From DEPARTMENT OF WOMEN’S AND CHILDREN’S HEALTH
DIVISION OF OBSTETRICS AND GYNECOLOGY
Karolinska Institutet, Stockholm, Sweden

LEVONORGESTREL EMERGENCY CONTRACEPTION
EFFECTS ON ENDOMETRIAL DEVELOPMENT
AND EMBRYO IMPLANTATION

Chun-Xia Meng
孟春霞

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Opportunities are just for those who are well-prepared.

机遇只垂青有准备的人。
ABSTRACT

Background: The dialog between the developing embryo and receptive endometrium is under the control of ovarian steroids and numerous biomolecules, some of which have been suggested as “markers of endometrial receptivity”. Unintended pregnancies are common. The standard treatment with levonorgestrel (LNG, 1.5mg either in a single dose or in two doses with 12 hours intervals) provides women with a safe means of preventing unwanted pregnancy after unprotected intercourse. However, its mechanisms of action when used for emergency contraception (EC) remain a matter of discussion.

Overall aim: To study the effects of LNG used for EC on markers of endometrial receptivity, embryo implantation and first-trimester tissues.

Papers I and II: Endometrial biopsies were taken from fertil women with a regular menstrual cycle (n=12 for paper I; n=22 for paper II) during cycle days LH+4 to LH+5. The stromal and epithelial cells were isolated and cocultured in 3-dimensional endometrial constructs. Immunostaining of estrogen receptor (ER)-α and β, progesterone receptor (PR)-(A+B), vascular endothelial growth factor (VEGF), leukemia inhibitory factor (LIF), interleukin (IL)-1β, and cyclooxygenase (COX)-2 were present in both cultured epithelial and stromals cells, whereas the expression of PR-B, androgen receptor (AR), integrin αvβ3, and mucin 1 were confined to epithelial cells. Treatment with LNG did not change the expression of any markers studied or impair blastocyst attachment to the endometrial construct. Mifepristone, used as a positive control, up-regulated the ER-β and PR-B expression, whereas it down-regulated the expression of stromal VEGF, epithelial integrin αvβ3 and mucin 1. Mifepristone also inhibited blastocyst attachment in vitro.

Paper III: First-trimester decidua and chorionic villi were collected from women (n=9) who had self-administered LNG (1.5mg) for EC after ovulation and subsequently became pregnant and who opted to interrupt the pregnancy with vacuum aspiration. Samples from comparable, unexposed women (n=9) were collected as controls. No significant differences in the expression of ERs, PRs, AR or Ki67 in the samples exposed and unexposed to LNG were seen using immunohistochemistry.

Paper IV: Endometrial biopsies were taken from fertil women (n=8) on cycle days LH+6 to LH+8: i) throughout the normal cycle and ii) after treatment with LNG (0.75mg daily during LH+1 to LH+4). The treatment significantly reduced PR-A and PR-B immunoreactivity in glandular epithelium, whereas it increased LIF stromal immunoreactivity and mRNA expression. Differences in the expression of other markers of endometrial receptivity were insignificant between the two groups.

Conclusions: Levonorgestrel used for EC does not affect markers of endometrial receptivity studied neither in vitro nor in vivo. Levonorgestrel does not impair embryo attachment to a 3-D endometrial cell culture construct. Post-ovulatory administration of LNG has no effect on the expression of ovarian steroid receptors or Ki67 in first-trimester tissues. New agents with improved effects on follicular development, ovulation and endometrial receptivity should be developed and available to increase the EC efficacy.

Key words: Emergency contraception, levonorgestrel, endometrial receptivity markers, embryo implantation, first-trimester tissues
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<td>ABC</td>
<td>Avidin-Biotin-Complex</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>COX</td>
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<tr>
<td>cPLA2</td>
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<tr>
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<td>colony-stimulating factor-1</td>
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<td>DAB</td>
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<td>DAPI</td>
<td>4′, 6-diamidino-2-phenylindole dihydrochloride</td>
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<tr>
<td>E₂</td>
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<tr>
<td>EC</td>
<td>emergency contraception</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<td>ECP(s)</td>
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<td>ethylenediaminetetraacetic acid</td>
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<td>Engelbreth-Holm-Swarm</td>
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<td>estrogen receptor(s)</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>Flt-1</td>
<td>fms-like tyrosine kinase</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
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<td>IL-1R</td>
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<tr>
<td>IL-1R AcP</td>
<td>interleukin-1 receptor accessory protein</td>
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<tr>
<td>IUD</td>
<td>intrauterine device</td>
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<tr>
<td>IVF(-ET)</td>
<td><em>in vitro</em> fertilization(-embryo transfer)</td>
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<tr>
<td>KDR/flk-1</td>
<td>kinase domain region</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>LH</td>
<td>luteinizing hormone</td>
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<tr>
<td>LIF(R)</td>
<td>leukemia inhibitory factor (receptor)</td>
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<tr>
<td>LNG</td>
<td>levonorgestrel</td>
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<tr>
<td>mg</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>(m)RNA</td>
<td>(messenger) ribonucleic acid</td>
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<tr>
<td>MUC</td>
<td>mucin</td>
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<tr>
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<td>phosphate buffered saline</td>
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<td>platelet-derived growth factor</td>
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<td>PG(s)</td>
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<td>PRAKO</td>
<td>progesterone receptor-A knockout</td>
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<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<td>SE</td>
<td>standard error</td>
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<tr>
<td>SPRM(s)</td>
<td>selective progesterone receptor modulator(s)</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>NK</td>
<td>natural killer</td>
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<tr>
<td>UPA</td>
<td>ulipristal acetate</td>
</tr>
<tr>
<td>VEGF(R)</td>
<td>vascular endothelial growth factor (receptor)</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>3-D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>17β-HSD</td>
<td>17β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
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1 INTRODUCTION

1.1 MENSTRUAL CYCLE AND HUMAN ENDOMETRIUM

The menstrual cycle averages 28 days in length, but can range from 21 to 35 days in healthy women of reproductive age. It can be divided into three phases with regard to the endometrium: the menstrual phase, the proliferative phase and the secretory phase (Figure 1). The phases occur in response to hormones associated with regulation of the ovarian cycle. Menstruation and the proliferative phase occur during the follicular phase of the ovarian cycle, while the secretory phase corresponds to the luteal phase. At each phase of the menstrual cycle, the histological appearance of the endometrium reflects prevailing hormone production by the ovaries.

The endometrium is composed of two layers. The functional layer is the upper two thirds of the endometrium, which is sloughed off at menstruation, and is the site of proliferation, secretion and degradation. The basal layer comprises the lower third, which is retained during menstruation, and serves as the source for regeneration of the functional layer. The proliferative phase of the endometrium is stimulated and sustained by estrogens secreted by the developing ovarian follicles. The proliferative phase continues until 1 day after ovulation, which occurs at about day 14 of an ideal 28-day cycle. With ovulation, the corpus luteum forms and secretes progesterone. Under the influence of progesterone, the endometrial glands enlarge and become corkscrew-shaped, with their lumen filled by secretory products. Mucoid fluid produced by the gland epithelium is rich in nutrients required to support development of embryo if implantation occurs, especially glycogen. The arteries elongate and spiral through tissues of the functional layer. Under the influence of progesterone, decidualization begins in the late luteal phase and involves increased mitosis and differentiation of stromal cells. In the absence of implantation, the transient corpus luteum undergoes regression which results in an abrupt decrease in estrogen and progesterone levels with subsequent shedding of the functional layer.

Figure 1. Hormonal regulation of the female menstrual cycle
Source: modified from www.theholisticcare.com
1.2 CELLULAR COMPOSITION OF HUMAN ENDOMETRIUM

The endometrium is a complex tissue which can be divided into two hormone-responsive compartments: an epithelial compartment consisting of luminal (surface) and glandular epithelium; and a stromal compartment filled with fibroblasts, endothelial cells, and leukocytes. Luminal epithelial cells provide a defensive barrier and also act as the crucial site of embryo attachment. Glandular epithelial cells secrete numerous autocrine/paracrine factors that are required for endometrial development and/or embryo implantation. Endothelial cells are present in the walls of arteries and veins in stroma, playing active role in endometrial angiogenesis. Leukocytes are involved in inflammatory reaction, and form part of the immune system. Their presence varies across the menstrual cycle and throughout pregnancy (Salamonsen et al., 2000). Endometrial leukocyte populations include: neutrophils and eosinophils (prior to menstruation), macrophages and mast cells (throughout the menstrual cycle), T lymphocytes (throughout the entire cycle, with an increase prior to menstruation), B lymphocytes (present perimenstrually), and natural killer (NK) cells. The major leukocyte population present in the endometrial stroma when decidualization, implantation, and placentation occur consists of NK cells (Critchley, 2005). Regulation of leukocyte aggregation, proliferation and activation within the endometrium is not yet well understood. Fibroblast cells, secreting matrix metalloproteinases (MMP), are involved in the remodelling endometrial extracellular matrix (ECM). As the name suggests, ECM forms the extracellular part of endometrium which provides structural support to endometrial cells in addition to performing various other important functions. Endometrial macrophages can induce proliferation of fibroblast cells through the secretion of many cytokines and growth factors (Braundmeier et al., 2006).

1.3 HUMAN IMPLANTATION

Fertilization normally occurs in the fallopian tube ampulla 24 to 48 hours after ovulation. Between day 3 and 4 (approximately on cycle day 18 of an ideal 28-day cycle) the zygote migrates through the fallopian tube until it reaches the uterine cavity at the morula stage (Adams EC et al., 1956; Croxatto et al., 1972; Norwitz et al., 2001). The transition from morula to blastocyst is accompanied by cellular differentiation: surface cells become the trophoblast and give rise to extraembryonic structure including the placenta, whereas inner cell mass gives rise to the embryo. The blastocyst remains free floating within the uterine cavity for approximately 3 days before hatching occurs, around day 7 after ovulation. Thereafter, implantation occurs (Figure 2).

Human implantation probably includes three consecutive stages: apposition, adhesion and invasion. Apposition, the initial contact of a blastocyst to the uterine wall, is unstable. The next stage, stable adhesion, is characterized by increased physical interaction between the blastocyst and uterine epithelium. Microvilli located on the apical surface of syncytiotrophoblasts are thought to interdigitate with uterine microprotrusions of the epithelial apical surface known as “pinopodes” (Norwitz et al., 2001) or “uterodomes” (Adams SM et al., 2002). Invasion begins with syncytiotrophoblast penetrating the uterine epithelium.

Following blastocyst attachment and penetration to the endometrial luminal epithelium, the trophoblast then invades through the stroma, interacting with the ECM of decidualized stromal cells before establishing the maternal vasculature and forming a
functional placenta. The establishment and maintenance of pregnancy depends on an estrogen-primed endometrium under the influence of progesterone after ovulation (Harper, 1992).

Successful implantation is the end result of complex molecular interaction between the hormone-primed uterus and a mature blastocyst. The estimated rate of implantation in natural cycles is 15% to 30% (Miller et al., 1980). It decreases with age in a nonlinear fashion until age 35, at which point there is an approximately 3% decrease per year (Spandorfer et al., 2000).

### 1.4 THE IMPLANTATION WINDOW (OR UTERINE RECEPTIVITY)

Implantation occurs only during the “implantation window”, a limited period when the human endometrium is receptive for the embryo to implant. It begins on approximately day 6 after the luteinizing hormone peak (LH+6) and completes by LH+10 (or day 20-24 of a 28-day cycle) (Harper, 1992; Wilcox et al., 1999). This process requires synchronization between the developing embryo and the receptive endometrium. The dialog between embryo and endometrium is under the control of sex steroids (estrogen and progesterone), a variety of molecules (growth factors, cytokines, vasoactive factors), and other hormones including human chorionic gonadotropin (hCG) (Kodaman et al., 2004). Although the complex process of embryo implantation remains to be clearly characterized, numerous cellular and molecular markers of endometrial receptivity have been identified.

Uterine receptivity is defined as “the temporally and spatially unique set of circumstances within the endometrium that allows for successful implantation of the embryo” (Giudice, 1999). The features of uterine receptivity include histological changes in which the endometrium becomes more vascular and edematous, the endometrial glands display enhanced secretory activity, and the development of pinopodes on the luminal surface of the epithelium (Norwitz et al., 2001). In addition,
multiple signals synchronize development of the blastocyst and preparation of the uterus.

1.4.1 Morphological change – pinopodes

Balloon-like protrusions formed by microvilli on the apical surface of endometrial luminal epithelium are known as “pinopodes”. “Uterodomes” has been proposed as an alternative name, since the uterine surface protrusions observed in the human are not pinocytotic (Adams SM et al., 2002). The stages of pinopode development have been defined as: developing, fully developed and regressing (Nikas, 1999). There are conflicting results regarding the duration of human pinopode presence. Some studies proposed a short life span around cycle day 19 to 22 (Develioglu et al., 1999; Nikas, 1999; Aghajanova et al., 2003). Others detected that pinopods were present throughout the mid- to late-secretory phase or until the 11th week of pregnancy (Acosta et al., 2000; Usadi et al., 2003; Quinn et al., 2007), however they displayed cycle-dependent morphological changes. This indicates that the morphology of pinopodes, rather than their presence or absence, is of great significance (Usadi et al., 2003). It is commonly accepted that pinopodes vary among individuals in terms of the number and timing of their appearance. During the window of implantation pinopode presence coincides with increased progesterone levels, down-regulation of progesterone receptor B (Stavreus-Evers et al., 2001), and expression patterns of several cytokines and molecules which have been suggested as markers of endometrial receptivity. These include: leukemia inhibitory factor (LIF) and its receptor (LIFR) (Aghajanova et al., 2003), heparin-binding epidermal growth factor-like growth factor (HB-EGF) (Stavreus-Evers et al., 2002), integrin αvβ3 (Nardo et al., 2003). Pinopodes have been suggested as a marker of human endometrial receptivity, however there is some debate about this perception (Quinn et al., 2007). To date, there is no direct evidence that fully developed pinopodes influence the timing of human implantation.

1.4.2 Ovarian hormone regulation

The synchronized implantation process has many aspects, in which the role of steroid hormones is the best understood. Many events in uterine growth and function are regulated by the interplay between estrogen and progesterone. In general, progesterone antagonizes estrogen proliferation and metabolism. Androgens are common precursors of estrogens (Figure 3). Androstenedione is converted to testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD). Rapidly demethylation at C-19 aromatizes testerone to estradiol, which is the major estrogen secreted by human ovary. Estradiol may also arise from androstenedione via estrone, and estrone itself is secreted in significant daily amounts. It induces proliferation of epithelial cells and stromal fibroblasts during the pre-ovulatory phase, whereas after ovulation luteal progesterone induces glandular differentiation as well as stromal fibroblast proliferation and differentiation (Punyadeera et al., 2003). The orchestrated changes in estrogen and progesterone concentrations are essential in creating a receptive endometrium to allow implantation.

Ovarian hormones act via their ligand-specific receptors which are members of the nuclear receptor superfamily. Estrogen and progesterone receptors are localized predominantly in the cell nucleus, although some nucleocytoplasmic shuttling does occur (Kodaman et al., 2004).
1.4.2.1 Estrogen and estrogen receptors (ERs)

The most potent form of mammalian estrogenic steroid in humans is $^{17\beta}$-estradiol (E$_2$), which is produced primarily by the ovaries and the placenta (Speroff et al., 2005). Implantation requires a pre-ovulatory increase in E$_2$ secretion. Transcriptional effects of E$_2$ are mediated by two subtypes of the estrogen receptor (ER): $\alpha$ and $\beta$. The crucial role of ERs in fertility is confirmed by unresponsiveness to estradiol in adult female ER-$\alpha$ knockout mice, which are infertile (Lubahn et al., 1993). However, the ER-$\beta$ knockout mice are still fertile (Krege et al., 1998). In all types of endometrial cells, expression of both ERs reaches the maximum level in the late follicular phase (Lessey et al., 1988; Snijders et al., 1992; Matsuzaki et al., 1999). During the early luteal phase ER expression declines, followed by an increase in the mid and late luteal phases (Snijders et al., 1992). These changes reflect the cyclic changes in estradiol (which increases ER expression) and progesterone (which decreases ER expression). The ER-$\alpha$ appears to be the dominant ER isoform, suggesting that estrogenic effects occur mainly through ER-$\alpha$ (Fujimoto et al., 1999; Matsuzaki et al., 1999; Wang et al., 1999). ER-$\beta$ may play a role in the modulation of the effects of ER-$\alpha$ (Weihua et al., 2000). The presence of ER-$\beta$ positive stromal cells located close to vascular smooth muscle cells during cycle days 24-26 suggests some specific role of this receptor during decidualization. ER-$\alpha$ is also present in the cells of the endometrial vascular wall with highest expression during the peri-ovulatory period (Lecce et al., 2001).

1.4.2.2 Progesterone and progesterone receptors (PRs)

Progesterone, produced by the corpus luteum, facilitates the action of estradiol in inducing the LH surge that leads to ovulation (Hoff et al., 1983; Batista et al., 1992)
and stimulates the proliferation and differentiation of stromal cells. It plays an essential role in the initiation and maintenance of pregnancy.

Two subtypes of human progesteron receptors (PRs) have been described. PR-A is a truncated version of PR-B, lacking 164 amino acids at the N-terminus (Tung et al., 1993). Due to the distinct methods of descriptive quantification, the cell-specific changes in PR expression described in various studies are not always consistent with one another. However, in general, PR expression in endometrial glandular epithelium peaks in the late follicular and early luteal phase (reflecting induction of PR by estrogen) with a drastic decline at the mid secretory phase, whereas in stromal cells it shows only minor fluctuations (Lessey et al., 1988; Snijders et al., 1992).

The adult female mice with null mutation of the PR gene (PRKO) displays significant defects in all reproductive tissues, suggesting PR is essential for the female reproductive activities associated with progesterone (Lydon et al., 1995). Ablation of PR-A (PRAKO) results in severe abnormalities in ovarian and uterine function. Analysis of urine function in PRAKO mice reveals a progesterone-dependent proliferative activity of PR-B in the epithelium and provides evidence that that tissue-specific reproductive effects of PR-B are due to specificity of target gene transactivation rather than differences in tissue-specific expression relative to PR-A (Mulac-Jericevic et al., 2000; Conneely et al., 2001).

The establishment of normal endometrial receptivity appears to be tightly associated with down-regulation of epithelial PR (Lessey, Yeh et al., 1996). Many, if not all, endometrial factors and cytokines are directly or indirectly regulated by progesterone (Okulicz et al., 1993; Lessey, Yeh et al., 1996; Meseguer et al., 2001; Catalano et al., 2007; Cameron et al., 1997; Gemzell-Danielsson et al., 1997; Ghosh et al., 1998; Marions et al., 1999; Qiu et al., 2002).

1.4.2.3 Androgen and androgen receptor (AR)

Endometrium is a target for androgen action either directly via the AR or indirectly via the effect of estrogen derived from androgen aromatization. Although there is a significant quantity of androgens in the endometrium, their function has not been clarified, except for being estrogen precursor. Treatment with androgen can antagonize E2 action in the endometrium, and this effect is most likely mediated by endometrial AR (Slayden et al., 2001). Therefore, androgen may serve as antiproliferative factor in the endometrium. Androgens are also found to induce prolactin production by human endometrial stromal cells in vitro (Narukawa et al., 1994).

The immunohistochemical localization of AR in the human endometrium, decidua and placenta of early pregnancy has been investigated (Prodi et al., 1980; Horie et al., 1992; Castracane et al., 1998; Slayden et al., 2001). The positive nuclear staining of AR is predominantly in endometrial stroma, and is more intensive in the proliferative phase than the secretory phase (Slayden et al., 2001). Endometrial AR expression is induced by estrogen and androgens (Fujimoto et al., 1994; Lovely et al., 2000; Apparao et al., 2002) and suppressed by progestins and epidermal growth factor (EGF) (Slayden et al., 2001; Apparao et al., 2002).

Unlike ERs and PRs, the role of AR in endometrial function and development towards endometrial receptivity remains poorly understood. Endometrial AR expression is elevated in women with polycystic ovarian syndrome which is associated with poor reproductive ability (Apparao et al., 2002). In women and non-human primates,
treatment with antiprogestins suppresses estrogen-dependent mitotic activity in the endometrial glands (Wolf et al., 1989; Cameron et al., 1996; Baird et al., 2003). However antiprogestins do not bind to the estrogen receptor. Androgens also suppress estrogen-dependent endometrial proliferation and AR expression is increased by antiprogestin treatment (Slayden et al., 2001). These data indicate AR may be involved in the mechanism by which antiprogestins induce endometrial antiproliferative effects in the presence of estrogens (Brenner et al., 2002; Brenner et al., 2003).

1.4.3 Cellular adhesion molecules

Cell Adhesion Molecules are glycoproteins located on the cell surface involved in binding with other cells or the ECM in a process called cell adhesion. During the window of implantation, human endometrium and embryo express adhesion molecules which contribute to the blastocyst attachment to the endometrium. Among these molecules, intergrins and mucins are well studied.

1.4.3.1 Integrins

Integrins are a family of transmembrane glycoproteins, consisting of two non-covalently linked subunits: α and β. To date, 18α and 8β subunits have been identified in mammals, assembling into 24 distinct integrins (Hynes, 2002). Integrins play key roles in development, immune responses, leukocyte trafficking, hemostasis, oncogenic transformation, thrombosis and inflammation (Hynes, 2002). Diverse integrins have been found in the endometrium and most of them are consecutively expressed throughout the entire menstrual cycle (Lessey, Castelbaum, Buck et al., 1994). Among them, the α6β1 subunit of integrin undergoes cycle-specific changes in endometrial epithelial cells with higher expression during the putative window of implantation (Lessey et al., 1992; Lessey, Castelbaum, Buck et al., 1994; Nardo et al., 2003), which suggests a role in the establishment of uterine receptivity. Moreover, the aberrant expression of intergrin β3 in the epithelial cells has been associated with certain diagnoses among infertile women, including luteal phase deficiency (Lessey et al., 1992; Lessey, Yeh et al., 1996), endometriosis (Lessey, Castelbaum, Savin et al., 1994), hydrosalpinx (Meyer et al., 1997), and unexplained infertility (Lessey et al., 1995; Lessey, Ilesanmi et al., 1996). Functional blockade of integrin α6β1 on the day of implantation reduced the number of implantation sites in mice (Illera et al., 2000). The cycle-dependent pattern of integrin α6β1 suggests its regulation by steroid hormones. Treatment with estrogen or androgens down-regulates integrin α6β3 expression in Ishikawa cells, whereas EGF and transforming growth factor (TGF)-α induce its expression (Somkuti et al., 1997; Apparao et al., 2002). High estrogen concentration during the proliferative phase acts via its receptors to suppress intergrin expression. However the luteal progesterone induces expression of epithelial integrin α6β3, probably by down-regulating ERs, increasing expression of paracrine factors (e.g. EGF), or stimulating the synthesis of its ligand osteopontin (Achache et al., 2006). These data suggest that endometrial integrin α6β3 is either directly involved in the process of implantation, or at least serves as an effective marker of endometrial receptivity.
1.4.3.2 Mucin (MUC) 1

Mucins are a family of highly glycosylated glycoproteins found in the apical cell membrane of human epithelial cells of a great number of tissues, including uterine tissue (Devine et al., 1992). Among the cloned human mucins, only MUC 1 and a low amounts of MUC 6 have been found in the endometrial epithelium (Gipson et al., 1997).

MUC 1 is also known as polymorphic epithelial mucin, episialin or epithelial membrane antigen. It is secreted by endometrial epithelium and its expression is regulated by steroid hormones and the implanting embryo. After ovulation the MUC 1 concentration increases several-fold (Hey et al., 1994), which may be induced by the combined presence of progesterone and estradiol at the receptive endometrium (Meseguer et al., 2001). While the human embryo increases MUC 1 expression in endometrial epithelial cells during the apposition phase of implantation in vitro, during the adhesion phase the embryo induces a paracrine cleavage of MUC 1 at the implantation site. This indicates that MUC 1 may act as an endometrial anti-adhesive molecule which must be locally removed by the human blastocyst during the adhesion phase (Meseguer et al., 2001). This is consistent with the finding in mice that both the protein and messenger ribonucleic acid (mRNA) levels of MUC 1 decrease to barely detectable levels by day 4 of pregnancy, before the timing for blastocyst attachment. It has been proposed that in MUC 1-expressing cells there is a delicate balance between the adhesion and anti-adhesion forces, which can be shifted towards adhesion by strengthening the integrin-mediated adhesion or towards anti-adhesion by increasing the level of MUC 1 expression (Wesseling et al., 1995). Women with unexplained infertility might have a genetic susceptibility to failure of embryo implantation due to small MUC 1 allele size (Horne et al., 2001). Failure of implantation was found to be associated with abnormal endometrial expression of MUC 1 and retention of PR, particularly in the epithelial cells (Horne et al., 2005). In women suffering from recurrent spontaneous miscarriage the concentration of MUC 1 in uterine flushings was significantly lower than in fertile women during the window of implantation (Hey et al., 1995).

The above findings strongly suggest that progesterone-dependent regulation of MUC 1 appears to be an important factor in determining endometrial receptivity, although its actual role remains to be determined.

1.4.4 Biomolecular markers of endometrial receptivity

Several cytokines and biomolecules have been identified as markers of endometrial receptivity.

1.4.4.1 Angiogenic factors and their receptors

Angiogenesis is the formation of new blood vessels, an essential process in tissue growth and development. Ovarian, uterine, and placental tissues of the female reproductive system exhibit growth and regression in a dynamic and periodic manner. The endometrium is a major source of angiogenic factors during the menstrual cycle and pregnancy (Reynolds et al., 1992). Angiogenesis is influenced by many other growth factors and other substances, such as prostaglandins.
Vascular endothelial growth factor (VEGF) is an endothelial cell-specific angiogenic protein, abundantly expressed in human endometrium. It is an estrogen-responsive angiogenic factor with expression varying throughout the menstrual cycle peaking during the mid secretory phase (Shifren et al., 1996; Sugino et al., 2002). Angiogenesis can be induced by VEGF, which also increases the permeabilization of blood vessels (Keck et al., 1989). The VEGF-A ligand system is one of five isoforms created by alternative splicing of VEGF mRNA. This system plays an important role in new blood vessel formation and induces proliferation, migration and differentiation of endometrial endothelial cells. The expression of VEGF in endometrium is induced by many hormones and molecules, including progesterone, hCG, EGF, and relaxin (Ancelin et al., 2002; Kawano et al., 2002; Palejwala et al., 2002; Sengupta et al., 2008). Developing human blastocysts in culture medium also stimulate VEGF synthesis (Krussel J et al., 2000; Artini et al., 2008).

Two VEGF receptors have been identified: VEGFR-1 (fms-liketyrosine kinase, Flt-1) and VEGFR-2 (kinase domain region, KDR/flk-1) (Neufeld et al., 1999; Krussel JS et al., 2003). In addition to vascular endothelial cells, VEGF receptors are also expressed in glandular epithelial cells, decidualized stroma in early pregnancy in vivo (Sugino et al., 2002), and isolated endometrial epithelial and stromal cells (Krussel JS et al., 1999).

It is believed that VEGF and its receptors play important roles in both human and animal implantation and maintenance of pregnancy (Ghosh et al., 1998; Krussel JS et al., 1999; Ghosh et al., 2000; Rabbani et al., 2001; Wang et al., 2003; Wollenhaupt et al., 2004; Ghosh et al., 2005; Artini et al., 2008).

1.4.4.2 Interleukin (IL)-1

Macrophage produces IL-1, as do epidermal, epithelial, lymphoid, vascular and other tissues. This polypeptide possesses the ability to modulate the proliferation, maturation and functional activation of various cells, and to mediate inflammatory and immune responses (Lomedico et al., 1984; Dinarello, 1988).

Two biochemically distinct but structurally related molecules of IL-1 have been cloned: IL-1\(\alpha\) and IL-1\(\beta\). Although both are biologically active, only IL-1\(\beta\) is functional (Dinarello, 1988). The IL-1 receptor (IL-1R) family consists of type I IL-1R, type II IL-1R and IL-1R accessory protein (IL-1R AcP) (Dinarello, 1988). Type I receptor mediates the biological effects of IL-1, whereas type II receptor acts as a negative regulator (Sims et al., 1994). Another member of the IL-1 family is the receptor antagonist IL-1ra, which can block the binding and consequently the biological activities of IL-1\(\alpha\) and IL-1\(\beta\) (Dinarello, 1988).

The protein expression of IL-1\(\beta\) was detected in the endothelial cells of spiral blood vessels and isolated stromal cells throughout the menstrual cycle, with increasing staining from proliferative to secretory phase observed (Simon et al., 1993). The presence of type I IL-1R mRNA and protein was also detected in glandular and luminal epithelial cells throughout the cycle, with an increase during the secretory phase (Simon et al., 1993). These findings indicate that the IL-1 system may be an important paracrine/autocrine mediator of intercellular interactions in the endometrium. Elevated levels of IL-1\(\alpha\) and IL-1ra together with lower expression of type II IL-1R were found in the peritoneal fluid and serum from women with endometriosis (Kondera-Anasz et al., 2005). This indicates that impairment of normal IL-1 activity in women with
endometriosis might play an important role in pathogenesis and development of the disease.

The contribution of IL-1 to the implantation process was established using murine models. Blockade of maternal endometrial type I IL-1R with recombinant human IL-1ra prevented implantation in mice by interfering with embryonic attachment, without adverse effects on blastocyst formation, hatching, fibronectin attachment, outgrowth, and migration in vitro (Simon et al., 1994). Moreover, the implantation rate for women undergoing in vitro fertilization embryo transfer (IVF-ET) who have detectable serum concentration of IL-1α and IL-1β on the day of hCG administration appears to be higher than for those without detectable concentration of IL-1 (Karagouni et al., 1998). The role of IL-1α in the endometrium has not been as clearly defined as that of IL-1β. It is postulated that the latter is one of the factors mediating communication between the implanting blastocyst and the endometrium. In humans, expressions of integrin β3 and leptin were up-regulated by IL-1β in endometrial epithelial cell culture (Gonzalez et al., 2001). Furthermore, the embryonic IL-1 system appeared to be involved in up-regulation of integrin β3 in epithelial cells in vitro (Simon et al., 1997). Therefore, IL-1 may reinforce the paracrine cross-talk between blastocyst and endometrial epithelium. Type II IL-1R luminal secretion significantly decreased within the implantation window, suggesting that IL-1 inhibition was alleviated by down-regulating the expression of type II IL-1R, thus the pro-implantation action of IL-1 was facilitated (Boucher et al., 2001). Moreover, IL-1β was found to stimulate secretion of matrix metalloproteinase (MMP)-9 and metalloproteinase as well as the invasion in vitro (Librach et al., 1994).

1.4.4.3 Leukemia inhibitory factor (LIF)

LIF is a pleiotropic glycoprotein expressed by multiple tissue types. The LIF receptor shares a common gp130 receptor subunit with the IL-6 cytokine superfamily (Auernhammer et al., 2000). LIF acts through a cell surface receptor complex that comprises the LIF receptor (LIFR) and the gp130-receptor chain (Achache et al., 2006).

Expression of LIF mRNA in the endometrial epithelium has been shown to occur in a menstrual cycle-dependent manner, with a dramatic increase in the mid- and late-luteal phase as well as in the decidual tissues of first-trimester pregnancy (Charnock-Jones et al., 1994; Kojima et al., 1994; Arici et al., 1995; Vogiagis et al., 1996). A similar secretion mode appeared in cultured endometrial epithelial cells (Laird et al., 1997). Immunohistochemical methods allow observation of LIF protein in the luminal and glandular epithelium as well as in the stroma. In contrast to the epithelial staining, stromal staining was detected without noticeable cyclic variation (Vogiagis et al., 1996).

Evidence for the role of maternal endometrial LIF in the implantation process derives from the inability of transgenic mice lacking the LIF gene to present receptive endometrium, although their blastocysts are viable (Stewart CL et al., 1992). Abnormal LIF levels in infertile women further supports its proposed role in human implantation. The deregulation of endometrial LIF secretion throughout the menstrual cycle was found in women with unexplained infertility, especially those with multiple failures of implantation (Hambartsoumian et al., 1998). Similarly, LIF concentration in uterine flushing on day LH+10 is significantly lower in women with unexplained infertility.
Moreover, potentially functional mutations in the LIF gene infrequently occurred in women with unexplained infertility (Steck et al., 2004).

A variety of cytokines such as IL-1, tumor necrosis factor (TNF-α), platelet-derived growth factor (PDGF), EGF, and TGF-β, insulin-like growth factors (IGFs) are potent inducers of LIF expression in cultured endometrial stromal cells in a concentration- and time-dependent manner, whereas interferon (IFN)-α is a potent inhibitor of LIF production (Arici et al., 1995; Perrier d'Hauterive et al., 2004). Although LIF expression reaches maximal levels during the secretory phase, when the endometrium is under progesterone influence, its regulation by steroidal hormones is not yet established. On one hand, no direct evidence of steroid hormones (estradiol and progestins) stimulatory effects on LIF mRNA or protein expression in endometrial cell culture have been found (Arici et al., 1995; Hombach-Klonisch et al., 2005). On the other hand, progesterone treatment significantly reduced LIF secretion by endometrial explants in vitro (Hambartsoumian et al., 1998). However, in vivo treatment with mifepristone, a progesterone antagonist, reduced LIF expression in glandular epithelial cells at the expected time of implantation (Gemzell-Danielsson et al., 1997). Findings regarding the effect of hCG, secreted by trophoblasts, on LIF expression are also contradictory. In cultured human endometrial epithelial cells, hCG increased LIF secretion from follicular phase to secretory phase (Perrier d'Hauterive et al., 2004), whereas another study did not find any significant effect of hCG on LIF secretion by endometrial cells (Uzumcu et al., 1998).

To date, the role of LIF in the process of implantation is not clear. The LIF receptor is expressed in endometrial epithelium throughout the menstrual cycle (Cullinan et al., 1996) and in human pre-implantation embryos (Charnock-Jones et al., 1994; van Eijk et al., 1996; Chen HF et al., 1999), suggesting the embryo may be a target of LIF. During the implantation window, high levels of LIF and its receptor in the epithelium exist simultaneously with the appearance of pinopodes (van Eijk et al., 1996). Timing and regulation of LIF expression in the endometrium and of its receptor expressed by the blastocyst suggests a potential role of LIF signaling in physiological and pathological processes involving human implantation and infertility.

1.4.4.4 Prostaglandins (PGs) and cyclooxygenase (COX)

Prostaglandins are a family consisting of four members, named PGD2, PGE2, PGF2, and prostacyclin (PGI2), which are generated from membrane phospholipids by the consecutive action of two enzymes: cytosolic phospholipase A2 (cPLA2) followed by COX (Achache et al., 2006). In the uterus, PGs are produced by both epithelial and stromal cells. The PGF2 concentration during the mid secretory stage is significantly higher than in the late proliferative or late secretory phases, whereas the PGE2 level does not show cyclic variation (Maathuis et al., 1978). Cultured endometrial stromal cells produce PGI2 in response to estrogen and this reaction can be blocked by progestins (Levin et al., 1992). Prostaglandins are involved in the control of cytokine release, cell growth, differentiation, and vasoactive effects (Kelly et al., 2001). Evidence for the role of PGs in blastocyst implantation in rodents was established by using an inhibitor of PG synthesis (Kennedy, 1977) and using cPLA2 knockout mice (Song et al., 2002).

Currently, three isozymes of COX have been identified: COX-1, COX-2, and COX-3 (a variant of COX-1) (Smith et al., 1996; Chandrasekharan et al., 2002). Gene targeting
in mice has established distinct functions for the COX isoforms. Targeted disruption of COX-2, but not COX-1, produces multiple failures in the reproductive processes of female mice, including ovulation, fertilization, implantation, and decidualization (Lim et al., 1997). It is found that COX-2-derived PGE₂ is essential for ovulation, whereas COX-2-derived PGF₂α is essential for implantation and decidualization (Lim et al., 1999; Matsumoto et al., 2001). A COX-2 inhibitor in combination with LNG could disturb the ovulatory process in a higher proportion of cases than LNG alone, thus this combination could be used for emergency contraception (EC), especially if the unprotected intercourse occurred during high-risk period (Massai et al., 2007).

During the implantation period in humans, expression of COX-2 was found in the luminal epithelium and the perivascular cells (Marions et al., 1999). Expression of COX-1 and COX-2 was also detected in cultured epithelial cells and stromal cells (Yang H et al., 2002). The pathology of endometriosis is associated with the misregulated COX-2 expression. Studies have found an increased expression of COX-2 in the endometrial epithelium or stromal cells in women with endometriosis (Ota et al., 2001; Chishima et al., 2002; Matsuzaki et al., 2004), indicating abnormal PG generation is correlated with pathological abnormalities in endometriosis.

In the human endometrium, COX-2 expression is regulated by various cytokines. Progesterone may inhibit the COX-2 synthesis in the endometrium (Critchley et al., 1999) and decidual cells of first-trimester pregnancy (Ishihara et al., 1995). It is distinctly up-regulated by IL-1β, IL-17A, TGF-β1 in the stromal cells (Huang et al., 1998; Wu et al., 2005; Chang et al., 2008; Hirata et al., 2008). Low concentration of granulocyte-macrophage colony-stimulating factor (GM-CSF) increases the COX-2 enzyme activity in luteal-phase stromal cells, whereas high concentration induces inhibition (Wang et al., 2006).

1.4.4.5 Tumor necrosis factor (TNF)-α
As it is named, TNF has been associated with in vitro and in vivo killing of tumor cells. It has been implicated in ovulation, corpus luteum formation and luteolysis, and aberrant levels of TNF are associated with diverse reproductive diseases such as recurrent spontaneous abortions and endometriosis (Haider et al., 2009). The gene and protein expression of TNF-α is present in the endometrium, and its activity is increased during the proliferative phase, declined in the early secretory phase, and increased again in the mid to late secretory phase (Tabibzadeh, 1991; Hunt et al., 1992). This expression fluctuation suggests positive associations with levels of female sex hormones which show similar cyclic fluctuations. The TNF-α mRNA and protein are also present in human placenta and endometrial cells at early and late stages of gestation (Chen HL et al., 1991). TNF-α exerts multiple influences on the cellular growth. Local estrogen biosynthesis in human endometrial glandular cells could be induced by TNF-α, which also directs estrogen metabolism to be more hormonally active (Salama et al., 2009).

1.4.4.6 Additional markers of endometrial receptivity
There are clearly many other cytokines and growth factors involved in endometrial functioning and in the process of implantation that merit more detailed discussion. These include: IL-6, IL-11, HB-EGF, the EGF family, TGF-β, colony-stimulating factor-1 (CSF-1), IGFs, insulin-like growth factor binding protein-1 (IGFBP-1), MMP,
calcitonin, homeobox genes (Giudice, 1999; Cavagna et al., 2003; Achache et al., 2006; Guzeloglu-Kayisli et al., 2009). However as these additional markers of endometrial receptivity were not included in this project, these will not be discussed here.

1.5 EMBRYO QUALITY / GRADE

Embryo quality definitely affects the implantation rate and pregnancy outcome. A three-part scoring system based on blastocyst expansion, inner cell mass and trophectoderm development has been developed to grade human blastocysts before transfer to women undergoing IVF (Gardner et al., 2000).

Briefly, blastocysts are graded as 1 to 6 based on their expansion degree and hatching status: 1. an early blastcyst with a blastocoel less than half of the embryo volume; 2. a blastocyst with a blastocoel no less than half of the embryo volume; 3. a full blastocyst with a blastocoel completely filling the embryo; 4. an expanded blastocyst with a blastocoel volume larger than that of the early embryo and with a thinning zona; 5. a hatching blastocyst with the trophectoderm starting to herniate through the zona; 6. a hatched blastocyst which has completely escaped from the zona.

For blastocysts graded as 3-6 (full blastocysts onward), development of the inner cell mass is assessed as: A. many cells tightly packed; B. several cells loosely grouped; C. very few cells. The trophectoderm is assessed as: A. many cells forming a cohesive epithelium; B. few cells forming a loose epithelium; C. very few large cells.

1.6 FIRST-TRIMESTER DECIDUA AND CHORIONIC VILLI

The decidua is an active tissue which forms the specialized endometrium of pregnancy. The biochemical dialogue between feto-placental and maternal units must pass through the decidua. Decidual cells are derived from endometrial stromal cells, under the sequential influence of estrogens and progesterone (Marx et al., 1999). Thus they appear during the luteal phase and continue to proliferate during early pregnancy, eventually lining the entire uterus including the implantation site. The decidual cells are characterized by the accumulation of glycogen and autocrine/paracrine agents (Handwerger, Harman et al., 1992; Handwerger, Richards et al., 1992).

Human placenta development is established by trophoblast invasion into the endometrium and its vasculature. Trophoblastic cells, which form the outer layer of the pre-implanting blastocyst, attach to the uterine epithelium, invade or destroy the epithelial barrier in endometrium, and ultimately establish direct contact with the maternal blood flow. The transportation as well as metabolic and endocrine function of the placenta resides primarily in the floating villi which are covered by two trophoblastic layers, the inner cytotrophoblast stem cells and the outer-layer of highly differentiated syntiotrophoblast (Korgun et al., 2006). Cells of the syncytium have irreversibly lost their ability to divide and grow, by fusion of cytotrophoblast cells. Vascularity, as well as vascular permeability, is enhanced in the decidualized endometrium.

1.7 IN VITRO MODELS FOR HUMAN BLASTOCYST IMPLANTATION

Significant advances in the understanding of embryo implantation have been made by using animal models, especially mice and non-human primates. However, the results
from animal studies cannot be extrapolated unconditionally to humans as the process of human implantation may be unique. For ethical and legal reasons, the implantation of a blastocyst in human endometrium cannot be studied in vivo. Therefore, the molecular and cellular events that mediate human embryo implantation remain largely unknown. In the absence of in vivo implantation sites, in vitro models mimicking the different stages of human embryo implantation during the first few days of pregnancy have recently been developed.

Solid-phase assay is used to investigate the function of specific proteins in supporting human trophast attachment and outgrowth by placing the embryo on a protein-coated coverslips (Chobotova et al., 2002). This simple type of solid-phase model is useful since it allows the function of specific proteins to be determined individually. However, as endometrium is a complicated tissue with interactive autocrine/paracrine signaling among various cell types, its absence in this assay limits the information output.

Separation of human endometrium into its epithelial and stromal components has been achieved through collagenase digestion. In contrast to stromal cell populations, the epithelial cells survive in short-term primary culture, but high-density epithelial cultures remain viable for longer period (Kirk et al., 1978). Monolayers of endometrial epithelial cells in growth chambers were found to present microvilli, cilia and cytoplasmic protrusions as observed in vivo (Lindenberg et al., 1984). When human blastocysts were added, they attached to the monolayer cell cultures and exhibited trophoblastic cells outgrowth (Lindenberg et al., 1986). Similar models consisting of human endometrial epithelial cells monolayers have been used to examine the importance of embryo-derived signals in regulating protein expression in humans (Simon et al., 1997; Gonzalez et al., 2000; Meseguer et al., 2001). However, as epithelial cells lose their structural polarity and differentiated function if they grow as monolayers (Schatz et al., 1990), these cultures can provide only limited information on blastocyst adhesion and penetration. Basement membrane material (Matrigel) has been demonstrated to promote a differentiated morphology of mouse mammary epithelial cells (Li et al., 1987) and human endometrial epithelial cells (Schatz et al., 1990; White et al., 1990). Matrigel, a urea extract of the basal lamina-rich Engelbreth-Holm-Swarm (EHS) tumor, mimics natural basal lamina and can be used as a substrate for functional epithelial cells in culture. Matrigel contains entactin-laminin, collagen type IV, heparin sulphate proteoglycan and some growth factors such as TGF-β, EGF, IGFs, basic fibroblast growth factor (bFGF) and PDGF, which occur naturally in the EHS tumor (Classen-Linke et al., 1997).

A monolayer culture of human stromal cells provided an in vitro model for studies on the process of human decidualization (Irwin et al., 1989). Human hatched blastocysts not only adhere to, invade and penetrate into a cultured endometrial stromal cell monolayer, but also produce higher levels of hCG compared to those cultured on plastic, indicating that stromal cells secrete factors that promote trophoblast survival and renewal (Carver et al., 2003).

In contrast to the in vivo estrogen responsiveness demonstrated by most cell types in the uterus, the in vitro culture of human or rodent endometrial epithelial cells alone were either unresponsive to estrogen or required high concentrations to achieve minimal responses (Inaba et al., 1988; Alkhalaf et al., 1991; Uchima et al., 1991). A considerable amount of experimental evidence supports the relationship between epithelial and stromal cells in the endometrium. Interactions between epithelial and stromal cells restored the estrogen responsiveness of epithelial cells in mice (Cooke et
al., 1986; Inaba et al., 1988) and in humans (Pierro et al., 2001), and also modified the effect of progesterone on protein secretion by epithelial cells (Mahfoudi et al., 1994). In addition, endometrial stromal cells were also demonstrated to regulate both growth and differentiation of primary epithelial cells in humans (Arnold et al., 2001).

As described above, in vitro models containing only epithelial cells or stromal cells cannot represent the in vivo physiological condition and is therefore insufficient for implantation studies.

A 3-dimensional (3-D) cell culture system has been described, in which the human endometrial stromal cells are seeded into a collagen matrix in culture well inserts, with a thin covering of basement membrane material (Matrigel); the epithelial cells are embedded on top and grow in a monolayer (Bentin-Ley et al., 1994; Kim et al., 2005). This culture system has been confirmed to imitate the day-to-day morphology of the cycle, including the presence of pinopodes (Bentin-Ley et al., 1995). This 3-D model has been useful for investigating interactions between endometrial stromal cells and epithelial cells, but studies in conjunction with blastocyst are rather limited. The attachment of blastocysts to the top of endometrial pinopodes was observed in all cases (Bentin-Ley et al., 1999).

1.8 EMERGENCY CONTRACEPTION (EC)

Despite the availability of effective contraceptive method, a large number of pregnancies are unintended. More than half of unintended pregnancies – an estimated 45.5 million worldwide – are resolved by induced abortion each year (Henshaw et al., 1999). Although the situations leading to contraceptive failure are diverse, many women know the reasons why they became pregnant (pill missing, condom slippage, etc). Emergency contraception provides women with a safe means of preventing pregnancy following unprotected sexual intercourse (FFPRHC, 2006). It is recommended for use in the following situations: breakage of condom, displacement of an intrauterine device (IUD), missed pills, unsuccessful withdrawal or non-use of contraception, including sexual assault. Any woman at risk of unwanted pregnancy may need these methods occasionally. Millions of unwanted pregnancies could be avoided if EC were widely accessible and properly used (Berer et al., 1995).

1.8.1 The fertile window and timing of EC

Although intercourse can take place at any stage of the ovarian cycle, there is only a limited time around ovulation when pregnancy will result. Fertilization must occur within 24 hours of ovulation, since after that time the oocyte deteriorates rapidly and fertilization then either fails or gives rise to a defective embryo (Baird, 2009). In contrast, spermatozoa can survive in the female reproductive tract for 5-6 days after intercourse (Muller et al., 2003). Therefore, the high-risk fertile phase extends from 5 days before ovulation to the day of ovulation (Wilcox et al., 1995; Wilcox et al., 2000). However, timing of the fertile window can be highly unpredictable, even if the menstrual cycle is regular. The possibility of late ovulation produces a persistent risk of pregnancy even into the sixth week of the cycle (Wilcox et al., 2001; Trussell et al., 2003). The estimated probability of conception with one act of intercourse relative to the day of menstrual cycle is listed in Figure 4. Therefore, EC should be recommended at any time during the cycle, when unprotected intercourse has occurred.
1.8.2 Theoretical targets of EC

Emergency contraception could theoretically prevent pregnancy by affecting the following reproductive processes (Croxatto et al., 2001):

- Follicle maturation and ovulatory process
- Sperm penetration into the cervix and migration into the fallopian tube
- Fertilization, zygote development and transport in the fallopian tube
- Pre-implantation development within the uterus
- Endometrial receptivity
- Blastocyst signaling, adhesion and invasion
- Corpus luteum function and responsiveness to hCG

1.8.3 Approaches to EC

Several approaches to EC have been described (Cheng et al., 2008), classified as pills containing synthetic hormones and insertion of an IUD. Hormonal pills are often referred to as “post-coital pills” or “morning-after pills” in the media and among laypeople. These pills include high-dose estrogens, danazol, combined oral contraceptives containing estrogen and progestin (Yuzpe Method), a progestin alone (LNG), and selective progesterone receptor modulators (SPRMs) like mifepristone. Ulipristal acetate, a new type of SPRMs, has recently been developed and come onto the market. Currently, the gold standard regimen of EC recommended by World Health Organization (WHO) is the LNG-only pill.

1.8.4 Efficacy of emergency contraceptive pills (ECPs)

Although the copper-bearing IUD is the most effective EC method, its wide-spread use is limited due to logistic and medial reasons. Administration of oral hormonal pills is usually considered more convenient, with almost no medical contraindications (FFPRHC, 2006).

Figure 4. Probability of clinical pregnancy with one act of intercourse relative to the day of menstrual cycle

Data from Wilcox et al., 2001; Trussell et al., 2003
The absolute efficacy of ECPs remains undetermined and depends on the specific formulation, doses of regimen, time interval between unprotected intercourse and treatment, as well as the risk of conception. At present, the proportion of pregnancies prevented by EC compared with the expected number without treatment has been reported to vary from 57% to 95% (Ho et al., 1993; WHO, 1998; WHO, 1999; Rodrigues et al., 2001; Arowojolu et al., 2002; von Hertzen et al., 2002; Xiao et al., 2002; Hamoda et al., 2004; Ngai et al., 2005). The earlier an ECP treatment is given, the more effective it is (WHO, 1998; Piaggio et al., 1999). While LNG is more effective than the Yuzpe regimen (WHO, 1998), a single dose of mifepristone (≥50mg) has higher EC efficacy than both (Ashok et al., 2002; Cheng et al., 2008).

1.8.5 Side effects of ECPs

The side effects of ECPs are rare, self-limiting and usually mild. Nausea (up to 60%) and vomiting (up to 20%) are the main side-effects of the Yuzpe regimen (Glasier et al., 1992). Since estrogen is the component which causes the nausea and vomiting, the LNG-only pill has significantly fewer side effects than the Yuzpe regimen (Ho et al., 1993; WHO, 1998; Lee et al., 1999). Mifepristone is better tolerated than the Yuzpe regimen with significantly fewer side effects except for a delay in the onset of menstruation (Ashok et al., 2002).

1.8.6 Easy accessibility of ECPs

Some people are concerned that easy availability of ECPs may encourage unprotected intercourse particularly among young women. A meta-analysis including eight randomized controlled trials has shown that advance provision of ECPs did not lead to increased frequency of unprotected intercourse, nor changes in contraceptive methods used (Polis et al., 2007). Similarly, we have shown that abortion-seeking women who had experience in using ECPs were more likely to have had adopted contraception during the cycle of conception and they had more general knowledge on contraception (Meng et al., 2009). There is also a study that shows EC use is not associated with increased risk for chlamydia infection (Stewart HE et al., 2003).

1.8.7 EC use in Sweden

Following the introduction and information provided by the Swedish Association for Sexuality Education Program, the Yuzpe regimen was approved for use in Sweden in 1993, and in 1995 it became possible for midwives to prescribe the Yuzpe regimen in a 4-tablet formula with each tablet containing 50mg ethinyl estradiol and 0.25mg of LNG. The use of Yuzpe regimen for EC continued until a licensed product containing LNG without estrogen became available in Sweden (NORLEVO®, NYCOMED) in May 2000. From April 2001 on, it has been available over-the-counter and free of charge in youth clinics. In May 2003, another brand of LNG-only pill (POSTINOR®, Schering AG) came to the Swedish market. In 2004, one tablet containing 1.5mg LNG substituted the two tablets of 0.75mg in Sweden. The insertion of a copper-bearing IUD for EC is also available but rarely used in Sweden.
1.9 YUPZE REGIMEN

The ECP regimen first approved by the United States Food and Drug Administration (FDA) is called the Yuzpe Regimen, named for Canadian Professor A. Albert Yuzpe, who in 1974 published the first study describing the efficacy and safety of post-coital contraception (Yuzpe et al., 1974). The Yuzpe regimen typically used consists of 200mg of ethinyl estradiol in combination with 1.0mg of LNG. Half the dose is taken within 72 hours after unprotected intercourse, and the other half is taken 12 hours later (Yuzpe et al., 1977). In the 1980s, licensed product of Yuzpe regimen became available for post-coital contraception in Canada, United Kindom and Finland. WHO added the Yuzpe regimen to its essential drugs list in 1996. The FDA declared the Yuzpe regimen safe and effective in 1997 and approved a Yuzpe-based EC kit in 1998. Based on the global need for EC, the widely used Yuzpe regimen has been evaluated intensively by the WHO.

There is good evidence that the post-coital contraceptive effect of the Yuzpe regimen is mainly due to a delay of an inhibition of ovulation and insufficient corpus luteum function (Ling et al., 1983; Swahn et al., 1996). Its direct effect on the endometrium is limited, if any (Ling et al., 1983; Kubba et al., 1986; Swahn et al., 1996).

1.10 LEVONORGESTREL (LNG)

Levonorgestrel is a synthetic progestational hormone with actions similar to those of progesterone (Figure 5) and about twice as potent as norgestrel (Jones et al., 1979).

![Figure 5. Molecular structures of progesterone, levonorgestrel, mifepristone, and ulipristal acetate](image)

The LNG-only pill is most commonly used for EC. The dosing strategy approved by the United States FDA is 0.75mg as soon as possible within 72 hours of an unprotected intercourse, with a second dose of 0.75mg taken 12 hours later. The second dose can also be taken 12-24 hours after the first without reducing the efficacy (Ngai et al., 2005; Hansen et al., 2007). A single dose of 1.5mg LNG is as effective as the split-dose without changing the efficacy or worsening any side effects (von Hertzen et al., 2002; Hansen et al., 2007), therefore this is the currently recommended regimen by WHO. Studies of efficacy show that LNG can also be used between 73 and 120 hours after an unprotected intercourse (Rodrigues et al., 2001; von Hertzen et al., 2002; Ngai et al., 2005).

Despite being widely used for EC worldwide, the mechanisms of action of the standard LNG regimen are not fully known. A number of studies have been conducted to address this issue. If administered at least 2 days prior to the LH surge, LNG causes either a delay or an inhibition of the LH surge, and therefore inhibits or delays ovulation in women (Durand et al., 2001; Marions et al., 2002; Croxatto et al., 2004;
However if administered only one day prior to ovulation, the LH surge occurs as expected but causes a significant shortening of the cycle length, indicating that LNG may also disrupt corpus luteum formation causing premature luteinisation of unruptured follicle (Okewole et al., 2007). However, others believe that peri- and post-ovulatory administration of LNG does not impair corpus luteum function or endometrial morphology (Durand et al., 2001). Levonorgestrel does not influence sperm acrosome reaction (Yeung et al., 2002; do Nascimento et al., 2007). It inhibits spermatozoa-oocyte fusion as well as decreases the curvilinear velocity of spermatozoa only at high concentration, and the contribution of these effects to EC is unlikely to be significant (Yeung et al., 2002). It appears that LNG does not impair the cervical mucus either, because viable spermatozoa were found in the genital tract 24-28 hours after intake of the pill (do Nascimento et al., 2007).

Another proposed mechanism is that LNG interferes with function or protein expression of the endometrium. Glycodelin-A is the major progesterone-regulated glycoprotein secreted into the uterine cavity by secretory-phase endometrial glands. Absence of contraceptive glycodelin-A during peri-ovulatory midcycle in endometrium is consistent with an open “fertile window” (Seppala et al., 2002). Levonorgestrel taken for EC 3-4 days prior to the LH surge was found to alter the luteal-phase secretory pattern of glycodelin-A (Durand et al., 2005). This may be one of the mechanisms of action of LNG when administered before the LH surge. However, another study declared that LNG taken before the follicular rupture does not influence the glycodelin-A level in uterine flushing 24-28 hours after LNG intake. (Durand 2005). Therefore, the role of glycodelin-A in the LNG EC mechanism remained to be determined.

If administered after ovulation has been triggered, levonorgestel does not have any detectable effect on the endometrium or progesterone level when given after ovulation (Spona et al., 1975; Durand et al., 2001; Marions et al., 2002; Marions et al., 2004). Neither does it interfere with post-fertilization events in Cebus monkeys or in rat (Muller et al., 2003; Ortiz et al., 2004). Evidence in humans is lacking due to the inaccessibility of human implantation sites. Debate whether post-coital effects of LNG are due to ovulation suppression or to an involvement in post-ovulatory events is extensive, therefore further research is needed. Increased knowledge about the mechanism of action could hopefully increase acceptability and availability of this method, thus reduce the number of induced abortions.

### 1.11 MIFEPRISTONE

Considering progesterone facilitates the action of estradiol in inducing the pre-ovulatory LH surge, and is essential for pregnancy initiation and maintenance, it was recognized that development of a therapeutic PR antagonist would be a significant advance in contraceptive technology. Antiprogestins could thus function as contraceptive agents by inhibiting ovulation, rendering the endometrium nonreceptive for implantation or inducing shedding of the endometrium and dislodging the implanting embryo (Spitz et al., 1996).

Mifepristone, also known as RU486, is a synthesized derivative of 19-norsteroid with high affinity for PR (Lahteenmaki et al., 1987), which presents less antiglucocorticoid properties than the original compound (Spitz, Bardin, 1993). In the presence of progesterone, mifepristone is an antagonist, but alone it exhibits endometrial
progestational effects at low doses and an antiproliferative (antiestrogenic) effect at higher doses (Wolf et al., 1989). It became the first available active antiprogestosterone and has been used successfully as a medical alternative for early pregnancy interruption. At low doses suitable for EC, it exhibits antiprogestin but not antiglucocorticoid action (Croxatto et al., 2001).

Taken within 72 hours of unprotected coitus, mifepristone is effective as an EC. A single dose of 10mg, 50mg or 600mg has been found to be equally effective in prevent pregnancies and the administration could be extended to 120 hours after coitus (WHO, 1999). Lower doses are associated with less disturbance of the menstrual cycle (WHO, 1999). In some countries, 25mg and 200mg are also available.

In contrast to Yuzpe regimen and LNG, the mechanism of action of mifepristone is well documented. The effects on follicular development and ovulation are dependent on dosage and the time of treatment during the menstrual cycle. Administration of mifepristone during the first 3 days of the follicular phase, when no dominant follicle is present, has no effect on follicular growth and fails to alter subsequent events of the cycle (Stuenkel et al., 1990). If taken during the mid and late proliferative phase, a variety of regimens with a single dose as low as 10mg have been shown to interrupt follicle development thus delay or inhibit the ovulation (Shoupe et al., 1987; Batista et al., 1992; Ledger et al., 1992; Marions et al., 2002; van der Stege et al., 2006). The effect is most pronounced when the dominant follicle reaches a diameter of 12mm (Croxatto et al., 1995). At low doses, 1-10mg, ovulation is delayed but not necessarily abolished (Croxatto et al., 1993; Spitz, Croxatto et al., 1993; Godfrey et al., 2004).

Mifepristone affects the endometrial receptivity in a dose-dependent manner. With a single dose of 200mg given shortly after the LH surge, mifepristone blocks the down-regulation of PR and significantly affects markers of human endometrial receptivity (Swahn et al., 1990; Maentausta et al., 1993; Beier-Hellwig et al., 1994; Gemzell-Danielsson et al., 1994; Cameron et al., 1997; Gemzell-Danielsson et al., 1997; Marions et al., 1998; Marions et al., 1999; Qiu et al., 2002; Sun et al., 2003). It therefore functions as an effective luteal-phase contraceptive method (Gemzell-Danielsson et al., 1993). However, low dose of mifepristone (10mg) taken in the early luteal phase does not cause any changes in the expression of integrin α4 and β3, COX-1 and COX-2, or pinopodes, but inhibited the down-regulation of PR in the endometrium (Marions et al., 2002).

As the research can not be performed in human in vivo, animal models provide evidence of the antiprogestins effects on post-fertilization events. Post-coital administration of mifepristone has been shown to inhibit egg development and implantation in rats (Psychoyos et al., 1987).

Although mid-dose of mifepristone has higher efficacy than LNG (Cheng et al., 2008), it is unlikely to become widely available as an EC in the foreseeable future because it can be used and is available as an abortifacient if combined with a prostaglandin analogue. Today, mifepristone in low or mid doses (10mg, 25mg or 50mg) for EC use is only available in China.

1.12 BARRIERS TO EC USE

Introduction of ECPs in many countries has generated much controversy and litigation. One of the main barriers to the wide-spread use of EC around the world is the lack of knowledge on the mechanism of action especially with regard to the effect on the
endometrium, endometrial function and embryo implantation. In regions such as Latin America where there are laws protecting human life following fertilization, it is commonly believed that ECPs may interrupt the development or implantation of a newly formed embryo. Therefore, an increased knowledge about the mechanisms of action and safety of EC is essential for the development of new methods as well as for optimizing the use of those already available. This knowledge may also influence individual and cultural acceptability of EC use.
2 AIMS OF THE STUDY

The overall aim of all studies presented in this thesis was to further understand the mechanisms of action of LNG used for EC and possible effects when administered at the time of or when ovulation has already occurred.

The specific aims were:

- To study the effect of LNG on markers of endometrial receptivity in a 3-D endometrial cell culture construct.
- To study the effect of LNG on embryo attachment to a 3-D endometrial cell culture construct.
- To study women exposed to LNG used for EC who chose to interrupt the ongoing pregnancy, with regard to the effect of LNG on the steroid receptors and Ki67 in first-trimester decidua and chorionic villi.
- To explore a new regimen of LNG used for EC by studying the effect of high-dose LNG on the markers of endometrial receptivity.
3 MATERIALS AND METHODS

3.1 ETHICS APPROVAL

The studies were approved by the local ethics committee at Karolinska University Hospital and Karolinska Institutet, Stockholm, Sweden. Permission for collecting the early pregnancy tissue samples from induced abortion was obtained from the Chinese Committee of Population and Family Planning.

3.2 SAMPLE COLLECTION

3.2.1 Endometrial biopsies (papers I, II and IV)

In our project, non-smoking, healthy women with proven fertility aged 22-44 years with a regular menstrual cycle (25-35 days) were recruited (n=12 for paper I, n=22 for paper II, n=8 for paper IV). Exclusion criteria included current use of an IUD or use of any hormonal contraception or glucocorticoids for at least 3 months prior to the study, abnormal results of Papanicolaous smears, or breast-feeding. Participating women were asked to monitor the urinary LH peak twice a day from cycle day 10 to 2 days after positive detection (LH+2) by using a rapid self-test (Clearplan, UK). Endometrial biopsies were taken from all participants during days LH+4 to LH+5 (paper I and II) or during LH+6 to LH+8 (paper IV) by using a Randall curette (Dimeda, Germany) without prior dilation of the cervix or local anesthesia.

3.2.2 Decidua and chorionic villi (paper III)

Women (n=9) aged 22-36 years with regular menstrual cycles (28-33 days) who were diagnosed at 6-9 weeks gestation and then opted to interrupt the pregnancy with vacuum aspiration were recruited. All of them had self-administered 1.5mg LNG for EC during the cycle days 16-23. Comparable, unexposed pregnant women (n=9) were recruited as controls. None of them had used any hormonal treatment or IUD for a minimum of 3 months prior to the last menstrual period. Samples from the decidua and chorionic villi were collected at the time of surgery at International Peace Maternity and Child Health Hospital in Shanghai, China. Samples were then formalin-fixed, paraffin-embedded and sectioned.

3.3 IMMUNOASSAY OR IMMUNOLITE (PAPERS I, II AND IV)

Morning urine samples from cycle day 10 to 2 days after the detected LH peak collected by the endometrial donors were validated by using either enzyme immunoassay employing SEROZYME (Serono Diagnostics, Ocean Scientific Inc, CA, USA) (papers I and II) or LH Immulite kit (SIEMENS, UK) employing IMMULITE 1000 system (Diagnostic Products Corporation, US) (paper III).

3.4 PREPARATION OF ENDOMETRIAL CELL CULTURE (PAPERS I & II)

Endometrial cell cultures mimicking the architecture of normal endometrium were prepared (Figure 6) as described by Bentin-Ley with minor modifications (Bentin-Ley et al., 1994; Bentin-Ley et al., 1999) (see paper I and II for details).
In brief, the fresh biopsies were minced into small pieces and incubated in trypsin-Ethylene-diaminetetraacetic acid (EDTA) and supplemented with pancreatin. The enzymatic reaction was arrested by supplement of fetal calf serum. This was followed by collagenase digestion and the epithelial glands and surface epithelium were collected in Ham’s F-10 medium. After sedimentation, the supernatant was removed and the cells were washed in Ham’s F-10. The mixture of cells was filtered through a cell strainer which allowed single stromal cells to pass through while the glandular tubules were restrained in the filter. The “stromal cell fraction” was sedimented and the pellet was mixed with Vitrogen 100. The mixture was placed in Millipore Cell Culture inserts and the inserts were placed in culture dish. After the collagen gel was polymerized, the upper surface was coated with a thin layer of murine basement membrane material (Matrigel). The suspension was then removed and culture medium (Alpha Modification of Eagle’s Medium, Invitrogen, Sweden) was added to the bottom of the dishes. The final concentration of estrogen and progesterone in the culture medium were 0.3nmol/l and 900nmol/l. The “epithelial gland fraction” was seeded on top of the basement membrane to cover up to 70% of the gel surface, allowing the glands to spread out and grow in a monolayer. Culture medium was then added into the insert. The cells were cultured in an incubator at 37°C with 5% CO2 in air and the medium was changed every other day. The cultures were examined under a Zeiss inverted phase contrast microscope. After 5 days of culture, the epithelial layer was confluent and ready for study use.

Figure 6. Establishment of the 3-dimensional human endometrial cell cultures. Human endometrial biopsies were taken from female volunteers 4 to 5 days after the luteinizing hormone (LH) peak. Epithelial cells and stromal cells were then isolated. Stromal cells were mixed with collagen into culture insert, followed by a thin layer of Matrigel, with endometrial epithelial cells seeded on the top. Surplus human blastocysts were placed on the surface of the epithelial cells.

3.5 TREATMENT WITH LNG

3.5.1 In vitro endometrial receptivity study (paper I)

When the epithelial cell layer was confluent, all cultures were treated with progesterone (10^{-5} mol/l) only; progesterone (10^{-5} mol/l) plus mifepristone (10^{-5} mol/l); or progesterone
(10⁻⁵mol/l) plus LNG (10⁻⁵mol/l) in each case using 5μl of ethanol as base. The modified Alpha medium was used and changed every other day. After an additional 5 days, the cultures were terminated by fixation in neutral-buffered 4% formaldehyde and dehydrated. The materials were embedded in paraffin wax and sectioned. Sections were stained with hematoxylin and eosin for morphologic observation by light microscope before further immunohistochemical analysis.

3.5.2 Embryo attachment study (paper II)

Human embryos from in vitro fertilization (IVF) treatment were donated with informed consent by couples treated for infertility. These embryos were either cryopreserved more than 5 years or were fresh surplus embryos not suitable for cryopreservation. The quality of embryos was evaluated as ‘excellent’ or ‘good’ by an embryologist. Embryos were initially cultured in S2 medium (Vitrolife Ltd, Gothenburg, Sweden) to the blastocyst stage and were placed on the epithelial cell layer at the onset of treatment described above. They were randomly assigned to the different treatment groups. After exposing the embryos along with culture media to the above treatment for 5 days, the implantation site was examined every 24 hours through a Zeiss stereo microscope. Identification of blastocysts was simple since the cell cultures were translucent. Blastocyst attachment was tested by washing thoroughly with phosphate buffered saline (PBS), and as a result unattached embryos were washed away. The endometrial cell cultures with attached embryos were fixed in 4% formaldehyde, dehydrated and a portion was paraffin-embedded and sectioned.

3.5.3 In vivo endometrial receptivity study (paper IV)

The study was conducted in two cycles: one control and one treatment cycle. Each subject served as her own control. During the treatment cycle, each woman received 4 doses of 0.75mg LNG (Postinor, Germany) at 24-hour intervals, with the first dose given on LH+1. One part of the endometrial biopsies was frozen in liquid nitrogen for gene analysis, and the remainder fixed in formalin solution, embedded and sectioned. For light microscopy the endometrial sections were stained with hematoxylin and eosin.

3.6 IMMUNOSTAINING

3.6.1 Immunohistochemistry (papers I, II, III and IV)

Antibody-mediated detection of all studied markers was performed using the standard Avidin-Biotin-Complex (ABC) method (Figure 7, for details see papers I, II, III and IV).

3.6.2 Immunofluorescence (papers I and II)

The immunofluorescence staining for mucin 1 (Abcam, United Kingdom), Cytokeratin-7 (clone LP5K, Abcam, United Kingdom), Cytokeratin (clone AE1/AE3, mouse monoclonal, DakoCytomation, USA), and Vimentin (Clone V9, mouse monoclonal, DakoCytomation, Denmark) used in papers I and II was carried out with a similar protocol as mentioned above (Figure 8), except that counterstaining was done by using 4’, 6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen, Leiden, The Netherlands).
Figure 7. The immunohistochemistry procedure

- paraffin-embedded sections
- deparaffinization + rehydration (xylene + graded ethanol)
- Yes (papers I, II) no (papers I, II, IV)
- ER-α, ER-β, PR-B, PR-(A+B), AR
- microwave heating
- IL-1β, LIF, VEGF, COX-2, TNF-α, MUC1 (AbD Serotec), Integrin αβ3
- deparaffinization + rehydration + antigen retrieval (2100-Retriever: DIVA DECLOAKER + HOT RINSE)
- 0.3% hydrogen peroxide (papers I, II)
- peroxidized solution (paper III, IV)
- incubation in blocking solution (species-specific serum / universal blocking reagent)
- incubation in diluted primary antibody overnight at 4°C
- incubation in secondary antibody
- application of diaminobenzidine (DAB)
- hematoxylin counterstaining
- dehydration
- mounting + sealing

Figure 8. The immunofluorescence staining procedure

- paraffin-embedded sections
- deparaffinization + rehydration (xylene + graded ethanol)
- 0.3% hydrogen peroxide
- incubation in blocking solution (species-specific serum)
- incubation in diluted primary antibody for 2 hours at room temperature (MUC1: Abcam; Cytokeratin; Vimentin)
- incubation in secondary antibody
- DAPI counterstaining
- mounting + sealing
3.6.3 Semi-quantitative scoring system (papers I, III and IV)

The immunostaining of all slides was assessed twice by two persons using a semi-quantitative subjective scoring system under a light microscope (Zeiss, Carl Zeiss AG, Germany). To avoid errors resulting from uneven staining, 10 fields of the whole section were scored at 200x magnification and the median value taken as a final score. In each field, the score was given depending on the signal intensity and percentage of positive cells (for details see papers I, III and IV). In cases of disagreement, slides were reviewed a third time, followed by a conclusive judgment.

3.7 REAL TIME REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR) (PAPER IV)

3.7.1 Tissue homogenization, RNA extraction and cDNA synthesis

The endometrial samples kept in liquid nitrogen were homogenized, and then the total ribonucleic acid (RNA) was extracted by using Trizol reagent. The RNA concentration was measured using a NanoDrop™ 1000 Spectrophotometer (Thermo scientific, MA, USA) and all samples had an OD 260/280 ratio higher than 1.7. Two microgram of total RNA, pretreated with RNaseOUT™ Recombinant Ribonuclease Inhibitor, was used for RT reaction, which was performed by using a SuperScript™ RNase H- Reverse Transcriptase according to manufacturer’s instructions. The synthesized double-stranded complementary deoxyribonucleic acid (cDNA) was stored at -70°C until use (for details see paper IV).

3.7.2 Real-time RT-PCR

The levels of mRNA encoding ER-α, ER-β, PR-(A+B), AR, IL-1β, LIF, VEGF-A, COX-2, TNF-α, integrin αv, integrin β3 and mucin1 in the endometrial samples were quantified by real time RT-PCR running on 7300 Real Time PCR System (Applied Biosystems, USA). Appropriate primers and probes were part of the TaqMan Gene Expression Assays purchased commercially (Applied Biosystems, USA) (see paper IV for details). Real-time RT-PCR amplification was performed in triplicate using 96-well optical PCR plates. The threshold cycles (C_T), at which an increase in reporter fluorescence above the baseline signal could first be detected, were determined. All experiments were carried out with negative control (RNase-free water instead of cDNA) to ensure absence of contamination. To correct for variations linked to differences in amount of RNA input, or for different levels of inhibition during reverse transcription or PCR, the expression levels of mRNA were normalized by using both 18S ribosomal RNA and GAPDH as housekeeping genes. The geometric mean of C_T for these two endogenous controls was used for normalization of the mRNA levels for the gene of interest (Vandesompele et al., 2002) and this value was subtracted from that target gene C_T of the respective sample, giving ΔC_T. The ΔC_T of the control group was then subtracted from that of the treatment group, giving ΔΔC_T. As the amount of product doubles in each cycle, the relative gene expression was calculated by the formula 2^-ΔΔC_T.
3.8 STATISTICAL ANALYSES

Comparison of the immunostaining scores between the control and treatment groups were performed employing SPSS software (version 17.0) using a non-parametric test, Mann-Whitney Rank Sum Test, for comparing two independent samples (papers I and III) or Wilcoxon Signed Ranks Test for comparing two dependent samples (paper IV). The results were presented as mean ± SE (standard error). The difference in blastocyst attachment rate between the control and treatment groups (paper II) was compared by using Fisher’s exact test. The quantitative data from real-time RT-PCR (paper IV) were log transformed and the paired t-test was used for statistical analysis. A p-value of <0.05 was considered statistically significant.
4 RESULTS

4.1 VALIDATION OF URINARY LH PEAK (PAPERS I, II AND IV)

There was a good agreement (difference less than 1 day) between the results of immunoassay (papers I and II) or immunolite (paper IV) and the calculated day based on uterine LH peak determined by self-test.

4.2 ESTABLISHMENT OF 3-D ENDOMETRIAL CELL CULTURES (PAPERS I AND II)

Endometrial biopsies were obtained from 22 donors, however 2 samples were too small to be included in this study. The endometrial stromal and epithelial cells were successfully isolated from 20 women. Out of these, 18 samples were used for cell culture since 2 did not yield a sufficient number of cells. From these cultures, 12 were used for paper I which consisted of 3 groups treated with progesterone along with LNG or mifepristone respectively. Four and three endometrial constructs from the mifepristone and LNG treated groups, respectively, were either lost or damaged during the process due to their fragile nature. Additionally, 11 of the 18 cultures were used in paper II. One culture was contaminated with fungi and the remainder were used in establishing the fine technique.

The hematoxylin-eosin stained endometrial construct showed stromal cells and monocytes embedded into the collagen matrices with the surface covered by an epithelial cell layer (Figure 9). The existence of epithelial cells on the top, and stromal cells underneath were confirmed by immunostaining of Cytokeratin and Vimentin, respectively (Figure10).

4.3 EFFECT ON MARKERS OF ENDOMETRIAL RECEPTIVITY

4.3.1 In vitro result (paper I)

As can be clearly seen from Table 1, treatment with LNG did not cause a significant change in any molecule studied in the cultures, when compared to the negative controls treated by progesterone. The positive control mifepristone up-regulated expression of ER-β and PR-B in the epithelial cells (P<0.05), whereas expression of stromal VEGF (P<0.05), epithelial integrin αvβ3 and MUC1 (P<0.01) decreased (see figure 1, 2 and3 in paper I). Expression of ER-β, PR-B, Integrin α1β3, and MUC1 was absent in cultured stromal cells, as seen by immunohistochemistry. Neither epithelial cells nor stromal cells were stained by TNF-α (for details see paper I).

4.3.2 In vivo result (paper IV)

Following treatment, the immunoreactivity of both P receptor (PR)-A and PR-B significantly declined in glandular epithelia, whereas stromal immunoreactivity and mRNA expression of LIF increased significantly compared to the controls. No significant differences in other molecules studied were found between the paired control and treatment groups (Table 1, details see paper IV).
Figure 9. Cross section of the 3-dimensional human endometrial cell culture as seen by hematoxylin-eosin staining (E: epithelial cells; S: stromal cells; C: collagen)

Figure 10. In the cultured endometrial construct, the epithelial cells are stained for Cytokeratin (A) whereas the stromal cells are stained for Vimentin (B) as seen by immunofluorescence. The nuclei were counterstained blue with 4’, 6-diamidino-2-phenylindole dihydrochloride DAPI.
Table 1. *In vitro* and *in vivo* changes in markers of endometrial receptivity in human endometrium following the treatment of LNG or mifepristone in comparison to controls (↑: increase, P<0.05; ↓: decrease, P<0.05; ↓↓: decrease, P<0.01); ↔: insignificant change; (-): no staining. LNG: levonorgestrel; Epi C: epithelial cells; SC: stromal cells; LEC: luminal epithelial cells; GEC: glandular epithelial cells.

4.4 EFFECT ON EMBRYO ATTACHMENT (PAPER II)

The attached embryos grew larger and contracted the collagen gel (Figure 11). They were positive for Cytokeratin-7 immunofluorescence staining. In the progesterone treated group, 10 out of 17 (59%) blastocysts attached to the cell culture, whereas in cultures exposed to LNG, 6 out of 14 embryos (43%) attached, which was not significantly different from that of control cultures. None of the 15 blastocysts in the culture exposed to mifepristone attached to the matrices (P<0.01, figure 12).

Figure 11. Human embryo (E) attached to the matrix (M) of endometrial construct and contracted the surface of the gel. The attached embryo was stained for Cytokeratin-7.
4.5 EFFECT ON FIRST-TRIMESTER DECIDUA AND CHORIONIC VILLI
(PAPER III)

As can be seen from Table 2, post-ovulatory administration of 1.5mg LNG used for emergency contraception did not cause any changes in the immunoreactivity of ER-α, ER-β, PR-B, PR-(A+B), AR, Ki67 in first-trimester decidua and chorionic villi, when compared to unexposed controls. The decidual epithelium and syncytiotrophoblast were absent for Ki67 staining.

Table 2. The immunoreactive change of ER-α, ER-β, PR-B, PR-(A+B), AR, and Ki67 in first-trimester decidua and chorionic villi exposed to post-ovulatory administration of 1.5mg levonorgestrel for emergency contraception, when compared to unexposed samples. (↔: insignificant change) LEC: luminal epithelial cells; GEC: glandular epithelial cells; SC: stromal cells; STB: syncytiotrophoblast; CTB: cytotrophoblast.
5 DISCUSSION

5.1 THE FEMALE DONORS

Endometrial donors included in the studies were non-smoking, healthy women with proven fertility. Since the implantation rate remains constant until the age of 35 at which time a linear decrease of 2.77% per year is observed (Spandorfer et al., 2000), female donors under 40 years are preferable in order to obtain normal endometrium with receptivity, (Bentin-Ley, Lopata, 2000). However, due to the difficulties in recruiting female endometrial donors fulfilling inclusion criteria, the age of the women involved in our project varied between 22 and 44 years. It cannot be denied that the older women may have poorer endometrial receptivity than those younger. In papers I and II, the treatment with either LNG or mifepristone was administered randomly into the cultured endometrium, and the embryos were assigned into different treatment groups randomly. In paper IV, the subjects served as their own controls. These could to some extent reduce any age-related differences between groups.

5.2 EFFECT ON MARKERS OF ENDOMETRIAL RECEPTIVITY

A considerable number of factors have been found to mirror endometrial receptivity, some of which are investigated in this project. It is the first in vitro study showing the effect of LNG on markers of endometrial receptivity in a well-established three-dimensional endometrial construct. Consistent with in vivo findings (Landgren et al., 1989; Marions et al., 2002), we found that LNG did not affect the expression of known markers of receptivity in cultured human endometrium. In addition, it is generally accepted that estrogen up-regulates the expression of ERs and PRs, whereas progesterone down-regulates their expression. In our in vivo study, LNG in a higher dose than standard EC regimen decreased PR expression and caused only minor changes (increased LIF expression) in other markers of endometrial receptivity, which are insufficient to impair the endometrial receptivity. These findings support the concept that from a physiological and pharmacological point of view, the use of synthetic progestin like LNG is highly unlikely to reduce endometrial receptivity, especially when administered occasionally.

Mifepristone was used as a positive control in our in vitro study since it has been previously shown to affect endometrial receptivity both in vivo (Maentausta et al., 1993; Qiu et al., 2002; Gemzell-Danielsson et al., 2003; Sun et al., 2003) and in vitro (Prange-Kiel et al., 2001). Both mifepristone and LNG act through the PR, and the former is considered an antagonist whereas the latter is an agonist. The increased expression of ER-β and PR-B in epithelium as well as decreased expression of stromal VEGF, epithelial integrin αvβ3, and mucin 1 caused by mifepristone are consistent with the in vivo findings by other studies in humans and animals (Maentausta et al., 1993; Gemzell-Danielsson et al., 1994; Ghosh et al., 1998; Marions et al., 2002; Qiu et al., 2002; Sun et al., 2003)

5.3 EFFECT ON EMBRYO ATTACHMENT

Since for many people, due to different religious believes, a human life is considered to start from the completion of fertilization, and therefore any acceptable drug should not
affect the post-fertilization events. In our cultured endometrial construct exposed to LNG treatment, the rate of blastocyst attachment did not significantly differ from the control. This study conducted in human endometrium supports the earlier findings in rodents and primates that post-coital administration of LNG does not interfere with post-fertilization processes such as embryo implantation (Muller et al., 2003; Ortiz et al., 2004).

As a positive control, antiprogestin mifepristone inhibited embryo attachment, which is consistent with results from animal studies in which high-dose of mifepristone was used (Psychoyos et al., 1987; Yang YQ et al., 1990; Dao et al., 1996).

5.4 EFFECT ON EMBRYO VIABILITY

The result of our in vitro study indirectly showed that LNG has no effect on the human blastocyst in terms of its viability. All 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 LNG from day 1 to 5. There was sufficient time for the embryo to undergo any degenerative changes LNG could cause to the unhatched blastocyst or the trophoblast cells of hatched embryos. We observed no changes in attachment rate in the LNG group compared with that of controls. This indirectly shows that both the drug and the endometrial cell products in the culture do not affect human embryo viability.

5.5 EFFECT ON ONGOING PREGNANCY

Despite lack of evidence, some physicians fear that the Yuzpe regimen or LNG-only pill may increase the risk of fetal malformation if administered to a woman in early pregnancy without awareness, or in the case of failed prevention. A meta-analysis of 12 available prospective studies did not find any statistically significant association between oral contraceptive use in early pregnancy and fetal malformation (Bracken, 1990). A prospective cohort study showed that there was no association between the use of LNG pills for EC and the risk of major congenital malformation, pregnancy complications or any other adverse pregnancy outcomes (Zhang et al., 2009).

5.6 HUMAN ENDOMETRIAL CELL CULTURES

In the process of understanding human endometrial receptivity and embryo implantation, different animal and human in vitro models have been developed. It is a well-documented fact that usage of animal models in understanding human endometrial receptivity has great limitations since the process of human implantation may be unique. A number of other initial models have involved the primary culture of human stromal monolayer or coculture of endometrial epithelial and stromal cells in monolayers, however this gives only limited information on blastocyst attachment, as the reciprocal interaction of different cell types in the endometrium is essential for proper function in vivo. Thus a 3-D endometrial construct, comprising endometrial stromal cells in collagen matrix with surface epithelial cells, was developed (Bentin-Ley et al., 1994; Bentin-Ley, Lopata, 2000). Our in vitro study shows that the molecular profile of this 3-D construct is similar to the receptive endometrium in vivo and exposure to mifepristone leads to a significant change in its molecular expression which inhibits blastocyst attachment to the model, whereas LNG has no effect. This
consistency with previous findings, with regard to the effects of LNG and mifepristone on endometrial receptivity and embryo attachment, confirmed the reliability of using the 3-D endometrial cell culture model to mimic the physiological endometrium. Bentin-Ley et al. developed the 3-D human endometrial cell culture model used in this study (Bentin-Ley et al., 1994; Bentin-Ley et al., 1995; Bentin-Ley et al., 1999; Bentin-Ley, Horn et al., 2000), and earlier studies have only provided information on the ultrastructure of human blastcyst-endometrial interactions in vitro. A similar culture model has previously been studied to investigate the effect of antiprogestin Org 31710 on the secretion of glycodelin and LIF in the culture medium; assess the immunohistochemical expression of PR, IL-1R type I, and integrin subunit β3 in epithelial cells; visualize endometrial pinopodes by scanning electron microscopy and observe human blastocyst attachment (Petersen et al., 2005). Compared to the work of Petersen et al., we examined more markers of endometrial receptivity and proved that this 3-D endometrial cell culture model to a large extent mimics the physiological environment. However, our model does have limitations, for example it lacks other important endometrial components such as endothelial cells, and it is well-documented that the interactions between various endometrial cells significantly contributes to endometrial receptivity.

5.7 CONTRADICTORY FINDINGS OF LIF ROLE IN IMPLANTATION

Although LIF has been shown to be critical for implantation in mice (Stewart CL et al., 1992), its importance and regulation pathway in human implantation remain unclear. LIF expression coincides with progesterone domination of the endometrium and treatment with antiprogestin mifepristone immediately after ovulation reduced immunoreactive LIF at the expected time of implantation (Gemzell-Danielsson et al., 1997), indicating that progesterone might be a major regulator of LIF expression. LIF may influence embryo implantation through autocrine-paracrine interaction (Cullinan et al., 1996) and can functionally mediate progesterone-dependent pinopode formation (Aghajanova et al., 2003). However, there are some contradictory findings regarding LIF and endometrial receptivity. The LIF content of endometrial secretion did not appear to reflect endometrial maturity (van der Gaast et al., 2009). There was no direct stimulatory evidence of steroid hormones (estradiol and progestins) on LIF mRNA expression or protein production by endometrial cells in culture (Arici et al., 1995). In a recent study, the clinical pregnancy rate after assisted reproductive techniques was significantly lower in patients receiving recombinant human LIF than in those receiving placebo (Brinsden et al., 2009). In vitro LIF inhibits the differentiation of cytotrophoblastic cells towards an invasive phenotype by inhibiting the secretion of metalloproteinases, increasing the deposition of fetal fibronectin into the extracellular matrix and inhibiting the differentiation of cytotrophoblastic cells into syncytium (Bischof et al., 1995). We found that high-dose LNG, administered orally, caused a significant increase in the LIF mRNA expression and stromal protein level, compared to the control.
5.8 DISCREPANCY BETWEEN PROTEIN AND GENE LEVELS DETECTED

In paper IV, the mRNA level of a gene encoding both PR subtypes did not vary to the same extent as the protein levels of both PR-A and PR-B. The fact that glandular epithelial cells are only one constituent of the endometrium may partly explain this. In preparation for real-time RT-PCR, the whole endometrial tissue was homogenized and analyzed together, making it difficult to identify the change in specific cell type. Discrepancies between mRNA and protein expression can also be biologically caused by post-transcriptional regulation and differences in their respective turnover rates (Hack, 2004; Cox et al., 2005). Technical imprecision of methods used to determine the expression levels could also explain the variation in results (Hack, 2004).

5.9 ULIPRISTAL ACETATE (UPA) – A POTENTIAL SPRM AS ECP

Ulipristal acetate (17 alpha-acetoxy-11 beta-[4-N, N-dimethylaminophenyl]-19-norpregna-4, 9-diene-3, 20-dione), also referred to as CDB-2914, VA2914, HRP-2000, RTI 3021-012, RU44675), is a novel orally-active SPRM. As shown in Figure 5, the chemical structure of UPA is similar to that of both mifepristone and progesterone. Both in vivo and in vitro studies have shown that UPA exhibits similar progesterone antagonist activity, but lower antiglucocorticoid activity (Attardi et al., 2002; Blithe et al., 2003; Passaro et al., 2003; Attardi et al., 2004).

It has recently been specifically developed for emergency contraceptive use, with enhanced specificity for PR. It was approved by the European Medicines Agency in May 2009 under the brand name “Ellaone” which contains 30mg of UPA. A single dose is recommended for use up to 120 hours after unprotected intercourse. The biological effects of UPA vary according to the dose given. Continuous administration of low-dose of UPA can induce amenorrhea and inhibit ovulation without down-regulating estradiol levels or inducing endometrial hyperplasia in women (Chabbert-Buffet et al., 2007). Single doses of UPA administered during the mid follicular phase suppress leading follicle growth, causing a dose-dependent delay in folliculogenesis and suppression of plasma estradiol. At higher doses, a new leading follicle is often recruited (Stratton et al., 2000). A recent study found that when given prior to the LH surge rise, UPA inhibited 100% of follicular rupture. If administered when the size of the leading follicle was ≥ 18 mm in diameter, follicular rupture failed to occur within 5 days following treatment in 44% to 59% of the subjects (Croxatto et al, abstract present at 8th Congress of the European Society of Gynecology). In conclusion, UPA inhibits or delays follicular rupture by postponing LH surge or possibly through direct ovarian inhibitory effect. Therefore UPA could prevent pregnancy when administered very late in the follicular phase, even if the LH levels have already begun to rise, when use of LNG or Yuzpe is too late for ovulation inhibition (Figure 13). When administered during early luteal phase, UPA causes a significant dose-dependent decrease in endometrial thickness, a delay in maturation with an increase in glandular PR expression (Stratton et al., 2000; Stratton et al., 2009). Mid-luteal administration of UPA at doses of 1-100mg antagonizes progesterone action on the endometrim in a dose-dependent manner, with no apparent antiglucocorticoid effects (Passaro et al., 2003). In addition, UPA is also being used in other applications including treatment of uterine fibroids (Wilkens et al., 2008).
A randomized controlled trial compared the efficacy and side effects of UPA with LNG (2 times of 0.75mg) and showed a trend toward higher effectiveness of UPA with similar side effect profile as LNG. This finding is consistent with a recent study yet to be published (Cameron et al, abstract present at 8th Congress of the European Society of Gynecology). When administered within 24 hours of unprotected intercourse, the EC efficacy of UPA was three times that of standard LNG (Ulmann et al, 2009, abstract present at 8th Congress of the European Society of Gynecology).

The above data indicate that UPA is a potent antiprogestin with weak anti-glucocorticoid activity and single doses are well tolerated for EC use (Stratton et al., 2000).

5.10 RELEVANCE FOR STUDYING EC MECHANISMS OF ACTION

Emergency contraception is the only method women can use to avoid becoming pregnant after having unprotected intercourse. Any woman of reproductive age many need EC at some point in order to avoid an unwanted pregnancy. However, mainly due to the incomplete knowledge on its mechanisms of action, EC is largely under-utilized worldwide. To date, it is known that pre-ovulatory administration of LNG (1.5mg), mifepristone (10mg) or UPA (30mg) used for EC acts mainly through delaying or inhibiting ovulation. However, when the LH level has already started to rise, only UPA or probably mifepristone as well could delay ovulation (Figure 14).
Increased knowledge on the mechanisms of ECPs has the potential to increase the acceptability and use of ECPs as well as optimize the use of methods already available, with the purpose to reduce the number of unwanted pregnancies and induced abortion.

Figure 14. Mechanisms of action of emergency contraceptive pills. UPA: ulipristal acetate (30mg); LNG: levonorgestrel (1.5mg); Mifepristone: 10mg
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6 SUMMARY AND CONCLUSIONS

The main findings of this thesis are:

I. This 3-D human endometrial cell culture construct expresses progesterone-regulated markers of endometrial receptivity as seen in the physiologic condition. Treatment with LNG did not affect the expression of these markers in contrast to mifepristone.

II. Levonorgestrel did not impair the attachment of human embryos to the \textit{in vitro} endometrial construct, whereas mifepristone inhibited blastocyst attachment.

III. Post-ovulatory administration of 1.5mg of LNG to women with a subsequent pregnancy did not affect the immunoreactivity of ovarian steroid hormone receptors or proliferation index Ki67 in the first-trimester decidua and chorionic villi.

IV. A high-dose LNG used for EC caused only minor alternations in the protein and mRNA levels of endometrial receptivity markers.

The present study provides evidence that our 3-D endometrial model could to a large extent mimic the functional characteristics of endometrium \textit{in vivo}. Therefore, this model could be used to clarify the roles of various molecules involved in the early stages of human embryo implantation. In addition, increased knowledge about the nature of receptivity and embryo-endometrium interactions at the molecular level can also be potentially used to improve fertility and develop contraceptive agents.

A number of factors mirror endometrial receptivity, some of which were studied in this project. Levonorgestrel has no influence on any of these markers either \textit{in vivo} or \textit{in vitro}, even in the case of increasing the dose. Pre-ovulatory administration of LNG is highly effective for EC, however, if taken at the time when LH level has already started to rise, it is insufficient to prevent ovulation and has no effect on the endometrium or other post-ovulatory events. Consequently it is ineffective to prevent pregnancy (Novikova \textit{et al.}, 2007). Although the lack of effect on implantation is positive from a religious and social acceptance perspective, it is unfortunate from a medical point of view since implantation-inhibited effect would improve the EC efficacy and thus increase the possibility of reducing unwanted pregnancies. Therefore, although LNG is well-tolerated and easily accessible, there is still a need to develop more effective EC methods. To ensure the highest efficacy and to cover the whole window of fertility, the ideal agents for EC also need to target the endometrium. The potential SPRM, UPA, which has higher EC efficacy than LNG, holds the potential to become the most commonly used ECP in the next decade.

Emergency contraception should be made available to women of reproductive age. Nevertheless, women seeking EC should also be counselled and offered a choice of effective and reliable methods of contraception for regular use, since regular contraceptive methods are more efficient than ECPs.
7 FUTURE PERSPECTIVES

7.1 IMPROVING THE 3-D ENDOMETRIAL COCULTURES

The 3-D cocultures of endometrial epithelial and stromal cells have been useful for studying complex interactions between the maternal endometrium and the blastocyst, though studies in conjunction with blastocyst implantation are limited. The endometrium is a complex tissue that consists of several distinct cellular compartments. The autocrine/paracrine interactions between different cell types of the endometrium are essential for its proper function in vivo. Therefore, the optimal in vitro model for investigating endometrial receptivity and the process of embryo implantation should contain all endometrial cell types, including endothelial and immune cells. Further development of this 3-D culture system should to a large extent imitate the normal endometrium morphologically as well as physiologically. Studies investigating markers of endometrial receptivity at the gene levels expressed in this cell culture model are recommended. Future studies are also required to clearly address the mechanisms of synergistic communication between maternal and embryonic cells. Thus it would provide an excellent in vitro model suitable for exploring molecular events in the process of human blastocyst implantation, as well as for reproducing in vivo pathological processes such as endometrial cancer invasion and endometriosis. Moreover, this model also has the potential to make an important contribution to development of new therapeutic and diagnostic strategies for implantation failure in the future.

7.2 STUDIES ON EC

The use of EC before occasional intercourse could be possibility developed as an “on demand” dual protection method if combined with a microbicide (Sitruk-Ware et al., 2007). ECPs applied prior to intercourse may induce a contraceptive effect by interfering with the ovulatory process and/or acting on the cervical mucus to prevent sperm penetration.

The mechanisms and effects of UPA for EC need further studies especially with regard to the pregnancy outcome in case of EC failure.

An antiproliferative effect of mifepristone has been observed when given to women of fertile age (Engman et al., 2008). A possible protective effect of SPRMs such as mifepristone and UPA against breast cancer would be of benefit when used for contraception and should be further investigated. Other health benefits when using SPRMs for contraception could be expected and should also be explored.
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