Mifepristone, but not levonorgestrel, inhibits human blastocyst attachment to an in vitro endometrial three-dimensional cell culture model

P.G.L. Lalitkumar¹, S. Lalitkumar¹, C.X. Meng¹, A. Stavreus-Evers², F. Hambiliki³, U. Bentin-Ley⁴ and Kristina Gemzell-Danielsson¹,⁵

¹Division of Obstetrics and Gynecology, Department of Woman and Child Health, Karolinska Institutet, Karolinska University Hospital, S-171 76 Stockholm, Sweden; ²Department of Women’s and Children’s Health, Uppsala University, Uppsala, Sweden; ³Department of Clinical Science, Division of Obstetrics and Gynecology, Karolinska Institutet, Stockholm, Sweden; ⁴Danish Fertility Clinic, Copenhagen, Denmark

⁵Correspondence address. Tel: +46-8-517-72128; Fax: +46-8-517-74314; E-mail: kristina.gemzell@ki.se

BACKGROUND: The use of fertility regulating drugs is limited among various socio-ethnic groups due to limited knowledge about their mechanism of action. This study investigates the effect of levonorgestrel and mifepristone on attachment of human embryos to an in vitro endometrial construct. METHOD: Three-dimensional endometrial constructs were established by co-culturing early luteal phase human endometrial stromal and epithelial cells. Expression of endometrial receptivity markers in this construct were examined by immunohistochemistry. Effects of mifepristone and levonorgestrel on viability and attachment of human blastocysts were investigated. RESULTS: Endometrial constructs expressed the factors involved in endometrial receptivity: estrogen receptor, progesterone receptor, vascular endothelial growth factor, leukemia inhibitory factor, interleukin-1, COX-2, MUC-1 and integrin-αVβ3. None of the 15 embryos cultured with mifepristone attached to the endometrial construct (P<0.01), whereas 10/17 in control, and 6/14 in levonorgestrel, groups attached. The attachment was confirmed by the positive expression of cytokeratin 7 at the attachment site. CONCLUSION: Mifepristone inhibits blastocyst attachment. Levonorgestrel did not impair the attachment of human embryos to the in vitro endometrial construct. This model could be used to understand endometrial receptivity and embryo-endometrial dialog and to develop new fertility regulating substances.

Keywords: 3D-endometrial cell construct; receptivity markers; mifepristone; levonorgestrel; human blastocyst

Introduction
Endometrial receptivity is part of the synchrony between hormonally primed endometrium and the preimplantation embryo. With priming of estrogen, progesterone acts on the endometrium leading to both morphological and biochemical changes through their receptors. Several reviews have been written on the molecular markers of receptive endometrium (Lessey 2000; Nikas 2000; Lindhard et al., 2002). The receptive endometrium shows a down regulation for estrogen receptor (ER) and progesterone receptor (PR) and appearance of cell adhesion molecules such as MUC-1 and integrin-αVβ3 (Lessey 2002). Additionally, leukemia inhibitory factor (LIF) and interleukin-1 beta (IL-1 beta) are involved in the molecular dialog between preimplantation embryo and the endometrium (Bulletti et al., 2005). Thus an ideal in vitro model to study the endometrial receptivity and blastocyst implantation should express these morphological and molecular features.

Early events in human pregnancy, such as apposition, adhesion and attachment of the preimplantation embryo towards the maternal receptive endometrium, remain an enigma due to ethical and technical constraints. There are few in vitro models for studying the above-mentioned events involved in blastocyst implantation (Bentin-Ley and Lopata 2000).

Mifepristone, a potent antiprogestin, and levonorgestrel, a synthetic progestational agent, are both used for emergency contraception (EC). Mifepristone inhibits blastocyst implantation by acting at the endometrial level to alter the molecular profile of the endometrium and the embryo, which is exposed to changed uterine milieu (Danielsson et al., 1997; Lalitkumar et al., 1998). If given immediately after ovulation, mifepristone is highly effective in preventing implantation and pregnancy (Gemzell-Danielsson et al., 1993). Levonorgestrel, a gestational compound, inhibits ovulation if administered during the preovulatory period. It also thickens the cervical mucus and acts as a barrier for sperm transport (Kessaru et al., 1974).
Both mifepristone and levonorgestrel in single doses have emerged as the most effective hormonal agents for EC (von Hertzen et al., 2002). The mode of action of levonorgestrel as EC and its effect on the reimplantation embryo and endometrium are not clearly understood and remains a matter of debate.

The aim of this study was to investigate the effects of mifepristone and levonorgestrel on human blastocyst attachment in an in vitro model that expresses characterized markers for endometrial receptivity. This study also examined the viability of human embryos exposed to levonorgestrel in the same model.

Materials and Methods

Ethics
This study was approved by the local ethics committee at Karolinska University Hospital, Solna, Stockholm and the Ethics Committee of Uppsala and Örebro, Sweden. Endometrial tissue for cultures and embryos (surplus embryos and those cryopreserved for ≥5 years) were obtained after informed consent from healthy fertile women and infertile women undergoing IVF respectively.

Endometrial biopsy
Endometrial biopsies were obtained from healthy volunteers (n = 22), 22–40 years of age, with normal menstrual cycles (25–35 days) and proven fertility. None of the women had used any hormonal or intrauterine contraceptive device for a minimum of 3 months prior to the biopsy. The biopsies were obtained by curettage using a Randall curette (Dinmed, Duttlingen, Germany) from the upper part of the endometrial cavity during cycle days LH + 4 to LH + 5. All subjects determined the LH peak in urine samples collected twice daily, from approximately cycle day 10 to LH + 2 by using a rapid self-test (Clearplan, Searle Unipath Ltd, Bedford, UK). A small sample of endometrium was taken for the morphological dating (data not shown).

Three-dimensional endometrial cell culture
Endometrial three-dimensional (3D) stromal and epithelial co-cultures mimicking endometrium were prepared as described by Bentin-Ley et al., (1994, 2000) with minor modifications. In brief, the endometrial tissues were minced with a scalpel and incubated with pancreatin–trypsin EDTA (0.05 g/ml of trypsin–EDTA solution, Sigma-Aldrich, Stockholm, Sweden) after which collagenase (150 IU ml⁻¹; Sigma-Aldrich) was added. The mixture of cells were filtered through a 40 micron mesh cell strainer (Falcon; BD Biosciences, USA) that allowed single stromal cells to pass through while the tissues were minced with a scalpel and incubated with pancreatin–trypsin–EDTA solution, Sigma-Aldrich, Stockholm, Sweden) after which collagenase (150 IU ml⁻¹; Sigma-Aldrich) was added. The mixture of cells were filtered through a 40 micron mesh cell strainer (Falcon; BD Biosciences, Belgium) that allowed single stromal cells to pass through while the glandular tubules were restrained in the filter. The stromal cells (0.5 × 10⁶ cells/ml) in the culture insert (Millipore Billerica, MA, USA) were embedded in 200 μl of purified sterile bovine collagen (3 mg/ml) in solution (PureCol, Inamed, Fremont, CA, USA). After forming the gel, murine basement membrane material (Matrigel; BD Biosciences) was coated on its top by adding 200 μl of matrigel and removing it with a polished Pasteur pipette and air drying for 5 min. Epithelial glands were seeded on the top of the matrigel to cover ~70% of the gel surface and were cultured with modified alpha-medium by adding 4 ml AmnioMax C100, penicillin–streptomycin (2000 IU), 0.2 ml l-glutamine (200 mmol/l, Life Technologies, Invitrogen AB, Stockholm, Sweden), 5 ml fetal calf serum (Invitrogen AB) and 0.5 g bovine serum albumin (Sigma-Aldrich) to 100 ml of the medium. The final concentrations of estrogen and progesterone were 0.3 and 900 nmol/l. The cells were cultured in an incubator at 37°C with 5% CO₂ in air. After 2 days of culture, the inserts were washed to remove the unbound cells and the medium was changed.

Treatment and embryo attachment
After 5–6 days of culture, when the epithelial cell layer was confluent, all cultures were treated with progesterone (10⁻⁸ M) along without or with mifepristone (10⁻⁵ M) or levonorgestrel (10⁻⁵ M) in 5 μl of ethanol as base. Only vehicle was added in the control group. Modified alpha medium was used for the culture of embryo—endometrial construct and the medium was changed every second day. Human embryos were initially cultured in S2 medium (Vitrolife Ltd, Gothenburg, Sweden) until blastocyst stage and were placed on the epithelial cell layer at the onset of treatment. Embryos were randomly assigned to the different treatment groups. After exposing the embryos along with the cell culture system to the above treatment for 5 days, the embryos were checked for any attachment with the culture matrix by washing thoroughly with phosphate-buffered saline (PBS). Embryo attachment to the cultures was recorded by light microscopy. They were fixed in 4% formalin and a portion was used to prepare paraffin blocks.

Immunohistochemistry
Endometrial cell culture sections of 15 μm were deparaffinized with xylene and rehydrated with graded ethanol. A microwave method of antigen retrieval was conducted to unmask the antigenic sites for factors such as ER-α, ER-β, PR-(A + B), PR-B and integrin α₃β₁ by placing the sections in 0.01 M sodium citrate buffer (pH = 6.0) and heating them for 10 min at 650 W in a microwave oven. All sections were subjected to quenching of endogenous peroxidase activity by placing slides in 0.3% hydrogen peroxide in methanol for 30 min at room temperature, and this was followed by incubation in respective blocking serum for 30 min. They were later incubated with the primary antibodies overnight in a humidified chamber at 4°C. Negative controls for both the primary and the secondary antibodies were included in every batch. The details of primary antibodies are mentioned in Table 1. The secondary antibody, consisting of biotinylated antibody (Vector Laboratories Ltd, Peterborough, UK), was applied for 30 min at room temperature. After rinsing with PBS, avidin–biotin–peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories Inc, Burlinghame, CA94010, USA) was added on the sections and incubated for 30 min at room temperature. Peroxidase DAB substrate kit (Vector Laboratories Ltd) was used to visualize the immunostain, and the sections were counter stained with hematoxylin. The protocol of integrin MUC-1 immunostaining was similar.

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Dilution</th>
<th>Host species</th>
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<td>ER-α</td>
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<td>1:20</td>
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<tr>
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<td>AbD Serotec, Oxford, UK</td>
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<tr>
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<td>1:2000</td>
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<td>PR-(A + B)</td>
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<td>Mouse</td>
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<tr>
<td>AR</td>
<td>DakoCytomation, Glostrup, Denmark</td>
<td>1:50</td>
<td>Mouse</td>
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<tr>
<td>IL-β</td>
<td>Abcam</td>
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<td>Mouse</td>
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<tr>
<td>VEGF</td>
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<tr>
<td>Integrin α₃β₁</td>
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<td>1:60</td>
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<tr>
<td>COX-2</td>
<td>Santa Cruz Biotechnology Inc., California, USA</td>
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except that after incubation with the primary antibody for 2 h at room temperature and a rinse in PBS, the sections were incubated in 4',6-diamidino-2-phenylindole dihydrochloride (Molecular Probes, Eugene, Oregon, USA) diluted 1:20 000 for 10 min. The sections were evaluated by indirect immunofluorescence microscopy. The cryosections (20 μm) with the attached embryos were subjected to immunohistochemistry using mouse monoclonal antibody against human cytokeratin 7 conjugated with fluorescein isothiocyanate (Abcam, Cambridge, UK). A negative control for all the antibodies were run by omitting the primary antibody or by using the isotype matched immunoglobulin G (data not shown).

Results

Cell culture
Endometrial stromal and epithelial cells were isolated from 20 women. Out of these, 18 were used for the cell culture as two biopsies did not yield a sufficient number of cells. Of these cultures, 11 were used in this study. One of the cultures was contaminated with fungi and the rest were used in establishing the fine technique. The transverse sections of the co-culture stained with hematoxylin showed stromal cells and lymphocytes embedded into collagen matrices with the surface covered with an epithelial cell layer (Fig. 1).

Expression of endometrial receptivity markers
The epithelial cells in the endometrial constructs were positive for ERs, PRs and androgen receptors as seen by immunohistochemistry. The immunoreactivity was mainly confined to the nucleus. The stromal cells were mostly negative for estrogen and androgen receptors, but slightly positive for PRs. The expression of interleukin-1β was very strong in the epithelial cells, whereas the stromal cells were only slightly positive. Both epithelial and stromal cells were positive for LIF and vascular endothelial growth factor (VEGF). The expression of cell adhesion molecule MUC-1 was detected only on the surface of the epithelial cells by immunofluorescence. Integrin αvβ3 was also expressed only by epithelial cells on the apical side. Stromal cells were negative for these factors. The representative micrographs are shown in Fig. 2.

Figure 1: Cross section of the in vitro endometrial construct stained with haematoxylin
This 3D culture system was developed by co-culturing human endometrial stromal cells (S) in collagen with an overlay of epithelial cells (E). Bar = 100 μm

Blastocyst attachment rate
A total of 46 embryos were used in this study. The culture treatments did not affect the hatching of any blastocysts. In the control group, 10 out of 17 (59%) blastocysts attached to the cell culture, whereas in cultures exposed to levonorgestrel, 6 out of 14 embryos (43%) attached, this was not significantly different from that of control cultures (Fig. 3). None of the 15 blastocysts in the culture exposed to mifepristone attached to the matrices (P < 0.01). One embryo in the control group and one in the levonorgestrel group left a hollow on the culture surface but did not attach to the matrix (Fig. 4). In some cultures, the embryos grew larger on attachment and contracted the gel including the epithelial cells (Fig. 5).

Confirmation of attached embryos by cytokeratin 7
The attached blastocysts were positively stained for the trophoblast marker cytokeratin 7 (Fig. 6). This confirms the observation made by inverted stereo zoom microscope that the group of cells attached to the cell culture is made up of embryonic trophoblast cells.

Discussion
Levonorgestrel is widely used for both regular and EC, even though its mechanism of action is still unclear (Gemzell-Danielsson and Marions 2004; Trussell and Jordan 2006). Many proposals have been made that it may affect follicular growth, ovulation, sperm motility, fertilization, blastocyst implantation and endometrial function (Croxatto et al., 2001; Marions et al., 2002). It is known that levonorgestrel administered before ovulation is more effective than post-ovulation treatment (Novikova et al., 2007). However, the critical factor in accepting any contraception by many political and religious constituencies is the belief that a new human life begins at the time that fertilization is completed and that the drug of choice should not affect the post-fertilization events. This study tries to answer one of the most important questions in the use of levonorgestrel as a contraceptive agent by showing that it has no direct effect on the attachment of human embryos to the endometrial construct. This study conducted in human samples, further supports the earlier findings in rodents and monkeys that post-coital administration of levonorgestrel does not interfere with post-fertilization processes required for embryo implantation, such as development of embryos, acquisition of endometrial receptivity and embryo attachment (Mulle et al. 2003; Ortiz et al. 2004).

Oral administration of 0.75 mg levonorgestrel in repeated doses during the early follicular phase suppresses proliferative activity in the endometrium and when administered in late follicular phase, it affects ovulation. However, it has no effect on the endometrium when administered in the secretory phase of the menstrual cycle (Landgren et al., 1989; Marions et al., 2002). In this study, we could not observe any differences in the human blastocyst attachment properties to endometrial constructs exposed to levonorgestrel compared with that of the control group. This supports the view that that endometrium exposed to levonorgestrel is still receptive for the human embryo implantation.
One of the mechanisms of action of contraceptive drugs is at the embryonic level. There is evidence that embryos collected from mifepristone exposed endometrium are nonviable (Ghosh et al., 1997). This is observed despite the fact that embryos exposed to mifepristone in vitro are viable and can give rise to live births (Wolf et al., 1990). Thus, degenerative changes observed in the embryo could be attributed to the altered molecular profile of endometrial secretory products present in the uterine cavity, where the preimplantation embryo develops (Catalano et al., 2003; Lalitkumar et al., 2005). In this study also, none of the embryos in the mifepristone group attached. After 5 days of culture with mifepristone, the embryos were either degenerated partially or lysed completely. The failure of these embryos to attach could be due to the effect of mifepristone on endometrial cell constructs, either leaving them non-receptive or leading them to a non-receptive state. The attachment failure could also be due to the altered molecular expression of the endometrial cells and their secreted products, affecting the viability and growth of embryos indirectly, since

**Figure 2**: Expression of endometrial receptivity markers in the in vitro endometrial construct as seen by immunohistochemistry

The nucleus of epithelial cells expressed steroid receptors ER-α (A) and ER-β (B). Both stromal and epithelial cells were positive for PR-A + B (C); a few epithelial cells were positive for PR-B (D) and androgen receptor (E). Expression of IL-β (F), VEGF (G), LIF (I) and COX-2 (K) were high in epithelial compartment. Immunostaining for integrin αvβ3 (J) and MUC-1 (L) was detected in the epithelial cells. The cells were negative for TNF-α (H). Bar = 100 μm

**Figure 3**: Blastocyst attachment rate in control, mifepristone and levonorgestrel exposed embryo–endometrial constructs

None of the embryos in the mifepristone group attached to the construct (*****p < 0.01 compared with the control; NS, non-significant compared with the control—Fisher’s exact test)
a direct effect of mifepristone on embryo viability has been ruled out.

This study indirectly showed that levonorgestrel has no effect on the human blastocyst in terms of its viability. All the embryos used in this study were unhatched blastocysts at the time of introduction into the culture. They hatched on Day 1 or 2 in the coculture system and were continuously exposed to levonorgestrel from Day 1 to 5. There was sufficient time for the embryo to undergo any degenerative changes levonorgestrel could cause to the unhatched blastocyst or the trophoblast cells of hatched embryos. We observed no changes in the attachment rate in the levonorgestrel group compared with that of controls. This indirectly shows that both the drug and the endometrial cell products in the culture do not affect the viability of human embryos.

Moreover, this study design with an in vitro model, in which the effect of levonorgestrel on human blastocyst development and attachment is investigated, has two important features to be noted. First, the 3D model with endometrial stromal and epithelial cells expresses the endometrial receptivity molecular markers mimicking human endometrium. The culture system with collagen matrix facilitates the stromal cells to grow in a 3D cellular structure with endocrine and paracrine communication leading to better homeostasis, as demonstrated earlier by Arnold et al. (2001). Progesterone and the drugs of choice in this study act though the classical PRs, even though their action through non-classical PRs could not be neglected. It was observed that epithelial cells in this study express both estrogen and PRs. Also the endometrial receptivity markers such as integrin α₃β₃, LIF, IL-1β, MUC-1 and VEGF were present in the in vitro endometrial constructs. Secondly, the effect of the contraceptive drug levonorgestrel on blastocyst attachment was tested and compared along with a well-established antifertility agent, mifepristone, as a positive control as it is known to inhibit both embryo viability and implantation in vivo. A similar culture model has been studied earlier to investigate the effect of antiprogestin Org 31710 on human blastocyst–endometrial interaction (Petersen et al., 2005). Thus to our knowledge, this is the best existing model available for studying human endometrial receptivity and embryo implantation.

Interestingly, we observed in two of the cultures, where the hatched blastocysts expanded but failed to attach, that the epithelial cells were displaced at the site of the embryos. Although this study does not deal with any molecular characterization of the embryonic factors in implantation, there are reports that human embryos produce gelatinase (Bischof et al., 1995). It is very likely that the epithelial cells are detached due to the degradation of the basement membrane rather than to cell lysis, as MMP-2 produced by the trophoblast cells is known to degrade collagen IV, a major content in the basement membrane (Puistola et al., 1989).

This study is limited to the attachment stage in understanding the blastocyst implantation process. Further studies with critical design and long culture conditions would throw more

Figure 4: Epithelial cells (E) at the site of an unattached, hatched blastocyst in the culture were found to be displaced, exposing the collagen gel (C)
Bar = 100 μm

Figure 5: Human embryos (E) attached to matrix (M) of endometrial constructs in the control (A) and levonorgestrel (B) groups
The embryos attached to the construct contracted the surface of the gels (C). Bar = 100 μm
light in understanding the molecular events in penetration of trophoblast cells into the matrix. Even though the histology of the endometrial construct in this study closely mimics human endometrium, its cellular composition is only partial with stromal and epithelial cells. This could be further improved with the incorporation of other cell types seen in the normal human endometrium as the role of factors produced by mononuclear and vascular cells in endometrial receptivity cannot be neglected. There is very limited information about the concentration of mifepristone or levonorgestrel at the endometrial cellular level when given orally for fertility control. But the dosage of these drugs used in this study is sufficient enough to inhibit the action of progesterone as shown by earlier studies (Catalano et al., 2003).

Future implications of this model could be to understand the gene expression of embryonic cells during the early interactions with the maternal system. This could be used to understand the process of human implantation events and to understand many unresolved clinical problems related to infertility. It may also be used to develop effective and more acceptable drugs for fertility regulation.

In conclusion, this study opens a broader avenue in accepting the use of a routinely used fertility regulating substance, levonorgestrel, by various groups as it does not affect the event of embryo–maternal attachment. Further studies with human endometrial samples exposed to levonorgestrel at different phases of the menstrual cycle, along with complete profiling of endometrial gene expression, may help to reveal the in-depth the molecular mechanism underlying the action of levonorgestrel as fertility regulating substance.

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