Aberrant expression of angiopoietins-1 and -2 and vascular endothelial growth factor-A in peri-implantation endometrium after gonadotrophin stimulation

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BACKGROUND: Ovarian stimulation affects normal endometrial development. The expression of angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2) and vascular endothelial growth factor-A (VEGF-A) and the vascular state in the peri-implantation endometrium in women with natural and gonadotrophin-stimulated cycles were compared.

METHODS: The expression of these angiogenesis-associated molecules in endometrial biopsies, collected on Day 7 after human chorionic gonadotrophin injection or luteinizing hormone surge in stimulated or natural cycles respectively, or at mid-luteal phase of women undergoing diagnostic laparoscopy, were analysed. RESULTS: Women with gonadotrophin-stimulation had lower Ang-1, but higher Ang-2, mRNA and protein expression (P<0.05), and increased concentrations of von Willebrand factor (vWF) and blood vessel density than those with natural cycles (P<0.05). Although stimulated cycles had higher VEGF-A mRNA expression (P = 0.023), VEGF-A protein expression was similar between the groups. Lower Ang-1/Ang-2 but higher Ang-2/VEGF-A mRNA ratios (P = 0.025) were found after gonadotrophin-stimulation. The ratios were negatively (P < 0.001) and positively correlated (P < 0.001) with estradiol levels, respectively. Cyclical changes in Ang-1 and Ang-2, but not in VEGF-A expression were noted. CONCLUSIONS: The decreased Ang-1 concentration and Ang-1/Ang-2 ratio and the increased Ang-2 concentration, with the increased vWF concentration and blood vessel density, in stimulated cycles suggests advanced endometrial angiogenesis after gonadotrophin-stimulation.

Keywords: angiogenesis; angiopoietin; endometrium; ovarian stimulation; VEGF

Introduction

Ovarian stimulation is used in almost all in vitro fertilization (IVF)/embryo transfer programs to obtain more follicles and thereby, create more embryos available for transfer. However, such stimulation has side effects. It affects luteal phase function (Macklon et al., 2006). Ovarian stimulation inevitably increases the circulating estradiol (E2). Excessive stimulation leads to ovarian hyperstimulation syndrome (OHSS) (MacDougall et al., 1993). The development and gene expression of the endometrium are also affected (Bourgain and Devroey, 2003; Horcajadas et al., 2007) resulting in diminished endometrial receptivity in stimulated cycles (Paulson et al., 1990). Gonadotrophin-stimulation has also been associated with advancement of endometrial development (Ubaldi et al., 1997; Kolibianakis et al., 2002). No pregnancy was found when such advancement on the day of oocyte retrieval was more than 3 days (Kolibianakis et al., 2002).

Vascular supply is important for endometrial development and growth of the implanted embryo. Cyclical variation in endometrial blood flow has been documented (Gannon et al., 1997). Endometrial blood flow has also been correlated with implantation (Jinno et al., 2001). The endometrial and sub-endometrial blood flow is significantly higher in pregnant patients with live birth than in those with miscarriage (Ng et al., 2007). Both the endometrial and sub-endometrial blood flow are lower in gonadotrophin-stimulated cycles than that in natural cycles of the same patient (Ng et al., 2004a), consistent with the observation that the endometrial development is affected in a high hormonal environment after stimulation.

Superphysiological high levels of E2 are a possible cause of the adverse development of endometrium during IVF treatment. Delayed glandular maturation and advanced stromal morphology with significantly more stromal vessels and stromal oedema in the endometrium have been noticed in women with E2 levels >20 000 pmol/l on the day of human chorionic gonadotrophin (hCG) injection (Basir et al., 2001b). Endometrial blood flow is negatively correlated with serum E2 levels only in IVF treatment (Ng et al., 2006).
Women with markedly elevated serum E$_2$ have suboptimal endometrial perfusion (Basir et al., 2001a; Ng et al., 2004b), and lowered implantation and pregnancy rates (Ng et al., 2000), but the qualities of their oocytes and embryos are not affected (Ng et al., 2003). A similar conclusion has been reached by others (Simon et al., 1995), though this was negated by a systematic review (Kosmas et al., 2004).

Angiogenesis is defined as the growth of new capillaries from pre-existing blood vessels (Folkman and Shing, 1992; Hanahan, 1997). Vascular endothelial growth factor (VEGF) is the most extensively studied angiogenic factor (Amoroso et al., 1997). Angiopoietin is a family of molecules that collaborates with VEGF in angiogenesis (Suri et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999; Thurston, 2003). Angiopoietin-1 (Ang-1) binds to Tie-2 receptor tyrosine kinase on the endothelial cells to enhance their recruitment and interaction with the peri-endothelial cells and extracellular matrix. It is important in promoting the maturation and stabilization of blood vessels (Suri et al., 1996). Ang-2 is a natural antagonist of Ang-1. Although Ang-1 is widely expressed in many tissues, Ang-2 is found predominantly expressed at sites of vascular remodeling, such as those in the female reproductive tract. The biological activity of Ang-2 is modulated by VEGF. At the angiogenic sprouting sites with abundant VEGF, Ang-2 stimulates formation of blood vessels. However, increased Ang-2 expression in the presence of a low level of VEGF is associated with vascular regression (Maisonpierre et al., 1997; Holash et al., 1999).

The equilibrium between endogenous pro- and anti-angiogenic factors is tightly regulated. In the female reproductive system, disruption of the equilibrium may occur in OHSS, dysfunctional uterine bleeding, endometriosis, pregnancy loss and intrauterine growth retardation (Geva and Jaffe, 2000). It is known that at the implantation site, there is an increased endometrial vascular permeability followed by progressive endothelial cell growth and angiogenesis. We hypothesize that gonadotrophin stimulation affects endometrial vasculature by disturbing the balance between the pro- and anti-angiogenic activity of Ang-1, Ang-2 and VEGF in the endometrium. The first objective of this report was to compare the expression of Ang-1, Ang-2 and VEGF-A, and the vascular state as determined by the endothelial cell marker, vWF, in the endometrium of women with natural and gonadotrophin-stimulated cycles. The second objective was to determine whether excessively high E$_2$ levels would further perturb the equilibrium in endometrial angiogenesis, possibly leading to the impairment of implantation rates in excessive responders after ovarian stimulation.

**Materials and Methods**

**Expression of Ang-1, Ang-2 and VEGF-A in peri-implantation endometrium of natural and stimulated cycles**

**Patient samples**

The Ethics Committee of the University of Hong Kong approved the research protocol. Written informed consent was obtained from women before collecting their endometrial samples for the study. Endometrial biopsies were collected by a Pipelle sampler (Unimar, Neuillyen-Thelle, France) and fixed for paraffin embedding or frozen in −80°C for RNA and protein extraction. Infertile females coming for IVF treatment were recruited when embryo transfer was not performed due to the failure of fertilization caused by male factors, absence of spermatozoa in testicular sperm extraction on the day of oocyte retrieval or when the serum E$_2$ level was $>20000$ pmol/l on the day of hCG injection. A total of 47 patients were recruited. The long protocol of ovarian stimulation regimen and the measurement of serum E$_2$ and progesterone levels were reported previously (Ng et al., 2000, 2001; Makkar et al., 2006). Briefly, patients were treated with Buserelin (Suprecur; Hoechst, Frankfurt, Germany) nasal spray four times a day from the mid-luteal phase of the preceding cycle, followed by human menopausal gonadotropin (hMG) (Pergonal; Serono, Geneva, Switzerland). When there were $\geq 3$ follicles with diameters greater than 15 mm and the diameter of the leading follicle was 18 mm, 10 000 IU of hCG (Profasi; Serono) was given intramuscularly and serum E$_2$ concentrations were measured on the day of hCG and 7-days post-hCG. No luteal support was given to the recruited patients because such treatment would increase the risks of OHSS in some of them who already had high concentrations of serum E$_2$. Patients with natural cycles were classified as Group N ($n = 15$) whereas those with stimulated cycles were classified as Group S ($n = 32$). Endometrial biopsies were collected on Day 7 after the luteinizing hormone (LH) surge in natural cycles and on Day 7 after hCG injection in stimulated cycles.

**Real-time PCR**

Total RNAs from endometrial biopsies were extracted by the Absolutely RNA MicroPrep Kit (Stratagene) according to the manufacturer’s protocol. RNA samples (300 ng) were reverse-transcribed into cDNA using the First-strand cDNA Synthesis Kit (Amersham Pharmacia, Buckinghamshire, UK). Real-time quantitative analysis of human Ang-1, Ang-2 and VEGF-A mRNAs were performed using an ABI 7500 Sequence Detector (PE Applied Biosystems, Foster City, CA). Multiplex quantitative polymerase chain reaction (qPCR) using 18S as an internal control for normalization of RNA loading was performed in a 20 μl reaction mixture containing 2 μl of sample cDNA, 10 μl 2X TaqMan Universal PCR Master Mix, 1 μl of 18S internal control and 1 μl of 20X assays-on-demand gene expression assay for human Ang-1, Ang-2 and VEGF-A (PE Applied Biosystems). Water instead of RNA was used as the no template control.

Quantitative analysis was done by the sequence detection software supplied with the ABI 7500 Sequence Detector. The threshold cycles ($C_t$) for each reaction were calculated and used for quantifying the amount of starting template in the reaction. The proportion of the gene of interest in a sample was presented as the difference in $C_t$ values ($\Delta C_t$) between the respective gene and that of 18S. The relative gene expression among samples was calculated by the $2^{-\Delta \Delta C_t}$ method (Livak and Schmittgen, 2001).

**Western blot**

The protein expression of Ang-1, Ang-2 and VEGF-A in Groups N and S were examined by western blot analysis. Aliquots containing 15 μg of endometrial lysates were fractionated by 12% SDS–PAGE and transferred to a PVDF membrane. The membranes were probed with anti-Ang-1 (1:500 dilution), anti-Ang-2 (1:500 dilution) (R&D Systems, Minneapolis, MN, USA) or anti-VEGF-A (1:500 dilution) (Zymed, San Francisco, CA, USA) antibodies in blocking solution overnight at 4°C. The secondary antibody was used horseradish peroxidase (HRP)-conjugated anti-goat antibody (1:5000, Amersham Pharmacia). The membrane was visualized by enhanced chemiluminescence according to the manufacturer’s recommendations.
endometrial expression of Ang-1, Ang-2 and VEGF-A mRNA across the menstrual cycle

Endometrial biopsies were taken from 36 women undergoing diagnostic laparoscopy for assessment of tubal patency. These women had regular menstrual cycles and had not received any anti-inflammatory or hormonal medication for at least 3 months prior to diagnostic laparoscopy. Their reproductive stage was determined by the day of the last menstrual period and confirmed by endometrial histology. Total RNA was extracted from the biopsies and subjected to the qPCR studies of the relative expression of Ang-1, Ang-2 and VEGF as described above.

Statistical analysis

Data regarding the expression of Ang-1, Ang-2 and VEGF-A, vWF concentration and H-scores were analysed using the SigmaPlot software package (Jandel Scientific, San Rafael, CA). Statistical analysis was performed by Kruskal–Wallis and Mann–Whitney U test followed by multiple pair-wise comparisons using the Student–Newman–Keuls method. Linear associations of the E2 and progesterone treatment effects with Ang-1, Ang-2 and VEGF-A expression were tested using Pearson’s correlation coefficient. P < 0.05 was considered statistically significant.

Results

Expression of Ang-1, Ang-2 and VEGF-A in peri-implantation endometrium of natural and stimulated cycles

Endometrial biopsies were obtained from 15 women in natural cycles and 32 women receiving gonadotrophin stimulation for IVF. There were no significant differences in the demographic data between the two groups of patients in terms of age, duration of infertility, doses and duration of gonadotrophin used and endometrial thickness. The serum E2 and progesterone concentrations in the stimulated cycles were significantly higher at LH/hCG day and at LH/hCG+7 days (Table I).

Using qPCR, Ang-1, Ang-2 and VEGF-A transcripts were detected in the endometrial tissues of the patients. In the peri-implantation period, there was a significant decrease in Ang-1 (P < 0.001) but an increase in Ang-2 (P < 0.001) and VEGF-A (P = 0.023) mRNA expression in endometrium after stimulation when compared to those with natural cycles. The Ang-1/Ang-2 mRNA ratio was significantly lower (P < 0.001), whereas the Ang-2/VEGF-A mRNA ratio was significantly higher (P = 0.025), in the stimulated cycles than in the natural cycles (Fig. 1).

Linear regression and Pearson Correlation analysis were used to analyse the relationship of Ang-1, Ang-2 and VEGF-A mRNA with hormonal parameters (Fig. 2). Serum E2 levels at LH/hCG days were negatively correlated with Ang-1 and the Ang-1/Ang-2 ratio and positively correlated with Ang-2, VEGF-A and the Ang-2/VEGF-A ratio (P < 0.05). Except for VEGF-A, significant relationships were also found when the E2 and progesterone levels at hCG/LH+7 were compared.

Using Western blot analysis, specific bands of Ang-1 and Ang-2 with the expected molecular sizes were detected in endometrial biopsies (Fig. 3A). After normalization with β-actin, significantly lower Ang-1 protein expression (P = 0.037) and significantly higher Ang-2 protein expression (P < 0.001) were detected in the endometrium of the stimulated cycle when compared to that of natural cycle. No significant difference was found in the protein expression of VEGF-A (Fig. 3B). The relative expression levels of Ang-1, Ang-2 and VEGF in patients with natural or stimulated cycles is summarized in Table II.

The spatial expression of Ang-1 and Ang-2 was studied by immunohistochemistry and semi-quantified by H-score (Table III). Ang-1 immunoreactivity was detected in the glandular and luminal epithelium, stromal cells and endothelial cells
of endometrium from natural cycles. The stromal signal of Ang-1 decreased significantly after gonadotrophin stimulation when compared to natural cycle ($P = 0.002$), while no difference was found in the glandular epithelium in these two groups of patients ($P = 0.14$). The Ang-1 signal was significantly ($P = 0.023$) higher in the glandular epithelium than in the stroma after gonadotrophin stimulation, but not in natural cycles. In samples from natural cycles, Ang-2 in the glandular epithelium was higher than that in stroma ($P = 0.01$). A similar difference was found in the samples from stimulated cycles ($P = 0.011$). However, no difference in the H-score was found in the expression of Ang-2 in the glandular epithelium nor in the stroma when comparing endometrium with or without gonadotrophin stimulation. No signal was found in the endometrium when the primary antibodies were omitted (data not shown). Representative photographies of Ang-1 and Ang-2 immunohistochemical staining results are shown in Fig. 3C.

When comparing the data between the moderate and excessive responders, no significant difference was found in the mRNA and protein expression of Ang-1, Ang-2 and VEGF-A and their ratios. While significantly lower Ang-1 mRNA levels, lower Ang-1/Ang-2 mRNA ratios and higher Ang-2 protein levels were found in both moderate and excessive responders when compared to patients with natural cycles, only excessive responders showed significantly higher Ang-2 mRNA levels, VEGF-A mRNA levels and Ang-2/VEGF-A mRNA ratios and lower Ang-1 protein levels than those having natural cycles (data not shown).

**Vascular states in peri-implantation endometrium**

The concentration of vWF in human endometrium was used as an objective and quantitative evaluation of the vascular state in the tissue (Lawson et al., 2005). The ELISA results demonstrated that stimulated cycles (median: 2.18 mU/ml, range: 1.06–3.79) showed significantly higher vWF concentrations compared to natural cycles (median: 0.58 mU/ml, range: 0.23–1.84) ($P < 0.001$). The concentration of vWF in stimulated cycles was significantly higher than in natural cycles ($P < 0.001$), indicating a more activated vascular state in stimulated cycles.

**Table I.** Demographic data and ovarian responses in patients of natural cycles and stimulated cycles.

<table>
<thead>
<tr>
<th></th>
<th>Natural cycles ($n = 15$)</th>
<th>Stimulated cycles ($n = 32$)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>31 (27–36)</td>
<td>32 (26–38)</td>
<td>0.239</td>
</tr>
<tr>
<td>Duration of infertility (Years)</td>
<td>6 (3–13)</td>
<td>4.75 (2–11)</td>
<td>0.134</td>
</tr>
<tr>
<td>HMG doses (ampoules)</td>
<td>–</td>
<td>23 (16–45)</td>
<td>–</td>
</tr>
<tr>
<td>HMG duration (days)</td>
<td>–</td>
<td>10 (7–16)</td>
<td>–</td>
</tr>
<tr>
<td>Serum E2 level on day of LH surge or hCG (pmol/L)</td>
<td>653 (428–1313)</td>
<td>21 001 (1216–61 608)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum PROGESTERONE level on LH or hCG +7 days (pmol/L)</td>
<td>368 (135–1046)</td>
<td>9823 (693–28 900)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endometrial thickness (mm)</td>
<td>10 (6–19)</td>
<td>12 (8–18)</td>
<td>0.081</td>
</tr>
</tbody>
</table>

Data are presented as median (range).

hMG, human menopausal gonadotrophin.
than that in natural cycles (median: 1.71 mU/ml, range: 0.94–2.39) ($P = 0.029$) (Fig. 4A).

Fig. 4B shows representative images of vWF immunohistochemistry on the endometrial paraffin sections from natural and stimulated cycles. Blood sinusoids and capillaries in the surface endometrium were stained brown. Spiral arterioles were rarely seen in the endometrial aspirates. The blood vessel density in the tissue was determined by the areas covered by blood vessel in microscopic sections. Results showed that the total area covered by blood vessels in the stimulated cycles (median = $17.26 \times 10^5$ pixels; range = $11.77 \times 10^5 – 21.51 \times 10^5$) was significantly higher than that in the natural cycles (median = $8.02 \times 10^5$ pixels; range = $5.86 \times 10^5 – 14.27 \times 10^5$, $P < 0.001$).

Figure 2: Linear regression and Pearson Correlation analysis of mRNA levels for Ang-1, Ang-2 and VEGF-A, as well as the Ang-1 to Ang-2 and Ang-2 to VEGF-A ratios with $E_2$ at LH/hCG day and LH/hCG+7 day and with $P_4$ at LH/hCG+7 day. Pearson correlation was performed and $P$-values are shown. $\beta$, correlation coefficient.
Expression of Ang-1, Ang-2 and VEGF-A mRNA in human endometrium across the menstrual cycle

To study the relative expression of endometrial Ang-1, Ang-2, and VEGF-A expression across normal menstrual cycle, the mRNA expression of Ang-1, Ang-2, and VEGF-A were examined in endometrium at the early-proliferative (EP), mid-proliferative (MP), late-proliferative (LP), early secretory (ES), mid-secretory (MS) and late secretory (LS) phases by

Table II. Summary statistics of Ang-1, Ang2, VEGF mRNA and protein expression in endometrial tissues of natural or stimulated cycles.

<table>
<thead>
<tr>
<th></th>
<th>Natural cycles (n = 15)</th>
<th>Stimulated cycles (n = 32)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang-1</td>
<td>1.178 (0.458–2.027)</td>
<td>0.578 (0.043–2.151)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ang-2</td>
<td>0.578 (0.155–1.42)</td>
<td>1.127 (0.282–4.192)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.589 (0.25–1.229)</td>
<td>0.808 (0.426–2.679)</td>
<td>0.023</td>
</tr>
<tr>
<td>Ang-1 to Ang-2 ratio</td>
<td>1.966 (0.817–9.802)</td>
<td>0.481 (0.065–1.933)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ang-2 to VEGF ratio</td>
<td>0.116 (0.025–0.209)</td>
<td>0.192 (0.040–0.420)</td>
<td>0.025</td>
</tr>
<tr>
<td>protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang-1</td>
<td>1.108 (0.888–1.252)</td>
<td>0.950 (0.767–1.370)</td>
<td>0.037</td>
</tr>
<tr>
<td>Ang-2</td>
<td>0.847 (0.636–1.083)</td>
<td>1.017 (0.664–1.479)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.784 (0.618–1.012)</td>
<td>0.841 (0.606–1.101)</td>
<td>0.487</td>
</tr>
</tbody>
</table>

**Expression of Ang-1, Ang-2 and VEGF-A mRNA in human endometrium across the menstrual cycle**

To study the relative expression of endometrial Ang-1, Ang-2, and VEGF-A expression across normal menstrual cycle, the
qPCR (Fig. 5). Significant differences were found in Ang-1 and Ang-2 expression, as well as in Ang-1 to Ang-2 and Ang-2 to VEGF-A ratios (Kruskal–Wallis test, \( P < 0.05 \)). No significant difference was found in VEGF-A expression among different phases of the cycle (Fig. 5C). The expression of Ang-1 peaked in MS (Fig. 5A), while that of Ang-2 increased significantly only in LS (Fig. 5B). The Ang-1 to Ang-2 ratio was significantly higher in ES and MS when compared to all other phases of the menstrual cycle (Fig. 5D). A significantly lower Ang-2 to VEGF-A ratio was obtained in LP when compared to ES, MS and LS (Fig. 5E).

**Discussion**

This is the first study comparing the expression of angiopoietins in the peri-implantation endometrium of women in natural and stimulated cycles. Angiogenesis is important in female reproductive events and a good blood supply towards the endometrium is required for implantation. The present report demonstrates a decreased expression of Ang-1 and an increased expression of Ang-2 in gonadotrophin-stimulated cycles. These changes affected the balance between pro- and anti-angiogenic factors in the stimulated endometrium leading to the formation of more blood vessels with high permeability, and to increased endometrial perfusion as reflected by higher endometrial vWF expression and blood vessel density after gonadotrophin stimulation.

Angiopoietins are vascular endothelial cell-specific growth factors critical in the later stages of angiogenesis after VEGF-induced formation of new capillaries (Geva and Jaffe, 2010).

### Table III. H-Score of Ang-1 and Ang-2 immunoreactivity in endometrial tissues of natural or stimulated cycles.

<table>
<thead>
<tr>
<th></th>
<th>Natural cycles ((n = 9))</th>
<th>Stimulated cycles ((n = 9))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glandular</td>
<td>1.9 (1.3–2.74)(^a)</td>
<td>1.72 (1.08–2.08)(^c)</td>
</tr>
<tr>
<td>Stromal</td>
<td>2.26 (1.18–2.74)(^*)</td>
<td>1.35 (0.36–1.68)(^*)</td>
</tr>
<tr>
<td>Ang-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glandular</td>
<td>1.26 (0.2–1.58)(^a)</td>
<td>0.54 (0.06–1.73)(^a)</td>
</tr>
<tr>
<td>Stromal</td>
<td>0.16 (0.02–0.44)(^b)</td>
<td>0.1 (0.06–0.42)(^b)</td>
</tr>
</tbody>
</table>

\(^a\)Data in the same row are significantly different \((P = 0.002)\).

\(^a\)–\(^c\)Data in the same column are significantly different \((P < 0.05)\).

**Figure 4:** Concentration of vWF in endometrial tissues. (A) Box plots of vWF concentrations in endometrial biopsies from natural (N) and stimulated (S) cycles. Data are presented as median with 10th, 25th, 75th and 90th percentiles and outliers (†). Statistical analysis was performed by Kruskal–Wallis test and \( P \)-values were shown. (B) Representative images showing vWF immunohistochemistry in endometrium from natural or stimulated cycles.

**Figure 5:** Bar graphs (mean ± SE) representing the relative expression of Ang-1, Ang-2 and VEGF-A mRNA, and the Ang-1 to Ang-2 and Ang-2 to VEGF-A mRNA ratios in human endometrium in the menstrual cycle. EP, early-proliferative \((n = 4)\); MP, mid-proliferative \((n = 4)\); LP, late-proliferative \((n = 7)\); ES, early-secretory \((n = 10)\); MS, mid-secretory \((n = 6)\); LS, late-secretory \((n = 6)\). Except for VEGF-A (C), significantly differences in Ang-1 levels (A), Ang-2 levels (B), Ang-1 to Ang-2 ratios (D) and Ang-2 to VEGF-A ratios (E) were found among different phases of the menstrual cycle (Kruskal–Wallis test). a–b, a–c, a–d are significantly different from each other \((P < 0.05)\).
Angiopoietins in a stimulated cycle

2000). Ang-1 induces migration, tube formation, sprouting and survival, but not proliferation of the endothelial cells (Eklund and Olsen, 2006). It is also an anti-permeability factor that overcomes the permeability brought by VEGF (Jones, 2003). On the contrary, the action of Ang-2 is context dependent. It destabilizes the vessel integrity by binding to Tie-2, the same receptor used by Ang-1, leading to vessel regression in the absence or in the presence of low levels of VEGF, and induces new blood vessel formation in the presence of abundant VEGF (Maisonpierre et al., 1997).

The present study showed significantly lower Ang-1, but higher Ang-2, mRNA and protein expression in gonadotrophin-stimulated women than in those with natural cycles. An increased Ang-2/VEGF-A ratio suggests the formation of more new blood vessels in women with stimulated cycles consistent with the higher concentration of the endothelial cell marker, vWF, and the higher blood vessel density in endometrium after stimulation. Though more blood vessels were formed after gonadotrophic stimulation, these newly formed blood vessels would interact less efficiently with the pericytes due to lower expression of Ang-1, and may become leaky leading to stromal edema in endometrium of women with stimulated cycles (Noci et al., 1997). We have demonstrated an increase in the length of endometrial stromal blood vessels and in stromal edema scores in women with high E2 levels when compared with those in natural cycles (Basir et al., 2001b). Endometrial edema increases towards the menstrual period, when the endometrium is not receptive to the implanting embryo (Johannisson et al., 1987). Thus the disproportionate increase in stromal edema in women after gonadotrophin stimulation may lead to an endometrial environment less favorable for implantation.

Maisonpierre et al. (1997) suggested that the Ang-1/Ang-2 ratio is more important than the absolute level of individual angiopoietins, and that excess Ang-2 leads to vessel destabilization. In the present study, the lower Ang-1/Ang-2 ratio in the stimulated cycles, and the negative correlation between Ang-1/Ang-2 ratio and E2 level on hCG day and hCG+7 day supported the adverse effect of the high E2 level on endometrial vasculature. The results are in line with the observation that daily administration of E2 to ovariectomized rats reduced tissue Ang-1 but increased Ang-2 mRNA expression (Ye et al., 2002). E2 is likely to act directly on the expression of Ang-1 and Ang-2 as their promoter regions contain 1 and 5 putative estrogen responsive element (ERE) sites, respectively (unpublished observations), though the regulatory role of these ERE sites on Ang-1 and Ang-2 expression remains to be confirmed.

The progesterone levels on hCG+7 day were negatively and positively correlated with Ang-1 and Ang-2, respectively. Indeed, abnormal angiogenesis after long-term progesterin-only contraception is associated with reduced Ang-1 expression (Krikun et al., 2004). Thus, it is possible that the high progesterone level after gonadotrophin stimulation also contributes to the aberrant expression of angiopoietins. However, progesterone is unlikely to contribute to the increase in edema in stimulated cycles as progesterone has little effect on vascular permeability (Ma et al., 2001). On the contrary, E2 promotes uterine vascular permeability (Ma et al., 2001).

The spatial expression of Ang-1 and Ang-2 protein in this study agrees with a previous report (Hirchenhain et al., 2003); Ang-1 is in the stroma, epithelium and blood vessels whereas Ang-2 is mainly in the glandular epithelium. The decreased stromal, but not epithelial, Ang-1 expression following ovarian stimulation suggests that the high steroid environment have differential effects on Ang-1 expression in various cell types. The immunoreactivity of Ang-1 was more intense in the secretory phase, especially in the stroma surrounding the blood vessels (Hewett et al., 2002), indicating that stromal Ang-1 may act on the subepithelial capillary plexus. On the other hand, Ang-2 expression was mainly localized to the glandular epithelium and endothelium. Although significantly higher endometrial Ang-2 expression was detected after gonadotrophin stimulation using Western blotting, no significant difference was found using the immunohistochemical staining method. The discrepancy could be due to the lower detection sensitivity of the staining method and the small sample size used in the experiment. Despite higher glandular Ang-2 expression, it is still unclear whether the increased production of epithelial-derived Ang-2 could reach the endometrial blood vessels as the bulk of endometrial epithelial VEGF is secreted apically into the uterine cavity (Hornung et al., 1998).

Unlike most of the vasculature with constant structure and function throughout life, the endometrial vascular bed undergoes cyclical changes (Ferenczy et al., 1979; Rogers et al., 1992; Rogers and Gargett, 1998). The human endometrial capillaries are surrounded by a loose and discontinuous basement membrane in the EP phase, and the whorled extensions of basement membrane with pericytes are not developed until early secretory to mid-secretory phase (Roberts et al., 1992). The coincidental exponential increase of Ang-1 in the early and mid-secretory phase in the normal human (this study; Hirchenhain et al., 2003) and rhesus macaque (Nayak et al., 2005) endometrium supports a role of Ang-1 in recruiting pericytes for vessel stabilization, which could be crucial to embryo implantation (Rogers, 1996). On the other hand, the expression of Ang-2 did not vary much in the menstrual cycle except in the late secretory phase when its expression increased significantly, probably for endometrial repair after menstruation. The differential expression of angiopoietins leads to the highest Ang-1 to Ang-2 ratio at the early and mid-secretory phases, favoring the maturation and stabilization of blood vessels formed in the proliferative phase. Compared with endometrium in the natural cycle, this ratio dropped 4-fold in the stimulated cycles, in line with the increased vasculature in the stimulated cycles.

The importance of VEGF in angiogenesis is well accepted. It is likely to play a role in endometrial repair in menstruation as its expression is high around the time of menstruation (Licht et al., 2003; this study). In the present study, the mRNA but not protein of VEGF-A increased after ovarian stimulation. A similar discrepancy between VEGF-A mRNA and protein expression in normal human endometrium has been reported (Punyadeera et al., 2006). The discrepancy in the protein and mRNA expression could be due to the secretion of VEGF mainly into the lumen of the endometrial glands (Hornung...
et al., 1998; Gargett et al., 1999). Though there was increased transcription of VEGF-A mRNA after gonadotrophin stimulation, the translated VEGF-A protein molecules could have been released to the glandular lumen causing an insignificant change in the VEGF-A protein content within the tissue. VEGF-A is unlikely to contribute significantly to the impairment of vasculature in the stimulated cycles because no correlation was found between the steroid levels on LH/hCG+7 days and the VEGF-A mRNA level, and the expression of VEGF-A varies only slightly in the secretory phase of the natural cycles. The latter observation supports a previous observation on lack of correlation between VEGF expression and endometrial angiogenesis (Gargett et al., 1999).

In coopted tumor vessels, dramatic induction of Ang-2 is related to vascular regression when the VEGF level is low, but is associated with robust angiogenesis when VEGF is up-regulated (Holash et al., 1999). Results in the present study showed that VEGF and Ang-2 mRNA were up-regulated in stimulated cycles leading to a higher Ang-2/VEGF-A ratio when compared with women in natural cycle. The observation matched with the present results showing a higher vWF concentration and blood vessel density after gonadotrophin stimulation, and with our previous findings that excessive responders had significantly more stromal vessels (Basir et al., 2001b). When the endometrium developed from the proliferative phase to the secretory phase, there was less than 2-fold increase in the Ang-2 to VEGF-A ratio. The change was much smaller than that of the Ang-1/Ang-2 ratio; a change of almost 10-fold was detected within the same period. Thus, the change in Ang-1 could be more important than that of Ang-2 in affecting angiogenesis in normal menstrual cycle.

The results in the present study demonstrated that gonadotrophin stimulation decreased Ang-1 concentrations and the Ang-1/Ang-2 ratio, and increased Ang-2 and VEGF-A concentrations at the mid-secretory phase. In the natural cycles, these changes also occurred during the transition from mid-secretory to late secretory phases. Thus, it is speculated that high steroid environment leads to advancement in the production of angiogenic factors resulting in impaired endometrial vasculature with more blood vessels formed and a higher edema score. However, comparison between the expression of Ang-1, Ang-2 and VEGF-A in moderate and excessive responders showed no difference on the expression of these genes, suggesting that their aberrant expression do not contribute significantly to the impairment of implantation rates in excessive responders after ovarian stimulation.

In summary, the decrease in Ang-1 and increase in Ang-2 expression and consequential decrease in Ang-1/Ang-2 ratio after ovarian stimulation may increase endometrial perfusion, leading to an impaired endometrial vasculature. These results may account for the increased edema found in our previous study (Basir et al., 2001b) and the higher vWF concentration and blood vessel density detected in the present study. However, the impairment of implantation and pregnancy rates in the excessive responder appears to be unrelated to the aberrant expression of Ang-1 and Ang-2. It remains to be determined whether the recent proposed minimal stimulation protocols, a combination of clomiphene citrate and gonadotrophins, with or without the addition of a GnRH-antagonist (Muasher et al., 2006), could produce a lower disturbance to the production of angiogenic factors.

Acknowledgements
We thank the clinical staff of the Department of Obstetrics and Gynecology, University of Hong Kong, for supplying the human endometrial samples.

Funding
This study was partly supported by the Committee on Research and Conference Grant of the University of Hong Kong and Competitive Earmarked Research Grants (HKU7514/05M and HKU7395/04M) of the Research Grant Council, Hong Kong.

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Submitted on April 12, 2007; resubmitted on December 15, 2007; accepted on January 2, 2008