antibody and binding only to the secondary antibody. Photographs were taken using a DMI4000B light/inverted microscope (Leica, Wetzlar, Germany).

3. Results

3.1. OVEC and UHEC monolayer culture

OVECs and UHECs were cultured under the same conditions. However, OVECs reached confluence faster than UHECs. The average time to reach confluence was 12 days and 16 days for OVECs and UHECs, respectively. **Figure 1** shows the different stages of cell growth in the oviduct and uterine horn. Cilia were seen in tissue culture of the oviduct and uterine horn.

3.2. Cell growth and counts

All the isolated cells reached confluence prior to harvesting (96–100%). The number of cells increased with time of incubation. Monolayer cells isolated using the trypsin–EDTA method took longer than those isolated using the collagenase method. However, the monolayer epithelial cell from both fluid collection and scraping

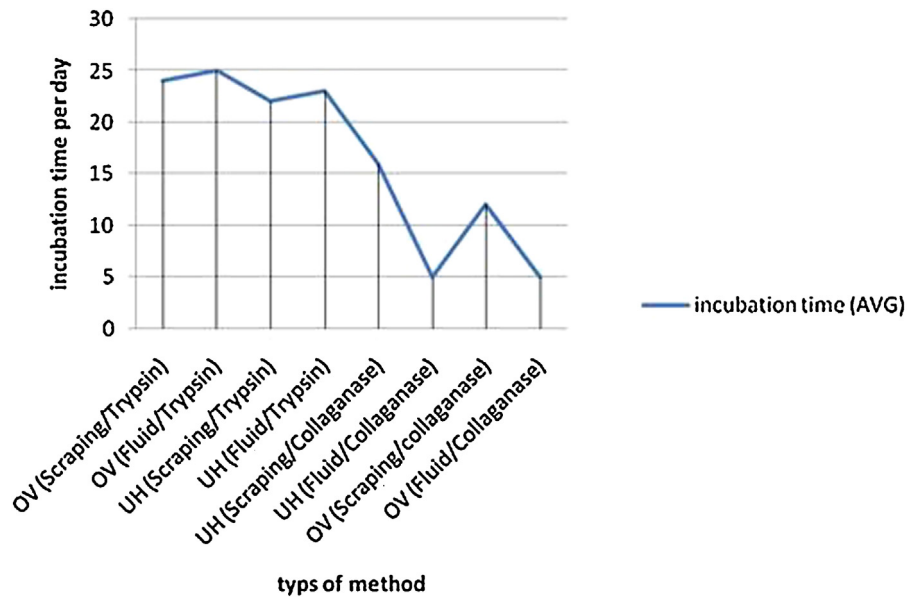


Fig. 1. This curve depicts the relationship between the methods of cell isolation and incubation time. We considered confluence as the end of incubation. AVG = the average incubation time; ND = not determined; OV = oviduct; UH = uterine horn.

cells did not show any significant difference in growth rate. The growth chart in Figure 1 illustrates the relationship between the cell isolation method and incubation time.

3.3. Immunohistochemical staining

The epithelial cells were stained immunohistochemically to assess their viability. The OVECs showed strong staining for cytokeratin (Figure 2).

4. Discussion

The OVECs and UHECs have a crucial role, but it is still poorly understood. Several studies have attempted to gain further knowledge. *In vitro* culture of epithelial cells is a basic step for investigating the role of the oviduct and uterus in infertility [20]. Epithelial cells are the source of active signals and secrete different components to maintain natural processes [11,12,21]. The techniques for isolation of epithelial cells have improved and developed rapidly.

The cells isolated using the trypsin–EDTA method took longer to reach confluence compared with those isolated using the collagenase technique. There was no particular reason for this difference. Counting of the cells and selecting the correct size of flask are recommended. The number of cells increased during incubation until reached confluence stage. Alterations in cell morphology, growth rate, response to stimuli, gene expression profile, and protein production were observed during cell culture passage [16,17]. Neumann et al [8] found that the level of mRNA expression was altered with increasing number of passages and presumed dedifferentiation of primary synovial fibroblasts. Moreover, alteration in mRNA expression

of adrenomedullin (*ADM*), heat shock 70-kDa protein 8 (*HSPA8*) and prostaglandin E synthase (*PGES*) in immortalized OVECs was reported [1,22].

Another issue arising during cell culture was the quality of the cells. Mixed populations and cell contamination were investigated. Immunohistochemical staining aimed to evaluate the quality of the cells and ensure that only epithelial cells were isolated and harvested. Epithelial cells showed cytokeratin staining, while other cell types did not. As depicted in Figure 2, >95% of the cells had a brown color, which indicated the presence of cytokeratin and that they were epithelial cells.

Contamination in some flasks was a major problem during primary cell culture. This contamination was probably due to the rapid growth of the cells. In addition, there was a possibility of contamination of UHECs with stromal cells. The stromal cells have a secretory role in the function of the uterus [10]. The culture of OVECs and UHECs established in this study could yield valuable information on gene and protein expression. This was shown in this study and is in agreement with other studies with similar findings [3,23]. OVECs and UHECs have also been used to gain a better understanding of the maternal interaction with gametes and embryos [21,22,24–26].

In conclusion, OVECs and UHECs were cultured and established. Both techniques used in this study, scraping and enzymatic techniques, were useful and showed no significant differences. Several issues were raised during cell culture and addressed appropriately. However, proper maintenance of all conditions during cell culture will prevent such issues. This cell culture model has the potential to study the secretory interactions of the female reproductive tract with spermatozoa, oocytes, or embryos in future. Further study is needed to apply this model and investigate the mechanism of cell interaction

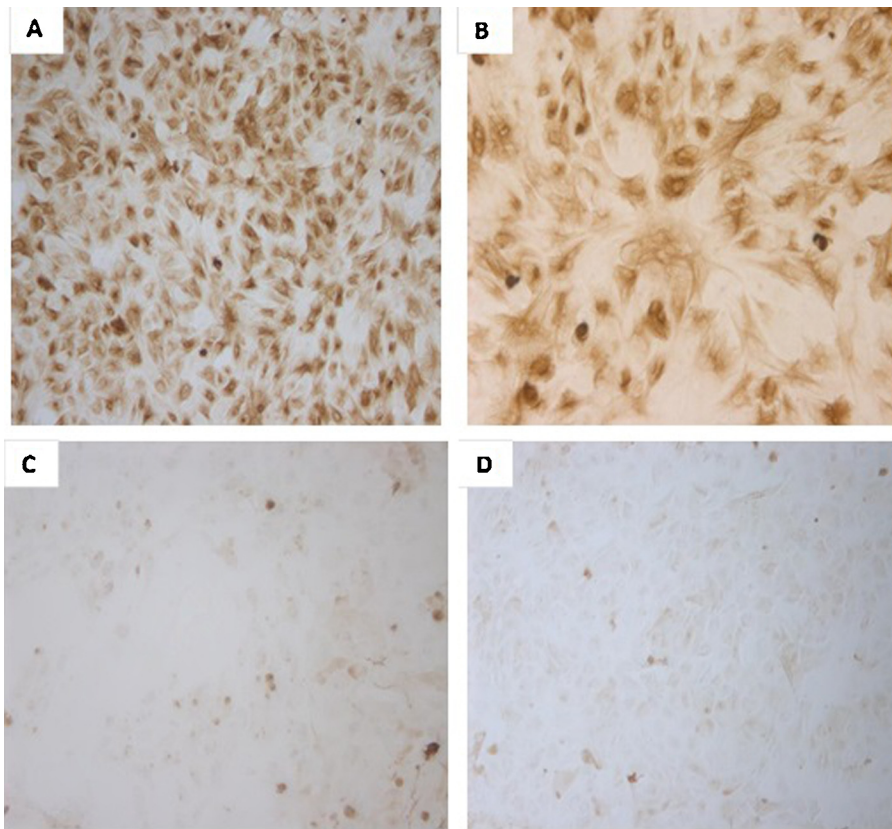


Fig. 2. Oviductal cells with immunohistochemical staining. Positively stained cells are shown in (A) at 10 \times and (B) at 20 \times magnification. Control cells are shown in (C) at 10 \times and (D) at 20 \times magnification.

with gametes and embryos in the fallopian tubes and uterus.

Conflicts of interest

The author declares no conflicts of interest.

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