Gene expression profiling of human peri-implantation endometria between natural and stimulated cycles

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Objective: To investigate the effect of high serum E_2 levels in gonadotropin-stimulated cycles (hCG+7) on the gene expression patterns of human endometrium compared with natural cycles on the seventh day of LH surge (LH+7) and elucidate the underlying molecular changes that may be related to endometrial receptivity.
Design: Observational study.
Setting: University Hospital.
Patients(s): Infertile patients with normal menstrual cycles undergoing IVF treatment.
Intervention(s): Gonadotropin stimulation and endometrial biopsy.
Main Outcome Measure(s): Gene expression by microarray and quantitative polymerase chain reaction (qPCR).
Result(s): Endometrial samples from natural (n = 5) and stimulated (n = 8) cycles were collected. Patients in the stimulated cycles were classified as moderate (n = 4) or excessive (n = 4) responders if their serum E_2 levels on the day of administration of hCG were $\leq$20,000 pmol/L or >20,000 pmol/L, respectively. The RNA transcripts were profiled by Affymetrix HG-U133A microarray. Clustering and principal component analysis demonstrated a significant difference ($\geq$2-fold) in the expression patterns of 411 genes among the three groups. Putative estrogen response elements or progesterone response elements were identified in the promoter regions of 49 differentially expressed genes of diverse biologic functions. The qPCR confirmed the microarray result in 47 endometrial samples.
Conclusion(s): High serum E_2 and/or progesterone modulate the gene expression profiles of human endometrium and may affect endometrial receptivity. (Fertil Steril® 2008;90:2152–64. ©2008 by American Society for Reproductive Medicine.)

Key Words: Endometrium, implantation, microarray, steroid hormones, stimulated cycle

Ovarian stimulation is used in the great majority of in vitro fertilization (IVF) programs (1, 2). Although more fertilizable oocytes are obtained after ovarian stimulation, excessive stimulation is associated with increased risk of ovarian hyperstimulation syndrome (OHSS). Recent data show that high serum estradiol (E_2) may have an adverse effect on implantation and pregnancy rates in stimulated IVF cycles (3–7). The detrimental effect can be abrogated with the use of a step-down regimen during ovarian stimulation to control the serum E_2 level (4). However, that observation could not be repeated in other studies (8, 9).

We previously reported that high serum E_2 levels, i.e., >20,000 pmol/L on the day of hCG administration affect implantation and pregnancy rates in stimulated IVF cycles (6). Endometrial morphometric analysis demonstrated a dyssynchronous development of the endometrium with delayed glandular maturation and advanced stromal morphology in hyperstimulated patients (10). They also had a significantly higher endometrial blood flow on day 7 after hCG injection compared with those having lower E_2 levels (11). More importantly, the embryo (6) and oocyte (12) quality appeared unaffected by the high serum E_2 level.

Implantation is a critical step in the establishment of a pregnancy. However, the molecular signals governing endometrial receptivity are not clearly known. In view of the complexity of endometrial receptivity in humans, microarray analyses have been used to study this biologic process from a global genomic perspective. Owing to ethical reasons, study on the human endometrium process with an implanting embryo is not possible. There are a few studies comparing the gene expression of endometrium throughout the cycle (13) and during the "implantation window" (LH/hCG + 5 days to 9 days) with those in the proliferative or early luteal phases of the cycle (14–18). In those studies, a large number of genes were found to be differentially expressed. Although the results demonstrated the developmental complexity of the endometrium, only a few genes, including osteopontin, apolipoprotein D, dickkopf/DKK1, and olfactomedin, were found to be up- or down-regulated in all of the studies (19).

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In the stimulated IVF cycles, most ovarian stimulation protocols use gonadotropins and GnRH analogues to prevent premature LH rise. Two recent studies compared the gene expression profiles in the luteal phase of natural and controlled ovarian stimulated cycles (20, 21). Again, only a few differentially expressed genes were found in common between the two studies, suggesting that the current controlled ovarian stimulation protocols could induce endometrial responses similar to those in natural cycles.

In the present study, we investigated the effect of high serum E_2 levels on the gene expression patterns of endometrium taken from patients who underwent ovarian stimulation with excessive responses and compared them with those from the natural cycle during the "implantation window." Analyses of the difference in global gene expression profiles between these groups allowed us to have a better understanding on the adverse effects of excessive ovarian stimulation on endometrial receptivity.

MATERIALS AND METHODS

Human Subjects

Forty-seven infertile women aged between 26 and 38 years, and attending the Assisted Reproduction Unit at the Department of Obstetrics and Gynaecology, Queen Mary Hospital, Hong Kong, for IVF treatment, were recruited for this study. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Hong Kong. A written informed consent was obtained from the patients before the start of the study. All of the women had a regular menstrual cycle and a normal uterus. No significant intrauterine or ovarian abnormalities were detected by transvaginal ultrasonography. Only women with a regular ovulatory menstrual cycle and a male factor infertility, who had not received any steroidal hormones for two or more months before this study and who agreed to use condoms for contraception during the study cycle were recruited for endometrial biopsies in the natural cycle.

For the stimulated cycle, ovarian stimulations were carried out as described previously using the long protocol (6). Briefly, the subjects were pretreated with a GnRH analogue, buserelin nasal spray (Suprecur; Hoechst AG, Hoechst, Germany), 150 μg four times a day, from the midluteal phase of the cycle before the treatment cycle. Human menopausal gonadotropin (Pergonal; Serono, Geneva, Switzerland) injection was started after confirmation of pituitary down-regulation. Then 10,000 IU hCG (Profasi; Serono) was administered when the leading follicle reached 18 mm in diameter and there were at least three follicles $>$15 mm in diameter. The serum E_2 level was measured on the day of hCG administration, and the patients were classified into either moderate (serum $E_2 \leq$20,000 pmol/L on hCG administration day) or excessive (serum $E_2 >$20,000 pmol/L on hCG administration day) responders as described previously (6). Subjects were recruited from women who did not have embryo transfer after IVF treatment because of failure of fertilization or risk of OHSS.

Tissue Collection

Endometrial biopsies in the natural cycles were taken on day LH+7. Blood was taken daily for serum E_2 and LH concentration, starting 18 days before the next expected menstruation until LH surge, which was defined as when the serum LH level was more than double the mean of the preceding values. Endometrial biopsies in the stimulated cycles were obtained 7 days after the hCG injection (hCG+7) from patients who did not have embryo transfer during IVF treatment because of fertilization failure or possible risk of developing OHSS. Biopsies were performed as an outpatient procedure from the fundal and upper part of the body of the uterus using Pipelle (CCD Laboratories, Paris, France). The biopsy was snap-frozen in liquid nitrogen and stored at -80°C until use. All of the endometrial biopsies were examined under microscope. Subnuclear vacuoles were found in about 20% of the glandular epithelial cells in all of the samples examined, and prominent glandular secretion was found in the lumen, confirming that the biopsies were in the same developmental phase. Serum E_2 and progesterone (P_4) levels were also measured on the day of the biopsy by commercially available chemiluminescence-based immunoassay kits.

RNA Isolation and Affymetrix Microarray

The total RNAs of 13 endometrial samples were isolated by RNeasy Mini Kit (Qiagen, Crawley, Sussex, UK) according to the supplier's protocol. The total RNA bound to the column was eluted in RNase-free water. The RNA quality was examined by Agilent 2100 bioanalyzer (Agilent Technologies Dentschland GmbH, Waldbronn, Germany). Ten micrograms of the total RNA from each sample was used for microarray analysis. Human genome–wide gene expression was examined with HG-U133A microarray GeneChip (Affymetrix, Santa Clara, CA), which is composed of more than 22,000 oligonucleotide probe sets. The details of this GeneChip are available at the manufacturer's web site (http://www.affymetrix.com). The GeneChip hybridization, signal scanning, data acquisition, and preliminary analysis were performed at the Genome Research Center of the University of Hong Kong according to the standard protocols recommended by Affymetrix. In brief, double-stranded cDNA was synthesized from 8 μg total RNA with the GeneChip T7 Oligo-(dT) Promoter Primer Kit (Affymetrix) and the SuperScript Choice System (Invitrogen, Carlsbad, CA). Before fragmentation, BioArray High Yield RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, NY) was used to synthesize the biotin-labeled cRNA by in vitro transcription. Then 15 μg labeled cRNA was used to hybridize the microarray chip. The chips were washed and stained using the GeneChip Fluidics Station 400, and the images were captured by GeneChip Scanner 3000 (Affymetrix).

Data Analysis

GeneSpring 7.2 software (Agilent Technologies, Palo Alto, CA) was used to analyze the microarray data. Per-chip normalization was carried out first with the Robust Multichip Average (RMA) analysis algorithm based on the expression