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In vitro Effects of Sex Hormones on the Proliferation of Endometrial Cells Isolated from Laying Hens

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Abstract: Researchers investigated the effects of Estrogen (E_2) and Progesterone (P_4) on the morphology, growth and proliferation of chicken endometrial cells *in vitro*. Type I collagenase digestion was used to isolate endometrial epithelial and stromal cells and the cells were identified by immunohistochemistry. MTT assays (MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) were performed to analyze the effects of the sex hormones on the proliferation of the endometrial cells *in vitro* and growth curves were constructed to demonstrate the growth rules of endometrial cells cultured *in vitro*. The epithelial and stromal cells tested positive for keratin CK18 and vimentin, respectively, in the immunohistochemical analysis. The MTT experiments showed that the growth rate of the chicken endometrial cells began to increase logarithmically on day 2, reaching the plateau phase on day 6 or 7. The endometrial epithelial cells treated with 100 nM E_2 and/or 100 nM P_4 proliferated at a significantly higher rate $p<0.05$) than the control cells. However, although, the stromal cells treated with 100 nM P_4 proliferated at a significantly higher rate ($p<0.05$) than the control cells, the E_2-treated endometrial stromal cells did not.

Key words: Endometrium, epithelium cell, laying hen, proliferation *in vitro*, stromal cell, sex hormone

INTRODUCTION

The uterus of laying hens is part of the oviduct and forms the eggshell. The uterus of laying hens is vesicular shaped and contains tubular glands that secrete calcium, pigment and the cuticle. The endometrium of laying hens consists of a layer of simple columnar epithelium and the underlying lamina propria. The uterine epithelium consists primarily of secretory glandular epithelial cells. The lamina propria consists of endometrial stromal cells and stroma. Endometrial glandular epithelial cells proliferate periodically in response to ovarian hormones. In different physiological cycles, the combined effect of E_2 (Estrogen) and P_4 (Progesterone) promote changes in endometrial cell morphology and biochemistry and also affect endometrial cell proliferation and secretion. E_2 regulates the development and differentiation of germ cells and their niches, the physiological behavior of estrogen is mediated by its nuclear receptors. estrogen receptors drive physiological changes by regulating different transcription factors and target genes (Hall and McDonnell, 1999). Pierro *et al.* (2001) showed that E_2 promoted the proliferation of human endometrial epithelial cells *in vitro* and that other sex hormones also affect the proliferation of endometrial cells *in vitro*.

The endometrial cells of rats (Ho *et al*., 2006), pigs (Zhang and Davis, 2000), rabbits (Wang *et al*., 2010), monkeys (Wei *et al*., 2005), humans (Wang *et al.,* 2012), sheep (Chen *et al*., 2012) and bovine (Donofrio *et al*., 2008) have been isolated and cultured but reports of cultured endometrial cells of laying domestic hens are scant. The objectives of our study were to culture the endometrial cells of laying hens and to investigate the effects of sex hormones on the proliferation of the cultured chicken endometrial cells *in vitro*. Researchers used type-I collagenase digestion to isolate epithelial and stromal cells from the endometrium and performed MTT experiments (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) to assess the effect of E_2 and P_4 on the proliferation of the endometrial cells *in vitro*. The study provides a model for the investigations of

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the proliferation, differentiation and metabolism of endometrial cells of laying hens *in vitro* and lays a theoretical foundation for studies of the sex-hormone-mediated regulatory mechanisms of endometrial cells.

MATERIALS AND METHODS

Animals and reagents: Hy-Line Brown hens aged 32 weeks were obtained from the qnimal experiment station at Shihezi University in Xinjiang, China. Only hens with laying rates >80% were used in the experiments. The DMEM/F12 medium was obtained from HyClone (Utah, USA), The mycoplasma-free Fetal Bovine Serum (FBS) was obtained from Sijiqing Biological. The type-I collagenase, penicillin and streptomycin solution and the MTT reagent were obtained from Solarbio. The 0.25% trypsin was obtained from Sigma. The Dimethyl Sulfoxide (DMSO) was obtained from Biosharp. The P_4 and E_2 were obtained from Sigma-Aldrich (St. Louis, MO, USA). The keratin CK18 monoclonal antibody, vimentin monoclonal antibody, streptavidin-peroxidase immunohistochemistry kit and DAB solution were obtained from Zhongshan (Beijing, China). Light microscope and inverted microscope connected to a PC via a Nikon digital system (Olympus CKX41) made in Japan. Microplate spectrophotometer (BioTek Power Wave XS2) made in USA.

Isolation and culture of endometrial epithelial cells and stromal cells

Scissors method: The uterus was collected from laying hens by sterile dissection. The blood vessels, connective tissues and epidermis were removed from the uterine tissue under sterile conditions and the endometrial layer of the uterus was washed three times with sterile PBS (phosphate-buffered saline). The endometrial tissue was cut into 1 mm^3 pieces using sterile scissors and the fragments were digested by immersion in a solution containing 1 mg mL^{-1} type-I collagenase (Solarbio) at 37°C for 60 min. The digestion was terminated by the addition of culture medium containing 10% FBS (Sijiqing Biological). The digestion mixture was filtered using a 200-mesh cell strainer and the filtrate was centrifuged for 5 min at 120x g. The precipitate was washed twice and suspended in culture medium containing 10% FBS. Cell viability was determined to be >90% by staining with 0.1% trypan blue. The number of cells was estimated using a hemocytometer and the cell density was adjusted to 6×10^5 cells mL^{-1}, the isolated cells were cultured in culture dishes. Subculturing of the confluent cultures was done to purify the stromal cells. The confluent cells were detached from the dishes using 0.25% trypsin (Sigma) at 37°C and transfer the cells with a sterile transfer pipette. The detached cells were centrifuged, mixed with DMEM/F12 (HyClone, Utah, USA) +10% FBS and reseeded. The purified cells were seeded into 5 cm culture dishes, 6 and 96-well plates at 37°C in air containing 5% CO_2, respectively. A glass cover slip was placed at the bottom of each well of the 6-well plates before adding the cell suspension. The culture medium was replaced the following day and the growth state of cells was observed using an inverted microscope (OLYMPUS CKX41, Japan) daily for 3-4 days. The identity of cells that adhered to the cover slip were determined using immunohistochemistry. The endometrial stromal cells were isolated using this method.

Scraping method: The uterus was collected from laying hens by sterile dissection. The endometrial epithelial cells were scraped from the uterine wall using a surgical blade and digested by immersion in a solution containing 1 mg mL^{-1} type-I collagenase at 37°C for 60 min. The remaining steps were identical to those of the Scissors Method. The endometrial epithelial cells were isolated using this method.

Immunohistochemical experiment: The morphologies of stromal cells and epithelial cells were observed using an inverted microscope daily. Stromal cells and epithelial cells adhering to the glass cover slips were identified using immunohistochemistry. The cover slips were gently washed three times with PBS and were fixed in 4% paraformaldehyde at room temperature for 20 min. The stromal cells and epithelial cells were analyzed using the anti-vimentin or anti-cytokeratin ck18 antibody (Zhongshan, Beijing, China) as the first antibody, respectively and reacted with the biotinylated secondary antibody and streptavidin-peroxidase conjugate provided in the SP kit (Zhongshan, Beijing, China). Then the slips of stromal and epithelial cells were stained with DAB solution (Zhongshan, Beijing, China) and observed under the light microscopy (OLYMPUS CKX41, Japan).

Growth curve analysis of cultured endometrial cells: The MTT assay has been used to test cytotoxicity of reagents, cell proliferation and viability. MTT, a yellow tetrazole is reduced to purple formazan in living cells. A solubilization solution (usually dimethyl sulfoxide, an acidified ethanol solution) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The cell growth curves of stromal cells and epithelial cells were determined based on the data from the MTT experiments. The epithelial and stromal cells were cultured in 96-well plates with each well containing approximately 10^4 cells in a volume of 200 μL of medium. The culture medium was replaced every 24 h. Eight wells were selected for cell growth analysis at the various time points. After 20 μL of 5 mg mL^{-1} MTT solution (Solarbio) was added to each of the eight wells, the cells were cultured for an additional 4 h at 37°C. The culture medium was aspirated and 150 μL of DMSO (Biosharp) was added to each well.

Table 1: Details of the experimental treatments of endometrial cells isolated from laying hens

	Groups		Treat groups									
Hormones	B	C	1	2	3	4	5	6	7	8	9	B
E_2 (nM)	-	-	10	50	100	-	-	-	100	10	100	-
P_4 (nM)	-	-	-	-	-	10	50	100	10	100	100	-

B = Blank group; C = Control group; - = not add that hormones (E_2 or P_4). The blank groups added PBS without cells. The treat groups consisted of nine different treatments

The plate was placed on an oscillator for 10 min to gently mix the contents of the wells and the absorbance of the contents of each well was measured at 490 nm using a microplate spectrophotometer (BioTek Power Wave XS2, USA). The procedure was repeated daily for 8 days and the data were analyzed using Microsoft Excel 2010 (Redmond, WA, USA).

Analysis of effects of sex hormones on the proliferation of endometrial cells: Stromal cells and epithelial cells derived from Scissors Method and scraping method were cultured in 96-well plates for 24 h. The cells consisted of the blank, control and test groups. The culture medium for the test groups were replaced with medium containing different concentrations of E_2 or/and P_4(Sigma-Aldrich) as shown in Table 1. Four wells were treated with each concentration of hormone. The blank wells were treated with PBS and the control cells were treated with culture medium that lacked sex hormones. Cells were cultured for an additional 3 days and cell proliferation was assessed using the MTT assay as described in growth curve analysis. The absorbance value for each well was measured at 492 nm using a microplate spectrophotometer. The data were analyzed using the SPSS, version 17.0, Statistical Analysis Software (IBM, Armonk, NY, USA).

Measurement and analysis: All photos were taken with a light microscope (Olympus CKX41, Japan) connected to a PC via a Nikon digital system.

Data were processed by SPSS Statistics version 17.0 (IBM, Armonk, NY, USA), mono factor analysis of variance and linear correlation were used for analysis.Values were considered significantly different at $p<0.05$.

RESULTS

Culture results of endometrial cells: The cells were observed daily using an inverted microscope . Both the epithelial cells and stromal cells adhered to the bottom of the cell culture plates after 36 h of growth. The cells obtained using the scraping method consisted primarily of epithelial cells mixed with a small number of stromal cells. The epithelial cells had an elliptical or irregular morphology with a clear border and were arranged closely (Fig. 1a). The cells obtained using the Scissors Method

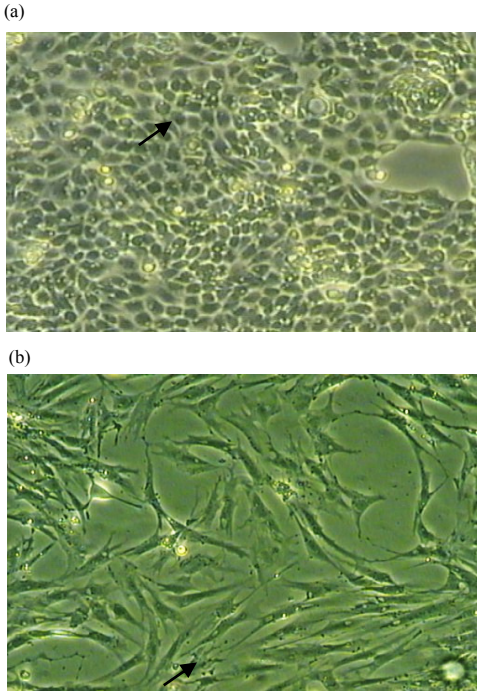

Fig. 1: The morphology of chicken endometrial epithelial and stromal cells. The morphology of endometrial epithelial and stromal cells cultured for 48 h under inverted microscope vision fields (100x); a) epithelial cells; b) stromal cells of chicken endometrium

consisted primarily of stromal cells that had a fusiform or irregular morphology with a prominent nucleus. The stromal cells were arranged in parallel or radial pattern (Fig. 1b).

Immunohistochemical analysis of endometrial cell migration: The immunohistochemistry results showed that both epithelial and stromal cells were isolated, based on the staining of the cytokeratin ck18 or vimentin proteins, respectively (Fig. 2a, b). The stromal and epithelial cells which represented brown cytoplasm and a bluish-violet nucleus signify a positive reaction for vimentin or cytokeratin ck18 and were considered viable endometrial stromal or epithelial cells, respectively. Almost 95% of the stromal cells and epithelial cells showed positive reaction.

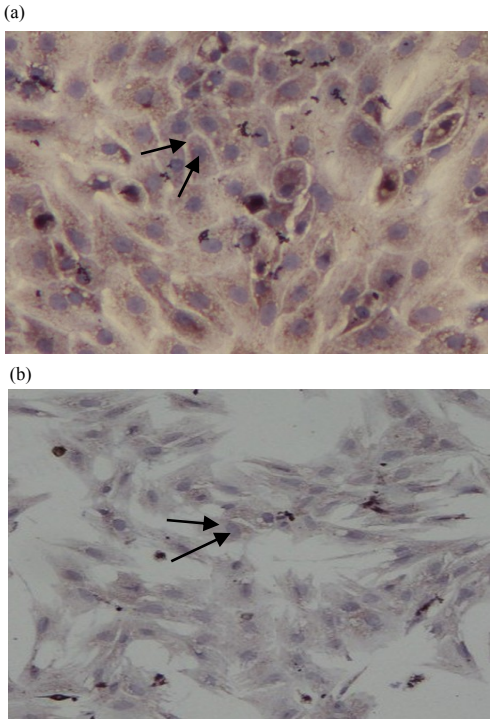

Fig. 2: Immunohistochemical results of chicken endometrial epithelial and stromal cells. Immunohistochemical results of endometrial epithelial and stromal cells under light microscopy (200x); a) the endometrial epithelial cells represented brown cytoplasm and a bluish-violet nucleus signify a positive reaction to cytokeratin ck18 antibody; b) the endometrial stromal cells also present a brown cytoplasm with a bluish-violet nucleus which showed a positive reaction to vimentin antibody

The cell growth curve measured by MTT Method: The growth curve of the endometrial epithelial cells increased logarithmically from day 2-5, reaching the plateau phase on day 7. The lag phase growth rate of the stromal cells was similar to that of the epithelial cells. However, the logarithmic growth phase occurred from day 2-5 and the growth curve reached the plateau phase on day 6 (Fig. 3).

Effects of sex hormones on the proliferation of endometrial cells *in vitro*: Based on the absorbance values of the different groups of cells, researchers found that E_2 and P_4 exhibited different effects on the proliferation of the epithelium and stromal cells (Table 2). As shown in Table 2, high levels of E_2 (100 nM) or P_4 (100 nM) significantly promoted the proliferation of the endometrial epithelial cells ($p<0.05$), compared to the control cells. The last three treatments were treated with

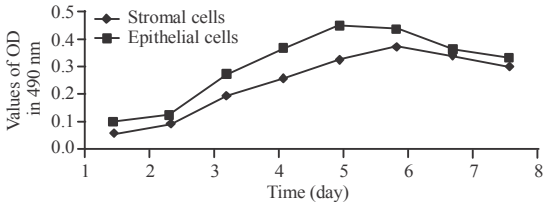

Fig. 3: Growth curves of endometrial epithelial and stromal cells isolated from laying hens

Table 2: Effects of Estrogen (E2) and Progesterone (P4) on the proliferation of epithelium cells and stromal cells isolated from chicken endometrium

Groups	Epithelium cells	Stromal cells
Control	0.442±0.083^{a}	0.359±0.018^{a}
E_2 (10 nM)	0.488±0.060^{ab}	0.383±0.056^{a}
E_2 (50 nM)	0.519±0.032^{ac}	0.460±0.093^{ab}
E_2 (100 nM)	0.562±0.058^{bcd}	0.468±0.101^{ac}
P_4 (10 nM)	0.487±0.062^{a}	0.449±0.054^{a}
P_4 (50 nM)	0.514±0.016^{a}	0.462±0.061^{a}
P_4 (100 nM)	0.549±0.046^{bcd}	0.553±0.052^{bcd}
E_2 (100 nM)+P_4 (10 nM)	0.553±0.065^{bcd}	0.469±0.064^{a}
E_2 (10 nM)+P_4 (100 nM)	0.554±0.054^{bcd}	0.577±0.040^{cd}
E_2 (100 nM)+P_4 (100 nM)	0.615±0.030^{d}	0.648±0.074^{d}

C = Control group. Values are presented as mean±SE (n = 4 each) and those with different superscripts within a column (a, b, c and d) significantly differ ($p<0.05$)

different concentrations of E_2 and P_4 (100 nM E_2+10 nM P_4, 10 nM E_2+100 nM P_4 and 100 nM E_2+100 nM P_4) and all of these three treatments also significantly increased the proliferation of the epithelial cells ($p<0.05$). The endometrial stromal cells treated with 100 nM P_4 or a combination of P_4 (100 nM) and E_2 (10 or 100 nM) proliferated at a significantly higher rate ($p<0.05$) than the control cells and no significant difference was observed between the various groups of stromal cells treated with 100 nM P_4 ($p>0.05$). No significant increase in growth was observed for the stromal cells treated with E_2 alone. These results suggest that both 100 nM E_2 and 100 nM P_4 can promote the proliferation of endometrial epithelial cells *in vitro* but only 100 nM P_4 can promote the proliferation of endometrial stromal cells *in vitro*.

DISCUSSION

There are many factors that can affect the physiology of the endometrium of laying hens. *In vivo* studies of the chicken endometrium are limited by many factors. Thus, an effective method of culturing chicken endometrial cells would enhance the study of the uterine physiology of chickens. Previous investigations have isolated and cultured the endometrial epithelial and stromal cells of cattle (Slonina *et al*., 2009), canine (Bartel *et al*., 2013), equine (Szostek *et al*., 2014) and rabbit (Chen *et al*., 2003). Park *et al*. (2003) isolated human endometrial

epithelial and stromal cells using a Centrifugation Method, Blauer *et al*. (2005) described an Organotypic Model to co-culture the epithelial cells of the normal human endometrium.

The chicken endometrial epithelial cells located in the mucosal folds of endometrium, researchers scraped the superstratum of endometrium gently with scalpel and isolated the epithelial cells by type I collagenase digestion. The stromal cells located below the epithelial cells so researchers used scissors method to cut the endometrium (the epicuticle of endometrium were removed) into pieces and used type I collagenase digestion to isolate cells. The cells that derived from Scissors Method contained a mixture of epithelial and stromal cells as the stromal cells is easier detached from the culture dish (with 0.25% trypsin at 37°C) than epithelial cells, so subculturing was done to purify the stromal cells. Cytokeratin ck18 is a kind of protein in epidermal cells and vimentin existing in mesenchymal cells extensively. The epithelial and stromal cells that derived from Scraping and Scissors Method were identified by immunohistochemistry using anti-cytokeratin ck18 and anti-vimentin monoclonal antibodies, respectively. These results are consistent with a previous study of the endometrial cells of rabbits (Chen *et al*., 2007). The cell growth curves showed that both the epithelial and stromal cells proliferated at a measurable rate during the lag phase of growth on day 1. The growth rate increased from day 2-5 or 6 during the logarithmic phase, reaching the plateau phase on day 6 or 7. These results are consistent with a previous study of the growth of cultured endometrial cells from rabbits (Song *et al*., 2006). Researchers found that the growth characteristics of chicken endometrial epithelial and stromal cells *in vitro* are similar. The findings provide a theoretical basis for further studies of the endometrial cells of laying hens.

E_2 and P_4 have a synergistic effect on the physiology of the endometrium *in vivo*. The regional microenvironment of the uterus changes regularly in response to the estrous and reproductive cycles. Studies have shown that sex hormones play a regulatory role in endometrial cell proliferation (Chang *et al*., 2011) and differentiation (Rider, 2006) as well as the expression of hormone receptors by endometrial cells (King and Critchley, 2010). Huang *et al*. (2013) showed that the secretion of E_2 and P_4 of geese blood plasma increased continually until it reached the peak from sexual maturity to production period. This change of sex hormones plays a vital role in the regulation of egg production. *In vitro* effects of sex hormones on the proliferation of chicken endometrial cells has not been widely studied. In mammals, a lot of similar researches have been investigated. Ayimuguli *et al*. (2011) found that 10 or 100 nM 17β-estrogen significantly increased the proliferation of yak endometrial epithelial and stromal cells. Falkowska-Podstawka *et al*. (2006) showed that a high concentration of progesterone (10^{-5}M) alone significantly enhanced bovine stromal cell growth. Slayden and Brenner (2004) demonstrated that the macaque endometrium treated with E_2 alone induces an artificial proliferative phase marked by extensive endometrial epithelial cell proliferation.

In our current study, researchers treated cultured endometrial epithelial and stromal cells isolated from laying hens with different concentrations of E_2 and/or P_4 and the results showed that compared with the control cells, 100 nM E_2 or 100 nM P_4 significantly increased ($p<0.05$) the proliferation of the endometrial epithelial cells whereas only treatment with 100 nM P_4 significantly increased ($p<0.05$) the proliferation of the endometrial stromal cells. These results are consistent with those of a previous study by Song *et al*. (2006). The study also found that treatments using 10 or 100 nM E_2 and 10 or 100 nM P_4 in combination significantly increased the proliferation of epithelial cells *in vitro* and that the combination treatments containing 100 nM P_4 significantly increased the proliferation of stromal cells *in vitro*. The findings of the study differed from those of similar studies in other species. Bar (2009) showed that estrogen mediated the folliculogenesis and egg production of chicken and E_2 and P_4 are involved in calcium metabolism for eggshell formation and ovipositioning. E_2 is essential in maintaining the function of chicken oviducts, it also triggers the formation of tubular glands and epithelium differentiation (Palmiter and Wrenn, 1971). The results likely reflected the differences between the physiology of the uterus of laying hens and that of other species.

CONCLUSION

The use of type I collagenase digestion is an effective method for isolating viable endometrial epithelial and stromal cells from laying hens. The growth cycle of the endometrial cells of laying hens in cell culture was approximately, 7 days. Treatment with 100 nM E2 and/or 100 nM P_4 significantly increased the proliferation of epithelial cells of laying hens *in vitro*. Treatment with 100 nM P_4 alone significantly increased the proliferation of endometrial stromal cells *in vitro* whereas treatment with E_2 alone did not. The findings provide a foundation for future studies of the physiology of endometrial cells of laying hens and provide a theoretical basis for the identification of sex-hormone-mediated regulatory mechanisms of the chicken uterus.

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