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Research Article

Effects of Steroid Hormones on Epithelial and Stromal Cells Culture and Proliferation of Laying Hens, *In vitro*

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Abstract

The aim of this study was to study the effect of steroid hormones on the morphology, growth and proliferation of endometrial cells of laying hens *in vitro*. Endometrial epithelial and stromal cells were isolated and cultured via type I collagenase digestion, which then were identified by using keratin CK18 or vimentin by immunohistochemistry and treated with steroid hormones, such as E_2 and P_4, and the growth curves *in vitro* evaluated by MTT assay. The results showed that endometrial epithelial and stromal cells were successfully isolated and cultured, both of which began to enter the logarithmic phase of growth after 24 h and reached the plateau phase at 7 days after inoculation. Compared to control group, the endometrial epithelial cells were significantly proliferated ($P<0.05$) by treating with E_2 (100 nmol/L) and P_4 (100 nmol/L), however the stromal cells were only significantly proliferated ($P<0.05$) by treating with P4 (100 nmol/L). From these results we can suppose that steroid hormones could positively effect on the proliferation of endometrial cells of laying hens, which would contribute to explore a method to examine the most suitable conditions for endometrial culture *in vitro* and help to improve further studies on the physiological and functional characteristics of the uterus in the poultry.

Keywords: *Endometrium, Epithelium cell, Laying hen, Sex hormone, In vitro*

Steroid Hormonların Yumurtacı Tavukların Epitel ve Stroma Hücre Kültürüne *İn vitro* Etkileri

Öz

Bu çalışmanın amacı, steroid hormonların *in vitro* yumurtacı tavukların endometriyal hücrelerinin morfoloji, büyüme ve proliferasyonu üzerine etkilerini araştırmaktır. Endometriyal epitel ve stroma hücreleri izole edildi ve tip I kollagenaz digesyon ile kültüre edildi ve sonrasında immunohistokimyasal olarak keratin CK18 veya vimentin ile tanımlandı. E_2 ve P_4 gibi steroid hormonlar ile muamele edildikten sonra MTT testi ile *in vitro* büyüme eğrisi değerlendirildi. Elde edilen sonuçlar endometriyal epitelyum ve stroma hücrelerinin başarılı bir şekilde izole ve kültüre edildiğini ve her ikisinin de 24 saat sonrası büyümenin logaritmik fazına girmeye başladıklarını ve inokulasyon sonrası 7 günde plato fazına eriştiklerini gösterdi. Kontrol grubu ile karşılaştırıldığında E_2 (100 nmol/L) ve P_4 (100 nmol/L) ile muamele edilen endometriyal hücrelerin anlamlı derece prolifere oldukları ($P<0.05$), ancak sadece P4 (100 nmol/L) ile muamele edilen stroma hücrelerinin anlamlı derecede proliferayon gösterdikleri ($P<0.05$) belirlendi. Elde edilen sonuçlardan steroid hormonların yumurtacı tavukların endometriyal hücrelerinin proliferasyonu üzerine pozitif etkisinin olabileceği kanısına varılmıştır. Bu durum *in vitro* endometriyal kültür için en uygun kondisyonun belirlenmesine katkıda bulunarak kanatlılarda uterusun fizyolojik ve fonksiyonel özelliklerinin çalışılmasına fayda sağlayabilir.

Anahtar sözcükler: *Endometriyum, Epitel hücresi, Yumurtacı tavuk, Seks hormonu, In vitro*

INTRODUCTION

In mammals, uterus is the organ, where the embryo gets attached itself and development of theembryo takes place in. However, the uterus of laying hens is vesicular shaped, which is a major place of formation of eggshell and contains tubular glands that secretes calcium, pigment and the cuticle. Uterus in different physiological cycles always occurs a series of changes in different morphological and functional traits including organization structure, thickness and hormone-secreting and so on. Especially, the uterus endometrium is the target tissue of ovarian steroid hormones and plays an important role in research on the reproductive physiology in most mammals and

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poultry, and the *in vitro* primary culture of endometrial cells of mammals have been widely used in the studies regarding the interaction between the embryo and endometrium are critical for embryonic development and survival [1-5].

As is well known, the endometrium consists of a single layer of epithelium plus the stroma on which it rests. At present, there are many reports in scientific literature, on the uterine epithelium cells in a variety of mammals, including rats [6], pigs [7], rabbits [8], monkeys [9], sheep [10]. Similarly, stromal cells are connective tissue cells of endometrium, which are also cells that support the function of the parenchymal cells of that organ. Numerous previous studies have also been found involving stromal cells, such as rat [11], humans [12,13], mouse [14], bovine [15,16] and ovine [17]. Meanwhile, more remarkable, some other studies showed that steroid hormone was an important factor affecting the proliferation of primary endometrial cells and stromal cells *in vitro*. Especially, ovarian steroid hormones such as estrogen (E_2) and progesterone (P_4) in different physiological cycles could induce obvious changes in endometrial cell morphology and biochemistry such as epithelium cell proliferation and secretion [18,19]. Besides, there were plenty of studies also showed that stromal cells derived from proliferative or secretory endometria, cultured in the absence of steroid hormones, grew as monolayers that showed only occasional areas of immunoreactive fibronectin. Treatment with physiological doses of estradiol and progesterone stimulated cell proliferation, resulting in multilayering with an increase of the saturation density [20-22]. But in practice, the primary cells culture is a complex process where cells are grown under the appropriate conditions established by artificial operation, generally outside their natural environment. Up to now, there are still few reports on establishment or another word an *in vitro* primary endometrial cells cultivation system and how the ovarian steroid hormones affect the growth of endometrial cells in the poultry.

Thus, in the present study, the aim of this study was to explore the methods of isolation and culture of endometrial cells of laying hens and observe the effects of steroid hormones on the proliferation of endometrial epithelial and stromal cells *in vitro*, we successfully isolated epithelial and stromal cells from the endometrium of laying hens by Type-I collagenase digestion. And then we evaluated the effects of steroid hormones, such as E_2 and P_4 on the proliferation of the endometrial epithelial and stromal cells *in vitro* by MTT experiments (MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]). This study attempted to provide an effective and convenient method for the isolation of these cells and an *in vitro* model for the study of obtaining and identifying the proliferation, differentiation, and metabolism of the uterine endometrial cells in laying hens in the future.

MATERIAL and METHODS

Ethics Statement

This study was approved by the Ethical Committee of Animal Experiments, Animal Science and Technology College, Shihezi University (Number: A2011085). All samples were collected in strict accordance with the committee's guidelines. During the experiment, effort was made to prevent animals from suffering.

Animals

Hyline layers were randomly chosen as the experimental animals which were in the same feeding conditions and lighting regime. All Hyline layers were in good physical condition without the uterine disease and at the peak of egg production rate of 80%. The experimental animals were kept in the animal experimental station of College of Animal Science and Technology of Shihezi University.

Reagents

DMEM/F12 mixed medium (HyClone Corporation, USA), non-original body fetal bovine serum (Hangzhou Sijiqing Biotechnology Materials Limited Company), Type I Collagenase (Beijing Solarbio Company), green streptomycin mixture (Beijing Solarbio Company), MTT reagent (Beijing Solarbio Company), dimethylsulfoxide (DMSO) (Anhui BIOSHARP Company), P_4 (Sigma, USA), E_2 (Sigma, USA), keratin CK18 (Cytoker Tin 18, Beijing Zsgb-Bio), monoclonal antibody, vimentin monoclonal antibody (Beijing Zsgb-Bio), SP immunohistochemistry kit and DAB coloring solution (Beijing Zsgb-Bio Company). If they are not special names, please write in lower case letters

Separation and Cultivation of Endometrial Epithelial Cells and Stromal Cells

For epithelial cells two types of cultural conditions were employed: 1. Endometrial stromal cells. Above all, the uterus was collected from laying hens by sterile dissection, and the blood vessels, connective tissues and epidermis in the uterine tissues were all removed under sterile conditions, and the endometrial layer of the uterus was washed three times for 10 min with sterile phosphate-buffered saline (PBS). And then the endometrial tissues were cut into 1 mm^3 pieces using sterile scissors, and soon after the fragments were digested by immersion in a solution containing Type I Collagenase (1 mg/mL) at 37°C for 60 min, and then the digestion was terminated using complete culture medium containing 10% FBS (Sijiqing, Zhejiang Tianhang Biotechnology Co., Ltd.), and the digestion mixture was filtered using a 200-mesh cell strainer and then the filtrate was centrifuged for 5 min at 120×g. The precipitate was washed twice and then suspended in complete culture medium. And finally, the cell viability was determined to be above 90% by the trypan blue exclusion method. Number of cells was

estimated using a hemocytometer and cell density was adjusted to 6×10^5 cells/mL, then the cells were subcultured in 3.5 cm dishes coated with complete culture medium. 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. In addition, the detached cells were centrifuged and mixed with DMEM/F12 (HyClone, Utah, USA) + 10% FBS and reseeded. The purified cells were seeded into 5-cm culture dishes, 6-well plates, and 96-well plates at 37°C in air containing 5% CO_2 respectively. 2. Endometrial Epithelial cells. The uterus was collected from laying hens by sterile dissection and the endometrium epithelial cells were scraped with a scalpel, and after the methods are similar to that of stromal cell culture.

Immunohistochemistry Assay

Both the morphologies of stromal and epithelial cells were observed daily using an inverted microscope. Stromal and epithelial cells adhering to the cover slips were identified using immunohistochemistry, and the cover slips were gently washed three times with PBS and fixed in 4% paraformaldehyde at 25°C for 20 min. Then, the primary antibodies, including anti-vimentin and anti-cytokeratin ck18 (Zhongshan, Beijing, China) were selected and added to identify stromal and epithelial cells respectively, and then added separately the secondary antibody and streptavidin-peroxidase conjugate according to the Histo-stain-Plus Kit (Zhongshan, Beijing, China). And last, both stromal and epithelial cells were stained with DAB solution (Zhongshan, Beijing, China) and observed under the light microscopy (OLYMPUS CKX41, Japan), respectively.

Analysis for Growth Curve of Both Stromal and Epithelial Cells

The cell growth curves of stromal and epithelial cells were determined by using the MTT assays. Both the epithelial and stromal cells were cultured in 96-well plates at a density of 5×10^5 cells/well in DMEM/F12 medium (0.2 mL) for 48 h at 37°C in 5% CO_2/air. All wells were then observed using inverted fluorescence microscope at various fixed time-points. Each of the wells were individually added into 20 µL of 5 mg/mL MTT solution (Solarbio), which were still cultured for an additional 4 h at 37°C in 5% CO_2/air. After removing the supernatant, 150 µL of DMSO (Biosharp) were added to dissolve the formazan crystals. The plate was shaken for 10 min, and then read on an ELISA reader (BioTek Power Wave XS2, USA) at 490 nm. At least three repeat wells per group were analyzed, and the procedure was repeated three times.

Effects of Sex Steroid Hormones on The Proliferation of Both Stromal and Epithelial Cells

Stromal and epithelial cells (1.5×10^4 cells/cm^2) in secondary cultures were seeded on a new 96-well, flat-bottom microplates (Falcon) and grown in various media, daily supplemented with 10^{-7} M additive reagents including PBS, E_2, P_4 and $E_2 + P_4$ at 37°C in 5% CO_2/air. The experiments were divided into four groups, including PBS-treated as the control group, E_2-treated group, P_4-treated group and $E_2 + P_4$-treated group, as shown in *Table 1*. After 72 h, both stromal and epithelial cells proliferation in the four groups were individually assessed by the MTT assay and by cell counting, as described in growth curve analysis. Plates were read within 30 min with a multiwell plate reader (Thermomax, Molecular Devices Corp., Menlo Park, CA). Data were expressed in optical density units. Experiments were conducted with replicates of eight wells per treatment condition. Similar experiments were conducted on at least three different occasions with cells prepared from two different uterus tissues.

Statistical Analysis

All statistical analyses were performed using one-way ANOVA test by the SPSS 19.0 statistical software (IBM SPSS Inc., Armonk, NY, USA).

RESULTS

Culture of Endometrial Stromal and Epithelial Cells

Dynamics and morphology characteristics of cell colonies were as follows: the epithelial cells appeared as polygonal or oval with a clear border, and the monolayer cells were arranged closely, full cytoplasm, nuclear round and then growed as radioactive, blaze or helix in shape. However, the shape of stromal cells was fusiform or polygon, parallel to the bundle or radial arrangement, and prominent cells nucleus *(Fig. 1)*.

Immunohistochemical Staining

After separation, selected wells containing both epithelial

Table 1. *Varies of experimental treatments of epithelial cells and stromal cells of laying hens in vitro*

Groups	E_2 (nmol/L)	P_4 (nmol/L)
0	-	-
1	10	-
2	50	-
3	100	-
4	-	10
5	-	50
6	-	100
7	100	10
8	10	100
9	100	100

The horizontal representing no added those hormones, such as E_2 or P_4. The control group added PBS without hormones, and the treatment groups consisted of nine different treatments that were added with varies of the concentration of steroid hormones respectively

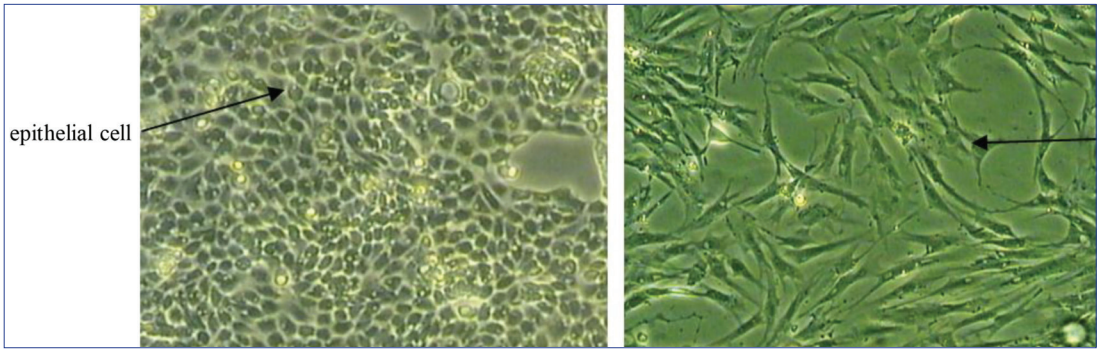


Fig 1. Morphology of endometrial epithelial cells and stromal cells of laying hen after 48 h cultivation using the inverted microscope vision fields (100×)

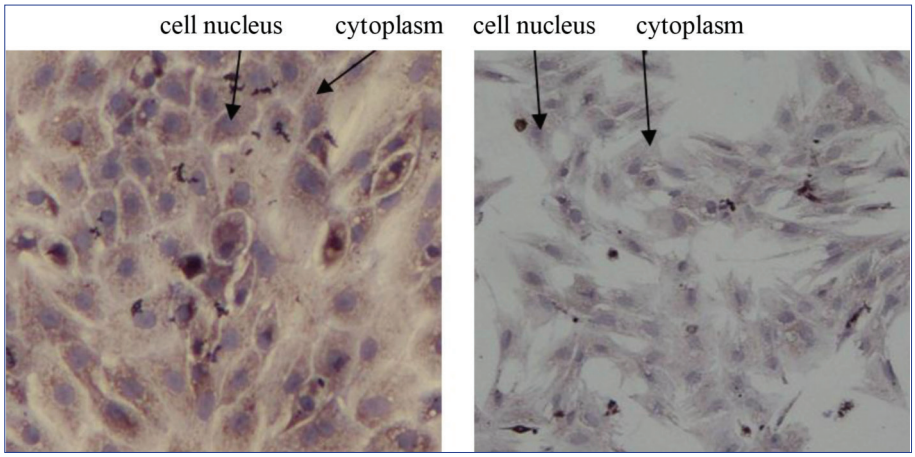
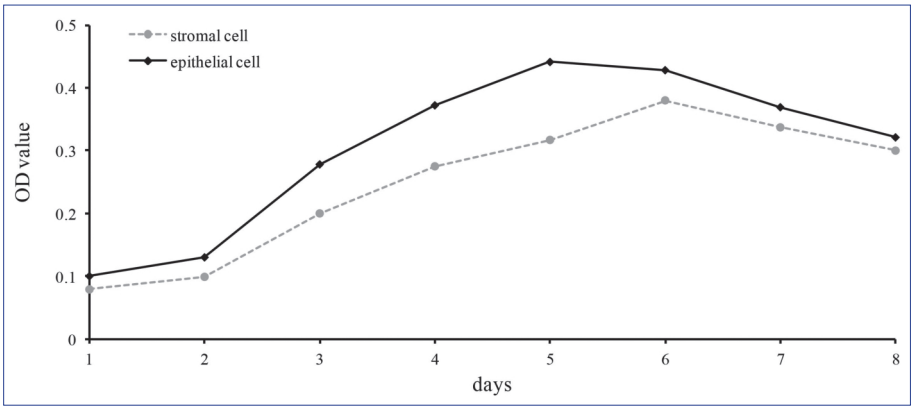


Fig 2. Results of immunohistochemical staining of laying hen endometrial epithelial and stromal cells using the light microscopy (200×)

Fig 3. Growth curves of endometrial epithelial and stromal cells of laying hen in different growth periods



and stromal cells were stained for the presence of either cytokeratmn or vimentin. Obtained the epithelial and stromal cells were stained positive for cytokeratin and vimentin respectively, however, their cytoplasm were dyed brown and nucleus were blue-purple *(Fig. 2)*. Thus, the immunohistochemical assay showed positive reaction of cytokeratmn and vimentin antigen in cultured epithelial and stromal cells, and the method of isolation and cultivation of the synovial cells *in vitro* is proved to be simple and feasible. This procedure was repeated 3 d after separation. Separated populations retained differentiated status and proved free of contamination by the above histochemical criterion.

Cell Growth Curve Measured by MTT Method

Endometrial epithelial and stromal cells were individually inoculated, isolated and cultured, and then grew slowly within the first 24 h after cell culture. There was a slight difference between the growth curve of epithelial and stromal cells, both of which endometrial epithelial cells growed to the logarithmic phase during 2-5 d after cells culture *in vitro* and then reached the plateau phase on the 7th d, whereas stromal cells were grown to logarithmic phase from the 2nd to 6th d after cells culture *in vitro,* and last reached the plateau phase on the 8th d *(Fig. 3)*.

Table 2. *Effects of E_2 and P_4 on the proliferation of epithelial and stromal cells of the laying hen*

Groups	Epithelium Cells	Stromal Cells
0	0.442±0.083^{a}	0.359±0.018^{a}
1	0.488±0.060^{ab}	0.383±0.056^{a}
2	0.519±0.032^{ac}	0.460±0.093^{ab}
3	0.562±0.058^{bcd}	0.468±0.101^{ac}
4	0.487±0.062^{a}	0.449±0.054^{a}
5	0.514±0.016^{a}	0.462±0.061^{a}
6	0.549±0.046^{bcd}	0.553±0.052^{bcd}
7	0.553±0.065^{bcd}	0.469±0.064^{a}
8	0.554±0.054^{bcd}	0.577±0.040^{cd}
9	0.615±0.030^{d}	0.648±0.074^{d}

All data 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)

Effects of Sex Hormones on The Proliferation of Endometrial Epithelial and Stromal Cells In Vitro

It was showed that the endometrial epithelial and stromal cells were carried different treatment with or without E_2, P_4 and E_2 + P_4 respectively *(Table 2)*. Compared to CG, there were no significant effect on the proliferation of endometrial epithelial and stromal cells using the lower level of E_2- or P_4-treated (10 or 50 nmol/L) (P>0.05), but there were markedly promoted the proliferation of the endometrial epithelial and stromal cells using the higher level of P_4-treated (100 nmol/L) (P<0.05). Meanwhile, we found that there was significantly stimulated the proliferation of the epithelial cells using the higher level of E_2-treated (100 nmol/L). And most notably, the combination of E_2- with P_4-treated appeared an additive effects in improving the proliferation of the endometrial epithelial and stromal cells (P<0.05). These results showed that the higher E_2 and P_4 (100 nmol/L) could enhance the proliferation of endometrial epithelial cells of laying hen, but there was only the higher P_4 (100 nmol/L) could increase the proliferation of endometrial stromal cells *in vitro.*

DISCUSSION

The uterus of the laying hen is, in essence, an eggshell gland and is a place where the eggshells was formed nucleation upon a fibrous scaffold (the eggshell membranes) followed by an interaction between the growing mineral crystals and the shell organic matrix. Likewise, the endometrial epithelium and stroma play an important role in the formation and secretion of thin chick egg white and ooporphyrin in the poultry. Thus, both the endometrial epithelial and stromal cells culture are important methods that can be used to explore the physiological function of the uterus of the poultry. Currently, previous studies have successfully isolated and cultured the endometrial epithelial and stromal cells in most mammals, such as cattle [23], canine [24], equine [25] and rabbit [26] and so on. It is also mentionable that Park et al.[27] have isolated and cultured human endometrial epithelial and stromal cells obtained by using a centrifugation method [27], and interestingly enough, Bläuer et al.[28] successfully constructed a novel organotypic culture model for normal human endometrium by co-culture *in vitro* [28]. Although there was still few studies reported the study of cultivation of endometrial epithelial and stromal cells in poultry. In the present study, the immunohistochemical detection of keratin and vimentin protein were conducted using keratin and vimentin antibody respectively, it showed that both of them were positive and identified as the endometrial epidermal and stromal cells, which indicated that the endometrial epithelial and stromal cells of laying hens were successfully isolated and cultured by means of collagenase (Type I) digestion. Moreover, the cell growth curves between the endometrial epithelial and stromal cells *in vitro* showed that both of them entered the latent period at 24 h after inoculation, and then growed into the logarithmic phase from the 2nd d cells and followed were grown slowly till the 5th-6th d and last reached the plateau phase was from the 7th d after isolation and culture *in vitro*. Similar cell growth curves in the study are also found the cultured endometrial cells in rabbits from Wang et al.[8] and Chen et al.[26] studies.

It is fairly well known that the endometrium is the inner epithelial layer, along with its mucous membrane, of the animal uterus, which consists of a single layer of columnar epithelium plus the stroma on which it rests. The endometrial epithelia and stroma are changed varies in thickness according to hormonal influences. Numerous studies have demonstrated that steroid hormones have an important regulatory role in the proliferation of endometrial epithelial and stromal cells [29], differentiation, and hormone receptor expression [30,31]. The microenvironment of the uterus would change regularly with the estrous cycle and the endocrine and reproductive function of the female animals, such as *in vivo* E_2 and P_4 on the regulation of endometrial proliferation, however, due to the limitations of *in vivo* studies, in recent years, more and more researches mainly focus on the isolation and culture of endometrial epithelial and stromal cells *in vitro* and the effects of steroid hormones on the proliferation of endometrial epithelial and stromal cells *in vitro*. But interestingly, studies have shown that E_2 mainly stimulates the proliferation of epithelial and stromal cells from the goat, and P_4 mainly promotes the epithelial cell proliferation; whereas in mice, E_2 stimulates proliferation of both the uterine epithelial and stromal cells *in vitro* [32]. Likewise, some other studies found that E_2 significantly promoted the proliferation of the yak endometrial epithelial and stromal cells and simultaneously stimulates endometrial cell proliferation and growth in the macaque [33,34], and Fałkowska-Podstawka et al.[35] study showed that a

high concentration of P_4 (10^{-5} mol/L) alone significantly enhanced bovine stromal cell proliferation [35]. Separately, Das' [36] study showed that P_4 was able to negatively regulate the proliferation of endometrial epithelial cells, but a certain amount of E_2 can promote the proliferation of endometrial epithelial cells in mice [36]. Besides those, Bar's [37] study showed that E_2 not only can mediate the folliculogenesis and egg production of chicken, but also combine wih P_4 are involved in calcium metabolism for eggshell formation and ovipositioning [37]. At the same time, the former is also essential in maintaining the function of chicken oviducts, which can trigger the formation of tubular glands and epithelium differentiation [38]. According to the results of the comparison, in the present study, we found that there were only a higher E_2 concentration (100 nmol/L) could significantly promote the proliferation of endometrial epithelial cells of laying hen *in vitro* ($P<0.05$), and a higher P_4 concentration (100 nmol/L) significantly stimulated the proliferation of endometrial epithelial and stromal cells of laying hen *in vitro* ($P<0.05$). But interestingly, the combination of a lower E_2 concentration (10 nmol/L) plus a higher P_4 concentration (100 nmol/L) or a higher E_2 concentration (100 nmol/L) plus a lower P_4 concentration (10 nmol/L) could also significantly promote the proliferation of epithelial and stromal cells of laying hen *in vitro*. Taken together, endometrial epithelial and stromal cells of laying hens can be successfully isolated and cultured *in vitro* by the means of collagenase type I digestion, and thus those would provide a kind of feasible research means for the regulatory mechanisms of steroid hormones mediated the function of the uterus in the poultry.

In conclusion, the described protocol proposes a technique to successfully establish primary cell cultures of endometrial epithelial and stromal cells of the laying hen. Moreover, we examined the possible effect of steroid hormones, such as E_2 and P_4, on the growth of endometrial epithelial and stroma cells, which could represent a valuable tool for the elucidation of secretion patterns by endometrial epithelial and stromal cells and also offer opportunities to study epithelial-stromal interactions *in vitro* in the poultry.

Conflict of Interest

There are no potential conflicts of interest.

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