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Original Article

Effects of gonadotropins (Gonal-F and Puregon) on human endometrial cell proliferation *in vitro*

Chi-Chen Chang [a,†], Yao-Yuan Hsieh [b,c,†], Kung-Hao Hsu [c,†], Chih-Sheng Lin [a,*]

[a] *Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan*
[b] *School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung, Taiwan*
[c] *Division of Infertility Clinic, Hsieh Yao-Yuan Womens' Hospital, Taichung, Taiwan*

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Abstract

Condensation: Both Gonal-F and Puregon, especially in their high-dosage administration, might inhibit the endometrial cell proliferation in the initial 48-hour culture. After 72-hour culture, Gonal-F persisted the inhibition of the endometrial growth, whereas Puregon reversed its effect to enhance endometrial growth.
Objectives: Endometrial proliferation or regeneration during menstrual cycle is regulated by sexual hormones. However, the effect of gonadotropins on the endometrial cell growth remains obscure. Herein, we aimed to investigate the effects of recombinant follicle-stimulating hormones (r-FSHs) (Gonal-F and Puregon) on the proliferation of human endometrial cells *in vitro*.
Materials and Methods: Human endometrial cells (RL95-2 cells) were obtained commercially and cultured in the serum-containing media in the presence of r-FSHs (Gonal-F and Puregon at concentrations of 0 mIU/mL, 200 mIU/mL, 400 mIU/mL, and 600 mIU/mL) up to 72 hours. According to the gonadotropin concentrations, all cultured endometrial cells were divided into four groups: (1) 0 mIU/mL (control); (2) 200 mIU/mL; (3) 400 mIU/mL; and (4) 600 mIU/mL. After 72-hour culture, endometrial cell proliferations were assessed overnight by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The influences of different r-FSH agents and dosages on endometrial cell proliferation in each group were evaluated and compared.
Results: In the four Gonal-F groups, the cell absorption (control and 200 mIU/mL, 400 mIU/mL, and 600 mIU/mL Gonal-F) after 24/48/72-hour cultures were as follows: (1) 0.57/0.7/0.82; (2) 0.56/0.66/0.78; (3) 0.55/0.64/0.77; and (4) 0.51/0.61/0.78. After 48 hours, higher dosage of Gonal-F appeared to significantly inhibit the endometrial cell proliferation. After 72-hour culture, all three dosages of Gonal-F appeared to inhibit the endometrial cell proliferation similarly. In Puregon groups, the cell absorptions were as follows: (1) 0.62/0.53/0.62; (2) 0.61/0.5/0.66; (3) 0.61/0.49/0.66; and (4) 0.64/0.49/0.66. Puregon administration displayed initial inhibition and subsequent stimulation effects on the endometrial cells.
Conclusions: Both Gonal-F and Puregon, especially in their high-dosage administration, appeared to inhibit the endometrial cell proliferation in the initial 48-hour culture. After 72-hour culture, Gonal-F persisted the inhibition of the endometrium, whereas Puregon reversed its effect by enhancing the endometrial growth. The differences might be because of the different formulations or molecular structures existing between alpha and beta follitropins.
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Keywords: Endometrium; Gonadotropin; Gonal-F; IVF; Puregon

* Corresponding author. Department of Biological Science and Technology, National Chiao Tung University, 75 Po-Ai Street, Hsinchu 300, Taiwan.
E-mail address: lincs@mail.nctu.edu.tw (C.-S. Lin).
† Chi-Chen Chang, Yao-Yuan Hsieh, and Kung-Hao Hsu contributed equally to this work.

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Introduction

Adequate endometrial development is one of the factors that play a significant role in early pregnancy. Endometrial growth is thought to depend on numerous factors, including hormones, lifestyle, uterine artery blood flow, posture, and others. Endometrial proliferation or regeneration during menstrual cycle is regulated by sexual hormones. A change in endometrial pattern and a decrease in endometrial and subendometrial blood flows are critical for the subsequent implantation of embryos. During the menstrual cycle, the endometrium undergoes cyclic proliferative and secretory changes in preparation for implantation. If this preparation is not sufficient, the implantation will fail.

The importance of endometrial development in *in vitro* fertilization (IVF) outcome has been previously reported. In the follicular phase, the growing follicles produce increasing amounts of estradiol that would induce proliferative endometrial changes. After ovulation, the corpus luteum produces progesterone that would initiate secretary changes. If implantation does not occur in the window of implantation, the endometrium will shed once the corpus luteum regresses. Thicker endometrium might improve the pregnancy rate [1]. With certain endometrial abnormalities (e.g. Asherman's syndrome) that prevent normal endometrial changes from occurring, implantation rates are low and abortion rates are high [2].

Numerous hormones might influence the endometrial proliferation and cyclic changes. Follicle-stimulating hormones (FSHs) have been known to directly act on endometrium, which results in the decidualization of endometrial cells [3]. FSH receptors have not been proven to be present in the human endometrium. However, myometrial and endometrial cancer cells have been known to contain FSH receptors [4,5]. Furthermore, other hormone receptors, such as LH/hCG receptor, have been shown to be present in human endometrium [6–8].

Recombinant FSH (r-FSH) has been routinely used for controlled ovarian hyperstimulation (COH) during assisted reproduction procedures. Two popular r-FSHs, follitropin alpha (Gonal-F; Serono, Rome, Italy) and follitropin beta (Puregon; Organon, Oss, The Netherlands), have been popularly used for years. There are numerous reports comparing their clinical applications in IVF. Most literatures demonstrated their comparable clinical results [9]. However, the effect of these recombinant gonadotropins on the endometrial cell growth remains obscure.

Reviewing the MEDLINE database, no investigators demonstrated the influence of r-FSH on endometrial proliferation. Ku et al [10] demonstrated the inhibitive effects of human menopausal gonadotropin (hMG) and urinary FSH (u-FSH, Metrodin; Serono) on endometrial cell proliferation. Besides this, there is no literature concerning the roles of recombinant gonadotropins in the endometrial proliferation *in vivo* or *in vitro*. Herein, we aimed to investigate the effects of r-FSH (Gonal-F and Puregon) on the proliferation of human endometrial cells *in vitro*. To the best of our knowledge, this is the first report about the related issue.

Materials and methods

Human endometrial carcinoma (RL95-2 cell line) was derived from Food Industry Research and Development Institute. Human endometrial cells were maintained in 90% of a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 medium containing 1.2 g/L sodium bicarbonate; 5 μg/mL bovine insulin; 15 mM HEPES (Gibco BRL, Gaithersburg, MD, USA); and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA). Cell growth was evaluated microscopically with an inverted-phase microscope. Human endometrial cells were cultured in the serum-containing media in the presence or absence of Gonal-F and Puregon for 72 hours.

According to the gonadotropin concentrations, all cultured endometrial cells were divided into four groups: (1) 0 mIU/mL (control); (2) 200 mIU/mL; (3) 400 mIU/mL; and (4) 600 mIU/mL. Human endometrium cells were counted by hemocytometer and seeded in 96-well plates (Corning, NY, USA) at 1×10^4 cells/well to 3×10^4 cells/well. Cells were grown at 37°C with medium alone (control) or 200 mIU/mL, 400 mIU/mL, and 600 mIU/mL of Gonal-F and Puregon. The culture procedures and determination and classification of gonadotropin dosages were modified after referring Ku et al's work [10].

The Cell Proliferation Kit I assay (Roche Applied Science, 68298 Mannheim, Germany) was used to assess the gonadotropins' influences on cell growth. After 72-hour culture, endometrial cell proliferations were assessed overnight by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to the culture wells for 4 hours, after which the cells were solubilized overnight at 37°C; absorbance at 595 nm was recorded using an enzyme-linked immunosorbent assay plate reader (Bio-Rad, Philadelphia, PA, USA). The influences of different r-FSH agents and dosages on endometrial cell proliferation in each group were evaluated and compared. All experiments were performed in triplicate using eight wells for each treatment. Optical densities were compared between the treatment groups and the controls incubated with medium alone. The intra- and interassay coefficients of variation were less than 5% and 6%, respectively.

Statistical analysis

Cell proliferation data were expressed as ratio influence of control culture proliferation. All results were normalized to cellular protein content, and the data were examined for equal variance and normal distribution before statistical analysis. The mean values were compared by analysis of variance with Fisher's least significant difference method for comparing groups [11]. Standard errors were within 10% of the mean of replicate wells. A p value less than 0.05 was considered statistically significant.

Results

In the four Gonal-F groups, the cell absorptions (control and 200 mIU/mL, 400 mIU/mL, and 600 mIU/mL Gonal-F) after 24/48/72-hour cultures were listed as follows: (1) 0.57/0.7/0.82; (2) 0.56/0.66/0.78; (3) 0.55/0.64/0.77; and (4) 0.51/0.61/0.78 (Figs. 1A–C). In the first 24 hours, low- or moderate-dosage (200 mIU/mL or 400 mIU/mL) Gonal-F appeared to have nonsignificant influences on the endometrial growth. In contrast, high Gonal-F dosage (600 mIU/mL) appeared to inhibit endometrial growth (Fig. 1A). In 48 hours, cell proliferation appeared to be negatively correlated with the Gonal-F dosage. Higher dosage of Gonal-F appeared to significantly inhibit the endometrial cell proliferation (Fig. 1B). After 72-hour culture, all three dosages of Gonal-F appeared to similarly inhibit the endometrial cell proliferation (Fig. 1C).

In Fig. 2, we observe the clear trend of initial inhibition of high-dosage Gonal-F administration for the endometrial cells. In proportion to the normal controls, the absorption ratios for the (1) control and (2) 200 mIU/mL, (3) 400 mIU/mL, and (4) 600 mIU/mL Gonal-F after 24/48/72-hour cultures were as follows: (1) $100 \pm 4.8/100 \pm 3.8/100 \pm 7.3$; (2) $98.4 \pm 0.9/94.9 \pm 3.7/94.5 \pm 0.8$; (3) $95.6 \pm 2.2/91.1 \pm 2.2/93.7 \pm 0.7$; and (4) $89.9 \pm 2.3/87.7 \pm 1.6/94.3 \pm 4.7$, respectively. High-dosage Gonal-F significantly inhibited cell growth compared with low-dosage Gonal-F. However, the inhibition of endometrial cells might be transient and might return to comparable levels as that in low-dosage Gonal-F (Fig. 2).

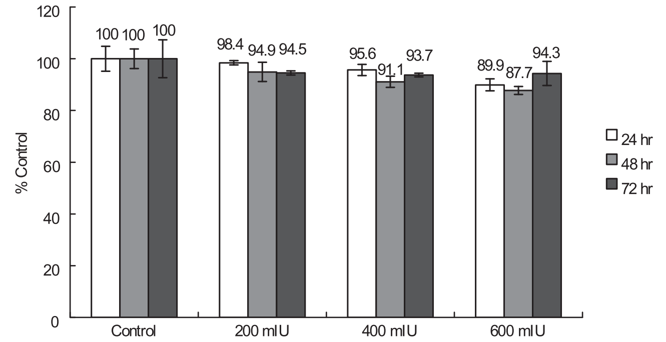

Fig. 2. Effects of different Gonal-F dosages on human endometrial cell growth. Results are expressed as a percentage of the control condition (culture medium with Gonal-F addition).

In the four Puregon groups, the cell absorptions after 24/48/72-hour cultures were listed as follows: (1) 0.62/0.53/0.62; (2) 0.61/0.5/0.66; (3) 0.61/0.49/0.66; and (4) 0.64/0.49/0.66 (Figs. 3A–C). In the first 24 hours, low-dosage Puregon (200 mIU/mL and 400 mIU/mL) appeared to have a nonsignificant influence on the endometrial cell proliferation. In contrast, high-dosage Puregon (600 mIU/mL) appeared to stimulate endometrial growth (Fig. 3A). After 48 hours, Puregon appeared to inhibit the endometrial cell proliferation.

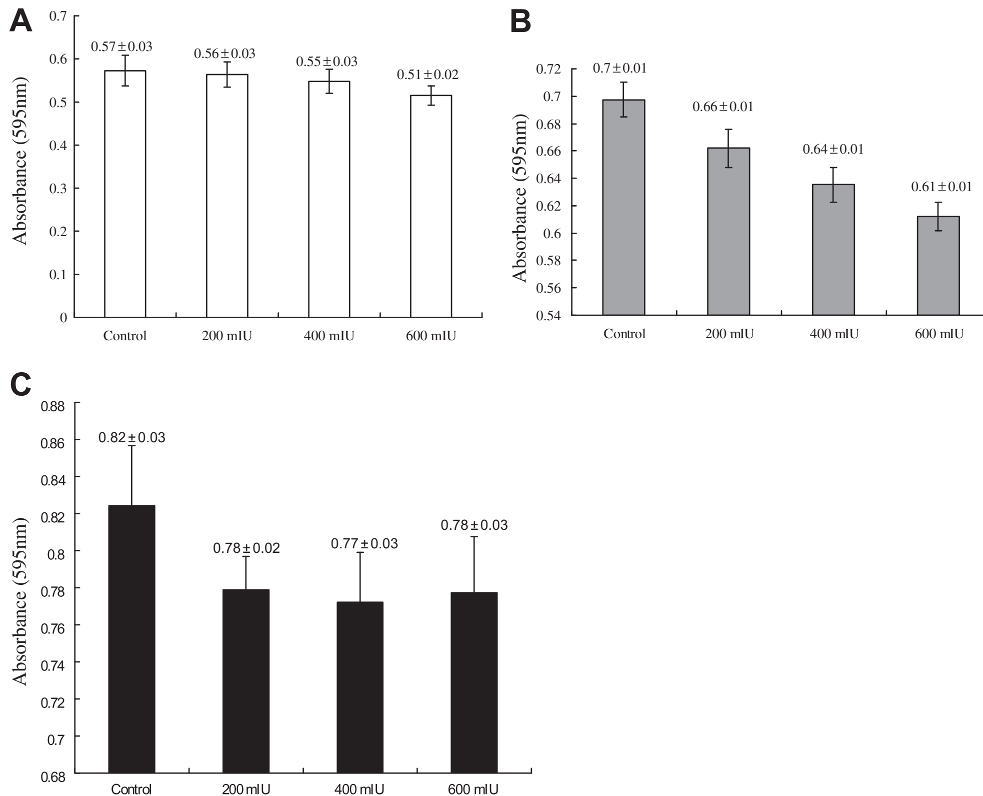

Fig. 1. Dose-dependent influence of Gonal-F, at different concentrations (0 mIU/mL, 200 mIU/mL, 400 mIU/mL, and 600 mIU/mL), on human endometrial cells for (A) 24 hr, (B) 48 hr, and (C) 72 hr, compared with the growth of controls treated with culture medium alone. (A) Culture for 24 hr with Gonal-F (NS difference among controls and 200-mIU/mL and 400-mIU/mL groups; $p < 0.05$ for 600-mIU/mL group compared with the other three groups.) (B) Culture for 48 hr with Gonal-F ($p < 0.05$ between 4 groups). (C) Culture for 72 hr with Gonal-F ($p < 0.05$ between each group) [NS difference among different Gonal-F dosage groups (200 mIU/mL, 400 mIU/mL, and 600 mIU/mL); $p < 0.05$ for Gonal-F groups compared with controls]. NS = nonsignificant.

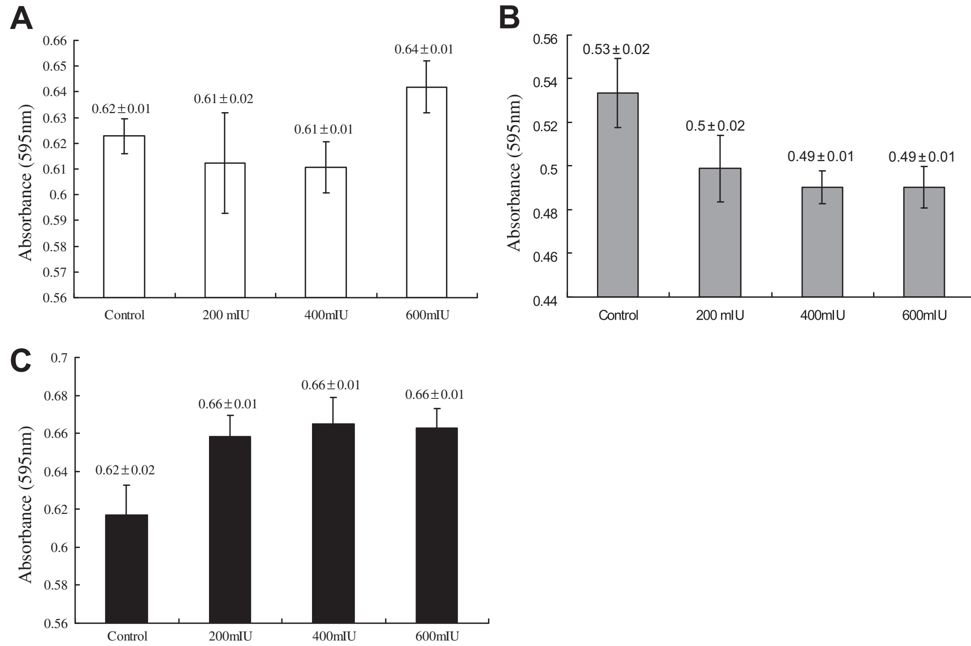

Fig. 3. Dose-dependent influence of Puregon, at different concentrations (0 mIU/mL, 200 mIU/mL, 400 mIU/mL, and 600 mIU/mL), on human endometrial cells for (A) 24 hr, (B) 48 hr, and (C) 72 hr, compared with the growth of controls treated with culture medium alone. Values represent mean ± standard error. (A) Culture for 24 hr with Puregon (NS difference between controls and 200-mIU/mL and 400-mIU/mL groups; $p < 0.05$ for 600-mIU/mL group compared with controls and 200-mg and 400-mg groups). (B) Culture for 48 hr with Puregon (NS difference among 200-mg, 400-mg, and 600-mg groups; $p < 0.05$ for 200-mIU/mL, 400-mIU/mL, and 600-mIU/mL groups compared with the control group). (C) Culture for 72 hr with Puregon (NS difference among 200-mg, 400-mg, and 600-mg groups; $p < 0.05$ for 200-mIU/mL, 400-mIU/mL, and 600-mIU/mL groups compared with the control group). NS = nonsignificant.

Furthermore, this inhibition is non-correlated with Puregon dosage (Fig. 3B). Higher dosage of Puregon appeared to significantly inhibit the endometrial cell proliferation (Fig. 3C). After 72-hour culture, all three dosages of Puregon appeared to similarly stimulate the endometrial cell proliferation (Fig. 3C).

In Fig. 4, we observe the trend of initial inhibition and subsequent stimulation effects of Puregon on endometrial cell proliferation. The absorption ratios for the (1) control and (2) 200 mIU/mL, (3) 400 mIU/mL, and (4) 600 mIU/mL Puregon after 24/48/72-hour cultures were as follows: (1) 100 ± 3.2/100 ± 8.7/100 ± 6.2; (2) 98.3 ± 0.7/93.5 ± 0.7/106.7 ± 1.7; (3) 98.1 ± 1/91.9 ± 3/107.8 ± 0.03; and (4) 103.1 ± 2.2/91.9 ± 6/107.4 ± 3.4, respectively. High-dosage Gonal-F significantly inhibited cell growth compared with low-dosage Gonal-F. However, the inhibition of endometrial cell proliferation might be transient and might reverse to stimulation effect after 72-hour culture (Fig. 2).

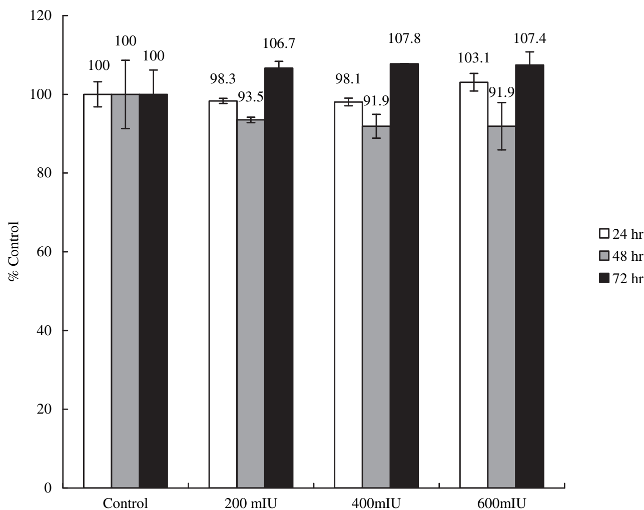

Fig. 4. Effects of Puregon on human endometrial cell growth. Results are expressed as percentage of the control condition (culture medium with Gonal-F addition).

Discussion

Numerous variables, such as age, embryo quality, and stimulation protocol, have a significant impact on assisted reproduction technique outcome. Evaluation of endometrial thickness and pattern might be beneficial to distinguish the fertile cycles from the infertile ones. Adequate proliferation and cyclic changes are necessary for successful implantation to occur. Thinner endometrium is often associated with implantation failure and abortion [12]. A minimum thickness of 10 mm during IVF was found to produce a higher pregnancy rate [12]. Therefore, during COH application, clinicians must pay close attention to endometrial development and follicle growth.

Numerous medicine administrations during COH might influence endometrial proliferation and pattern. The r-FSH preparations have been introduced in IVF markets for around one decade. Till date, there are two major r-FSHs [follitropin alpha (Gonal-F) and follitropin beta (Puregon)] used in clinical applications. Most literatures compared and demonstrated their clinical folliculogenesis and pregnancy rates [9]. There

was no difference in endometrial response after the administration of both agents [9].

Concerning the influence of other medicines on endometrium, clomiphene citrate (CC) administration during COH has been well known to be associated with thin endometrium [13]. Thicker endometrium was identified among women treated with gonadotropin when compared with those treated with CC [14]. A similar negative endometrial effect was not observed in the present CC + gonadotropin cycles. The endometrial thickness with CC + gonadotropin was similar to that in the simple gonadotropin cycles. The supraphysiological estradiol level reached during CC + gonadotropin stimulation was able to correct for the negative endometrial effects of CC alone.

Other medicine applications might also influence the endometrial proliferation. Estrogen supplementation during stimulation with CC has been shown to improve endometrial development and to result in thicker endometria and improved morphology [15,16]. Administration of ethinyl estradiol might reverse the deleterious effect of CC on endometrial development during the follicular phase [17]. Obesity and hypertension have been found to increase endometrial thickness independently [18]. In contrast, the estrogen or progesterone receptors were correlated with endometrial pattern but not with endometrial thickness [19]. Pre-retrieval hCG administration does not improve the endometrial thickness [20].

In this study, we first demonstrated the possible adverse effects of gonadotropin on the endometrial growth. Our application of r-FSH might prevent the contamination of urinary compounds in FSH/hMG on the endometrial proliferation and the subsequent evaluation. These findings were compatible with those of Ku et al [10], who demonstrated the adverse effects of hMG and u-FSH on endometrial proliferation. Concerning the gonadotropin concentration on the endometrial proliferation, after repeat experiments, we observed their different presentation between Gonal-F and Puregon on the endometrial proliferation. The difference in the effects of Gonal-F and Puregon on endometrium might be because of their different molecular structures. These findings were also different from those of Ku et al [10], which revealed the non-correlations between different concentrations of u-FSH/hMG and inhibition on endometrial cell proliferations.

In this study, the 3-day culture instead of a longer-period culture is considered because of the limited cell growth and the medium replacement during routine laboratory culture. Furthermore, we also observed differences between Gonal-F and Puregon, which might be the result of the intra- and interexperiment variations. We should also emphasize the possibility of an underlying difference between the human endometrial cells and the human cancer (RL95-2) cell line. Considering the donation limitation and the unavailability of fresh human endometrial cells, we adopted the endometrial tumor cell lines instead of the collection and culture of fresh human endometrial cells. Although greater numbers of evaluations are needed to validate these findings, it is logical to suspect that the different r-FSHs might exhibit different influences on the endometrial proliferation. Our preliminary investigation provides evidence that exogenous gonadotropin might directly or indirectly influence endometrial growth and the subsequent uterine receptivity. Furthermore, gonadotropin-induced suppression of endometrial growth might be of special concern for clinicians during ovarian hyperstimulations. It also suggested the necessity of exogenic estrogen addition for the individuals accepting ovarian hyperstimulation, especially when their ultrasonography showed thin endometrium after mid-follicular phase (<7 mm).

It is well known that the pituitary-derived FSH is a two-subunit (alpha and beta) glycoprotein. Up to 20 different FSH molecules with varying degrees of glycosylation can be purified [21], forming a spectrum of isoforms with differences in charge, bioactivity, and elimination half-life [22]. FSH is secreted by the pituitary as a microheterogeneous population in terms of different charges, molecular weights, and *in vitro* and *in vivo* bioactivities, with the secretion of polymorphic FSH being linked to hormone receptor [23,24]. More glycosylated molecules possess a longer half-life as compared with the desialylated molecules, which, probably because of increased plasma clearance, have a significantly decreased *in vivo* bioactivity [25,26]. Therefore, the possible underlying mechanism of our finding is so complex and obscure. However, the possible etiology might be the result of the different components or numerous factors between two r-FSHs, including the different presentations of r-FSH structures, amino acid sequences, affiliations, formulations, potencies, pH values, overall charge, isoforms, post-translational modification, receptor-binding affinity, plasma half-life, and others [27]. Furthermore, differences between the human endometrial cells and the endometrial cancer cell line (RL95-2 cell line) might have existed. Further application on the human endometrial cells is essential for the clarification of the related issues.

In conclusion, our preliminary results highlighted a statistically significant difference in the co-culture of different types of r-FSH on the endometrial growth. Both Gonal-F and Puregon, especially in their high-dosage preparation, appear to inhibit the proliferation of endometrial cells in the initial 48-hour culture. High-dosage gonadotropin administration might transiently inhibit the endometrial cell proliferation *in vitro*, which suggests its inhibitory role in endometrial growth during COH. After 72-hour culture, Gonal-F persisted its inhibition on endometrial proliferation, whereas Puregon reversed its effect by enhancing endometrial growth. The differences might be because of the different formulations, molecular structures, and potencies of the alpha and beta follitropins, as well as the subsequent stimulation or resistant effects on the hormone receptors of the endometrial cells. Despite its limited case numbers and conflicting outcomes between different r-FSHs, this study provided the preliminary database for the future surveys on the effects of gonadotropin on endometrium during COH.

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