Roles of Estrogen and Progesterone in Endometrial Hemodynamics and Vascular Endothelial Growth Factor Production

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Background: The endometrium becomes receptive to the embryo after sequential actions of estrogen and progesterone. The purpose of this study was to examine the effects of estrogen and progesterone on endometrial hemodynamics and on secretion of vascular endothelial growth factor (VEGF) from endometrial epithelial cells (EEC).

Methods: Six early postmenopausal women taking sequential estrogen and progestin [days 1–11: estradiol valerate (estrogen) 2 mg daily; days 12–21: estradiol valerate 2 mg plus norethisterone acetate (progestin) 1 mg daily] were recruited. Three-dimensional power Doppler angiography (3D-PDA) was performed before hormone treatment (phase 0), on days 10–11 of hormone treatment (phase E), and on days 18–20 of hormone treatment (phase E + P). Ishikawa EEC were treated with or without 17-β-estradiol and progesterone for 24 hours, followed by determination of VEGF concentrations in the supernatants.

Results: The endometrial volume was significantly increased in phase E and phase E + P as compared with that in phase 0. The vascularization index, flow index, and vascularization flow index in the subendometrial region, as measured by 3D-PDA, were significantly higher in phase E + P than in phase 0, but there were no significant differences in these indices between phase 0 and phase E. While treatment of EEC with 17-β-estradiol had little enhancing effect on VEGF production, progesterone alone or in combination with 17-β-estradiol significantly increased VEGF secretion from EEC.

Conclusion: Our data suggested that progesterone could stimulate VEGF secretion from EEC and subsequently increase subendometrial vascularity and blood flow. [*J Chin Med Assoc* 2009;72(4):188–193]

Key Words: endometrium, estrogen, hemodynamics, progesterone, ultrasonography

Introduction

In response to sequential estrogen and progesterone secretion from the ovary, the endometrium proliferates and then differentiates to become receptive to the embryo.[1] Significant elongation, branching, and dilatation of endometrial vessels are induced by coordinated action of estrogen and progesterone, thereby providing adequate blood supply to the receptive endometrium. In women with unexplained subfertility, endometrial and subendometrial vascularity have been found to be significantly reduced.[2] The junctional zone between the endometrium and myometrium, called the subendometrial zone, is rich in blood supply and plays an important role in the implantation process.[3] However, the effects of estrogen and progesterone on the hemodynamics of the subendometrial zone have not been studied.

Most of the studies examining uterine perfusion have applied pulsed wave Doppler.[4,5] Spiral arteries,

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terminal branches of the uterine arteries that supply the subendometrial region, have low flow rates and tortuous vasculature, making it difficult to accurately measure spiral artery blood flow using pulsed wave Doppler.[6] In contrast to pulsed wave Doppler, which analyzes the frequency shift of blood velocity information, the recently developed power Doppler ultrasound uses the amplitude component of the signals received to represent the number of moving blood cells.[7] Power Doppler ultrasound has been found to be superior to pulsed wave Doppler in situations of low blood flow, because it is more sensitive, less angle-dependent and not susceptible to aliasing. In combination with 3-dimensional (3D) ultrasound, power Doppler provides a unique tool to accurately quantify subendometrial blood flow.

Vascular endothelial growth factor (VEGF), an identified diffusible angiogenic factor, is emerging as an important factor for neovascularization of the reproductive tract.[8] It acts through several tyrosine kinase receptors, inducing both proliferation of endothelial cells and vascular permeability.[9] VEGF is one of the key angiogenic factors in the endometrium, where VEGF expression is upregulated in the mid-luteal phase.[10] This suggests that a concerted action of estrogen and progesterone is necessary for maximal VEGF production.

The purpose of this study was to examine the roles of estrogen and progesterone in endometrial angiogenesis, using *in vivo* and *in vitro* approaches. Early postmenopausal women taking cyclic estrogen and progestin were recruited for serial examinations of endometrial hemodynamics by 3D power Doppler angiography (3D-PDA), since these patients had no intrinsic fluctuations of ovarian steroid secretion and their endometrium would still be responsive to hormonal treatments. Then, we analyzed the effects of estrogen and progesterone on VEGF production from Ishikawa endometrial epithelial cells (EEC), which are positive for estrogen and progesterone receptors (ER and PR). Our data may help clarify the physiologic roles of estrogen and progesterone in endometrial angiogenesis that are important for embryo implantation.

Methods

Experimental design and recruitment of cases

We recruited postmenopausal women who had undergone menopause no more than 3 years previously and begun to take hormone treatment for alleviation of vasomotor symptoms and prevention of osteoporosis between December 2005 and November 2006. Informed consent was obtained from all participating patients. The menopausal status was confirmed by serum levels of estradiol and follicle-stimulating hormone, i.e. estradiol < 25 pg/mL and follicle-stimulating hormone > 20 mIU/mL. Patients with fibroids measuring > 3 cm in diameter, endometrial lesions, or adnexal abnormalities were excluded from the study. The recruited cases received cyclic hormone therapy, Sevina (Synmosa Biopharma, Taipei, Taiwan), to relieve menopausal symptoms and prevent osteoporosis. One course of Sevina treatment consists of 2 mg estradiol valerate (estrogen) daily from the 1st day to the 11th day, and 2 mg estradiol valerate plus 1 mg norethisterone acetate (progestin) daily from the 12th to 21st day. All cases received transvaginal ultrasonography and 3D-PDA on the day before Sevina treatment (phase 0), on days 10–11 of Sevina treatment (phase E), and on days 18–20 of Sevina treatment (phase E + P).

Ultrasound data acquisition and analysis

Data were acquired using a GE Voluson 730 Expert ultrasound system (GE Healthcare, Zipf, Austria) equipped with a 2.8–10-MHz transvaginal transducer. Identical fixed preinstalled power Doppler ultrasound settings were used in all cases: frequency 3–9 MHz, pulse repetition frequency 0.6 kHz, gain −5.0, wall motion filter "low 1". All scans were conducted by a single ultrasonographer (L.H. Chen). Each case was scanned in the supine position with knees flexed and hips abducted. A longitudinal view of the endometrial cavity was acquired and the volume mode entered.[6] Then, the resultant truncated sector defining the area of interest was adjusted, and the sweep angle set to 75° to ensure that the entire endometrium and subendometrium were included. A 3D data set was then obtained. Analysis of stored ultrasound volumes was done using the VOCAL™ (Virtual Organ Computer-aided AnaLysis) imaging program. Endometrial volume and subendometrial power Doppler flow indices were calculated. Three vascular indices were generated, i.e. vascularization index, flow index and vascularization flow index.[11] Vascularization index represents the ratio of color voxels to all voxels in the region of interest and reflects the density of vessels in the volume analyzed. Flow index is the mean intensity of all the power Doppler voxels in the volume of interest (the sum of the weighted color voxels divided by the number of all color voxels in the region analyzed) and indicates the energy reflected from the blood corpuscles in the vessels of the volume. Vascularization flow index is the sum of the weighted color voxels divided by all voxels in the region analyzed and shows both the density of vessels and the density of blood corpuscles in the vessels. Subendometrial flow indices were

obtained by applying a 5-mm shell outside the endometrial contour and analyzing the shell functions of the VOCAL.

Cell culture

Ishikawa (ECACC No. 99940201) EEC line was purchased from the European Collection of Cell Cultures (ECACC; Salisbury, UK) through the Union Biomed Corporation (Taipei, Taiwan). Expression of ER and PR are positive in Ishikawa cells.[12] Cells were cultured at 5% CO_2 and 37°C in minimum essential medium (MEM; Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) (Biological Industries, Kibbutz Beit Haemek, Israel). The cells were subcultured every 2 days by trypsinization (trypsin-EDTA solution; Life Technologies). For experiments on the effects of estradiol and progesterone, cells were cultured in a phenol-red-free MEM-alpha medium (Life Technologies) supplemented with 10% charcoal/dextran stripped FCS.

VEGF measurement

Cells were seeded at 1×10^4/100 μL/well in 96-well plates. After overnight incubation, different concentrations of 17-β-estradiol and progesterone (Sigma, St Louis, MO, USA) were added to each well. After 24 hours of treatment, the supernatants were collected and stored at –70°C for future analysis. The concentrations of VEGF in these samples were measured using the VEGF immunoassay Quantikine kit (R&D Systems, Minneapolis, MN, USA). An equal volume of each sample was added to each well of a 96-well ELISA plate that was precoated with an anti-VEGF antibody and then incubated for 2 hours at room temperature. The plate was then washed with buffer 3 times, followed by incubation for another 2 hours at room temperature with a peroxidase-conjugated secondary antibody. After washing 3 times with buffer, the substrate solution was added and the color was developed for 20 minutes at room temperature. Subsequently, the reaction was stopped by adding stop solution, and the optical density of the samples was read at 450 nm with correction of wavelength at 540 nm.

Statistical analysis

Data were expressed as mean ± standard error of the mean, and analyzed by repeated-measures analysis of variance (ANOVA) for the ultrasound data and 1-way ANOVA for the VEGF data, using SAS (SAS Institute Inc., Cary, NC, USA), followed by Fisher's *post hoc* least significant difference test. A p value < 0.05 was designated as statistically significant. All analyses were performed on a Pentium IV-based personal computer.

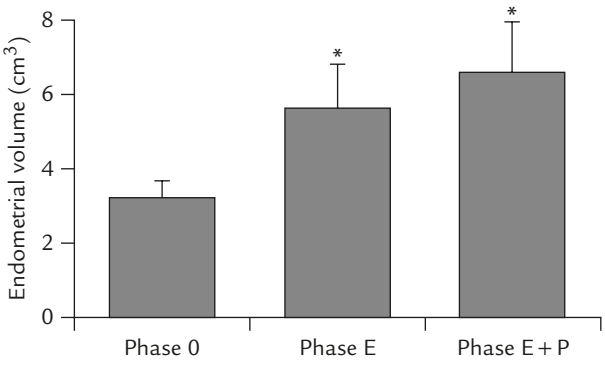

Figure 1. Endometrial volume in different phases of hormone treatment. Phase 0 = before hormone treatment. Phase E = estrogen phase of hormone treatment. Phase E + P = estrogen plus progestin phase of hormone treatment. *$p < 0.05$ compared to phase 0 ($n = 6$).

Results

Effects of estrogen and progestin on endometrial volume

A total of 6 patients were recruited. The mean age was 54 years. Mean parity was 2. Average time after menopause was 1.8 years. Before hormone treatment, mean serum estradiol, follicle-stimulating hormone, and luteinizing hormone were 16.3 pg/mL, 46.8 mIU/mL, and 52.4 mIU/mL, respectively. Endometrial volume, as measured by 3D ultrasound, was significantly increased after treatment with estrogen for 10–11 days (phase E) compared with that before treatment (phase 0). After treatment with estrogen for 11 days and estrogen plus progestin for another 7–9 days (phase E + P), the endometrial volume remained as high as that in phase E and was significantly higher than that in phase 0 (Figure 1; $p < 0.05$ between phase E and phase 0, $p < 0.05$ between phase E + P and phase 0, $p > 0.05$ between phase E and phase E + P; $n = 6$).

Effects of estrogen and progestin on subendometrial vascular flow

3D-PDA is a very useful tool to quantify hemodynamics in low-blood-flow subendometrial vessels because it is very sensitive. As shown in Figures 2A and 2B, the density of subendometrial vessel volumes (vascularization index) was significantly increased in phase E + P compared with those in phase 0 and phase E ($p < 0.05$ between phase E + P and phase 0, $p < 0.05$ between phase E + P and phase E; $n = 6$). Furthermore, the flow index and vascularization flow index were significantly higher in phase E + P than in phase 0 (Figures 2C and 2D; $p < 0.05$ between phase E + P and phase 0; $n = 6$).

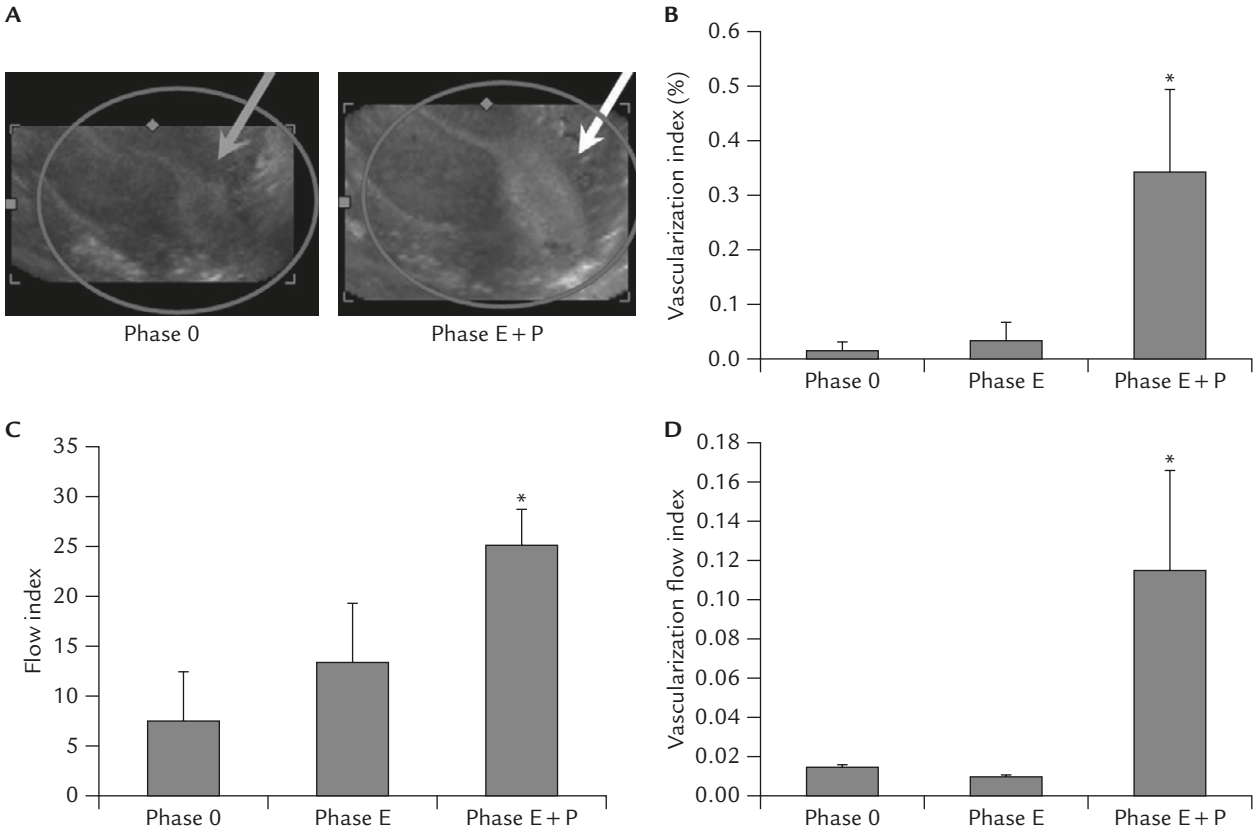

Figure 2. Three-dimensional power Doppler analysis of subendometrial blood flow in different phases of hormone treatment. Phase 0 = before hormone treatment. Phase E = estrogen phase of hormone treatment. Phase E + P = estrogen plus progestin phase of hormone treatment. (A) Representative ultrasound images. Arrow indicates subendometrial region. (B) Vascularization index. (C) Flow index. (D) Vascularization flow index. *$p < 0.05$ as compared with phase 0 ($n = 6$).

Effects of estrogen and progesterone on VEGF secretion from EEC

Since VEGF is the main angiogenic factor in the endometrium,[10] the effects of estrogen and progesterone on VEGF secretion from Ishikawa EEC were examined. Ishikawa EEC were cultured in the presence or absence of 17-β-estradiol and progesterone for 24 hours, followed by collection of the supernatants for VEGF measurements. While treatment of cells with 17-β-estradiol had little enhancing effect on VEGF production, progesterone significantly increased VEGF secretion from EEC (Figure 3; $p > 0.05$ between the 17-β-estradiol-only group and vehicle-treated control group, $p < 0.05$ between the progesterone-only group and vehicle-treated control group; $n = 5$). Treatment of cells with 17-β-estradiol plus progesterone also significantly enhanced VEGF secretion as compared with the vehicle-treated control group, but the enhancing effect of 17-β-estradiol plus progesterone on VEGF secretion was similar to that of progesterone alone ($p < 0.05$ between the 17-β-estradiol

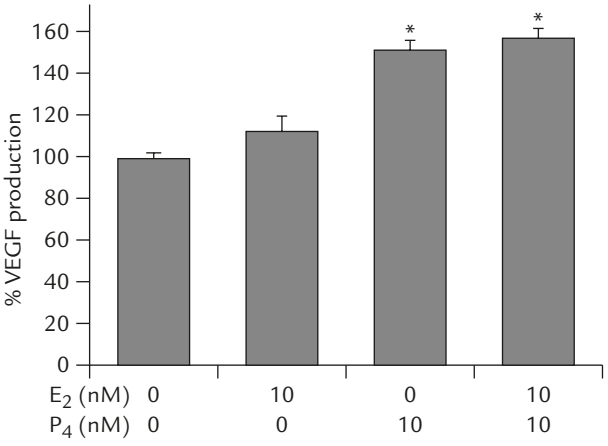

Figure 3. Effects of 17-β-estradiol (E_2) and progesterone (P_4) on vascular endothelial growth factor (VEGF) production in endometrial epithelial cells. Cells were treated with or without E_2 and P_4 for 24 hours, followed by determination of VEGF concentrations in the supernatants. 100% VEGF production was defined as the level of VEGF production in cultures treated with vehicles for 24 hours. *$p < 0.05$ compared with vehicle-treated controls ($n = 5$).

plus progesterone group and vehicle-treated control group, $p > 0.05$ between the 17-β-estradiol plus progesterone group and progesterone-only group; $n = 5$).

Discussion

Establishment and maintenance of the vasculature of the endometrium is one of the most critical determinants of pregnancy outcomes.[13] However, the roles of estrogen and progesterone in endometrial angiogenesis remain controversial.[14] Here, we demonstrated that estrogen treatment (phase E) could increase endometrial volume in early postmenopausal women and the addition of progesterone (phase E + P) did not further increase endometrial volume. In contrast, subendometrial vascularity and blood flow were significantly increased in phase E + P but not in phase E, compared with those in phase 0. Furthermore, VEGF production in ER- and PR-positive Ishikawa EEC was enhanced by progesterone but not by 17-β-estradiol. These results suggest that endometrial proliferation is stimulated by estrogen and endometrial angiogenesis is induced by progesterone.

Estrogen acts as a cell mitogen, and progesterone, in contrast, acts as a differentiation factor.[15] Furthermore, estrogen induces PR expression in the endometrium.[16] In this study, we demonstrated that progesterone could enhance VEGF production in PR-positive Ishikawa EEC, and that subendometrial vascularity and blood flow were significantly increased in phase E + P of cyclic hormone therapy. We proposed that after priming of endometrium with estrogen in the first 11 days of hormone treatment (phase E), PR expression in the endometrium could be induced, and therefore, the addition of progestin in the subsequent phase E + P significantly increased endometrial VEGF production, vascularity, and blood flow. Our concept was supported by a previous study showing that human endometrium was more responsive to progestin in the late proliferative phase when the endometrium had been exposed to endogenous estradiol for several days.[17]

To investigate endometrial angiogenesis, many radiographic tools have been introduced. Recently, functional imaging by magnetic resonance imaging has improved multiparametric measurement of endometrial angiogenesis in mice.[18] In addition, 18F-fluoro-deoxyglucose uptake in positron emission tomography has been applied to measure endometrial blood flow.[19] Furthermore, endometrial blood flow has been determined by the clearance of radiolabeled xenon-133 following its instillation into the uterine cavity.[20] The aforementioned radiographic tools, however, require intravenous or intrauterine injection of chemicals. In contrast, 3D-PDA represents an appropriate, informative and noninvasive tool for the assessment and quantification of endometrial hemodynamics.[11] 3D-PDA has been applied to predict endometrial cancer[21] and assess uterine receptivity.[2,22] In the present study, we aimed to study the effects of estrogen and progesterone on endometrial hemodynamics. Early postmenopausal women were recruited because they had no endogenous production of ovarian steroids and their endometrium was still responsive to hormone treatment. Another good option would be reproductive-age women taking gonadotropin-releasing hormone agonist (GnRH-a) for endometriosis or uterine leiomyoma. However, the use of cyclic estrogen and progestin in these cases may offset the beneficial effect of GnRH-a on the disease. Although natural progesterone has been used by some groups in postmenopausal hormone therapy, it induces irregular bleeding episodes more frequently than progestins (synthetic progestogen).[23] Therefore, progestin was used in this study. In this study, hemodynamics in the subendometrial region were measured because the intraendometrial vascularity was very low, even in the E + P phase.

In normal menstrual cycle, VEGF mRNA has been shown to increase relative to early proliferative-phase expression by 1.6-, 2.0-, and 3.6-fold in midproliferative, late proliferative, and secretory endometrium, respectively.[24] This is consistent with our findings that 17-β-estradiol induced a mild, albeit statistically insignificant, elevation in VEGF secretion from EEC, and progesterone stimulated a robust and statistically significant increase in VEGF secretion. Treatment of human myometrial microvascular endothelial cells (HMMEC) with VEGF has been demonstrated to promote HMMEC proliferation and induce formation of elongated tubular structures in HMMEC, an index of angiogenesis *in vitro*.[25] High coexpression of the 2 VEGF receptors, Flk-1/KDR and Flt-1, has also been observed in capillaries during the mid-secretory phase, suggesting that endometrial microvasculature is more responsive to VEGF in the mid-secretory phase than in other phases.[26] Therefore, the vascularity in mid-secretory-phase endometrium becomes higher because of enhanced endometrial VEGF secretion and upregulated expression of VEGF receptors in endometrial capillaries.

To determine the effects of estrogen and progesterone on endometrial hemodynamics in young reproductive-age women, patients receiving frozen-thawed embryo transfer may be recruited in the future because they will take sequential estrogen and progesterone following GnRH-a downregulation for

endometrial preparation. Furthermore, since there are 2 subtypes of PR (PR-A and PR-B), future studies will need to ascertain which subtype of PR is responsible for mediating the effect of progesterone on endometrial VEGF secretion and angiogenesis. Finally, 3D-PDA may be applied to ascertain the best luteal phase support regimen in assisted reproductive technology by comparing endometrial hemodynamics during the implantation window in patients taking different luteal-phase support regimens.[27]

In conclusion, using 3D-PDA and VEGF determination, the present study demonstrated that progesterone could stimulate VEGF secretion from EEC and subsequently increase subendometrial vascularity and blood flow. Therefore, uterine receptivity for the embryo may be increased by progesterone-induced endometrial angiogenesis. Further studies are required to explore more applications of 3D-PDA in assisted reproductive technology.

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