Too much of a good thing?
Experimental evidence suggests prolonged exposure to hCG is detrimental to endometrial receptivity

Jemma Evans1,2,* and Lois A. Salamonsen1,2

1Prince Henry’s Institute, Monash Medical Centre, PO Box 5152, Clayton, Melbourne, VIC 3168, Australia 2Faculty of Medicine, Nursing and Health Sciences, Monash University, VIC 3800, Australia

*Correspondence address. E-mail: jemma.evans@princehenrys.org

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STUDY QUESTION: Does prolonged exposure of the endometrium to hCG, as experienced after ovulation induction in an assisted reproduction technology (ART) cycle, affect functional measures of endometrial receptivity?

SUMMARY ANSWER: Prolonged endometrial hCG exposure detrimentally affects the manner in which the endometrium can respond to hCG secreted by the blastocyst.

WHAT IS KNOWN ALREADY: Prolonged hCG exposure down-regulates endometrial LH–CG receptor (LHCGR) expression in a baboon model. HCG exposure during the proliferative phase of oocyte-donation cycles and frozen embryo transfer cycles is associated with a lower pregnancy rate.

STUDY DESIGN, SIZE, DURATION: LHCGR was examined in endometria of women undergoing ART cycles (GnRH agonist/antagonist) and across the menstrual cycle in normally cycling fertile women. To determine whether prolonged hCG exposure affects the subsequent endometrial response to hCG, endometrial epithelial cells (HES cell line and primary cultures of human endometrial epithelial cells) were exposed to a low dose of hCG (0.5–5 IU) for up to 5 days, to mimic the chronic exposure during an ART cycle, and subsequently exposed to an acute ‘blastocyst mimic’ dose of hCG (20 IU).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Endometrial tissues were collected at hCG +2 (n = 37) from women undergoing ART between August 2006 and August 2008, and across the cycle from women with known fertility (n = 40). LHCGR localization and staining intensity were determined by immunohistochemistry and semi-quantitative scoring. HES cells were treated with hCG as above and analyzed for LHCGR localization (immunocytochemistry), phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 (western immunoblotting), adhesion to trophoblast-like matrices (adhesion assays) and tight junction integrity (trans-epithelial resistance assessment).

MAIN RESULTS AND THE ROLE OF CHANCE: Endometrial epithelial LHCGR staining was significantly lower in women stimulated with a GnRH agonist protocol who did not become pregnant in that cycle versus the natural menstrual cycle (P < 0.05). Chronic low-dose hCG exposure in vitro mediated a down-regulation and internalization of the LHCGR in endometrial epithelial cells. Prolonged exposure to chronic low-dose hCG (3–5 days) abrogated ERK 1/2 phosphorylation, adhesion to extracellular matrices and changes in tight junction integrity in response to a subsequent acute high dose (20 IU) of hCG.

LIMITATIONS, REASONS FOR CAUTION: Studies using cell lines and primary cultures of cells in vitro are not fully representative of the complex endometrial milieu in vivo.

WIDER IMPLICATIONS OF THE FINDINGS: These data reinforce the clinical observations that precocious or prolonged hCG exposure may detrimentally affect endometrial receptivity and provide a mechanistic basis for these clinical findings. The data appear to support the notion that in women for whom ART has not succeeded, a different, minimally stimulated approach without exposure to exogenous hCG may improve outcomes.

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Prolonged hCG alters endometrial receptivity

Introduction

It is estimated that ~17% of couples in developed countries seek medical advice for infertility (Cahill and Wardle, 2002) and an increasing number of pregnancies are achieved with the aid of assisted reproduction technologies (ARTs). These technologies include intrauterine insemination (IUI), oocyte donation, IVF and ICSI. The success rates for ARTs, however, are still not optimal with around one in five initiated ART procedures resulting in a live birth, and the demand for reproductive assistance is growing each year. It is therefore important to understand more fully the basic biology underlying development of both the embryo and the endometrium in ART cycles, to optimize the clinical outcome.

ART methods, except IUI, rely on multi-follicular development followed by ovulation induction with an ovulatory trigger, generally hCG, before oocyte collection and fertilization. It is increasingly appreciated that such exogenous hormonal stimulation for ARTs may be detrimental to endometrial receptivity (Kolibianakis et al., 2002; Bourgain and Devroey, 2003; Kolibianakis et al., 2003; Devroey et al., 2004; Kolibianakis et al., 2005; Papanikolaou et al., 2005). Interestingly, the ovulation trigger, hCG, which is generally thought to have a ‘pro-implantation’ effect (Licht et al., 2001; Sherwin et al., 2007; Banerjee et al., 2009; Evans et al., 2009; Paiva et al., 2011) is becoming a prime target in the search for substances with a negative role in endometrial receptivity (Kosmas et al., 2007; Prapas et al., 2009; Fatemi et al., 2010; Kyrou et al., 2012).

Traditionally, hCG was considered to act via LH–CG receptors (LHGRs) on the corpus luteum to prevent luteolysis and maintain progesterone production in the early stages of pregnancy. However, with the identification of an endometrial LHGR, the role of hCG signaling in the endometrium, likely to facilitate implantation, is now appreciated. Clinical studies (cited above), however, have raised concerns regarding the effect of hCG on the endometrium when it is used for ovulation induction, i.e. precocious exposure to hCG in the absence of pregnancy. Studies examining oocyte recipients have shown that prolonged exposure to hCG in the proliferative phase negatively affects the pregnancy rate (Prapas et al., 2009). Similarly, ‘natural’ ovulation, determined by monitoring the LH surge, results in higher implantation and ongoing pregnancy rates in normo-ovulatory women versus those in whom ovulation is triggered with hCG in IUI and frozen embryo transfers (Kosmas et al., 2007; Fatemi et al., 2010; Shapiro et al., 2011). These clinical data indicate a likely detrimental effect of early or prolonged hCG exposure on endometrial receptivity.

The aims of this study were to determine whether the LHGR is altered in the endometrium of women undergoing ARTs versus the normal endometrium and whether the localization and functionality of the LHGR is altered by prolonged hCG stimulation. We demonstrated the down-regulation of the LHGR in the ART endometrium versus normally cycling endometrium. Further, the LHGR becomes internalized and fails to activate downstream signaling, relaxation of endometrial epithelial tight junctions and mediation of endometrial epithelial cell adhesion to trophoblast-like extracellular matrices following prolonged exposure (versus acute exposure) to hCG. Prolonged exposure thus negatively affects functional measures of endometrial receptivity. These basic studies should help to inform future clinical practice.

Materials and Methods

Ethics statement

Ethical approval for all tissue collections was obtained from Institutional Ethics Committees at Southern Health and Monash Surgical Private Hospital. Written informed consent was obtained from all subjects prior to tissue collection. Patient age, BMI, number of previous ART cycles, cumulative FSH dose and pregnancy outcome information were subsequently collected (Table I).

Human tissue collection and patient details

Human endometrial biopsies were obtained by curettage from normal cycling women at different stages of the menstrual cycle, following laparoscopic sterilization or assessment of tubal patency (proliferative n = 10, early-secretory n = 10, mid-secretory n = 10, late secretory n = 10). Tissue biopsies were also collected (between August 2006 and August 2008) from women undergoing routine stimulation protocols for IVF on the day of oocyte retrieval (hCG + 2, infertile GnRH agonist n = 9; infertile GnRH agonist n = 28). The infertile agonist-stimulated group was further subdivided according to pregnancy outcome (infertile GnRH agonist n = 12, infertile agonist not pregnant n = 16).

In the GnRH agonist group (n = 28), the oral contraceptive pill (OCP) was commenced between Day 1 and Day 7 of the preceding cycle for at least 21 days, with the administration of the GnRH agonist (Synarel, 0.4 mg per day) 15 days after commencing OCP. After 10–14 days, ovarian stimulation was commenced with 125–225 IU recombinant FSH daily (dose used was dependent on age, and a BMI >30 kg/m²) until the visualization of at least three follicles ≥17 mm diameter, when ovulation was triggered with 250 μg recombinant hCG. In this group, 12 women subsequently became pregnant and 16 did not. Of those who became pregnant, eight underwent IVF and four had ICSI; two of this group had endometriosis, four had polycystic ovary (PCO) and two had polycystic ovarian syndrome. Of the women who did not become pregnant (n = 16), 1 underwent IVF and 15 underwent ICSI; 3 had PCO.

In the GnRH antagonist group (n = 9), ovarian stimulation commenced on Day 2 of the menstrual cycle with 125–225 IU recombinant FSH daily, as above, until at least one follicle ≥14 mm was visualized. GnRH antagonist (cetrorelix acetate, 0.25 mg) was then administered up to and including the day of hCG administration. When at least three follicles ≥17 mm were visualized, ovulation was triggered with 250 μg of recombinant hCG. In this group, three women had IVF, six underwent ICSI; two women had endometriosis. None of this cohort became pregnant in the cycle of sampling.

Oocytes were retrieved by transvaginal ultrasound-guided aspiration 2 days after hCG administration (hCG + 2). Endometrial biopsies were

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taken by Pipelle on the day of oocyte retrieval. All infertile women underwent fresh embryo transfer with embryos generated from their own oocytes. The luteal phase of all cycles was supplemented with vaginal administration of 8% progesterone gel (Cirnone, Serono) from hCG + 5.

For the collection of endometrial samples during the menstrual cycle, patients with uterine abnormalities such as leiomyomas, endometrial polyps, endometriosis, or those who had received steroid hormone therapy in the last 6 months were excluded. Menstrual cycle stage in women with normal cycles was confirmed by histological dating by an experienced pathologist (Noyes and Haman, 1953).

### Immunohistochemistry for an LHCGR in endometrium

Paraffin sections (5 μm) were dewaxed in Histosol (Sigma Chemical Co., St Louis, MO, USA) and rehydrated. Antigen retrieval was performed by boiling sections in 0.01 M citrate buffer. Endogenous peroxidase activity was blocked by incubation of sections with 3% hydrogen peroxide, followed by blocking of non-specific antibody binding by incubation with 10% non-immune swine serum/2% human serum in Tris-buffered saline (TBS). The sections were blocked by the incubation of sections with 3% hydrogen peroxide, followed by blocking of non-specific antibody binding by incubation with 10% non-immune swine serum/2% human serum in Tris-buffered saline (TBS). The application of 3,3′-diaminobenzidine (Dako) enabled the visualization of antibody binding. Sections were counterstained with hematoxylin, dehydrated and mounted with DPX. Immunoglobulin (Ig)G controls (rabbit IgG) at the same final concentrations as primary antibody showed no staining. For the assessment of immunohistochemical staining, the amount and intensity of immunostaining within each cellular compartment (glandular and luminal epithelium) was analyzed and allocated a score: 0 (no staining); 1 (minimal staining); 2 (strong staining); 3 (intense staining).

### Culture of endometrial epithelial cells

The HES human endometrial cell line (kindly gifted by Dr Douglas Kniss, Ohio State University, Columbus, OH, USA; Banerjee et al., 2009) was routinely maintained in a 1:1 mix of Dulbecco’s modified Eagle’s medium (DMEM):F12 (Invitrogen, Mulgrave, VIC, Australia) supplemented with 1% L-glutamine, 1% penicillin/streptomycin and 10% fetal calf serum (complete media). HES cells were chosen for these studies as they express the LHCGR.

### Cell culture hCG dosing

To determine the effect of prolonged hCG exposure on functional measures of endometrial receptivity, we designed a cell culture model to mimic exposure in ART cycles (below). In stimulated cycles, ovulation is triggered with a bolus of 5000–10,000 IU hCG. Given the body fluid volume and the prolonged half-life and reduced clearance rate of hCG versus LH, we estimated that the endometrium would be exposed to 0.5–5 IU hCG for the 5 days from ovulation trigger to placement of embryos into the uterine cavity. These doses were applied in the in vitro experiments. The 20 IU dose of hCG was chosen as this likely reflects the level of hCG secretion from an embryo in vivo (Lopata and Oliva, 1993).

### Table I Data for women who underwent assisted reproduction treatment and were included in a study on the effect of prolonged exposure to hCG on endometrial receptivity.

<table>
<thead>
<tr>
<th></th>
<th>GnRH antagonist, not pregnant</th>
<th>GnRH agonist, not-pregnant</th>
<th>GnRH agonist, pregnant</th>
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<tbody>
<tr>
<td>Number of subjects</td>
<td>9</td>
<td>16</td>
<td>12</td>
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<tr>
<td>Age (years)</td>
<td>39.4 ± 0.8b (32.4–43.8)</td>
<td>34.9 ± 1b (28.8–41.5)</td>
<td>35.6 ± 1b (28.6–39.5)</td>
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<tr>
<td>BMI (kg/m^2^)</td>
<td>23.6 ± 1.1b (19.6–32.4)</td>
<td>27.6 ± 1.5b (20.5–39.9)</td>
<td>26.0 ± 1.2 (20.0–35.4)</td>
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<tr>
<td>Collected oocytes</td>
<td>8.6 ± 1.2b (4–18)</td>
<td>8.6 ± 1.2b (2–20)</td>
<td>14.6 ± 2.7b (6–45)</td>
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<tr>
<td>Peak estrogen (pg/ml)</td>
<td>2578 ± 379b (1350–6046)</td>
<td>2756 ± 526b (583–8803)</td>
<td>5556 ± 1347b (2115–17 854)</td>
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<tr>
<td>Cycle number</td>
<td>5.5 ± 1.5b (1–19)</td>
<td>4.9 ± 1.3b (1–21)</td>
<td>2.9 ± 0.9 (1–11)</td>
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<td>Cumulative FSH (IU)</td>
<td>3161 ± 330 (1250–5400)</td>
<td>2858 ± 276 (1350–4500)</td>
<td>2429 ± 299 (1200–4950)</td>
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<tr>
<td>Parity</td>
<td>2 ± 0.4 (0–6)</td>
<td>0.8 ± 0.2 (0–2)</td>
<td>0.9 ± 0.3 (0–3)</td>
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<tr>
<td>Live births</td>
<td>0.7 ± 0.2 (0–2)</td>
<td>0.3 ± 0.1 (0–1)</td>
<td>0.4 ± 0.1 (0–1)</td>
</tr>
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</table>

*Data are mean ± SEM (range). Superscripts a and b represent. a = bp < 0.05.

Primary culture of endometrial epithelial cells

Endometrial epithelial cells were isolated from endometrial curettage samples (n = 2) taken from women according to standard protocols. Briefly, endometrial tissue was chopped and incubated in phosphate-buffered saline (PBS, Invitrogen) containing collagenase III (7.5 IU/ml, Sigma, Sydney, NSW, Australia) and DNAse I (100 mg/ml, Worthington, Lakewood, NJ, USA) in a shaking water bath at 37°C with shaking at 130 r.p.m. for 40 min. Digestion was stopped by the addition of excess DMEM/F12 (Invitrogen), and samples vacuum-filtered through 45-μm filters. Endometrial epithelial cells were washed off filters and further digested as above for 25 min. Cells were pelleted by centrifugation at 235g for 10 min, vigorously resuspended in complete media, plated and allowed to settle for up to 7 days. Cultures were checked visually daily for contamination of the epithelial cell cultures by stromal cells. None of the cultures used were contaminated by stromal cells.
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Figure 1 Experimental model of chronic low-dose and acute high-dose hCG exposure. A model (with minor modifications for different end-points) was constructed to mimic endometrial prolonged hCG exposure during an assisted reproduction technology (ART) cycle followed by exposure to acute hCG as secreted by the blastocyst. Cells (cell line and primary) were seeded and 24 h later serum-starved in Dulbecco’s modified Eagle’s medium/transferrin, sodium selenite, linoleic acid (DMEM/F12/TSL) for 24 h to synchronize (A, Day −1 and Day 0, respectively). They were then exposed to a chronic low dose of hCG (0.5–5 IU) for up to 5 days (A; general model) before treatment with an acute high dose of 20 IU hCG (B, D and E). Cells not previously exposed to hCG were also treated with an acute high dose of hCG (20 IU) at this time to determine the short-term effects of hCG. Control cells were maintained in DMEM/F12/TSL for long-term experiments (Fig. 1D).

Adhesion assays
HES cells were seeded into a 12-well plate at a density of $1 \times 10^5$ cells per well, allowed to attach and subsequently incubated in DMEM/F12 with TSL overnight. Cells designated ‘chronic low-dose hCG’ were treated with 2.5 or 5 IU hCG for 3 days. All but control wells were then treated with acute high-dose hCG (20 IU) for 24 h (Fig. 1B). Cells were trypsinized, $5 \times 10^4$ cells per well were seeded into plates coated with extracellular matrices [fibronectin, collagen I or collagen IV (Millipore), three wells/treatment] and allowed to adhere for 45 min. Non-adhered cells were removed by washing with PBS containing calcium and magnesium. Attached cells were stained with crystal violet, washed, detached with solubilization buffer (50:50 mix of 0.1 M Na$_2$HPO$_4$, pH 4.5, and 50% ethanol) and absorbance measured at 450 nm. Adhesion was calculated as a percentage of untreated control cells (n = 6).

Immunocytochemistry for an LHGR
HES cells were seeded onto chamber slides at a density of $5 \times 10^4$ and allowed to attach overnight prior to incubation in DMEM/F12 media supplemented with TSL (10 µg/ml) and treatment with chronic low-dose hCG for 5 days (Fig. 1C). Cell monolayers were subsequently exposed to ice-cold methanol for 10 min and stored in PBS. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide, and non-specific binding of antibody blocked by incubation with 10% non-immune horse serum/2% human serum in PBS. Primary antibody was applied overnight (1:20, LHGR K15, Santa Cruz, Sapphire Bioscience, Waterloo, NSW, Australia) at 4°C. Cells were washed sequentially in 0.2% Tween–PBS and PBS followed by the application of horse anti-goat biotinylated antibody (Vector, Abacus ALS, QLD, Australia) at 1:200 for 1 h. This was followed by further wash steps in 0.2% Tween–PBS and PBS and the application of streptavidin 488 (green) or 568 (red, Molecular Probes, Invitrogen) at 1:200 for 2 h at room temperature. Wash steps in 0.2% Tween–PBS and PBS were followed by the removal of chambers from the slides and mounting in Vectashield containing DAPI nuclear counterstain (Dako).

Western immunoblotting
HES cells were seeded into six-well plates ($1 \times 10^5$ cells/well) and allowed to attach overnight. Primary endometrial epithelial cells were seeded into 24-well plates and allowed to settle and expand for 7 days. Cell monolayers were subsequently: (i) incubated in serum-free DMEM/F12 media for short-term experiments or (ii) incubated in DMEM/F12 media supplemented with TSL for long-term experiments (Fig. 1D).

(i) Short-term experiments: HES cells were serum-starved overnight and allowed to attach with 20 IU hCG for 0, 5, 10, 15, 20 or 30 min.

(ii) Long-term experiments: HES and primary endometrial epithelial cells were incubated in media with TSL overnight, and then treated with chronic low-dose hCG (0.5–5 IU) for 5 days with daily replacement of hCG-containing media. All but control wells were then treated with acute high-dose hCG (20 IU) for 10 min (time of maximal signal activation as established by short-term experiments, Fig. 1D).

Cells were lysed in buffer (10 mM HEPES, 10 mM MgCl$_2$, 5 mM KCl, 0.1% Triton X-100 with protease and phosphatase inhibitors), and the lysates
termicer of lytsates was assessed by BCA assay (Bio-Rad). Twenty five micro gram of protein from each sample was run on a 12% sodium dodecyl sulfate polyacrylamide gel prior to immunoblotting of proteins to PVDF membranes (Amersham, Rydalmore, Vic, Australia). Pre-stained molecular weight markers (See-Blue, Bio-Rad) were run on gels in parallel with protein samples. Membranes were washed twice in TBS–0.2% Tween and non-specific binding was blocked by incubation in 5% skim milk/TBS–0.2% Tween for 1 h followed by the application of phospho-extracellular signal-regulated kinase (ERK) 1/2 antibody (pERK 1/2, 1:1000, Cell Signaling, Genesearch PTY, Arundel, QLD, Australia) overnight at 4°C. Immunoblots were washed in TBS–0.2% Tween prior to the application of secondary antibody (goat anti-rabbit horse-radish peroxidase conjugated antibody, 1:5000, Dako) for 1 h at room temperature. Immunoblots were then washed and exposed to enhanced chemiluminescence substrate (Amersham) before the detection of chemiluminesence by X-ray film. Immunoblots were stripped off bound primary antibody, washed and blocked in 5% skim milk in TBS–0.2% Tween before probing for total ERK 1/2 (loading control, 1:1000, Cell Signaling) as above. Semi-quantitative densitometric analysis was performed (n = 5 for HES cell line, n = 2 for primary endometrial epithelial cells).

TER assays
Bi-axial chambers (12 mm, 0.4 μm pore, Sigma) were coated with fibronectin according to the manufacturer’s protocol (BD Bioscience) prior to seeding of HES cells (5 x 10^5 cells per insert). Cells were allowed to attach overnight in complete media and then incubated in DMEM/F12 with 0.1% csFCS in both the basal and apical chambers. Cells designated ‘chronic low-dose hCG’ were treated with 0.5–5 IU hCG, added to the apical chamber for 5 days with daily replenishment of hCG. All except control wells were therefore examined at 10 min. TER measurements were taken daily commencing from the day of seeding. TER was quantified using a Millipore Millicell-Electrical Resistance System (+/– 5 IU hCG demonstrated less intense staining and also re-localization of the LHCGR in HES cells exposed to 20 IU hCG, maximal phosphorylation of ERK 1/2 was observed at 10 min, reducing to low levels by 30 min (Fig. 4A, lanes 3 and 6, respectively). All subsequent short-term stimulations were therefore examined at 10 min.

Statistical analysis
All statistical analyses used PRISM version 4.03 for Windows. All data were tested for normality prior to analysis. For normally distributed data, an unpaired t-test was applied. For non-normally distributed data, a Mann–Whitney test was performed. Significance was taken as P < 0.05. All data are presented as mean ± SEM of at least n = 5 individual experiments.

Results

LHCGR immunolocalization in the normal cycling endometrium and the ART endometrium
Limited LHCGR immunostaining was noted in the glandular epithelial cells of proliferative phase endometria (arrows, Fig. 2A), but its intensity increased as the menstrual cycle progressed, with intense immunostaining observed from the early secretory phase of the menstrual cycle onwards (arrows, Fig. 2B–D). During the mid-secretory phase, the LHCGR also localized to the perivascular cells surrounding the endometrial spiral arteries (open arrow, Fig. 2C). As secretory transformation of the endometrium continued from the mid- to the late-secretary phase of the menstrual cycle, the LHCGR increasingly localized to the stromal cells radiating out from cells surrounding the spiral arteries (open arrowheads, Fig. 2D).

In contrast, little LHCGR protein was observed in endometria taken on hCG +2 from women undergoing GnRH antagonist (Fig. 2E) or GnRH agonist (Fig. 2F and G) treatment for ovarian hyperstimulation. Limited patchy staining of the luminal epithelium (arrowheads, Fig. 2E), glandular epithelium (arrow, Fig. 2E) and stroma (open arrowhead, Fig. 2E) was seen in tissue from GnRH antagonist-treated women (none of whom were pregnant). By contrast, intense immunostaining for the LHCGR was observed in the endometria of women treated with the GnRH agonist protocol (sampled at LH +2) who did not become pregnant, specifically localized around the spiral arteries (open arrow, Fig. 2F). This localization close to the spiral arteries is similar to that observed in the mid-secretory phase in normal women (approximately LH +6–10), suggesting these endometria are histologically advanced. However, in comparison with the secretory phase in the normal cycle, immunostaining of the glandular epithelial cells in these tissues (GnRH agonist not pregnant) was very faint (arrows, Fig. 2F). Immunostaining for the LHCGR of the glandular epithelium in endometria of GnRH agonist-treated women who did become pregnant (arrows, Fig. 2G) was more intense than in those who did not become pregnant (arrows, Fig. 2F).

Semi-quantitative immunohistochemical scoring substantiated that staining intensity for the LHCGR was significantly reduced in the glandular epithelial cells of GnRH agonist-stimulated women who did not subsequently become pregnant versus normally cycling endometria in the early- and late-secretory phases (Fig. 2I, a versus b, P < 0.05). Similarly, LHCGR staining was significantly reduced in luminal epithelial cells of the same women versus normally cycling endometria in the early- and mid-secretory phases (Fig. 2J, a versus b, P < 0.05). The negative control (rabbit IgG) demonstrated no staining (Fig. 2H).

hCG treatment mediates down-regulation and re-localization of the LHCGR in endometrial epithelial cells
In untreated HES cells, the LHCGR was localized to the cell surface (Fig. 3A, and following 5 days serum starvation, Fig. 3B). However, after treatment with 1 IU hCG for 5 days, this cell surface staining was reduced in intensity (white arrows, Fig. 3C). Cells treated with 5 IU hCG demonstrated less intense staining and also re-localization to the nucleus in a number of cells (Fig. 3D).
Treatment of cells with the acute high dose of 20 IU hCG increased ERK 1/2 phosphorylation (1.51 ± 0.23-fold versus control, Fig. 4B, lane 4). However, cells that had received a chronic low dose of hCG (0.5 or 5 IU) for 5 days, subsequently treated with the acute high-dose hCG (20 IU), did not phosphorylate ERK 1/2 to the same extent (1.12 ± 0.06 and 1.17 ± 0.19-fold, respectively, Fig. 4B, lanes 2 and 3, lane 2 versus lane 4, P < 0.05).

These data were confirmed in primary endometrial epithelial cells (Fig. 4C, n = 2) where chronic low-dose hCG (0.5 IU) for 5 days followed by high-dose hCG (20 IU) likewise failed to elicit the maximal ERK 1/2 phosphorylation response mediated by acute high-dose hCG only (0.5 and 20 IU, 0.97-fold versus 20 IU only, 1.37-fold).

**hCG-mediated adhesion of endometrial epithelial cells to trophoblast-like matrices is abrogated by pretreatment with hCG**

Treatment of endometrial cell monolayers (HES cells) with acute high-dose hCG (20 IU) for 24 h enhanced endometrial epithelial cell adhesion to fibronectin (gray bar, Fig. 5A, P < 0.05), collagen I (gray bar, Fig. 5B, *P < 0.05) and collagen IV (gray bar, Fig. 5C, P < 0.05) versus control (Fig. 5A–C). Chronic low-dose hCG treatment (2.5 or 5 IU) for 3 days abrogated the adhesive response to an acute high dose of hCG (20 IU, Fig. 5A–C).
hCG-mediated relaxation of endometrial epithelial tight junctions is abrogated by pre-exposure to hCG

Treatment of polarized endometrial epithelial cell monolayers with chronic low-dose hCG (2.5 or 5 IU) initially reduced tight junction integrity versus control (100%) and this persisted for 3–4 days (Fig. 6). Tight junction integrity was restored to control levels after this time despite the continued presence of hCG (Fig. 6, Day 5). Treatment of cell monolayers not previously exposed to hCG with acute high dose (20 IU hCG) significantly reduced epithelial tight junction integrity (Fig. 5, *P*, 0.05). However, cells previously exposed to chronic low-dose hCG which were subsequently exposed to high-dose hCG (20 IU) could not relax epithelial tight junctions (20 IU).

Discussion

Implantation is a highly complex process requiring a close synchrony between the development of the endometrium and the embryo. Furthermore, the endometrium can respond to embryonic signals, such as hCG, to facilitate its preparation for implantation. This study demonstrates clearly that prolonged exposure to hCG, as would be experienced following ovulation induction in ART cycles, can exert a detrimental effect on functional measures of embryo-endometrial interactions. We demonstrate a down-regulation of the LHCGR in the ART endometrium compared with the normal cycling endometrium and both down-regulation and re-localization of the LHCGR in endometrial epithelial cells following prolonged treatment with hCG. Furthermore, pre-exposure of endometrial cells to chronic low-dose hCG renders the cells refractory to acute hCG, as would be experienced at the time of implantation: these cells exposed to a chronic low dose of hCG fail to activate downstream signaling, and fail to enhance adhesion or to relax tight junctions, as observed in the acute high-dose situation.

This study supports the in vivo baboon data which indicate that hCG down-regulates its own receptor in the endometrium (Cameo et al., 2006; Sherwin et al., 2010). Herein we demonstrate alterations in LHCGR localization in human ART endometrium exposed to hCG for ovulation induction, with reduced epithelial LHCGR staining but intense LHCGR staining around the endometrial spiral arteries, as observed in the hCG-exposed baboon endometrium (Cameo et al., 2006; Sherwin et al., 2010). Rapid down-regulation of the LHCGR following exposure to hCG has also been demonstrated in the ovary and testis (Conti et al., 1976; Sharpe, 1976; Rebois and Fishman 1984; Lak-kakorpi et al., 1993; Peegel et al., 2005). However, ART endometria are exposed to a complex hormonal milieu including exogenous hormones of the OCP, FSH, GnRH antagonist, precocious progesterone and supra-physiological levels of estrogen in addition to the ovulation trigger of hCG. We cannot therefore conclude that epithelial down-regulation of the LHCGR in the ART endometrium is exclusively due to an hCG-mediated mechanism. We observed no significant differences in LHCGR staining between GnRH agonist or GnRH antagonist cycles. Importantly, in the natural cycle, we demonstrate maximal luminal epithelial LHCGR in the early–mid secretory phase of the cycle, indicating that the endometrium is optimally primed to respond to embryonic signals, including hCG, which is an essential consideration when replacing frozen–thawed embryos back into natural cycles. These changes in the LHCGR across the cycle, while relatively small, are significantly different and important in facilitating receptivity.

Treatment of HES cells with low doses of hCG (1–5 IU) for 5 days resulted in a down-regulation and re-localization of the full-length LHCGR.

Figure 3 Localization of LH–CG receptor (LHCGR) in endometrial epithelial cells (HES) after prolonged hCG exposure. The LHCGR localizes to the cell surface of control cells maintained in serum (A, red, arrows) and in control serum-starved cells (B, arrows). In cells exposed to 1 IU (C) and 5 IU (D) of hCG for 5 days, staining for the LHCGR is less intense (arrows) with re-localization of LHCGR to the nucleus after treatment with 5 IU hCG (D, arrowheads). Red, Streptavidin 568; blue, DAPI.
In the ovary, treatment with hCG elevates mRNA expression of splice variants of the LHCGR, concomitant with a down-regulation of the full-length transcript (Lakkakorpi et al., 1993). Additionally, truncated forms of the LHCGR which are missing the trans-membrane and C-terminal domains exhibit intracellular localization and may cause mis-routing of the full-length receptor away from the cell membrane (Dickinson et al., 2009). Down-regulation and re-localization of the LHCGR in endometrial epithelial cells in the present study may thus reflect changes in alternative processing of LHCGR mRNA in response to hCG, similar to that observed in ovarian cells. Clearly, further investigation is required to determine the regulation of LHCGR splice variants in the endometrium under the influence of hCG.

Unlike the ‘classic’ hCG–LHCGR signaling pathway in the ovary, where coupling to Gαs mediates cAMP release, LHCGR signaling in the endometrium activates PI3-kinase and ERK-1/2 phosphorylation via Gq-coupled signaling (Srisuparp et al., 2003; Banerjee et al., 2009). In agreement, we demonstrate maximal ERK-1/2 phosphorylation

Figure 4 Activation of ERK 1/2 phosphorylation after chronic and acute exposure of HES and primary endometrial epithelial cells to hCG. Treatment of HES cells with 20 IU hCG-mediated maximal extracellular signal-regulated kinase (ERK) 1/2 phosphorylation (pERK) at 10 min (A, lane 3; B, lane 4). Chronic treatment with 0.5 IU hCG for 5 days and then acute treatment with 20 IU hCG for 10 min resulted in significantly reduced pERK versus cells without chronic treatment (B, lane 2 (0.5 IU chronic, 20 IU acute) versus lane 4 (20 IU acute only). *p < 0.05, n = 5). Likewise, in primary endometrial epithelial cells (C, n = 2), chronic treatment with 0.5 IU hCG for 5 days appeared to reduce pERK 1/2 following subsequent acute treatment with 20 IU hCG for 10 min, compared with cells treated with the acute dose only. Data are presented as mean ± SEM where appropriate.

Figure 5 Adhesion of endometrial epithelial cells (HES) to extracellular matrices after chronic and acute exposure to hCG. Treatment with an acute dose of hCG (20 IU for 24 h) enhanced adhesion of HES cells to fibronectin, collagen I and collagen IV (gray bars, A, B and C, respectively, n = 6) versus control (100%). Pretreatment with 2.5 or 5 IU hCG for 3 days followed by treatment with 20 IU hCG for 24 h significantly decreased adhesion to extracellular matrices versus cells treated with acute-dose hCG (20 IU) only (C versus II. A, B and C, all *p < 0.05, n = 6). Data expressed as mean ± SEM, n = 6 separate experiments.
after 10 min of acute hCG treatment (Srisuparp et al., 2003), but this was suppressed by chronic pretreatment with low doses of hCG, likely via receptor down-regulation. Similarly, silencing of the endometrial epithelial LHCGR abrogates hCG-mediated ERK-1/2 phosphorylation (Banerjee et al., 2009). As phosphorylation of ERK-1/2 regulates its translocation into the nucleus and induction of a wide array of genes, pre-exposure to hCG likely renders the endometrium unable to activate transcription and translation of certain ‘pro-implantation’ factors by blastocyst-derived hCG.

Following apposition, the blastocyst must firmly adhere to the endometrial luminal epithelium. We showed here that acute hCG enhanced adhesion of endometrial epithelial cells to the extracellular matrices, fibronectin and collagens I and IV, which are present on the surface of the blastocyst, an effect abrogated by pre-incubation with hCG. Similarly, to enable intrusive penetration of the luminal epithelium by the trophoblast, tight junctions between the epithelial cells must be relaxed. This process, which is initiated in the secretory phase of the menstrual cycle (Murphy et al., 1992; Rogers and Murphy, 1992; Murphy 2000), is here shown to be enhanced by hCG. However, this relaxation does not occur in cells pre-exposed to hCG. Together, these data suggest that chronic activation of the endometrial epithelial LHCGR by hCG affects cellular functions essential for implantation. Thus, de-sensitization and down-regulation of the LHCGR following pre-exposure to hCG appears to render the endometrium incapable of responding to a ‘blastocyst mimic’ dose of hCG in terms of these essential functional changes.

In conclusion, down-regulation of LHCGR, inhibition of signaling and abrogation of functional responses of the endometrium following chronic exposure to hCG may help explain why ovarian stimulation protocols are detrimental to implantation (Ertzeid and Storeng, 2001; Kosmas et al., 2007; Prapas et al., 2009; Fatemi et al., 2010; Shapiro et al., 2011; Kyrou et al., 2012). The data support the proposal that replacement of frozen–thawed embryos back into natural cycles, where LH levels were being monitored, would improve pregnancy rates (Fatemi et al., 2010; Kyrou et al., 2012). Clinics may be reluctant to follow these suggestions because of the higher number of visits required for monitoring and the relative unpredictability in the timing of the natural LH surge versus ovulation induction with hCG (Weissman et al., 2011). However, the observation of lower pregnancy rates in normo-ovulatory women treated with hCG (Kosmas et al., 2007; Fatemi et al., 2010; Kyrou et al., 2012) together with the data presented in this study indicates that prolonged hCG exerts a negative effect on endometrial receptivity. A considerable body of evidence now indicates that the use of hCG in ART cycles should be carefully re-considered and tailored to suit the individual woman, thus optimizing reproductive outcomes.

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Authors’ roles

J.E. conceived the study, performed experiments and wrote the manuscript. L.A.S. helped plan studies and edited the manuscript.

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Conflict of interest

None declared.

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