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Effect of gonadotropins on human endometrial stromal cell proliferation in vitro

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Abstract It has long been known that endometrial regeneration and proliferation is regulated by sex steroids, cytokines and various growth factors. The mechanisms responsible for such organized growth are still under investigation. Human chorionic gonadotropin/luteinizing hormone (hCG/LH) receptors have been found to be localized in human endometrium by immunocytochemistry. Gonadotropins have been widely used for hyperstimulation during in vitro fertilization (IVF) procedures; however, the direct effect of gonadotropin on the endometrium has not been adequately investigated yet. This study attempted to define the effect of gonadotropins on the proliferation of human endometrial stromal cells in vitro. Human endometrial stromal cells were obtained from hysterectomy specimens and cultured in serum-containing media for up to 72 h. The effects of adding 7.5, 15, 30, 150 mIU/ml of human menopausal gonadotropin (hMG) and follicle stimulating hormone (FSH), and 10, 100, 1,000, 10,000 mIU/ml of hCG on cumulative [^{3}H]-thymidine incorporation in endometrial stromal cells were assessed. This study demonstrated that FSH and hMG induced significant inhibition in [^{3}H]-thymidine uptake at all concentrations, respectively (P<0.05). In contrast to the above two hormones, hCG exerted inhibitory effect at concentrations of 1,000 and 10,000 mIU/ml (P<0.05). There was no evidence of dose-response correlations in all three gonadotropin experiments. These data imply that gonadotropins at the concentrations studied inhibit the proliferation of human endometrial stromal cells, at least, in short-term culture in vitro. Accordingly, we cannot negate the possibility that administered gonadotropin during ovarian hyperstimulation may directly influence the proliferation of human endometrial cells.

Keywords Endometrial stromal cell proliferation · In vitro culture · Gonadotropins

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Introduction

The endometrium is the innermost lining of the uterus. Its biological role is to provide an environment for the implantation and development of the embryo. In order to achieve this task, the endometrium has to go through regular cyclic changes. It is well known that these changes are regulated by sequential ovarian steroids.

The presence of human chorionic gonadotropin/luteinizing hormone (hCG/LH) receptors in the non-pregnant human uterus has first been demonstrated by immunocytochemistry [1]. Epithelial and stromal cells of the endometrium, myometrial cells, and vascular smooth muscle cells have showed immunostaining with a monoclonal antibody to rat luteal hCG/LH receptors. hCG binding sites were also identified in human endometrium using hormone binding assay [2]. Another report confirmed the presence of hCG/LH receptor in human endometrial stromal cells at mRNA and protein level [3].

FSH has been known to directly act on human endometrium, resulting in the decidualization of endometrial stromal cells in vitro [4]. FSH receptors are also implicated to be present in human myometrium [5], although they have not yet been identified in human endometrium. In addition, endometrial cancer cells have been known to contain FSH receptors [6].

Gonadotropins have been used extensively in in vitro fertilization (IVF) programs for obtaining many oocytes. The high estrogen levels frequently produced by gonadotropin stimulation will probably affect the growth of the endometrium during hyperstimulation. Because gonadotropin receptors are present in the endometrium, it is possible that the gonadotropins administered during hyperstimulation can modify estrogen-induced endometrial growth.

In the current investigation, we sought to determine whether gonadotropins directly influence the proliferation of endometrial stromal cells in vitro.

Materials and methods

Human endometrial stromal cell preparation

Endometrium was obtained from five women with regular menstrual cycles (26–30 days) between the ages of 30 and 40 undergoing hysterectomies for uterine leiomyomas. Samples were collected during the proliferative phase of their menstrual cycle, as determined by the onset of the last menstrual period. All of the patients gave their informed consent. Samples obtained from women with a diagnosis of endometriosis, subsequently proven endometrial pathology, or those who had taken hormone preparations within 3 months of the surgery, were not included.

The tissue obtained was immediately placed into culture medium and cell separation was performed within 60 min. The basic medium employed throughout this investigation was phenol red-free Dulbecco's modified Eagle's medium (DMEM, pH 7.4; Gibco, Grand Island, USA) supplemented with 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco). After removal of all blood clots, the remaining tissue was minced, placed in a culture medium supplemented with 10% fetal bovine serum (FBS; Gibco) and 0.25% type I collagenase (Gibco), and then agitated gently in a 37°C water bath for 20 min. The resulting suspension was centrifuged in a medium supplemented with 10% FBS. To separate stromal cells from glands, the suspension was filtered through a 38 µm sieve. The filtrate was centrifuged at 400 g for 10 min, resuspended in the medium, plated in plastic culture dishes (10 cm^2, Nunclon, Copenhagen, Denmark), and incubated at 37°C in 5% CO_2 for 30 min. The remaining erythrocytes and nonadherent epithelial cells were aspirated. The adherent stromal cells were mechanically detached from the dishes, washed twice, and resuspended. The predominance of spindle-shaped stromal cells was identified using a phase contrast microscope, after which the cells were counted using a hemocytometer. The purity of the attached cells was determined by immunocytochemistry using antivimentin and anticytokeratin antibodies. The total yield for the experiments described in this investigation was 3.1×10^7 stromal cells per biopsy (range 1.4×10^7 to 4.6×10^7). The cells were suspended in a medium supplemented with 10% FBS and plated into culture dishes. The dishes were incubated under sterile tissue culture conditions in 5% CO_2 and 95% air at 37°C.

Preliminary experiments

In an effort to establish the appropriate conditions for assessing cell proliferation and to characterize this culture system, we performed a preliminary set of experiments. Cells from passages three and four were harvested with 0.25% trypsin-1 mM EDTA (Gibco), neutralized with 10% FBS/DMEM (Gibco). First, the cells were cultured at concentrations of 10,000, 20,000, 30,000, 40,000, and 50,000 cells per well. Ten percent FBS/DMEM was supplied as a medium, and the optimal cell number per well was determined on a 72-h [^{3}H]-thymidine incorporation curve. Then, the minimal serum requirement in the nutrient media to sustain stromal cell growth was assessed with serum-free media (5 µg/ml insulin/5 µg/ml transferrin/DMEM) as a control. Cells from passages three and four were harvested, and $1–3\times10^4$ cells/well were seeded into 96 well plates (Nunclon). Fetal bovine serum and calf serum at concentrations of 2.5%, 5%, and 10% were supplied to the nutrient medium after the appropriate cell concentration was determined from the above experiment. Cells were grown over a 72-h period, harvested at 24, 48, and 72 h after plating, and cumulative counts per minute (CPM) were measured.

Gonadotropin experiments

We used the same cells as with the latter preliminary experiment and plating efficiencies were in the range of 70% to 80%. We used a phenol red-free medium in order to minimize the effect of steroids. There was a negligible difference in sex steroid concentrations before and after charcoal stripping. Since this experiment was performed over a short period of time, and showed no evidence of decidualization, we did not perform additional charcoal stripping in order to avoid unnecessary contamination. Cells were examined daily by phase-contrast microscopy and were incubated for 24 h before the addition of the experimental media, which were determined through the above experiments (gonadotropins in 2.5% CS/DMEM, CS: calf serum). Human menopausal gonadotropin (hMG: Pergonal, Serono, Aubonne, Switzerland) and follicle stimulating hormone (FSH: Metrodin, Serono) at final concentrations of 7.5, 15, 30, and 150 mIU/ml, and human chorionic gonadotropin (hCG: Profasi, Serono) at final concentrations of 10, 100, 1,000, and 10,000 mIU/ml, were added to the wells. Control cells were cultured in 2.5% CS/DMEM only. To determine whether the addition of gonadotropins was cytotoxic to the stromal cells, we determined cell viability using trypan blue staining. More than 90% viability was maintained in both the control and gonadotropin-exposed cells after 72 h of incubation.

[^{3}H]-Thymidine incorporation

Stromal cell proliferation, as reflected by deoxyribonucleic acid synthesis, was measured after the addition of 1 µCi of [^{3}H]-thymidine (Amersham, New York, USA) to each microwell. After 72 h in the culture, media containing unattached cells and unabsorbed thymidine were aspirated. Fifty microliters of 0.2% EDTA (Sigma, St. Louis, USA) was added to each well to detach the cells immediately before harvesting. The stromal cells in the microwells were trapped onto glass fiber filter paper using an automatic cell harvester (Skatron, Lier, Norway). Each filter paper disc corresponded to one individual well in the tissue culture plates. These were air-dried and placed into liquid scintillation counting tubes, to which 2.0 mL of liquid scintillation cocktail (Packard, Meriden, USA) was added. [^{3}H]-Thymidine incorporation was measured using a liquid scintillation analyzer (Packard).

Statistical analysis

Mean CPMs for gonadotropin incubation were compared to those for the control wells. In order to standardize the inter-experimental variation resulting from different cell concentrations, we analyzed the ratio of CPM after the addition of each gonadotropin over the CPM of the corresponding control. All assays were performed in quadruplicate with cells from a single biopsy. Each gonadotropin experiment was repeated at least three times. Cells from different patients were randomly allocated to individual experiments, and were never mixed during individual experiments. The Wilcoxon signed rank test was employed where appropriate. Regression analyses were performed using the least square technique. A P-value of <0.05 was considered to be statistically significant.

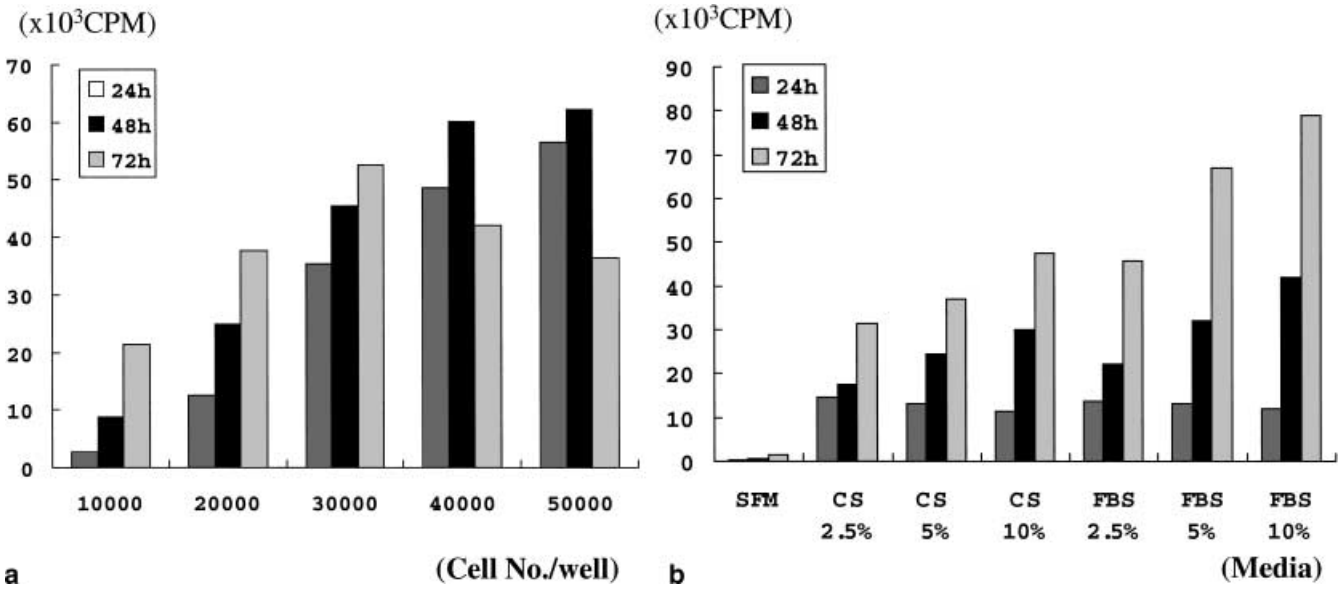

Fig. 1 **a** Endometrial stromal cell concentrations and [^{3}H]-thymidine incorporation during 72 h of culture in 10% FBS/DMEM. There was a correlation for up to 30,000 cells/well at 72 h**,** but not for higher concentrations. **b** Various nutrient media and their concentrations were tested using 1~3×10^{4}cells per well. Stromal cell proliferation in a serum-free medium (SFM) was significantly lower than in the other sets of serum containing media over a 72-h period (P<0.01). (*CPM* counts per minute, *DMEM* Dulbecco's modified Eagle's medium, *CS* calf serum/DMEM, *FBS* fetal bovine serum/DMEM)

Results

Preliminary experiments

The effects of cell concentrations on growth characteristics for this model were as follows: A continuously increasing CPM curve was acquired when 10,000–30,000 cells per well were incubated.

Figure 1 a presents the association between stromal cell concentration and DNA synthesis over a 72-h period of culture. After 24 and 48 h of culture, a linear relationship was demonstrated with an excellent correlation achieved between initial cell concentration and [^{3}H]-thymidine incorporation (r^2=0.9472, P=0.0052; r^2=0.9738, P=0.0018, respectively). At 72 h, however, there was a correlation for up to 30,000 cells/well**,** but not for higher concentrations. Thus, 3×10^4 cells were to be added to each microwell for the gonadotropin experiments.

Figure 1b shows the effects of varying concentrations of calf serum and fetal bovine serum added to the basic nutrient media (serum-free media: SFM) on stromal cell proliferation. The results are depicted as cumulative radioactivity over a 72-h period. Little proliferation was noted in the absence of either calf serum or fetal bovine serum, a finding that was confirmed by phase contrast microscopy. The addition of 2.5%, 5%, or 10% calf serum or fetal bovine serum produced a significant increase in cumulative [^{3}H]-thymidine incorporation over control values (serum-free media) during a 72-h period (P<0.01). The lowest concentration of calf serum was chosen as the minimal serum supplement for gonadotropin experiments.

Follicle stimulating hormone

On the basis of the results of the preliminary experiments, gonadotropin experiments were designed so that the nutrient medium was supplemented with 2.5% calf serum.

The effects of FSH were studied at four concentrations of 7.5, 15, 30, and 150 mIU/ml. FSH suppressed cell proliferation at all concentrations. Cell proliferation levels were 64.6%, 57.2%, 55.5%, and 46.6% of the control at each corresponding concentration (P<0.05) (Fig. 2a). No significant correlation could be determined between the FSH concentrations studied and cumulative [^{3}H]-thymidine incorporation.

Human menopausal gonadotropin

Results of hMG-induced thymidine incorporation demonstrated an inhibitory effect at 7.5, 15, 30, and 150 mIU/ml concentrations. Cell proliferation indicated at levels of 72.5%, 58.6%, 49.4%, and 56.1% of the control at each corresponding concentration (P<0.05) (Fig. 2b). There was no dose-response relationship between the concentration of hMG and cumulative [^{3}H]-thymidine incorporation.

Human chorionic gonadotropin

Human chorionic gonadotropin at concentrations of 10, 100, 1,000, and 10,000 mIU/ml showed endometrial stromal cell proliferation with values of 115.6%, 92.6%,

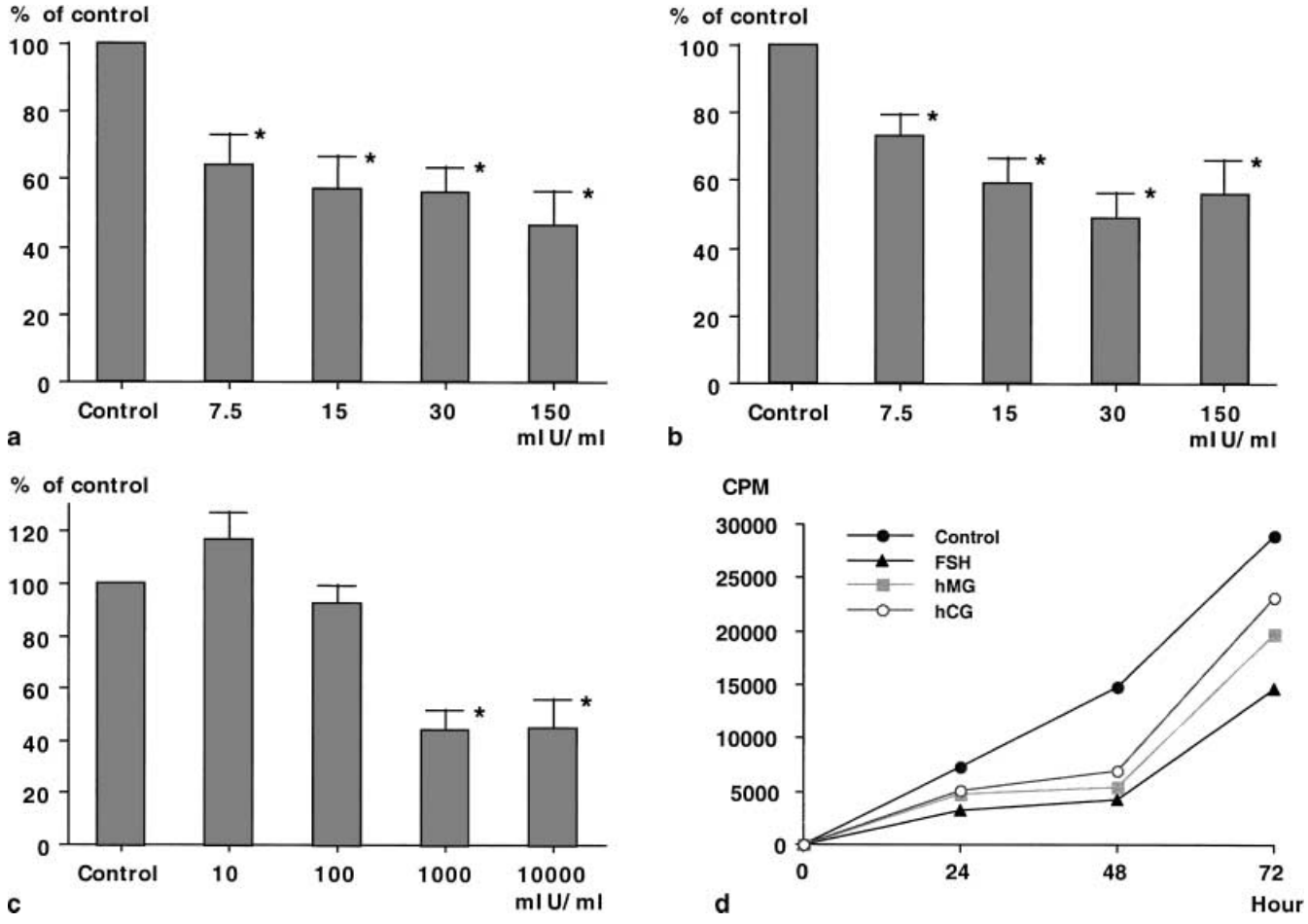

Fig. 2 The effects of adding gonadotropins to 2.5% CS/DMEM on the proliferation of human endometrial stromal cells in vitro at 72 h. The ratios of CPM after the addition of FSH (**a**), hMG (**b**), and hCG (**c**) over the CPM of the corresponding control were demonstrated. FSH and hMG showed significant inhibition at all concentrations (*: $P<0.05$), while hCG inhibited cell proliferation at 1,000 and 10,000 mIU/ml only (*: $P<0.05$). There was no dose-response relationship between the concentration of gonadotropins and cumulative [^{3}H]-thymidine incorporation (**a-c**). The inhibitory potencies of FSH and hMG were compared at 150 mIU/ml (hCG at 10,000 mIU/ml) (**d**). However, there was no significant difference in [^{3}H]-thymidine incorporation. This curve (**d**) is representative data, and no SEM values were drawn. (*CPM* counts per minute, *DMEM* Dulbecco's modified Eagle's medium, *CS* calf serum/DMEM, *SEM* standard error of mean)

44.1%, and 45.1% of the control, respectively. These results indicated that hCG exerted an inhibitory effect on the stromal cell proliferation at 1,000 and 10,000 mIU/ml ($P<0.05$) (Fig. 2c).

Comparison of FSH with hMG

Since hMG consists of LH and FSH, a comparison between the potency of FSH and hMG was attempted in order to indirectly investigate the role of LH. A concentration of 150 mIU/ml was employed for comparison of the two gonadotropins. As for hCG, a concentration of 10,000 mIU/ml was used. Inhibition by FSH, hMG and hCG resulted in cell proliferation at 50.3%, 68.3%, and 79.9% of the control at 72 h, respectively. Figure 2d shows a somewhat greater inhibition by FSH than hMG. However, the differences were not statistically significant.

Discussion

Since early 1990s, many reports have revealed the presence of gonadotropin receptors in nongonadal tissues. The presence of LH/hCG receptors has been demonstrated in the human uterus and its related tissues: normal endometrium [1, 2, 3], ectopic endometrial implant [7], adenomyosis [8], and endometrial carcinoma [9]. The clinical significance of gonadotropin action in the corresponding tissue has yet to be defined.

Our study shows that gonadotropins used for ovarian hyperstimulation have a direct influence on the proliferation of human endometrial stromal cells in vitro. Ever since the separate culturing of stromal cells became possible [10], it has been well established that human endometrial stromal cell proliferation is regulated by sex steroids, various growth factors, and cytokines [11, 12, 13]. However, the direct effects of gonadotropins on the proliferation of human endometrial stromal cells has not yet

been explored. Our data showed that gonadotropins inhibited the proliferation of human endometrial cells in vitro. The validity of our conclusions is strengthened by the results of our preliminary experiments, which were designed to establish the appropriate conditions for assessing cell proliferation.

We made use of three types of gonadotropins, which are commonly used for IVF: FSH, hMG, and hCG. Firstly, we tried to analyze the action of ligands for LH/hCG receptors. hCG was added to stromal cells to elucidate its direct action since the receptors for LH/hCG have already been localized in the human endometrium, including stromal cells [1, 3]. As for LH, few pure LH preparations are not being used for clinical use. Therefore, hMG was employed in this study and indirect assessment of its LH action was attempted. The role of LH could be inferred from our comparison study of the above three gonadotropins, as will be discussed later.

There have been many reports regarding the presence of LH/hCG receptor in human endometrium [1–3, 14]. They proved the existence of those receptors using various techniques. However, very recently, Stewart et al. [15] reported that there was no mRNA for gonadal LH/hCG receptors in the human endometrium. They suggested the possibility that there may be a variant receptor, in the form of a unique uterine receptor. Further studies are needed to reveal the exact characteristics of LH/hCG receptors present in the human endometrium.

FSH receptors have not been proven to be present in the human endometrium. However, there were reports on the presence of FSH receptor in human myometrium and endometrial cancer cells [5, 6]. This hormone was included in the cell proliferation assay because a report was published in which the decidualization of human endometrial stromal cells was induced by adding FSH, hMG, or hCG in vitro [4]. This showed that FSH can also act directly on human endometrial stromal cells and promote cell differentiation. However, the study did not address the quantitative assessment of the effect of gonadotropins on the proliferation of stromal cells. In our experiment, FSH was found to inhibit the proliferation of human endometrial stromal cells, as did hMG and hCG. These findings suggest the possibility of the existence of FSH receptors in human endometrial stromal cells.

On the other hand, we cannot negate the possibility that the contaminant in Metrodin, rather than FSH itself, is responsible for the above effect. This possibility also applies to the cases of Pergonal and Profasi, since urinary gonadotropins have other contaminant constituents that cannot be totally eliminated by the purification process [16]. Additional investigation using recombinant FSH, LH, and hCG will be necessary to elucidate the pure effect of gonadotropins on the proliferation of human endometrial stromal cells.

We performed an experiment (Fig. 2d) to compare gonadotropins. There was no significant difference in the inhibitory potency of FSH and hMG. Metrodin contains a minute amount of LH in contrast to Pergonal, and these two hormones exhibited comparable levels of inhibition upon cell proliferation. This suggests that FSH is much more potent in terms of suppressing cell proliferation than LH. Our findings that hCG suppressed cell proliferation only at high concentrations further supports the above rationale.

One of the most significant advances in assisted reproduction has been the adoption of superovulation strategies using exogenous gonadotropins. Hyperstimulation, which is used for follicular recruitment and development, probably influences the ability of the endometrium to achieve implantation. Our investigation provides evidence that exogenous gonadotropin itself, as well as the ovarian steroids produced by gonadotropin, may directly affect endometrial growth and/or uterine receptivity. It can be inferred that the gonadotropin-induced suppression of endometrial growth may prevent excessive overgrowth of the endometrium. This overgrowth may be induced by the supraphysiological level of estrogen that is frequently observed during hyperstimulation for IVF. In addition, hCG is widely used as the surrogate of LH surge to trigger ovulation in COH. Recently, the in vivo and in vitro effects of hCG on the endometrium have been extensively discussed [17]. The possible direct effects of hCG on the human endometrium, not mediated by ovarian hormones, are probably a consequence of stimulation of endometrial hCG/LH receptors. Since the half-life of hCG differs markedly from that of LH, possible pharmacological effects of hCG on the endometrium could alter endometrial receptivity in COH.

In conclusion, in this study, gonadotropins used for ovarian hyperstimulation were found to inhibit the proliferation of human endometrial stromal cells in vitro. This result may extend our knowledge regarding the action of gonadotropins. Further research on the combined effects of gonadotropins and ovarian steroids on stromal cells, with or without epithelial cells, in vitro will be necessary.

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