Mid-luteal endometrial intracrinology following controlled ovarian hyperstimulation involving use of a gonadotrophin releasing hormone antagonist

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BACKGROUND: There are concerns of reduced pregnancy rates with the use of gonadotrophin-releasing hormone antagonists (GnRH antagonists) in IVF/ICSI cycles. Sex steroids and their metabolizing enzymes in the endometrium may play a vital role in embryo implantation. This study has evaluated the levels and localization of sex-steroid receptors and metabolizing enzymes, 3β-hydroxysteroid dehydrogenases (3βHSD) and selected 17β-HSD (17βHSD), in mid-luteal endometrium of women treated with GnRH antagonist (Cetrorelix) and recombinant FSH (rFSH; Gonal-F) with luteal phase progesterone supplementation. METHODS: Mid-luteal phase endometrial biopsies were obtained from oocyte donors undergoing ovarian stimulation and from control women with regular periods. Immunohistochemistry and real-time quantitative–polymerase chain reaction (QRT–PCR) were used to compare protein and mRNA expression of progesterone receptor (PR), estrogen receptor α (ERα), estrogen receptor β (ERβ), androgen receptor (AR), 3βHSD1, 3βHSD2, 17βHSD2 and 17βHSD5. RESULTS: Cetrorelix–rFSH treatment caused a mid-luteal suppression of PR protein expression in the endometrial stroma, surface epithelium and glands, although expression in the glands of control samples was variable. In contrast, the treatment caused an increase in PR staining in perivascular cells. No other significant differences in protein expression were observed between the two groups. mRNA levels of AR, ERα, 3βHSD1 and 17βHSD2 were significantly reduced in the treatment group. PR mRNA levels were also reduced by GnRH antagonist–rFSH treatment, but the difference was not significant. CONCLUSIONS: Changes in the expression of sex-steroid receptors and metabolizing enzymes may lead to alterations in the activity and intracellular availability of estrogens, progestogens and androgens in endometrium of women treated with Cetrorelix and rFSH. Their impact on embryo implantation merits further evaluation.

Keywords: endometrium; GnRH antagonists; recombinant FSH; sex-steroid receptors; steroid metabolizing enzymes

Introduction

Gonadotrophin-releasing hormone antagonists (GnRH antagonists), e.g. Cetrorelix and Ganirelix, are now widely used in assisted conception treatments (Albano et al., 2000; Olivennes et al., 2000; The European and Middle East Orgalutran Study Group, 2001; The North American Ganirelix Study Group, 2001). They cause rapid suppression of LH levels and have been found to reliably prevent premature LH surges as a part of controlled ovarian hyperstimulation (COH) in IVF/ICSI treatment (Albano et al., 2000). Their use shortens the treatment cycle and also reduces the total amount of required gonadotrophins. Furthermore, they appear to reduce the incidence of ovarian hyperstimulation syndrome (Ludwig et al., 2001; Al-Inany et al., 2006). However, in comparison to the ‘long protocol’ with GnRH agonists, with the use of GnRH antagonists there is an ongoing debate regarding pregnancy rates. Whereas some studies have found similar pregnancy rates (Ludwig et al., 2001), others have raised concerns about a drop in pregnancy rates (Al-Inany et al., 2006).

Despite the advances in assisted conception practices, pregnancy rates are ~20–25%. In stimulated cycles, the endometrium is exposed to supraphysiological steroid hormone levels during the follicular phase and this might be responsible for an altered steroid receptor expression profile in the early luteal phase (Papanikolaou et al., 2005). After treatment with recombinant FSH (rFSH) and a GnRH antagonist, endometrial histological advancement at the time of oocyte retrieval was significantly lower compared to the ‘long protocol’ with GnRH agonists, with the use of GnRH antagonists there is an ongoing debate regarding pregnancy rates. Whereas some studies have found similar pregnancy rates (Ludwig et al., 2001), others have raised concerns about a drop in pregnancy rates (Al-Inany et al., 2006).
and is up-regulated by progesterone (Maentausta et al., 2002). There are limited data however on the state of the endometrium during the putative window of implantation. Although not clearly defined, the window of implantation is described as from Day 5 to Day 10 after the LH surge (Sharkey and Smith, 2003).

GnRH antagonists and rFSH may impact the processes of implantation through direct effects on the endometrium or indirectly through sex steroid availability and activity.

Estrogens (Norwitz et al., 2001; Ma et al., 2003), progesterogens (Lessey, 2003) and probably also androgens (Apparao et al., 2002) are thought to play vital, but as yet not fully defined, roles in the complex mechanisms underlying endometrial development leading up to and after embryo implantation. These hormones act via their cognate receptors. An alteration of the receptor expression profile could lead to changes in the function of the respective steroid hormone.

Intracellular ligand availability could also determine endometrial receptivity. The enzyme 3b-hydroxysteroid dehydrogenase/8S-4S-isomerase (3bHSD) is involved in the biosynthesis of all classes of active steroids. Pregnenolone is converted to progesterone under the effect of 3bHSD in the human endometrium and this might be crucial for implantation and maintenance of pregnancy. In the secretory phase, 3bHSD is moderately expressed in the glandular epithelium of the endometrium (Rhee et al., 2003).

The enzymes 17bHSD2 and 17bHSD5 have been identified in the human endometrium. The 17bHSD5 transforms not only androstenedione to testosterone and estrone to estradiol (E2), but also progesterone to 20-hydroxyprogesterone. In the endometrium, its expression has been localized to the surface epithelium and the vascular endothelium (Pelletier et al., 1999). The 17bHSD2 has a major role in the inactivation of E2 to estrone. It is also responsible for converting androgens to less potent forms, while also activating progesterone under the effect of 3bHSD in the human endometrium and this might be crucial for implantation and maintenance of pregnancy. In the secretory phase, 3bHSD is moderately expressed in the glandular epithelium of the endometrium (Rhee et al., 2003).

The enzymes 17bHSD2 and 17bHSD5 have been identified in the human endometrium. The 17bHSD5 transforms not only androstenedione to testosterone and estrone to estradiol (E2), but also progesterone to 20-hydroxyprogesterone. In the endometrium, its expression has been localized to the surface epithelium and the vascular endothelium (Pelletier et al., 1999). The 17bHSD2 has a major role in the inactivation of E2 to estrone. It is also responsible for converting androgens to less potent forms, while also activating progesterone. It is expressed in endometrial glandular epithelium, and is up-regulated by progesterone (Maentausta et al., 1993). The availability of various androgenic ligands to bind to the androgen receptor (AR) may be influenced by the local presence of 17bHSD2 (Burton et al., 2003). The activity of 17bHSD2 has been localized predominantly in the glandular epithelium but also in the endometrial stroma. It has been postulated that the antiestrogen action of progesterone in the endometrial glands is mediated through this enzyme (Casey et al., 1994; Burton et al., 2003).

The aim of this study was to compare the physiological mid-luteal endometrium with the endometrium during the putative window of implantation after treatment with rFSH and GnRH antagonists, by mimicking the exact conditions that would be expected to occur in an IVF/ICSI treatment cycle. We aimed to determine the endometrial intracrinochemistry in relation to expression of sex-steroid receptors and steroid metabolizing enzymes during the putative window of implantation.

Materials and Methods
First, we studied the sex-steroid receptor expression during the mid-secretory phase (Table 1). Sex-steroid receptor protein expression was studied with immunohistochemistry (IHC), and the mRNA expression was studied with real-time quantitative–polymerase chain reaction (QRT–PCR). Second, we evaluated the steroid metabolizing enzymes expression during the mid-secretory phase (Table 1). Enzyme expression was studied with IHC, and mRNA expression was studied with QRT–PCR.

Patient characteristics
Institutional ethical approval was obtained and all women gave informed written consent. The study group consisted of parous women volunteers who had come forward to donate oocytes. These women underwent a cycle of ovarian stimulation as per the Edinburgh Assisted Conception Unit’s protocol (Thong et al., 2003). The rFSH (Gonal-F; Serono, UK) was commenced on Day 4 of the menstrual cycle. All donors commenced ovarian stimulation at the dose of 150 IU. GnRH antagonist, Cetrorelix, was commenced at a dose of 0.25 mg daily on Day 7 or 8 of their cycle once two or more follicles had reached the size of 11 mm diameter. Ovarian response was monitored by transvaginal ultrasound from Day 4. When the three largest follicles measured ≥17mm, oocyte maturation was triggered by the administration of a single s.c. injection of 10 000 U human chorionic gonadotrophin (hCG) (Profasi; Serono). Oocyte retrieval was performed 35–36 h after hCG administration. Progesterone (Cyclogest; Alpharma, UK) vaginal pessaries (200 mg) were administered 12 hourly starting 2 days after oocyte retrieval and up to the day of endometrial sampling. A pipelle endometrial biopsy (EB) was conducted 8–10 days after hCG administration.

The control group consisted of 12 healthy parous women with regular menses (25–35 days). Endometrial biopsies from eight of these women were available for the IHC studies. The women were asked to provide a urine sample on alternate days from Day 10 of their last menstrual period (LMP). Urinary LH levels were measured. An EB was performed 6–10 days after the peak of a urinary LH surge (Fig. 1). Only parous women who attended the gynaecology clinics with requests for sterilization or other complaints excluding menstrual problems or infertility were included in the control group.

From the control subjects, endometrial tissue from a subset of five parous women was available for QRT–PCR studies. In these women, the LMP, endometrial histology and serum progesterone levels were all consistent with mid-luteal phase of the cycle. This subset was chosen on the basis of mRNA quality. Since we were comparing mRNA expression in the mid-secretory phase during the putative window of implantation, it was appropriate to include mid-secretory phase endometrium from fertile women with confirmed ovulation.

Endometrial biopsies were conducted during the mid-luteal stage of the cycle (Fig. 1). Serum progesterone level was checked at the time of the EB to confirm mid-luteal stage. All endometrial samples were histologically classified as per the Noyes criteria (Noyes et al., 1950). Endometrial tissue was fixed in 4% paraformaldehyde then embedded in paraffin for immunohistochemical analysis. In addition, endometrium was also frozen at the point of tissue collection in liquid nitrogen.

### Table 1: Details of study biopsy analyses

<table>
<thead>
<tr>
<th></th>
<th>Control, mean age (years) (n)</th>
<th>Study, mean age (years) (n)</th>
<th>P-value</th>
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<tr>
<td>Sex-steroid receptors and mRNA expression</td>
<td>IHC 38 (8) 31 (5)</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>QRT–PCR 41 (5) 31 (4)</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>Steroid metabolizing enzymes and mRNA expression</td>
<td>IHC 35 (6) 31 (5)</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>QRT–PCR 41 (4) 30 (3)</td>
<td>0.97</td>
<td></td>
</tr>
</tbody>
</table>
and stored at −70°C. RNA was extracted from frozen endometrial tissue.

The expression of progesterone receptor (PR), estrogen receptor α (ERα), ERβ and AR and enzyme expression of 3βHSD and 17βHSD5 were studied with IHC. The 3HSD antibody recognizes both human 3βHSD1 and 3βHSD2 enzymes. No documented antibodies were commercially available for 17βHSD2 enzyme and hence no immunochemistry was performed for this enzyme.

The PR, ERα, ERβ and AR mRNA and 3βHSD types 1 and 2 and 17βHSD types 2 and 5 mRNA levels were studied with QRT–PCR.

**Immunohistochemistry**

Paraffin sections (5 μm in thickness) were dewaxed in histoclear for 10 min and then rehydrated in descending grades of alcohol to distilled water (dH₂O). The sections were washed in dH₂O. After this, an antigen retrieval step was performed. After a 10-min wash in 0.01 M phosphate-buffered saline (pH 7.4) (PBS, Sigma, Dorset, UK), endogenous peroxidase activity was blocked. This was done by incubating the sections in 3% hydrogen peroxide in dH₂O for 10 min at room temperature. Tissue sections were then washed for 10 min in PBS. This was followed by 15 min incubation with avidin (Vector Laboratories Ltd, Peterborough, UK) at room temperature. After a rinse in PBS for 2 min, the sections were incubated with biotin (Vector Laboratories Ltd) for a further 15 min at room temperature. Following a 2-min wash in PBS, normal horse serum (NHS, Vector Laboratories) was applied to each tissue section. This was followed by incubation for 20 min in a humidified chamber at room temperature. The excess serum was removed and the primary antibody was applied. The negative control for the primary antibody was substituted with mouse immunoglobulin G (mIgG1, Sigma) or rabbit pre-immune serum at the same concentration.

After the primary antibody incubation, the sections were washed between each stage for 10 min in PBS. The secondary antibody was then applied. To identify positive staining, the peroxidase substrate diaminobenzidine (Dako, Cambridge, UK) was used as chromogen for demonstration of epitope. Tissue sections were washed in dH₂O and counterstained with Harris’s haematoxylin (a non-specific purple nuclear stain), dehydrated in ascending grades of alcohol and mounted from xylene using pertex mounting medium. A similar protocol was used for immunostaining of all receptors.

Table 2 summarizes the incubation conditions for immunolocalization of each of the epitopes studied. Commercially available antibodies were used for immunolocalization of endometrial PR, ERα, ERβ and AR. The 3βHSD rabbit polyclonal antibody was raised against recombinant human 3βHSD2 and recognizes both human 3βHSD1 and 3βHSD2 with similar affinity (S.E. McDonald and J.I. Mason).

### Table 2: IHC protocols

<table>
<thead>
<tr>
<th>Protein of interest</th>
<th>Antigen retrieval</th>
<th>Avidin–biotin pretreatment</th>
<th>Primary antibody</th>
<th>Negative control</th>
</tr>
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<tbody>
<tr>
<td>PR</td>
<td>Pressure cook (PC)</td>
<td>No</td>
<td>Monoclonal mouse anti-PR antibody</td>
<td>Mouse IgG</td>
</tr>
<tr>
<td>ERα</td>
<td>Microwave (MW)</td>
<td>No</td>
<td>Monoclonal mouse anti-ERα antibody</td>
<td>Sigma</td>
</tr>
<tr>
<td>ERβ</td>
<td>Buffer—0.01 M Na Citrate</td>
<td>No</td>
<td>Monoclonal mouse anti-ERβ antibody</td>
<td>Sigma</td>
</tr>
<tr>
<td>AR</td>
<td>Buffer—0.01 M Na Citrate</td>
<td>Yes</td>
<td>Monoclonal mouse anti-AR antibody</td>
<td>Mouse IgG</td>
</tr>
<tr>
<td>3βHSD</td>
<td>No antigen retrieval performed</td>
<td>Yes</td>
<td>Polyclonal rabbit anti-3βHSD (recognizes both isoforms)</td>
<td>Pre-immune serum</td>
</tr>
<tr>
<td>17βHSD5</td>
<td>Pressure cook (PC)</td>
<td>Yes</td>
<td>Monoclonal mouse anti-17βHSD-5</td>
<td>Mouse Ig</td>
</tr>
</tbody>
</table>

**Figure 1:** Timing of EB in the study and control group

OR, oocyte recovery, EB, endometrial biopsy

- **Study group**
  - Gonal-F
  - Cetrolex
  - Progesterone
  - Day 1 start of menses
  - Day 4 start of Rec–FSH
  - Day 8 scan: cetrolex 0.25 mg/day
  - hCG
  - OR
  - EB

- **Control group**
  - Day 1 start of menses
  - Urinary LH surge
  - EB
unpublished observations). A mouse monoclonal antibody against human 17βHSD5 (Lin et al., 2004) was a generous gift from Dr Trevor Penning (University of Pennsylvania, Philadelphia, USA). Although we used a mouse monoclonal against human 17βHSD2 in an earlier study (Burton et al., 2003), neither this nor any commercial 17βHSD2 antibody were currently available, and hence no immunocytochemistry was performed for this enzyme.

**RNA extraction and reverse transcription**

Frozen samples of endometrium stored at −70°C were homogenized and then total RNA was extracted using Trizol (Invitrogen Life Technologies Ltd, UK) according to the manufacturer’s instructions. The genomic DNA was removed by subjecting the RNA to DNase treatment. After extraction, the concentration and quality of RNA were assessed using an Agilent bioanalyzer (Agilent Technologies, South Queensferry, West Lothian, UK). The reverse transcription (RT) reaction was performed as described previously (Henderson et al., 2003; McDonald et al., 2006). In brief, a 10μl volume of reaction solution containing the following: 1x Taqman RT buffer, magnesium chloride, deoxyNTPs, random hexamers, Multiscribe reverse transcriptase, RNase inhibitor and nuclelease-free water (reagents from Applied Biosystems, Cheshire, UK) was used. An amount of 200 ng of template RNA was added. The RT reaction was conducted at 25°C for 60 min, 48°C for 45 min and 95°C for 5 min for one cycle. An RT-negative control had template RNA but no multiscribe enzyme included, and an RT H2O had template RNA replaced by nuclease-free water. Negative controls were included in every run. The samples were then stored at −20°C.

**Quantitative real-time PCR**

The primer/probe sets were designed using the Primer Express program (PE Applied Biosystems) as described previously (Henderson et al., 2003) or purchased from PE Applied Biosystems’ Assay on Demand service. Where possible these were chosen to span an intron to further reduce the chance of spurious readings due to genomic DNA contamination. The sequences of the primer/probe sets and their location within the specified cDNAs are given in Table 3. The 18S primers and probe were purchased from PE Applied Biosystems. A Taqman real-time PCR mix was then prepared containing final concentrations of Taqman universal PCR master mix (PE Applied Biosystems), and forward and reverse primers (300 nM) and probe for the sequence of interest (200 nM; PE Applied Biosystems). Wells were sealed with optical caps and the PCR was run on the Perkin-Elmer ABI Prism 7900 (PE Applied Biosystems) using standard conditions.

Taqman QRT–PCR was carried out with primers and probes specific for the PR, ERα, ERβ, AR, 3βHSD types 1 and 2 and 17βHSD types 2 and 5. The validated primers and probes for 3βHSD1, 3βHSD2 and 17βHSD5 were ‘Assay on Demand’ products supplied by PE Applied Biosystems and these primers were intron-spanning.

**Scoring, data presentation and statistical analysis of immunoreactivity**

The immunostaining intensity of epitopes in all tissue sections was assessed in a semi-quantitative manner on a four point scale: 0, no staining; 1, mild/minimal immunostaining; 2, moderate immunostaining and 3, intense immunostaining. All tissue sections were scored blind by at least two observers. The semi-quantitative IHC data were analysed using the Mann–Whitney test. The QRT–PCR data were log transformed and then the t-test was used to test for statistical significance.

**Results**

**Endometrial histology**

In the patients treated with a rFSH–GnRH antagonist, almost all biopsies with adequate tissue showed histological features consistent with mid-secretory phase of the cycle. In one biopsy, there were features suggestive of advancement of the dates and this was reported to be consistent with early to mid-secretory phase endometrium.

We examined the immunoexpression of PR, ERα, ERβ, AR, 3βHSD and 17βHSD5 at five cellular locations, i.e. endometrial glands, stroma, surface epithelium, vascular endothelium and perivascular cells. The mRNA transcripts of PR, ERα, ERβ, AR, 3βHSD1, 3βHSD2, 17βHSD2 and 17βHSD5 were evaluated.

**Table 3: Steroid receptor primer and probe sequences used for amplification by real time QRT–PCR**

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence</th>
<th>Position</th>
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<tbody>
<tr>
<td>AR forward</td>
<td>GTACCCTGCGCGCATGTT</td>
<td>951–1016</td>
<td>L29496</td>
</tr>
<tr>
<td>AR reverse</td>
<td>CCGATTCTGGTTCGACA</td>
<td>951–1016</td>
<td>L29496</td>
</tr>
<tr>
<td>AR probe</td>
<td>AGACAGGCGCTATGCCGTCCCA</td>
<td>951–1016</td>
<td>L29496</td>
</tr>
<tr>
<td>ERB1 forward</td>
<td>CGCCGCTACCTCTGATGCT</td>
<td>1459–1480</td>
<td>AB006590</td>
</tr>
<tr>
<td>ERB1 reverse</td>
<td>CGCCATTCTGGCCTATGGTGGT</td>
<td>1529–1552 (r)</td>
<td>AB006590</td>
</tr>
<tr>
<td>ERB1 probe</td>
<td>ATGATGTCTATGCTTGGCATACTCGCA</td>
<td>1499–1525 (r)</td>
<td>AB006590</td>
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<tr>
<td>ER forward</td>
<td>TGGACGCGCTTCGATGCCCG</td>
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<tr>
<td>ER reverse</td>
<td>CTATGGCGCTACTATGCCATCCC</td>
<td>1529–1541</td>
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</tr>
<tr>
<td>ER probe</td>
<td>TGTCCTAACCTTGGGACAGGAACC</td>
<td>1572–1600</td>
<td>NM_000125</td>
</tr>
<tr>
<td>PR forward</td>
<td>CTGGTGGCGCTCACTTTTGGTT</td>
<td>2151–2170</td>
<td>NM_000926</td>
</tr>
<tr>
<td>PR reverse</td>
<td>TGCGGTGAAAACATGTTTGCTT</td>
<td>2209–2233</td>
<td>NM_000926</td>
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<tr>
<td>PR probe</td>
<td>AGCCAAAGCCTAAAGCCGAGATTCATATT</td>
<td>2170–2199</td>
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</tr>
<tr>
<td>17βHSD-2 forward</td>
<td>TGGACGAGCATGGGAGGA</td>
<td>731–803</td>
<td>L11 708</td>
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<tr>
<td>17βHSD-2 reverse</td>
<td>GGTCCAGGCGGGCTTATGAT</td>
<td>731–803</td>
<td>L11 708</td>
</tr>
<tr>
<td>17βHSD-2 probe</td>
<td>CCCCAATGGAAAAGGCTGGAATCTT</td>
<td>731–803</td>
<td>L11 708</td>
</tr>
<tr>
<td>3βHSD1</td>
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<tr>
<td>3βHSD2</td>
<td>Assay on demand</td>
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<td>Hs0065123_ml</td>
</tr>
<tr>
<td>17βHSD5(AKR1C3)</td>
<td>Assay on demand</td>
<td></td>
<td>Hs00366267_ml</td>
</tr>
</tbody>
</table>

The positions of the sequences are given within the cDNA identified by the accession number; r denotes reverse strand.
**Immunohistochemistry**

**Sex-steroid receptors**

PR immunoexpression (Table 4, Figs 2A and 3) Compared with endometrium from untreated women, endometrium exposed to Gonal-F and Cetrorelix showed significantly reduced immunoexpression in stroma (P < 0.05) and surface epithelium (P < 0.05). Conversely, PR immunoreactivity was significantly increased in the perivascular cells (P < 0.05). The difference in PR immunoexpression in the glands was significant (P < 0.05), but we observed a marked variability in expression among the biopsies in the control group. ERα immunoexpression (Table 4, Figs 2B and 3) Mild immunoexpression of ERα was observed at most cellular locations. No ERα immunoreactivity was observed in the vascular endothelium. No significant differences were observed in endometrial ERα immunostaining between the two groups of women. ERβ immunoexpression (Table 4, Figs 2C and 3) ERβ immunoexpression at most cellular locations was consistently strong with or without treatment with GnRH antagonist and rFSH. Stromal expression was less intense, but overall no significant differences were observed in endometrial ERβ immunostaining between the two groups of women. AR immunoexpression (Table 4, Fig. 2D) AR immunoreactivity was negligible in the glandular epithelium with or without COH treatment. Stromal immunoreactivity of AR was moderate, but overall no significant differences were observed in endometrial AR immunostaining between the two groups of subjects.

**Steroid metabolizing enzymes**

3βHSD immunoexpression (Table 4, Figs 2E and 4) Irrespective of COH, expression of 3βHSD in the glands, surface epithelium and endothelium was at a low level. No immunoreactivity was seen in stroma or in perivascular cells. No significant differences were observed in endometrial 3βHSD immunostaining between the two groups of subjects.

17βHSD5 immunoexpression (Table 4, Figs 2F and 4) Moderate to intense 17βHSD5 immunoreactivity was observed in the endometrial glands, and surface epithelium with or without treatment. No immunoreactivity was observed in stroma, however, negligible immunoexpression was observed in perivascular cells. Moderate immunoreactivity was observed in the vascular endothelium. No significant differences were observed in endometrial 17βHSD5 immunostaining between the two groups of subjects.

**Quantitative real-time PCR (Table 5, Fig. 5)**

**Sex-steroid receptors**

QRT–PCR demonstrated a statistically significant reduction in the amount of endometrial ERα mRNA (P = 0.02) and AR mRNA (P=0.01) in COH treated women compared with the controls. Although the level of PR mRNA was reduced in the COH group, the difference was not significant (P = 0.12). No significant differences were observed in the amount of ERβ mRNA (P = 0.96) between the two groups.

**Steroid metabolizing enzymes**

The 3βHSD1 mRNA (P = 0.01) and 17βHSD2 mRNA (P = 0.02) levels were significantly reduced in the COH group. In relation to 3βHSD2 and 17βHSD5 QRT–PCR, sufficient RNA was only available in 1 and 2 study samples, respectively. Hence in these cases, the sample size was too small for analysis. However, expression of adrenal/gonadal-specific 3βHSD2 transcripts is predicted to be minimal in endometrium and the relative 3βHSD1/3βHSD2 transcript ratio observed in the one sample is supportive of a minimal 3βHSD2 contribution to endometrial 3βHSD.

**Discussion**

The use of rFSH and a GnRH antagonist is an effective and reliable regime for controlled ovarian stimulation as a part of IVF/ICSI treatment cycles. However, its effects on the endometrium especially in relation to embryo implantation have not been evaluated in detail. Although histological advancement of the chronological stage of the endometrium has been observed at the time of oocyte retrieval (Kolibianakis et al., 2002), the endometrial sex-steroid receptor protein and mRNA expression levels during the putative window of implantation, to our knowledge, have not been reported. Furthermore, we are unaware of any reports describing expression
of steroid metabolizing enzymes and their mRNA transcripts during the window of implantation in the mid-luteal phase of the cycle.

Here, we report a significant difference in PR protein expression in women treated with rFSH and a GnRH antagonist. Significant down-regulation of PR protein expression in the endometrial stroma and surface epithelium was observed. A significant up-regulation was observed in the perivascular cells in women treated with Cetrorelix and Gonal-F. PR immunoreactivity in the endometrial glands was reduced in the treatment group, although the expression in the controls was variable. No significant differences were observed in the protein expression of ERα, ERβ, AR, 3βHSD or 17βHSD5. Quantitatively, there was a significant reduction in the levels of ERα mRNA, AR mRNA, 3βHSD1 mRNA and 17βHSD2 mRNA in the treatment group. No significant difference was observed in the amount of ERβ mRNA transcripts between the two groups of women, and although PR mRNA was reduced in the treatment group, the difference was also not significant.

Healthy fertile women were recruited as the control group. We acknowledge that women undergoing assisted conception are a different group with alterations in physiological steroid levels and possibly in the endometrium. Hence, recruiting untreated parous women as a control group may not be the

Figure 2: Immunoreactivity scores in endometrial glands, stroma, surface epithelium, endothelium and perivascular cell compartments of women in the mid-luteal phase of cycle (control group) and in women who have received COH and a GnRH antagonist (study group) (A) PR, (B) ERα, (C) ERβ, (D) AR (E) 3βHSD enzyme and (F) 17βHSD5 enzyme. Note decreased PR in endometrial stroma ($P = 0.019$), surface epithelium ($P = 0.019$) and glands ($P = 0.003$), and increased PR in perivascular cells ($P = 0.023$). Box-and-whisker plots: box represents the 25th and 75th percentiles, whiskers represent the 10th and 90th percentiles and the heavy bar represents the median. C, control; S, study

Figure 3: Immunohistochemical localization of steroid receptors in human endometrium of women in the mid-luteal phase of cycle (control group) and in women who have received rFSH and a GnRH antagonist (study group) (A) PR immunostaining in endometrium from untreated women in mid-luteal phase—intense stromal (S) and surface epithelial (SE) immunoreactivity (A) inset: note low level of perivascular immunostaining (arrows). (B) PR immunostaining in the endometrium from women treated with a GnRH antagonist and rFSH—reduced stromal and surface epithelial immunostaining. (B) inset: more intense perivascular cell immunostaining (arrows). (C) ERα immunostaining in endometrium from women treated with rFSH and a GnRH antagonist—mild immunostaining at all cellular locations. (C) inset: absent immunostaining in the vascular endothelium (arrow). (D) ERβ immunostaining in endometrium from women treated with rFSH and a GnRH antagonist—strong immunoreactivity in all cell types. (D) inset: strong ERβ immunostaining in vascular endothelium. Scale bar = 20 microns. G, glands
ideal equivalent comparison. However, our aim was to try and understand the differences between the physiological state of embryo implantation, which occurs in the mid-luteal phase in a natural ovulatory cycle, and in rFSH and a GnRH antagonist treated endometrium. Hence, it was appropriate to include untreated fertile ovulatory women in the control group.

In the subjects in the control group, an LH surge was detected using an alternate day urinary LH protocol. We acknowledge that urinary LH measurement may not be the most accurate way of checking the timing of ovulation. However, it is possible to identify the window of implantation on the basis of an alternate day urinary LH dating protocol. First, the window of implantation is thought to extend over Days 5–10 after the LH surge. Hence, even with urinary LH dating, we are likely to identify the putative window of implantation. Second, it is theoretically possible that the difference of 1 or 2 days could influence the result. We have however dated the endometrium not only by LH dating, but also by histological dating and mid-luteal serum progesterone concentration at the time of EB. We are thus confident that the endometrium was sampled during the mid-luteal stage and certainly during the window of implantation. There are no data to suggest that a difference of 1 or 2 days is likely to have a major impact on the sex-steroid receptor expression. Indeed with the exception of one study looking at the PR and ER expression at LH+7

### Table 5: Summary of sex steroid and steroid metabolizing enzyme mRNA expression

<table>
<thead>
<tr>
<th>Sample</th>
<th>mRNA expression</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR</td>
<td>mRNA expression</td>
<td>0.12</td>
</tr>
<tr>
<td>ERα</td>
<td>mRNA expression</td>
<td>0.02*</td>
</tr>
<tr>
<td>ERβ</td>
<td>mRNA expression</td>
<td>0.96</td>
</tr>
<tr>
<td>AR</td>
<td>mRNA expression</td>
<td>0.01*</td>
</tr>
<tr>
<td>3βHSD1</td>
<td>mRNA expression</td>
<td>0.012*</td>
</tr>
<tr>
<td>3βHSD2</td>
<td>mRNA expression</td>
<td>Sample size too small</td>
</tr>
<tr>
<td>17βHSD2</td>
<td>mRNA expression</td>
<td>Sample size too small</td>
</tr>
<tr>
<td>17βHSD5</td>
<td>mRNA expression</td>
<td>Sample size too small</td>
</tr>
</tbody>
</table>

*P < 0.05 denotes statistical significance (t-test after log transformation).
*indicates reduction of mRNA level in the study compared with control group.
after treatment with ganirelix (Simon et al., 2005), where no alteration in the steroid receptor expression was reported, we are not aware of any data describing the steroid receptor expression in the mid-luteal phase after treatment with rFSH and a GnRH antagonist.

Luteal phase progesterone supplementation was administered to the oocyte donors (study group) to mimic an actual treatment cycle. Evidence exists to support the use of luteal phase progestogens after any form of down-regulation in an IVF/ICSI treatment cycle (Beckers et al., 2003) and most centres offer this routinely. Administering progesterone supplementation in one group (study) and not in another (control) may add an element of bias. However, our aim was to compare the differences in the mid-luteal phase endometrium from fertile untreated subjects, i.e. the physiological state with the mid-luteal endometrium of women routinely treated with a GnRH antagonist and rFSH.

This study has shown that under the effect of a GnRH antagonist and rFSH with luteal phase progesterone supplementation, there is a significant down-regulation of PR in the surface epithelium. The observation of PR down-regulation in the surface epithelium is in agreement with other studies evaluating PR content in the secretory phase of a normal menstrual cycle. It has been suggested that under the influence of progesterone, PR declines in the epithelium at the beginning of the window of implantation (Lessey et al., 1996). It therefore appears that any hormonal fluctuations that may result in the serum due to treatment with Cetrorelix and Gonal-F have little impact on intracellular PR in surface epithelium. In the secretory phase of a normal menstrual cycle, significant PR expression has been detected in the endometrial stroma (Lessey et al., 1988). However, under the effect of a GnRH antagonist and rFSH and with progesterone supplementation, we found a significant down-regulation of PR in endometrial stroma. Several genes have been localized to the endometrial stroma and significant proportions are expressed during the secretory phase (Yanaihara et al., 2004). Studies have also shown an important role for progesterone in the endometrial stromal cell in induction of extracellular matrix in relation to implantation (Nakamoto et al., 2005). A significant

**Figure 5:** Quantitative evaluation of sex-steroid receptors and steroid receptor metabolizing enzymes mRNA expression of women in the mid-luteal phase of cycle (control group) and in women who have received COH and a GnRH antagonist (study group)

(A) PR, (B) ERα, (C) ERβ, (D) AR, (E) 3βHSD1 enzyme and (F) 17βHSD2 enzyme. All endometrial tissue samples were compared with an internal control (comparator) obtained during the mid-luteal phase of the menstrual cycle. ERα mRNA (P = 0.02) and AR mRNA (P = 0.01) levels were significantly reduced in endometrial tissue from women treated with a GnRH antagonist and rFSH. 3βHSD1 mRNA (P = 0.01) and 17βHSD2 mRNA (P = 0.02) levels were significantly reduced in the endometrial samples from women treated with a GnRH antagonist and rFSH. Note scale(Y axis) in E and F differs from A–D.
down-regulation of stromal PR could thus influence gene expression during the secretory phase and adversely impact endometrial receptivity. PR protein has not been identified in the vascular endothelium (Krikun et al., 2005), but they are abundantly expressed in the perivascular cells throughout the menstrual cycle (Perrot-Applanat et al., 1988; Critchley et al., 2001). In this study, PR was significantly up-regulated in the perivascular cells of women treated with Cetrotrelix and Gonal-F. Progesterone acting on the perivascular cells has been implicated in the modulation of endometrial blood flow. Furthermore, cytokine control in the perivascular cells is thought to be controlled by progesterone (Kelly et al., 2002). These factors may well be important in the process of embryo implantation, and alterations of PR expression could influence these processes. PR expression in the glands of untreated women was variable. In regular cycling women, in the secretory phase, significant PR content was maintained in the stroma, but diminished in the glandular epithelium (Lessey et al., 1988). In this study, in some control biopsies, moderate glandular PR expression was maintained however, as evident in the box and whisker plot, there was marked variability in expression between biopsies. The number of women included in our study was small and this may have contributed to the apparent trend towards an increased mid-secretory PR expression in glands in untreated women. Furthermore, the women included in the control group were different from those who provided the study biopsies in the treatment group. Whereas ideally, we would have liked to recruit the same oocyte donors as controls to further reduce the bias, practically it was only reasonable to seek an altruistic oocyte donor on a single occasion. In the control group, the mid-luteal phase was confirmed through consistency of reported LMP, circulating serum progesterone levels and histological dating. It is interesting to note that with rFSH and a GnRH antagonist treatment, glandular PR immunostaining was diminished. This observation would be consistent with PR expression in the secretory phase of an untreated cycle. PR mRNA levels are known to vary during the human menstrual cycle. In the late proliferative phase, glandular PR mRNA levels are significantly higher but it reduced in the secretory phase. In the stroma, PR mRNA remains unchanged (Lau et al., 1996). Although there was some reduction in PR mRNA levels in the treatment group compared with the controls, the difference was not significant. However, PR changes in whole biopsies may not reflect the subtle changes in steroid receptor expression that exist between individual cell types.

In regular cycling women, ERα and ERβ have been identified in the endometrial epithelium, stroma, glands and perivascular cells (Saunders and Critchley, 2002). Only ERβ and not ERα is expressed in the vascular endothelium (Critchley et al., 2001). We did not observe any significant difference in immunostaining of either ERα or ERβ in treated women. Controlled ovarian stimulation is known to lead to supraphysiological levels of E2 and progesterone. These are thought to affect the endometrial receptivity through the predominantly gestational effects of endometrial phase advancement and premature luteinization (Kolb and Paulson, 1997). Hence, it appears that ER-mediated signalling appears to be of less importance in relation to implantation compared with PR mediated effects on the endometrium. It is interesting to note however that quantitative RT–PCR showed significantly reduced ERα mRNA levels in the treatment group.

AR expression is influenced by levels of circulating estrogens and androgens. Epithelial AR is up-regulated by estrogens and androgens and is inhibited by progestins (Slayden et al., 2001; Apparao et al., 2002). AR is expressed in the endometrial stromal cells. The intensity of expression declines from proliferative phase to mid-secretory phase. In late secretory phase, AR expression is diminished in all cell types (Mertens et al., 2001). We found no significant difference in protein expression of AR in women treated with FSH and a GnRH antagonist, however, QRT–PCR showed significantly reduced AR mRNA levels in the group of women treated with a GnRH antagonist and rFSH. So far, there are very limited data on effects of AR and AR-induced gene expression in humans. Studies in pigs show AR in the pig endometrium during the window of implantation and demonstrate the functional, albeit complex, interactions of androgens and estrogens in the regulation of uterine endometrial gene expression and cell growth in vitro (Kowalski et al., 2004). Further studies are needed to evaluate AR-induced gene expression in humans and the potential impact on embryo implantation.

The 3βHSD is weakly expressed in the glandular epithelium of the proliferative phase and moderately expressed in the glandular epithelium of secretory phase of the endometrium (Rhee et al., 2003). In this study, irrespective of presence or absence of treatment, immunostaining of 3βHSD in the glands, surface epithelium and endothelium was at a low level. No immunostaining was seen in stroma and perivascular cells. No significant difference was observed in endometrial 3βHSD immunostaining between the two groups of subjects. This suggests that the pre-ovulatory supraphysiological levels of estrogen and progestogen that result from COH and use of GnRH agonists do not lead to any significant alteration in the levels of 3βHSD protein during the window of implantation. The currently available antibody against 3βHSD recognizes both forms of human 3βHSD enzymes, types 1 and 2. Hence it is not possible to comment on changes in the amounts of 3βHSD1 protein. However, RNA studies indicate that 3βHSD1 mRNA transcripts may be changing. We observed a significant reduction in 3βHSD1 mRNA in women treated with a GnRH antagonist and rFSH. The 3βHSD1 is responsible for the conversion of inactive pregnenolone to active progesterone and of dehydroepiandrosterone to androstenedione. A reduction in 3βHSD1 transcripts will ultimately lead to a reduction of intracellular progesterone. In the presence of altered PR expression, as we observed with IHC, it is likely that the reduced ligand availability for binding to PR leads to a disturbance in the dynamics of ligand-receptor interaction. This may affect progesterone-mediated signaling pathways including alterations in gene expression profiles thereby affecting the receptivity of the endometrium.

The 17βHSD5 transforms androstenedione to testosterone and also progesterone to the inactive 20-hydroxyprogesterone, and in the endometrium its immunostaining has been
localized to the surface epithelium and the vascular endo-
thelium (Pelletier et al., 1999). Our findings are in agreement
with previous reports. We did observe moderate to intense
17βHSD5 immunoexpression in endometrial surface ep-
ithelium and vascular endothelium. However, in this study we
also observed moderate 17βHSD5 immunoexpression in the
endometrial glands. No immunoexpression was observed in
stroma and negligible immunoexpression was observed in the
perivascular cells. No significant difference was observed in
the 17βHSD5 immunoexpression between the two groups of
subjects. We observed a significant reduction of 17βHSD2
mRNA in women treated with rFSH and a GnRH antagonist.
Since 17βHSD2 is involved in the inactivation of E2 to estro-
ne and converting androgens to less potent forms, it is
likely that higher levels of intracellular E2 and androgens
persist thereby further disturbing the balance between estrogen,
progesterone and androgens. This may further affect the endo-
metrial development leading to suboptimal endometrial
receptivity.

We have identified only one previous report comparing the
effects of a GnRH antagonist treatment on mid-luteal phase
endometrium to the state of the mid-luteal endometrium in a
natural ovulatory cycle (Simon et al., 2005). However, that
study only examined ER and PR expression 7 days after an
LH surge. The GnRH antagonist used in that study was Ganir-
elix. In this study, we used Cetrorelix and to our knowledge this
is the first report describing the ER, PR and AR expression as
well as the steroid metabolizing enzymes expression in a
GnRH antagonist treated mid-luteal phase endometrium.

The reason, why so few studies have addressed the mechan-
isms of endometrial receptivity in IVF/ICSI cycles during the
window of implantation is due to the difficulty in obtaining, for
detailed studies, endometrial biopsies at this phase of the treat-
ment. Hence, most of the relevant studies have been performed
on only small numbers of subjects.

Conclusions
In summary, to our knowledge, this is the first report describing the
effects of a GnRH antagonist and rFSH on sex-steroid
receptor and steroid metabolizing enzymes expression in mid-
secretory phase endometrium. This study has shown that under
the effect of rFSH and a GnRH antagonist with progesterone
supplementation, significant alterations occur in endometrial
intracrinology at a time when the endometrium would be
expected to be most receptive for implantation. The impact of
these observations on embryo–endometrial interaction
requires further evaluation.

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