TOWARDS DEVELOPING A NEW CONTRACEPTIVE PILL: EFFECTS OF MIFEPRISTONE ON REPRODUCTIVE TISSUES AND MENSTRUAL CYCLE

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ABSTRACT

Existing hormonal contraception is highly effective and widely used; however, there is a move towards developing novel compounds that do not have some of the adverse health risks associated with the oestrogen content. Mifepristone, a progesterone receptor antagonist, has potential to be developed as a safe and effective oestrogen-free contraceptive pill. The current studies investigate various effects of mifepristone on the hypothalamic-pituitary-ovarian axis and reproductive tissues.

The first study investigates the effect of daily low-dose mifepristone on proliferation markers [phospho-histone H3 (pH3) mitosis marker] and steroid receptors [oestrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR)] in the endometrium. There was a significant down-regulation in pH3 and PR expression following mifepristone treatment whereas AR expression was up-regulated. Since androgens antagonize oestrogen-effects on the endometrium, mifepristone-induced AR up-regulation could play a role in its anti-proliferative effects.

The second study investigates the effects of daily low-dose mifepristone on endometrial parameters [microvasculature, vascular endothelial growth factor (VEGF) and glucocorticoid receptor (GR)] which may be associated with changes in endometrial vascular function. The majority (15/16) of subjects were amenorrhoeic, mean oestradiol (E2) concentrations remained in the mid-proliferative range and most (9/16 subjects) endometrial samples showed proliferative histology. GR expression was induced in the nuclei of glands and surface (luminal) epithelium and there was a significant increase in micro-vessel density and decrease in stromal VEGF following treatment. Glucocorticoids can modulate angiogenesis and the high incidence of
mifepristone-induced amenorrhoea may be related change in the regulation of vascular function.

The third study investigates the effects of daily low-dose mifepristone on vaginal morphology, histology, steroid receptor and natural anti-microbial [Serine Leukocytic Protease Inhibitor (SLPI)] content. There was no change in vaginal thickness, steroid receptor and SLPI content and distribution following mifepristone treatment. The absence of changes, in contrast to other oestrogen-free hormonal contraception, is reassuring.

The fourth study investigates the effect of three single doses (10, 25 and 200 mg) of mifepristone on menstrual cycle and the feasibility of timing administration as a once-month contraceptive pill based on the length of previous menstrual cycles (calendar). Only 45% of women were in the peri-ovulatory (correct timing) phase of the cycle on the day of drug administration and an increasing dose of mifepristone was associated with an increasing chance of having a delayed period (P<0.001). It is not possible to use the calendar approach to identify the correct time of administration of mifepristone and mifepristone disrupts menstruation in a dose-dependent manner. The endometrial mechanisms and contraceptive efficacy of low (≤ 10 mg) once-a-month dose need to be investigated in future studies.

In conclusion, endometrial and vaginal effects reported demonstrate safety of daily low-dose mifepristone treatment whereas a once-a-month administration based on the calendar disrupts menstruation and is unlikely to provide effective and reliable contraception.
DECLARATION OF ORIGINAL WORK

This is to declare that the entire work within this dissertation has been prepared through my own effort under the supervision of Professor David Baird, University of Edinburgh.

The work was carried out under the umbrella of the Contraceptive Development Network (CDN), an international research network on new contraceptive methods involving 5 centres (University of Edinburgh, University of Hong Kong, University of Capetown, Shanghai Institute of Family Planning and Technical Instruction, and Ogun State University Teaching Hospital in Nigeria). It was jointly funded by Medical Research Council (MRC) and Department for International Development (DFID) and directed by Professor David Baird. The co-ordinating centre for the Network was at the Centre for Reproductive Biology at the University of Edinburgh.

Full-time multidisciplinary (clinicians, scientists, research nurses, laboratory technicians, statistician and other support staff) staff at the CDN provided excellent framework for design and co-ordination of various studies, research governance, good clinical practice, data collation and monitoring, statistical support and presentation of results. Full details of my involvement in various studies are described in relevant chapters and any resultant publications appended.
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>AAG</td>
<td>α1 acid glycoprotein</td>
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<tr>
<td>ACTH</td>
<td>adreno-corticotrophic hormone</td>
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<tr>
<td>AR</td>
<td>androgen receptor</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>Cr</td>
<td>creatinine</td>
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<tr>
<td>E1G</td>
<td>oestrone glucuronide</td>
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<td>E2</td>
<td>oestradiol</td>
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<tr>
<td>ER</td>
<td>oestrogen receptor</td>
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<td>FSH</td>
<td>follicle stimulating hormone</td>
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<td>GR</td>
<td>glucocorticoid receptor</td>
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<td>H&amp;E</td>
<td>haematoxin and eosin</td>
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<td>HPA</td>
<td>hormone response element</td>
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<td>HRE</td>
<td>hypothalamo-pituitary-adrenal axis</td>
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<td>HRT</td>
<td>hormone replacement therapy</td>
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<tr>
<td>IUD</td>
<td>intra-uterine device</td>
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<td>LDM</td>
<td>low-dose mifepristone</td>
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<td>LH</td>
<td>luteinising hormone</td>
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<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
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<tr>
<td>NBF</td>
<td>neutral buffered formalin</td>
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<tr>
<td>NR</td>
<td>nuclear receptor</td>
</tr>
<tr>
<td>NS</td>
<td>not significant</td>
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<tr>
<td>P</td>
<td>p value</td>
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P4  progesterone
PCOS polycystic ovary syndrome
PdG pregnanediol glucuronine
PG prostaglandin
pH3 phospho-histone H3
PM postmenopausal
PR progesterone receptor
PRA progesterone receptor antagonist
PR-A progesterone receptor subtype A
PR-B progesterone receptor subtype B
PRE progesterone response element
RBA relative binding affinity
S significant
SD standard deviation
SEM standard error of mean
STI sexually transmitted infection
TVUSS transvaginal ultrasonography
VEGF vascular endothelial growth factor
Chapter 1

REVIEW OF LITERATURE

Progesterone, one of the key sex steroid hormones, modulates a variety of reproductive functions including differentiation of the endometrium, control of implantation, maturation of the mammary epithelium, modulation of GnRH pulsatility, oocyte release from the ovary and maintenance of the uterus in a quiescent state by inhibiting myometrial contractility (Csapo and Pulkkinen 1978; Conneely, Mulac-Jericevic et al. 2001). Although these actions have led to major pharmacological applications of progestins (synthetic progestogen; progestagen is a compound which produces effects similar to progesterone) in contraception and hormone replacement therapy (HRT), the most outstanding development in reproductive pharmacology has been the introduction of mifepristone, the first potent synthetic anti-progestagen. Its first clinical application was to induce termination of pregnancy and although the therapeutic promise is extensive (Table 1.1), the ethical and political issues surrounding its abortifacient use have largely overshadowed its development.

This focus of this review is the development of mifepristone as a novel method of female contraception.
Table 1.1: Clinical Applications of Progesterone Receptor Antagonists

<table>
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<td>1. Medical termination of pregnancy</td>
<td>100-600</td>
</tr>
<tr>
<td>2. Medical management of foetal demise</td>
<td>50-600</td>
</tr>
<tr>
<td>3. Labour induction</td>
<td>50-200</td>
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<tr>
<td>4. Contraception</td>
<td>10-200</td>
</tr>
<tr>
<td><strong>Long-term administration of mifepristone</strong></td>
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<tr>
<td>1. Leiomyoma</td>
<td>5-50</td>
</tr>
<tr>
<td>2. Endometriosis</td>
<td>5-100</td>
</tr>
<tr>
<td>3. Contraception</td>
<td>0.5-5</td>
</tr>
<tr>
<td>4. Cushing’s syndrome</td>
<td>≥ 200</td>
</tr>
<tr>
<td>5. Major depression with psychotic features</td>
<td>≥ 200</td>
</tr>
<tr>
<td>6. Alzheimer’s disease</td>
<td>≥ 200</td>
</tr>
<tr>
<td>7. Steroid receptor containing tumours (breast, ovary, endometrium, prostate, meningioma)</td>
<td>≥ 200</td>
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Source: refer other reviews and references therein (Baird, Brown et al. 2003; Spitz 2003; Chabbert-Buffet, Meduri et al. 2005; Fiala and Gemzel-Danielsson 2006)
1. The medical and social history of mifepristone

The discovery of progesterone in 1929 (Allen. 1929) was soon followed by efforts to develop an anti-progesterone. It was the discovery of progesterone receptor (PR) (O’Malley, McGuire et al. 1969) that led to widespread investigation into potential PR ligands. During their quest to synthesise a new glucocorticoids, chemists at Roussel Uclaf (Paris) discovered a novel method of producing 11-β substitutes of the steroid skeleton (Belanger, Philibert et al. 1981). They identified a 11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-propinyl-4,9-estradiene-3-one (RU38486; abbreviated RU486) which demonstrated a greater affinity for progesterone and glucocorticoid receptors than their naturally occurring ligands. RU486 (non-proprietary name mifepristone) was quickly put to test and the anti-progesterone activity was confirmed by demonstration of early pregnancy termination (Herrmann, Wyss et al. 1982). Medical abortion was first approved in France in 1988, followed by approvals in the United Kingdom (1991) and Sweden (1992). However, it was not until the turn of the century that medical abortion was approved in several other European countries and the United States. Mifepristone is now licensed in combination with a prostaglandin as an abortifacient in many countries across the world (Ulmann 2000; Fiala and Gemzel-Danielsson 2006). However, international anti-abortion politics (Ulmann 2000; Joffe and Weitz 2003) have kept the pharmaceutical industry away from developing this promising compound for treatment of other medical conditions.
2. Chemical structure of mifepristone.

Mifepristone (RU38486) is a beta-aryl-substituted, 19-nortestosterone-derived compound which lacks the C-19 methyl group of natural progesterone and glucocorticoids (Figure 1.1).

The two main structural modifications of steroid skeleton are the phenylaminodimethyl group perpendicularly grafted onto the 11-β position and the 1-propynyl side-chain substitution at the 17-α position. The high binding affinity for PR is attributed to both these variations whereas the size and positioning of the 11-β substitution determines the anti-progesterone activity and the 17-α side-chain modulates anti-glucocorticoid activity (Evans 1988; Garcia, Benhamou et al. 1992). Although compounds with varied anti-progesterone and anti-glucocorticoid activity have been developed, to date no pure progesterone antagonist totally devoid of anti-glucocorticoid activity has been described.
Figure 1.1: Structural formula of RU38486 (abbreviated RU486)

Chemical name: 11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-propynyl-4,9-estradiene-3-one
Non-proprietary name: Mifepristone
Molecular weight: 429.58
Molecular formula: C_{29}H_{35}NO_{2}
3. Pharmacokinetics of mifepristone

Only an oral preparation of mifepristone is available commercially for clinical use and dose of 0.5-800 mg has been administered in various therapeutic studies. Mifepristone is rapidly absorbed (70%) from the gut and peak levels appear in plasma within 1-3 hours. It undergoes extensive first-pass hepatic metabolism and its bioavailability is reduced to 40%.

Mifepristone binds to serum $\alpha_1$-acid glycoprotein (AAG), orosomucoid (Steingold, Matt et al. 1990) and the high-affinity, limited-capacity binding explains long half-life (20-40 hours) and divergent dose-dependent pharmacokinetics. Mifepristone follows linear-order kinetics initially, but, when the binding capacity of AAG is saturated at oral dose 100 mg, it follows zero-order kinetics (Swahn, Wang et al. 1986; Heikinheimo, Lahteenmaki et al. 1987). Doses of mifepristone exceeding 100 mg do not lead to higher plasma levels, but more susceptibility to metabolism. There is no difference in serum profiles between men, non-pregnant and pregnant women, or between morning and evening administration of the drug (Shi, Ye et al. 1993).

Three metabolites of mifepristone have been identified (Heikinheimo, Kontula et al. 1987; Heikinheimo, Lahteenmaki et al. 1987): mono-demethylated (RU42633), di-demethylated (RU42848) and hydroxylated (RU42698) metabolites. The metabolism of RU486 to RU42633 and RU42698 is rapid but di-demethylation to RU42848 occurs much more slowly and to a much lesser extent. The metabolites are less active but their abundance allows them to participate in the global action of the compound. These metabolites are excreted in the urine (10%) and in the faeces via the biliary system (90%).
4. Cellular mechanism of action

4.1 Nuclear Receptor Superfamily

The effect of steroid hormones is mediated through specific intracellular proteins, the steroid receptors, which belong to the superfamily of nuclear receptors (NR) (Evans 1988). Like all other NR's, steroid receptors have a modular structure with distinct regions corresponding to independent functional and structural domains that can be exchanged between related receptors without loss of function (Brosens, Tullet et al. 2004)(Figure 1.2).

**Figure 1.2: Functional domains of nuclear steroid receptors**

![Functional domains of nuclear steroid receptors](image)

The steroid receptors comprise five regions (Brosens, Tullet et al. 2004; Gronemeyer, Gustafsson et al. 2004)

1. A/B region: The amino terminus (NH2) contains a transactivation function (AF-1 and AF-3), which is of variable length and sequence in the different family members, and is recognized by co-activators and/or other transcription factors.

2. C region: The central DNA-binding domain (DBD) has two zinc-finger motifs that are common to the entire family followed by a C-terminal extension (CTE). The CTE contains amino acids required for the recognition of specific target DNA sequences called hormone response elements (HRE) and receptor dimerization.
3. D region: The hinge is quite variable within the NR superfamily. It acts as a bridge between the DBD and ligand-binding domain (LBD), enabling the NR to adopt different conformations by means of rotation. This region might also include localization signals that determine the intracellular localization of the NR.

4. E region: The carboxy-terminal (COOH) ligand-binding domain (LBD), whose overall architecture is well conserved between the various family members, nonetheless diverges sufficiently to guarantee selective ligand recognition. This domain also has the ligand-induced transactivation function (AF2) that is crucial for interaction with co-regulators and heat shock proteins. It is crucial for receptor dimerisation. A glycin at position 722 in this region of human PR is critical for mifepristone binding (Benhamou, Garcia et al. 1992).

There are two isoforms/subtypes each of the steroid receptors, oestrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR), which differ structurally and this multiplicity contributes to signal diversity and specificity of the respective ligands.

The two isoforms of the human PR (Clarke & Sutherland, 1990); an amino-terminal truncated PR-A (94 kDa) and the full-length PR-B (114 kDa), arise from a single gene and function as transcriptional regulators of progestin-responsive genes. The additional domain at the amino terminus of PR-B encodes a third transactivation function (AF3) which allows binding of a subset of co-activators to PR-B that are not efficiently recruited by progestin-bound PR-A. The structure and reproductive functions of the PR and its two isoforms are well characterised (Conneely and Lydon 2000; Conneely, Lydon et al. 2000; Leonhardt and Edwards 2002; Conneely, Mulac-Jericevic et al.
Human genital skin fibroblasts contain both the full-length (110 kDa) androgen receptor isoform (AR-B) and an amino-terminally truncated (87 kDa) isoform (AR-A). The two isoforms are expressed in a wide variety of human foetal and adult tissues (Wilson and McPhaul 1994; Wilson and McPhaul 1996). Although AR isoforms are structurally analogous to those of the PR, their reproductive function is not well characterised.

The two ER subtypes, ERα and ERβ, are encoded by different genes (Kuiper, Enmark et al. 1996; Mosselman, Polman et al. 1996). ERα and ERβ have a highly homologous DNA binding domain (DBD) but differ in the ligand-binding domain (LBD) and the transactivation function (AF-1). Although both subtypes can bind 17β-oestradiol with high affinity, they are differentially expressed in many tissues and have distinct transactivation properties (Kuiper, Carlsson et al. 1997; Paech, Webb et al. 1997).

4.2 Receptor Binding Affinity (RBA) of mifepristone

Mifepristone binds with high affinity to both the PR (RBA 506 %, progesterone 100 %) and the GR (RBA 685 %, dexamethasone 100%) (Elger, Bartley et al. 2000). It has a relatively low-affinity to the AR (RBA AR 13%, PR 103-105%, and GR 80%) (Attardi, Burgenson et al. 2002). The mineralocorticoid receptor (MR) which is structurally similar to GR demonstrates no affinity. In vivo and vitro studies clearly demonstrate virtually no affinity to the ER (Philibert 1984; Zou, Marschke et al. 1999).

Mifepristone is a type 2 progesterone receptor antagonist (PRA); it is a pure antagonist, but in the absence of progesterone, the steroid receptor-mifepristone complex may be
transcriptionally active on some genes (Spitz and Bardin 1993) and partial agonistic effect has been reported (Gravanis, Schaizon et al. 1985; Terakawa, Shimizu et al. 1988). Mifepristone antagonises glucocorticoid and androgen actions. Although mifepristone has virtually no binding to ERα and ERβ in-vitro, it acts as a weak (IC₅₀ >1µM) antagonist via ERα and as a fairly potent (IC₅₀ ~ 200 nM) antagonist via ERβ from an ERE-driven reporter in cells that do not express PR (Zou, Marschke et al. 1999). The underlying mechanism of ERβ antagonism is unclear, the hypothesis is that mifepristone is metabolized in these cells to a compound that is able to bind to ERβ with higher affinity than the parent compound (Zou, Marschke et al. 1999).

4.3 Transcription of steroid hormone action

Steroid receptors are held in a large multi-subunit complex containing heat-shock proteins which maintain the receptor in active conformation ready for ligand binding. Ligand-bound receptors, undergo conformational changes, dissociate themselves from heat-shock proteins, undergo dimerisation and are assembled on target gene promoters to activate or repress gene expression. Recruitment of ligand-bound nuclear receptors to target gene promoter regions occurs directly through specific DNA sequences (HRE) or indirectly through other DNA-binding transcription factors (Brosens, Tullet et al. 2004). Ligand-bound nuclear receptors also recruit co-activators and co-repressors which promote or repress transcription respectively. Genetic and biochemical data have revealed a plethora of factors that mediate nuclear receptor function and it is beyond scope of this manuscript to review all aspects of transcriptional regulation.
4.4 Transcription of progesterone and mifepristone

When expressed individually in cultured cells, PR-A and PR-B display different transactivation properties (Conneely, Mulac-Jericevic et al. 2003; Mulac-Jericevic and Conneely 2004). Agonist-bound PR-B functions as a strong activator of transcription of several PR dependent promoters and in a variety of cell types in which PR-A is inactive. Further, when both isoforms are co-expressed in cultured cells, in cell and promoter contexts in which agonist bound PR-A is inactive, the PR-A can repress the activity of PR-B (Vegeto, Shahbaz et al. 1993) and other steroid receptors including ER (McDonnell and Goldman 1994), AR, GR and MR (McDonnell, Shahbaz et al. 1994; Saatcioglu, Claret et al. 1994). PR-A and PR-B proteins also respond differently to progesterone antagonists. While antagonist bound PR-A is inactive, antagonist bound PR-B can be converted to a strongly active transcription factor by modulating intracellular phosphorylation pathways (Beck, Weigel et al. 1993; Sartorius, Groshong et al. 1994).

The binding of both progesterone and mifepristone produces conformational changes in the form of PR that permits it to bind to DNA. The two isoforms of human PR, PR-A and PR-B form homo and/or hetero-dimers (A:A, B:B or A:B) which bind to progesterone response elements (PRE) in the promoter region of progesterone genes. The mifepristone-PR dimer complex has a higher affinity for PRE than the progesterone-PR dimer complex. In the case of progesterone, this binding increases the transcription of these genes, producing progesterone effects whereas in the case of mifepristone, an inhibitory function in the carbox-terminal region of LBD renders this DNA-bound receptor transcription inactive. This is the basis of the progesterone
antagonistic action of mifepristone underlying its abortifacient and contraceptive actions.

Progesterone and mifepristone also interact with the transcription machinery by association with co-activators and co-repressors (Liu, Auboeuf et al. 2002; Smith and O'Malley 2004). Co-activators amplify transcriptional regulation and include steroid receptor co-activator (SRC) family members and receptor-interacting protein 140 (RIP 140). Co-repressors include nuclear receptor co-repressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). There is an intracellular equilibrium of co-activators and co-repressor in a tissue-specific manner which is modulated by agonist or antagonist ligands.

5. Toxicology in animals and safety in human trials

Roussel Uclaf conducted a comprehensive toxicology program in the mid-1980s and demonstrated the safety of the compound for short-term human use (Deraedt, Vannier et al. 1985). No long-term toxicity and carcinogenicity studies were performed and information on the safety of mifepristone after repeated and long-term administration is provided by various research studies investigating its therapeutic role in breast cancer, meningioma, endometriosis, uterine leiomyomata in doses ranging from 0.5 to 800 mg daily. Common side effects observed during long-term treatment with doses of up to 200 mg daily include fatigue, nausea, anorexia and vomiting. Weight loss, skin rashes, cessation of menses in premenopausal women, transient thinning of the hair and hot flushes have also been reported (Grunberg, Weiss et al. 1991; Lamberts, Koper et al. 1991; Grunberg 1994). Uncommon side effects include decrease in libido and gynaecomastia (Grunberg 1994), biochemical hypothyroidism (Heikinheimo, Ranta et
al. 1997), transient elevation in hepatic enzymes (Murphy, Kettel et al. 1993; Cameron, Thong et al. 1995; Murphy, Morales et al. 1995; Kettel, Murphy et al. 1998; Eisinger, Meldrum et al. 2003) [Onapristone, which is closely related structurally to mifepristone, was withdrawn from clinical trials because of its effect on hepatic enzymes (Klijn, Setyono-Han et al. 2000)] and low serum potassium levels (Romieu, Maudelonde et al. 1987; Chu, Matthias et al. 2001). Although, hypoadrenalism secondary to glucocorticoid antagonism has been reported with doses exceeding 200 mg per day (Laue, Lotze et al. 1990; Lamberts, Koper et al. 1991), it is an uncommon occurrence in humans with an intact pituitary–adrenal axis.

Mifepristone crosses the placental barrier (Hill, Selinger et al. 1991) and although abnormalities have been reported in surviving foetuses following administration of mifepristone-prostaglandin regimen for medical abortion, the data on mifepristone-only administration is reassuring (Sitruk-Ware, Davey et al. 1998; Sitruk-Ware 2006).

6. Anti-fertility effects

The key anti-fertility effects are the suppression of ovulation and endometrial disruption (Batista, Cartledge et al. 1992; Ledger, Sweeting et al. 1992; Croxatto, Salvatierra et al. 1993; Cameron, Thong et al. 1995; Danielsson, Swahn et al. 1997; Croxatto, Kovacs et al. 1998; Brown, Cheng et al. 2002; Baird, Brown et al. 2003). Other reproductive targets include induction of luteolysis (Schaison, George et al. 1985; Ottander, Hosokawa et al. 2000), and possible interference with tubal (Christow, Sun et al. 2002) and sperm function (Serres, Yang et al. 1994; Yang, Serres et al. 1994; Yang, Serres et al. 1996).

6.1. Effect on hypothalamo-pituitary axis, follicular development and ovulation
The effects of mifepristone on hypothalamo-pituitary axis, follicular development and ovulation vary depending on dose, timing and duration of administration. Administration of mifepristone during the follicular phase of the menstrual cycle delays the oestrogen rise and the luteinizing hormone (LH) surge, but has no effect on endometrial development during the implantation period, whereas, administration of an adequate dose immediately after ovulation mifepristone will significantly inhibit endometrial development.

**Follicular administration**

Short-term (3mg/kg in the first 3 days) administration in the early follicular phase does not alter the ovarian or menstrual cycle (Stuenkel, Garzo et al. 1990), whereas, a similar administration after ultrasound documentation of a dominant follicle arrested follicular development, suppressed oestradiol levels and delayed/inhibited onset of LH surge and ovulation (Liu, Garzo et al. 1987). The mechanism by which mifepristone interferes with the mid-cycle LH surge is complex and possible involves a direct reduction in sensitivity of the pituitary gonadotropin to the positive feedback effects of oestrogen (Baird, Thong et al. 1995).

A daily low-dose administration of 5 mg or 10 mg of mifepristone throughout one menstrual cycle prevents the leading follicle from achieving maturity and from producing the circulating oestradiol levels necessary to trigger the LH surge (Ledger, Sweeting et al. 1992; Croxatto, Salvatierra et al. 1993). A daily low-dose administration of 1-5 mg for duration of 3-15 days after selection of dominant follicle impairs follicular growth (Batista, Cartledge et al. 1992; Batista, Cartledge et al. 1994; Croxatto, Salvatierra et al. 1995). Further reduction in daily dose (1 or 2 mg) may
allow full follicular growth, but, is still sufficient to inhibit ovulation (Ledger, Sweeting et al. 1992; Croxatto, Salvatierra et al. 1993; Baird, Thong et al. 1995; Cameron, Thong et al. 1995).

The mechanism by which mifepristone interferes with follicular development is not clear. Mifepristone interrupts follicular growth after selection of the dominant follicle (Messinis, Krishnan et al. 1997) and hypotheses include an increase in progesterone receptors in the theca and granulosa cells of the dominant follicle (Iwai, Nanbu et al. 1990), decrease in follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Permezel, Lenton et al. 1989) and reduction in the sensitivity of the selected follicle to FSH (Messinis, Krishnan et al. 1997). The hypotheses are based on studies of varying methodology and dose and it is possible that all three mechanisms play a role depending on dose and time of administration.

Only two studies have reported endocrine effects of administration of single dose mifepristone in the follicular phase. A single dose of 5 mg administered when the lead follicle reached a diameter of 12–14 mm retarded its growth for 12–48 h after treatment (Croxatto, Salvatierra et al. 1995). A single dose of 10 mg administered when lead follicle was >15 mm, or approximately 2 days prior to the LH surge, delayed or inhibited the LH surge in all subjects (Marions, Hultenby et al. 2002). A dose-related delay in menstruation has been reported when 3 single doses of mifepristone were given for emergency contraception (1-week delay in menses; 600-mg = 36%, 10 mg = 18%) (WHO 1999). It is however impossible to ascertain the effect of dose on follicular growth and LH surge as mifepristone was administered at random.
Luteal-phase administration

Administration during the early-luteal phase (peri-ovulatory, after initiation of LH surge, LH + 0 to LH + 2) does not prolong the follicular phase and ovulation is not arrested (Liu, Garzo et al. 1987; Brown, Williams et al. 2003).

Mid-luteal phase administration of mifepristone increases the amplitude and frequency of LH pulses due to block in the suppressive effect of progesterone (Garzo, Liu et al. 1988; Shoupe, Mishell et al. 1990). This is followed by a decrease in the secretion of total LH and pulse amplitude suggesting direct pituitary action.

Late luteal administration of single-dose mifepristone reduces both frequency and amplitude of LH pulses suggesting a hypo-thalamo-pituitary action (Garzo, Liu et al. 1988).

Intermittent administration

Intermittent administration of dose below 10 mg does not inhibit ovulation (Gemzell-Danielsson, Westlund et al. 1996; Marions, Danielsson et al. 1998; Cheng, Weng et al. 2001). Higher doses may prolong the follicular phase and inhibition of ovulation is inconsistent (Spitz, Croxatto et al. 1993; Chen and Xiao 1997; Cheng, Weng et al. 2001; Godfrey, Mawson et al. 2004; Pei, Xiao et al. 2007). Once-a-week treatment did not influence the length of the luteal phase (Gemzell-Danielsson, Westlund et al. 1996).
Table 1.2 and 1.3: The effect of mifepristone administration on ovarian and endometrial function.

**Single dose**


2. Mid-luteal 200 mg, (Shoupe, Mishell et al. 1987)

3. Late-luteal 400, 600 mg, (Dubois, Ulmann et al. 1988; Lahteenmaki, Rapeli et al. 1988; Couzinet, Le Strat et al. 1990)

**Intermittent administration:** 2.5 mg (Gemzell-Danielsson, Westlund et al. 1996), 5 mg (Gemzell-Danielsson, Westlund et al. 1996; Marions, Danielsson et al. 1998; Cheng, Weng et al. 2001), 10 mg (Spitz, Croxatto et al. 1993; Cheng, Weng et al. 2001; Godfrey, Mawson et al. 2004), 25 mg (Chen and Xiao 1997; Pei, Xiao et al. 2007), 50 mg (Spitz, Croxatto et al. 1993; Pei, Xiao et al. 2007)

**Short-term administration:**

1. Early follicular: 3mg/kg daily for 3 days (Stuenkel, Garzo et al. 1990)

2. Late follicular: 3mg/kg daily for 3 days (Stuenkel, Garzo et al. 1990)

3. Mid-luteal: 3 mg/kg daily for 3 days (Garzo, Liu et al. 1988); 25-100 mg daily for 4 days (Schaison, George et al. 1985; Swahn, Johannisson et al. 1988)

**Continuous daily low-dose mifepristone:** 0.1 mg (Danielsson, Swahn et al. 1997), 0.5 mg (Danielsson, Swahn et al. 1997), 1 mg (Batista, Cartledge et al. 1992; Croxatto, Salvatierra et al. 1993; Croxatto, Kovacs et al. 1998), 2 mg (Ledger, Sweeting et al. 1992; Cameron, Thong et al. 1995; Brown, Cheng et al. 2002; Baird, Brown et al. 2003), 5 mg (Ledger, Sweeting et al. 1992; Croxatto, Salvatierra et al. 1993; Cameron, Thong et al. 1995; Brown, Cheng et al. 2002; Baird, Brown et al. 2003; Lakha, Ho et al. 2007), 10 mg (Croxatto, Salvatierra et al. 1993)
Table 1.2: Effect of single dose luteal and intermittent administration of mifepristone on ovarian cycle, endometrium and menstruation

<table>
<thead>
<tr>
<th>Dose</th>
<th>Follicular development</th>
<th>Ovulation</th>
<th>Endometrium</th>
<th>Menstruation</th>
<th>Pregnancy per cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>single dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>follicular</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>early luteal</td>
<td>no effect</td>
<td>no effect</td>
<td>retard secretory changes</td>
<td>no effect</td>
<td>3/269</td>
</tr>
<tr>
<td>mid-luteal</td>
<td>not applicable</td>
<td>no effect/reduced LH</td>
<td>not tested</td>
<td>bleed within 72 hours</td>
<td>not tested</td>
</tr>
<tr>
<td>late luteal</td>
<td>not applicable</td>
<td>reduced LH</td>
<td>not tested</td>
<td>Disruption</td>
<td>32/215</td>
</tr>
<tr>
<td>weekly dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 mg</td>
<td></td>
<td>no effect</td>
<td>no effect</td>
<td>minor asynchrony</td>
<td>no effect</td>
</tr>
<tr>
<td>5.0 mg</td>
<td>no effect/retard</td>
<td>no effect</td>
<td>asynchrony</td>
<td>no effect/delay</td>
<td>7/109</td>
</tr>
<tr>
<td>10.0 mg</td>
<td>no effect/retard</td>
<td>no effect/suppress</td>
<td>asynchrony</td>
<td>no effect/delay</td>
<td>6/124</td>
</tr>
<tr>
<td>2.0 mg</td>
<td>no effect/retard</td>
<td>no effect/suppress</td>
<td>asynchrony</td>
<td>no effect/delay</td>
<td>0/234</td>
</tr>
<tr>
<td>50.0 mg</td>
<td>no effect/retard</td>
<td>no effect/suppress</td>
<td>asynchrony</td>
<td>no effect/disrupt</td>
<td>0/222</td>
</tr>
</tbody>
</table>
Table 1.3: Effect of continuous administration of mifepristone on ovarian cycle, endometrium and menstruation

<table>
<thead>
<tr>
<th>Dose</th>
<th>Follicular development</th>
<th>Ovulation</th>
<th>Endometrium</th>
<th>Menstruation</th>
<th>Pregnancy per cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>short-term</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>early follicular</td>
<td>no effect</td>
<td>no effect</td>
<td>no effect</td>
<td>no effect</td>
<td>not tested</td>
</tr>
<tr>
<td>late follicular</td>
<td>arrested/retarded</td>
<td>suppress/delay</td>
<td>not tested</td>
<td>no effect</td>
<td>not tested</td>
</tr>
<tr>
<td>mid-luteal</td>
<td>not applicable</td>
<td>reduced LH output</td>
<td>pre-menstrual changes</td>
<td>bleed within 72 hours</td>
<td>not tested</td>
</tr>
<tr>
<td>long-term</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 mg/day</td>
<td>no effect</td>
<td>no effect</td>
<td>no effect</td>
<td>no effect</td>
<td>not tested</td>
</tr>
<tr>
<td>0.5 mg/day</td>
<td>no effect</td>
<td>no effect</td>
<td>minor changes</td>
<td>no effect</td>
<td>5/141</td>
</tr>
<tr>
<td>1.0 mg/day</td>
<td>arrested/retarded</td>
<td>inhibited (20-50%)</td>
<td>proliferative</td>
<td>no effect</td>
<td>not tested</td>
</tr>
<tr>
<td>2.0 mg/day</td>
<td>arrested/retarded</td>
<td>inhibited (90-100%)</td>
<td>proliferative</td>
<td>amenorrhea</td>
<td>0/50</td>
</tr>
<tr>
<td>5.0 mg/day</td>
<td>arrested/retarded</td>
<td>inhibited (95-100%)</td>
<td>proliferative</td>
<td>amenorrhea</td>
<td>0/50</td>
</tr>
<tr>
<td>10.0 mg/day</td>
<td>arrested/retarded</td>
<td>Inhibited (100%)</td>
<td>proliferative</td>
<td>amenorrhea</td>
<td>not tested</td>
</tr>
</tbody>
</table>
6.2. Effect on endometrial development and implantation

Pre-ovulatory administration

There was no effect on endometrial morphology following administration 50 mg daily on cycle days 7-10 (Swahn, Johannisson et al. 1988). There was minimal effect on endometrial development (on cycle day LH+6-8) following administration of single dose of 10 mg 2 days prior to ovulation (Marions, Hultenby et al. 2002). Expression of the β3 integrin subunit in the glandular epithelial cells was decreased in 2/6 subjects, whereas, other markers of receptivity were the same as observed in control cycles.

Peri-ovulatory administration

Single dose (200-800 mg) administration in the early luteal phase (LH+0 to LH + 2) causes changes in endometrial secretory activity, expression of steroid receptors, especially progesterone receptors, integrins and leukaemia inhibitory factor (LIF) at the expected time of implantation (Li, Dockery et al. 1988; Swahn, Bygdeman et al. 1990; Maentausta, Svalander et al. 1993; Gemzell-Danielsson, Svalander et al. 1994; Cameron, Critchley et al. 1997; Danielsson, Swahn et al. 1997; Dockery, Ismail et al. 1997; Critchley, Tong et al. 1999; Hapangama, Critchley et al. 2002; Brown, Williams et al. 2003) without affecting vaginal bleeding patterns (Swahn, Bygdeman et al. 1990; Gemzell-Danielsson, Swahn et al. 1993; Gemzell-Danielsson, Svalander et al. 1994; Hapangama, Critchley et al. 2002; Brown, Williams et al. 2003). Similar changes in endometrial secretory profiles of vascular endothelial growth factor, transforming growth factor β-1, leukemia inhibitory factor and glycodelin have been observed in the rhesus monkey following mifepristone administered on day LH+2 (Ghosh, Kumar et al. 1998; Lalitkumar, Sengupta et al. 1998). Mifepristone inhibits progesterone-
induced down-regulation of ER and PR (Swahn, Bygdeman et al. 1990; Maentausta, Svalander et al. 1993; Cameron, Critchley et al. 1997; Critchley, Tong et al. 1999; Hapangama, Critchley et al. 2002). It also affects expression of calcitonin (Kumar, Zhu et al. 1998) and prostaglandin dehydrogenase (Cameron, Critchley et al. 1997) and release of PGF2 through the uterine fluid (Gemzell-Danielsson and Hamberg 1994). The changes are likely to prevent implantation and contraceptive efficacy of such an approach has been demonstrated in two studies (Gemzell-Danielsson, Swahn et al. 1993; Hapangama, Brown et al. 2001). Peri-ovulatory administration also changes uterine contractility to a pattern more usually seen in the late luteal phase (Gemzell, Swahn et al. 1990), perhaps adding to the contraceptive effect.

The effect of 10 mg had a similar but much less marked effect (Marions, Hultenby et al. 2002). The endometrium was slightly out of phase, and the down-regulation of progesterone receptor concentration was inhibited in five of the six women. The effect on markers for endometrial receptivity was less marked. It is difficult to know if the effects with lower dose are sufficient to inhibit implantation. The efficacy of 10 mg dose mifepristone when used for emergency contraception (WHO 1999) and the possibility to extend the interval between the unprotected sexual intercourse and treatment to 120 hours (Ashok, Wagaarachchi et al. 2001; Hamoda, Ashok et al. 2004) suggest that this may be the case.

Post-ovulatory (luteal-phase) administration

Mid-luteal administration of a single dose (50-200 mg) of mifepristone induces endometrial bleeding within 72 hours (Schaison, George et al. 1985; Shoupe, Mishell et al. 1990; Greene, Kettel et al. 1992) and endometrium shows shrinkage of glandular
lumen and leukocyte infiltration and vascular infiltration (Swahn, Johannisson et al. 1988).

The mechanism of endometrial bleeding following mid-luteal administration is poorly understood. Luteolysis is dose-dependent (Schaison, George et al. 1985), however, endometrial bleeding occurs whether or not luteal regression is induced by the compound, indicating that mifepristone acts directly upon the endometrial tissue. A direct vascular effect has been suggested (Johannisson, Oberholzer et al. 1989) and our group has proposed a role for modulation of local prostaglandin production (Cheng, Kelly et al. 1993; Hapangama, Critchley et al. 2002). The progesterone levels in the peri-ovulatory phase are relatively low and consequently, expression of endometrial ER and PR is maximal, whereas, the opposite is true in the mid-luteal phase of the menstrual cycle. Therefore peri-ovulatory administration will prevent progesterone-induced changes, whereas, mid-luteal administration will antagonise progesterone-induced changes in the endometrium. When luteolysis is incomplete, a second bleed has been reported at expected time of menstruation (Schaison, George et al. 1985; Garzo, Liu et al. 1988; Swahn, Johannisson et al. 1988).

Late-luteal (Dubois, Ulmann et al. 1988; Lahteenmaki, Rapeli et al. 1988; Couzinet, Le Strat et al. 1990) phase administration disrupts the endometrium to a degree likely to prevent implantation.

Intermittent administration

Intermittent (weekly) administration of mifepristone disrupts endometrial maturation (Gemzell-Danielsson, Westlund et al. 1996; Cheng, Weng et al. 2001). The number of endometrial glands and glandular diameter decreased during weekly administration of
2.5 or 5mg of mifepristone for 8 weeks, but this decrease was not significant for the 2.5 mg group. Despite only minor effects on endometrial morphology, both regimens reduced secretory activity (Gemzell-Danielsson, Westlund et al. 1996).

6.3. Effect on corpus luteum

Administration of high doses of mifepristone during the mid- to late-luteal phase results in shedding of the endometrium and vaginal bleeding within a few days of the treatment. If premature menstruation is not accompanied by luteolysis (approximately 20 - 60% of cases), the endometrial and ovarian cycles are desynchronized and a second bleeding episode occurs at the time of the expected menstruation (Schaison, George et al. 1985; Garzo, Liu et al. 1988; Swahn, Johannisson et al. 1988). The manner in which mifepristone induces luteolysis in certain situations is not precisely known, but could be indirect through withdrawal of LH support (Mais, Kazer et al. 1986), as evidenced by a reduction in amplitude and frequency of LH pulses and blunting of the pituitary LH response to GnRH (Schaison, George et al. 1985; Garzo, Liu et al. 1988). It may also depend on the age of the corpus luteum (Swahn, Johannisson et al. 1988).

6.4. Effect on oocyte maturation and fertilization

Mifepristone treatment does not alter oocyte maturation and fertilization potential. 100 mg mifepristone was administered 1 h before induction of ovulation with injection of 5000 IU of human chorionic gonadotropin (hCG) in 20 subjects in whom follicular growth was stimulated with clomiphene for 5 days (Messinis and Templeton 1988). Laparascopy (for tubal sterilization) was performed 34 h after hCG and all follicles with a diameter of >15 mm were aspirated and collected oocytes submitted to in vitro
fertilization. 20 women similarly stimulated with clomiphene but not receiving mifepristone served as a control group. The number of retrieved oocytes, the rate of fertilization and the cleavage rate did not differ between the mifepristone group and controls (Messinis and Templeton 1988).

6.5. Effect on tubal milieu and function

The tubal microenvironment is of great importance to ensure normal embryo development (Smotrich, Stillman et al. 1996). Too rapid or too slow tubal transport could also be expected to cause desynchronization between the embryo and the tube, and/or blastocyst and endometrium. The role of progesterone in regulating tubal transport has been confirmed (Mahmood, Saridogan et al. 1998) and mifepristone reversed the inhibitory effect of high doses of progesterone on ciliary movement. ER and PR expression in the fallopian tube is regulated by the changing ovarian steroid hormones (Amso, Crow et al. 1994; Shah, Nandedkar et al. 1999). Following 200 mg of mifepristone on LH+2, PR expression increased in epithelial and stromal cells (Christow, Sun et al. 2002). There was also an effect on ER expression, although less pronounced and restricted to the epithelial cells. Modulation of progesterone regulated tubal microenvironment (Smotrich, Stillman et al. 1996) and tubal transport (Mahmood, Saridogan et al. 1998) may add to contraceptive mechanism of mifepristone.

6.6. Effect on the human spermatozoa

Progesterone triggers the acrosome reaction of capacitated human spermatozoa in vitro and a rise in intracellular calcium is one of the first events observed following the binding of progesterone to the sperm cell. Mifepristone inhibits this calcium influx and
subsequent acrosome reaction (Serres, Yang et al. 1994; Yang, Serres et al. 1994). It can also interfere with the ability of sperm to fertilise eggs (Yang, Serres et al. 1996).

7. Contraceptive Approaches

7.1. Daily pill

Our group reported the first large contraceptive efficacy study of low-dose mifepristone in 2002 (Brown, Cheng et al. 2002). 90 subjects, 40 in Edinburgh and 50 in China were randomised to 2 or 5 mg mifepristone daily for 120 days. The study design is illustrated in Figure 1.3.

Figure 1.3: Low-dose mifepristone for 120 days - study design
Briefly, subjects maintained a daily menstrual diary and a weekly urinary steroid (metabolites of oestrogen and progesterone) profile. Transvaginal ultrasound (TVUSS) and safety checks (symptom reporting, medical examination, haematology and biochemical investigations) were carried out pre-treatment and at 30, 60, 90, 120 and 150 days of treatment. Pipelle endometrial biopsies were collected at 3 time-points; pre-treatment mid-proliferative phase of menstrual cycle and 60 and 120 days following administration of mifepristone. Only 10 women in Edinburgh and all subjects in Shanghai used mifepristone as their only method of contraception.

The principal outcomes are summarised in Table 1.4 (Brown, Cheng et al. 2002).

**Table 1.4: Low-dose mifepristone for 120 days - principal outcomes**

<table>
<thead>
<tr>
<th></th>
<th>Edinburgh (n=50)</th>
<th>Shanghai (n=40)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 mg</td>
<td>5 mg</td>
</tr>
<tr>
<td>An-ovulation</td>
<td>90 %</td>
<td>95 %</td>
</tr>
<tr>
<td>Amenorrhea</td>
<td>65 %</td>
<td>88 %</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>0/10</td>
<td></td>
</tr>
</tbody>
</table>

The different thresholds for endometrial disruption (0.5 mg) and suppression of ovulation (2 mg), raise the prospect of endometrial contraception; prevention of implantation without disruption of ovulation. The contraceptive efficacy of 0.5 mg daily was investigated in 32 subjects (Marions, Viski et al. 1999). 5 pregnancies occurred in 141 at-risk treatment cycles, which is significantly less than if no contraception was used. Although the results are disappointing, they indicate the contribution of endometrial mechanisms to the contraceptive mechanism of low-dose mifepristone.
7.2. Weekly pill

Intermittent administration of dose below 10 mg does not inhibit ovulation (Gemzell-Danielsson, Westlund et al. 1996; Marions, Danielsson et al. 1998; Cheng, Weng et al. 2001) and although the endometrium is disrupted, the pregnancy rate is disappointingly high (Marions, Danielsson et al. 1998; Cheng, Weng et al. 2001). A recent Chinese study has reported high efficacy using 25-50 mg weekly dose (Pei, Xiao et al. 2007).

7.3. Once-a-month pill

There are two once-a-month contraceptive approaches using administration of a single dose of mifepristone (200-800 mg); administration in the early and late luteal phase of the menstrual cycle respectively. Late-luteal administration is a post-implantation contraceptive (Dubois, Ulmann et al. 1988; Lahteenmaki, Rapeli et al. 1988; Couzin, Le Strat et al. 1990; Swahn, Bygdeman et al. 1999) which reduces its wider acceptability and all three studies investigating its use reported pregnancy rates between 5-19%. On the other hand, early-luteal (peri-ovulatory, within 48 hours of the LH surge) administration, markedly retards endometrial development to a degree which is likely to prevent implantation (Swahn, Bygdeman et al. 1990; Maentausta, Svalander et al. 1993; Gemzell-Danielsson, Svalander et al. 1994; Cameron, Critchley et al. 1997). The biggest advantage of the peri-ovulatory once-a-month approach is that it does not alter the length of the menstrual cycle (Swahn, Bygdeman et al. 1990; Gemzell-Danielsson, Swahn et al. 1993; Gemzell-Danielsson, Svalander et al. 1994; Hapangama, Brown et al. 2001; Brown, Williams et al. 2003) thereby facilitating its continued use. Between 12-35% women experience light vaginal bleeding commence
within a few days of mifepristone and lasting 2-3 days (Swahn, Bygdeman et al. 1990; Gemzell-Danielsson, Swahn et al. 1993; Hapangama, Brown et al. 2001).

The contraceptive efficacy of a once-a-month administration of mifepristone has been demonstrated in two studies (Gemzell-Danielsson, Swahn et al. 1993; Hapangama, Brown et al. 2001). 21 Swedish women used 200 mg mifepristone on day LH + 2 every month as their only contraceptive method for 1-12 months (Gemzell-Danielsson, Swahn et al. 1993). There was only 1 pregnancy out of 124 “at risk” cycles, giving a probability of pregnancy of 0.008. The subsequent menstrual cycle was not disrupted, although 35% of women did report slight vaginal bleeding 2–3 days after treatment. In another study, the contraceptive efficacy of 200 mg of mifepristone administered once-a-month for 6-7 consecutive menstrual cycles on day LH + 2 in 32 women from Edinburgh was compared to the natural fecundity of a control group, comprising 20 women who were trying to conceive (Hapangama, Brown et al. 2001). There were two pregnancies out of a total of 178 cycles in the treatment group (probability of pregnancy 0.01) and 12 pregnancies out of a total of 50 cycles in the control group (probability of pregnancy 0.25-0.32).

The timing of administration of mifepristone is critical to the success of the periovulatory once-a-month approach. Administration during the follicular phase of the menstrual cycle (too early), inhibits follicular development, delays the mid-cycle LH surge and prolongs the length of the cycle (Croxatto, Salvatierra et al. 1995; Marions, Hultenby et al. 2002) whereas administration in the mid to late luteal phase (too late) induces bleeding within a few days and this is often followed by a second bleed at the time of the expected menses (Shoupe, Mishell et al. 1987; Dubois, Ulmann et al. 1988;
Lahteenmaki, Rapeli et al. 1988; Couzinet, Le Strat et al. 1990; Swahn, Bygdeman et al. 1999). Thus a single dose of mifepristone administered outside the critical peri-ovulatory time results in irregular bleeding which many women find unacceptable.

In the two studies which demonstrated the efficacy of the method, the peri-ovulatory window of administration was identified by the mid-cycle peak of LH in urine using urine test sticks (Ovu-quick, Organon) in Sweden (Gemzell-Danielsson, Swahn et al. 1993) and a home-use fertility monitor (Persona, Unipath Ltd) in Edinburgh (Hapangama, Brown et al. 2001). Both approaches are expensive and probably too demanding on user compliance to be used routinely. In the Edinburgh study for example, subjects failed to perform tests on 24% of the days required, with 42% of cycles having at least one missed test at time of highest fertility (Hapangama, Glasier et al. 2001). 11.2% of LH surges were missed as a consequence of imperfect use of the monitor.

Our group has demonstrated that mifepristone given on the day of the LH surge (LH+0), retards endometrium sufficiently to prevent implantation without interfering with ovulation (Brown, Williams et al. 2003). This observation widens the peri-ovulatory ‘window of opportunity’ for administering mifepristone to at least 3 days (LH+0 to LH+2) and allows more flexibility to investigate a cheaper and simpler method of timing administration.

7.4. Emergency contraception

Administration of a single dose (600 mg) of mifepristone within 72 hours of unprotected intercourse is an effective emergency contraceptive (Glasier, Thong et al. 1992; Webb, Russell et al. 1992). A large multicentre study has reported efficacy of
much lower doses (10, 50 and 100 mg) (WHO 1999). Whereas the 600 mg dose prevented all the expected pregnancies, the lower doses prevented only 85% and the most likely explanation is the reduced ‘endometrial contraceptive’ effect with lower doses. Other studies have since confirmed contraceptive efficacy of 200 (Ashok, Wagaarachchi et al. 2001) and 10 (Hamoda, Ashok et al. 2004) mg administered within 120 hours of unprotected intercourse.

8. Endometrial effects of mifepristone

A variety of endometrial effects have been reported depending on study methodology and background endocrine milieu at time of administration. These include proliferative (oestrogen-like), anti-proliferative (anti-oestrogen) and secretory (progesterone-like) effects.

8.1 Effects on proliferation and histology


Endometrial histology following 60 and 120 days of treatment is summarised in Table 1.5 (Baird, Brown et al. 2003).
### Table 1.5: Low-dose mifepristone for 120 days - endometrial histology

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-treatment proliferative phase</th>
<th>Day 60 mifepristone treatment</th>
<th>Day 120 mifepristone treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edinburgh 2mg</td>
<td>24 proliferative</td>
<td>20 proliferative</td>
<td>15 proliferative</td>
</tr>
<tr>
<td></td>
<td>2 not assessable</td>
<td>5 cystic dilatation/inactive</td>
<td>9 cystic dilatation/inactive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 not assessable</td>
<td>2 not assessable</td>
</tr>
<tr>
<td>5mg 23 proliferative</td>
<td>10 proliferative</td>
<td>10 cystic dilatation/inactive</td>
<td>12 proliferative</td>
</tr>
<tr>
<td></td>
<td>1 not assessable</td>
<td>9 cystic dilatation/inactive</td>
<td>1 secretory</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 complex hyperplasia</td>
<td>2 not assessable</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 menstrual type</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 not assessable</td>
<td></td>
</tr>
<tr>
<td>Shanghai 2mg</td>
<td>14 proliferative</td>
<td>15 proliferative</td>
<td>10 proliferative</td>
</tr>
<tr>
<td></td>
<td>6 not assessable</td>
<td>1 cystic dilatation/inactive</td>
<td>2 cystic dilatation/inactive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 menstrual type</td>
<td>1 secretory</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 secretory</td>
<td>1 menstrual</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 not assessable</td>
<td>6 not assessable</td>
</tr>
<tr>
<td>5mg 16 proliferative</td>
<td>13 proliferative</td>
<td>11 proliferative</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 not assessable</td>
<td>1 menstrual type</td>
<td>1 secretory</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 not assessable</td>
<td>1 cystic dilatation/inactive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 not assessable</td>
<td></td>
</tr>
</tbody>
</table>

Work carried out in the 1930s (Allen, 1929; Markee 1940)) established that ovarian steroids, oestradiol (E2) and progesterone (P4), were responsible for the changes in endometrial structure and function throughout the menstrual cycle. The majority of circulating oestrogen is derived from developing ovarian follicles and the remainder from extra-ovarian aromatisation of adrenal precursors. Unopposed oestrogen in the follicular phase of the cycle induces proliferative changes in the endometrium. Following ovulation, progesterone secreted by the corpus luteum induces secretory changes that make the uterus receptive to implantation. Endometrial shedding
(menstruation) is initiated by the fall in concentration of progesterone that follows luteal regression.

The degree of ovarian suppression following long-term mifepristone treatment is dose-dependent. Ovarian suppression is also clearly related to the level of background ovarian activity at the start of administration (Brown, Cheng et al. 2002). Chinese subjects had lower baseline ovarian activity (lower E2) compared to the Edinburgh subjects (Brown, Cheng et al. 2002). Following mifepristone administration, the Chinese subjects showed a significant fall in E2 levels whereas the Edinburgh subjects continued to secrete oestrogen in the mid-follicular range (Brown, Cheng et al. 2002)

Hyperplastic changes

A functional blockade of progesterone following administration of mifepristone raises concerns of risk of endometrial hyperplasia and pre-malignant changes secondary to effect of prolonged unopposed oestrogen. The risk appears to be largely dose-dependent.

In women, following high doses of mifepristone (50 mg/day for 6 months) variable effects such as atypical cystic changes have been described in endometrium (Murphy, Kettel et al. 1995). The endometrium demonstrated numerous mitotic figures; however, was no conclusive evidence of atypical hyperplasia. Endometrial polyps (Martineau and Levental 2000; Grunberg, Weiss et al. 2006) and hyperplasia (Grunberg, Weiss et al. 1991; Grunberg 1994; Newfield, Spitz et al. 2001; Grunberg, Weiss et al. 2006) have been reported following long-term administration of high-dose mifepristone for inoperable meninigiomas (200 mg/d) and intractable Cushing's
syndrome (400 mg/d). There were no cases of atypia and the hyperplasia regressed on cessation of mifepristone treatment (Newfield, Spitz et al. 2001).

Hyperplastic endometrial effects have also been observed with lower doses of mifepristone. Administration of 1mg mifepristone daily for 150 days was associated with increased endometrial thickness and dilated glands in 25 and 34% of the monophasic cycles respectively (Croxatto, Kovacs et al. 1998). Our group reported a significant increase in endometrial thickness (TVUSS) of the Edinburgh subjects by 60 days of mifepristone treatment whereas the Shanghai subjects showed a significant decrease. The endometrii in both groups were histologically proliferative and a small number of samples showed cystic dilatation (Baird, Brown et al. 2003). Endometrial thickness determined by ultrasound examination correlates poorly to histology-defined proliferation as it may also represent tissue oedema, fluid collection or benign stromal thickening (Fleischer, Wheeler et al. 1999; Mourits, Van der Zee et al. 1999; Liedman, Lindahl et al. 2000). One Edinburgh subject in the 5-mg group showed features of complex hyperplasia after 60 d of treatment, but after 120 days this subject’s endometrium had become inactive. No cases showed any evidence of cytological atypia (Baird, Brown et al. 2003).

A high incidence of simple endometrial hyperplasia (28%) was observed in women receiving 5 or 10 mg mifepristone daily for 6 months, for the treatment of symptomatic leiomyoma (Eisinger et al., 2003). Data from this study have since been re-evaluated (Steinauer, Pritts et al. 2004) and the authors (Eisinger, Bonfiglio et al. 2005) have now reported a longer follow-up. Simple hyperplasia was seen in 13.9 % at 6 months and 4.8 % at 12 months. All cases of hyperplasia occurred in the 10 mg group. No
endometrial sample showed cytologic atypia. The same group have now reported a further study of 52 subjects with symptomatic leiomyoma treated with mifepristone 5 mg daily for 26 weeks (Fiscella, Eisinger et al. 2006). There were no cases of endometrial hyperplasia.

The precise mechanism for these hyperplastic effects on the endometrium remains unknown. Mifepristone does not bind to ER (Philibert 1984; Zou, Marschke et al. 1999). High-dose mifepristone stimulates the HPA axis and there is an increase in ACTH and cortisol. On occasion this is accompanied by elevation of androstenedione, oestrone, testosterone and oestradiol (Lamberts, Koper et al. 1991; Heikinheimo, Ranta et al. 1997; Heikinheimo, Ranta et al. 2000). It is thus possible that endometrial aromatization of these adrenal androgens may enhance the estrogen milieu locally. However, recent studies have shown that mifepristone inhibits aromatase induction in human breast adipose tissue (Schmidt and Loffler 1997) and blocks medroxyprogesterone acetate-induced aromatase activity in endometrial stromal cells (Tseng, Mazella et al. 1986). Continuous administration of mifepristone in daily doses of 10 mg and below are not associated with increases in cortisol (Croxatto, Salvatierra et al. 1993). Hence the endometrial effects noted with these doses are not related to aromatization but could be a consequence of an unopposed oestradiol effect on the endometrium. Other explanations relate to the effects of the ER and PR isoforms. ERβ is anti-proliferative in several models (Weihua, Saji et al. 2000) and often functions as a transdominant repressor of ERα transcriptional activity (Hall and McDonnell 1999). Mifepristone can act as a potent ERβ antagonist (Zou, Marschke et al. 1999) and post-transcriptional effects of unopposed ERα could explain the observed oestrogenic effects. Mulac-Jericevic et al.(Mulac-Jericevic, Mullinax et al. 2000) have shown that
selective ablation of PR-A results in a gain of progesterone-dependent proliferative activity mediated through PR-B. PR-B increases whereas PR-A diminishes oestradiol responsiveness in the uterus. The precise effect on the endometrium may thus be dependent on the ratio of the different isoforms of ER and PR.

Anti-proliferative effects

Despite the mixed histopathological reports, at a cellular level, anti-proliferative effects have been reported in both animal (van Uem, Hsiu et al. 1989; Wolf, Hsiu et al. 1989; Hodgen, van Uem et al. 1994; Katkam, Gopalkrishnan et al. 1995; Zelinski-Wooten, Slayden et al. 1998) and human (Croxatto, Salvatierra et al. 1993; Cameron, Critchley et al. 1996; Croxatto, Kovacs et al. 1998) studies. Mifepristone does not bind to ER and the mechanism of this anti-proliferative effect, first described in 1989 as “non-competitive anti-estrogenic effect” (Wolf, Hsiu et al. 1989), remains largely unexplained. Several hypotheses which have been formulated to account for these observations include inhibition of the oestrogen receptor gene transcription by the PR-A isoform (McDonnell and Goldman 1994), reduced endometrial blood supply due to atrophy of spiral arteries (Chwalisz, Brenner et al. 2000), blockade of progesterone-dependent growth factors (Koji et al., 1994), inhibition of angiogenesis (Greb, Heikinheimo et al. 1997; Grow, Reece et al. 1998), apoptosis modulation via growth factors such as NkKB (Slayden, Hirst et al. 1993; Han and Sidell 2003), cell cycle blockade (Cameron, Critchley et al. 1996; Heikinheimo, Hsiu et al. 1996) and antioxidant effect of mifepristone (Parthasarathy, Morales et al. 1994).

More recently, a role for endometrial AR has been proposed based on robust data in primate and human studies (Brenner, Slayden et al. 2002; Brenner, Slayden et al.
Exogenous and endogenous androgens can suppress oestrogen-induced endometrial proliferation (Futterweit and Deligdisch 1986; Miller, Bedard et al. 1986; Rose, Dowsett et al. 1988; Tanaka, Umesaki et al. 1999) and androstenedione can inhibit human endometrial cell growth and secretory activity in vitro (Tuckerman, Okon et al. 2000). A recent study in postmenopausal women confirmed the anti-oestrogen, anti-proliferative effects of testosterone (Zang, Sahlin et al. 2007).

Administration of mifepristone is associated with an increase in AR (Slayden, Nayak et al. 2001) and increase androgen-AR transcription can mediate these anti-proliferative. Further evidence of the role played by androgens in this anti-proliferative effect is the observation that the pure anti-androgen, flutamide, blocks the anti-proliferative effects of the progesterone receptor antagonists (PRA) ZK137316 and ZK230211 in the endometrium (Slayden and Brenner 2003). Flutamide also blocked the hyalinizing degeneration of the spiral arteries induced by PRA (Slayden and Brenner 2003). Endometrial parameters (mean ± sem) from this study are reported in Table 1.6.

Table 1.6: Primate flutamide study – endometrial parameters.

<table>
<thead>
<tr>
<th></th>
<th>E2</th>
<th>E2 + PRA</th>
<th>E2 + PRA + Flutamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (mg)</td>
<td>360 ± 32</td>
<td>64 ± 10*</td>
<td>265 ± 92</td>
</tr>
<tr>
<td>Thickness (mm)</td>
<td>3.3 ± 0.4</td>
<td>1.1 ± 0.3*</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>Stromal Compaction</td>
<td>45.5 ± 3.4</td>
<td>142.3 ± 63.7*</td>
<td>54.9 ± 4.6</td>
</tr>
<tr>
<td>Mitotic Index</td>
<td>6.3 ± 0.6</td>
<td>0.3 ± 0.3*</td>
<td>5.2 ± 3.8</td>
</tr>
</tbody>
</table>

*a=P<0.05 compared to other values in the same row
The role of AR in E2-induced epithelial cellular proliferation was investigated in the immature rat uterus (Weihua, Ekman et al. 2002). The authors propose a pathway in which, in response to E2, there is sequential activation of ERα followed by elevation of the levels of AR, increased IGF-1 (insulin growth factor 1) secretion, and epithelial cellular proliferation. When administered together with E2, flutamide significantly inhibited E2-induced proliferation of the luminal epithelium (Weihua, Ekman et al. 2002).

There is no data on the anti-proliferative effects of long-term (>30 days) mifepristone administration. Cellular proliferation in paraffin embedded mifepristone-treated endometrial samples has been previously assessed by direct counting of mitotic cells in H&E section (Baird, Brown et al. 2003) and immunohistochemistry for proliferating cell nuclear antigen (PCNA) (Cameron, Critchley et al. 1996) and Ki67 (Cameron, Critchley et al. 1996; Baird, Brown et al. 2003) proteins. There was a significant reduction in mitotic count and Ki67 expression (Baird, Brown et al. 2003). Because Ki67 protein is expressed during several phases of the cell cycle, e.g., G1, S, G2 and M, counts of Ki67 positive nuclei in paraffin sections are numerically greater than mitotic counts, though the Ki67 is usually well correlated with the mitotic index (Gerdes, Lemke et al. 1984; Endl and Gerdes 2000). Mifepristone may block completion of the cell cycle at G2-M interphase (Heikinheimo, Hsiu et al. 1996) so that Ki67 protein persists for some time after cell division has been arrested. Therefore Ki67 counts may fail to reveal the impact of mifepristone treatment on suppression of oestrogen-dependent proliferation (Cameron, Critchley et al. 1996). Direct counting of mitotic cells in H&E section is time-consuming process that requires highly skilled observers (Hall and Levison 1990). A new marker of phosphorylated proteins
associated with mitosis, phospho-histone H3 (pH3) has been validated in paraffin embedded endometrial tissues and shows an excellent correlation with the mitotic count (Brenner, Slayden et al. 2003). This marker which directly assesses current status of cellular mitosis has not been used to investigate proliferation in mifepristone-treated endometrial samples.

8.2 Expression of endometrial sex steroid receptors

Steroid receptor expression across the menstrual cycle

Steroids interact with their target organs via specific nuclear receptors. The expression of endometrial sex steroid receptors (PR, ER, AR), all of which are nuclear proteins, varies both temporally and spatially across the menstrual cycle (Garcia, Bouchard et al. 1988; Lessey, Killam et al. 1988; Snijders, de Goeij et al. 1992; Critchley, Brenner et al. 2001; Slayden, Nayak et al. 2001). The expression of ER and PR are under dual control of oestrogen and progesterone. Both endometrial ER and PR are up-regulated during the follicular phase by oestrogen and subsequently down-regulated in the luteal phase by progesterone acting at both the transcriptional and the post-transcriptional levels (Chauchereau, Savouret et al. 1992). The cyclical changes are mostly limited to the superficial layers of the endometrium, whereas stroma and glands of the basal layer express ER and PR throughout the cycle.

AR is expressed predominantly in the endometrial stroma, and there is considerably higher intensity of AR expression during the follicular as compared to the luteal phase (Slayden, Nayak et al. 2001). It is absent from the glandular and luminal compartments.
GR is expressed in the fibroblasts, lymphocytes and endothelial cells of the human endometrial stroma. It is absent from the glandular and luminal compartments (Bamberger, Milde-Langosch et al. 2001). These findings have been validated by co-localisation of GR using western blot analysis and immunohistochemistry.

**Steroid receptor expression after mifepristone treatment**

Short term treatment with PR antagonist, either during the menstrual cycle, or with combined oestrogen therapy, leads to elevations of the two main uterine steroid receptors, ER and PR (Neulen, Williams et al. 1990; Slayden and Brenner 1994; Cameron, Critchley et al. 1996; Slayden, Zelinski-Wooten et al. 1998). Similar observation has been reported following administration in the early luteal phase (Maentausta, Svalander et al. 1993; Cameron, Critchley et al. 1996). This follows a blockage of progesterone-induced down-regulation of these proteins.

ER and PR expression following chronic treatment has been shown to be increased, decreased or unchanged depending on the dose and duration of treatment (Murphy, Kettel et al. 1993; Murphy, Kettel et al. 1995; Danielsson, Swahn et al. 1997; Slayden, Zelinski-Wooten et al. 1998). However, there is no data on steroid receptor expression following administration of low-dose mifepristone for more than 30 days. GR expression has not been investigated in mifepristone treated samples.

**9. Effect on vagina**

The data on effects of mifepristone on the vagina is limited to primate studies. Mifepristone exhibits tissue specificity with significant endometrial effects in both animal and human studies and a lack of any discernable effect on oviduct (Slayden,
Hirst et al. 1993; Brenner and Slayden 1994; Slayden and Brenner 1994) and vagina (Grow, Williams et al. 1996).

The state of the vaginal micro-environment affects a woman's risk of human immuno-deficiency virus (HIV) transmission. Several human and non-human primate studies have shown long-acting progestagen treatment increases the transmission of HIV (Rehle, Brinkmann et al. 1992; Ungchusak, Rehle et al. 1996; Nagachinta, Duerr et al. 1997), simian immuno-deficiency virus (SIV) (Marx, Spira et al. 1996), and other sexually transmitted infections (STI's) (Morrison, Bright et al. 2004; MacLean 2005). The underlying mechanisms are poorly understood. Experiments on hysterectomised rhesus monkeys suggest that the vagina, rather than the cervix or uterus, is the main portal of viral entry (Miller, Alexander et al. 1992; Miller 1998).

The mechanisms by which existing contraceptive could affect HIV/STD transmission through vagina remain obscure. Epithelial thickness and integrity modulate the ease of access of virus to immune cells and sub-epithelial vasculature (Mingjia and Short 2002). Oestrogen-induced surface keratinisation and hyperplasia protects Rhesus monkeys against SIV inoculation (Smith, Baskin et al. 2000; Smith, Mefford et al. 2004), whereas oestrogen-deficient women such as those who are post-menopausal (HIVStudyGroup 1992; Aaby, Ariyoshi et al. 1996) or on long-acting gestagens, are at increased risk of HIV, presumably as a result of vaginal thinning.

Although SIV has been shown to cross intact vaginal epithelium of rhesus macaques (Marx, Spira et al. 1996), progesterone treatment enhances SIV vaginal transmission by causing profound vaginal atrophy (Marx, Spira et al. 1996; Hild-Petito, Veazey et al. 1998). Hormonal contraception does not have similar effects on human vaginal
thickness (Mauck 1998; Bahamondes, Trevisan et al. 2000; Eschenbach, Patton et al. 2000; Miller, Patton et al. 2000) despite significant fall in oestradiol levels (Miller, Patton et al. 2000).

Vaginal epithelial thickness is regulated by the levels of circulating oestrogen. Epithelial thickness is maximal at time of ovulation (Sjoberg, Cajander et al. 1988; Ma, Lu et al. 2001; Farage and Maibach 2006) and decreases in the luteal phase and post-menopause (Farage and Maibach 2006). The response of human vaginal epithelium to progestagen-induced hypo-oestrogenism is variable. A small but significant decrease (10%) in thickness has been demonstrated in one study (Ildgruben, Sjoberg et al. 2005) whereas most other studies have demonstrated no change (Mauck, Callahan et al. 1999; Bahamondes, Trevisan et al. 2000).

Paradoxically, vaginal epithelial hyperplasia has been reported in users of depot medroxyprogesterone acetate (DMPA), oral contraceptive pill and progesterone implants (Ildgruben, Sjoberg et al. 2003).

Besides epithelial thickness and integrity, other biological variables such as vaginal microflora (Klebanoff and Coombs 1991; Klebanoff, Hillier et al. 1991; Martin, Richardson et al. 1999), immune cell populations (Spira, Marx et al. 1996; Hu, Gardner et al. 2000) and natural anti-microbials (Draper, Landers et al. 2000; Pillay, Coutsoudis et al. 2001; King, Critchley et al. 2003) also play an important role in innate defences of the reproductive tract.

The two major families of natural anti-microbials are defensins and whey acidic protein (WAP) motif containing proteins (King, Critchley et al. 2003). Secretory leukocyte protease inhibitor (SLPI) is a WAP motif containing protein which is present
at mucosal surfaces, where it regulates proteolytic enzymes during inflammatory events. In addition to inhibiting several serine proteases, SLPI has antimicrobial actions and acts as component of the innate immune system to protect epithelial surfaces from infection. The role of natural anti-microbial molecules in the acquisition of STI's has not been fully established. Levels of SLPI in vaginal fluid collected from posterior fornix are decreased in women with lower genital tract infection (Draper, Landers et al. 2000) and it has been suggested that high concentrations of SLPI in vaginal fluid and saliva may limit the spread of HIV during vaginal delivery (Pillay, Couttsoudis et al. 2001) and breast-feeding (McNeely, Dealy et al. 1995; Farquhar, VanCott et al. 2002). Endogenous and exogenous sex-steroid hormones, in the form of a combined oral contraceptive or levonorgestrel intrauterine system have been shown to influence gene transcription of SLPI and other natural anti-microbials in the endometrium (Fleming, King et al. 2003). Although SLPI has been identified in vaginal fluid, its localisation and role within the vaginal epithelium has not been studied.

The effect of low-dose mifepristone on the human vaginal morphology, steroid receptor and SLPI content and distribution has never been characterised.

10. Effects on menstrual physiology

Regular menstrual bleeding is the outwardly manifestation of cyclical ovarian function. Following puberty the average woman in developed countries may expect to menstruate over 400 times until the ovaries fail at the time of menopause. In contrast, in less well developed countries and in previous centuries, the majority of women were amenorrhoeic throughout most of their lives due to late puberty, pregnancy and
prolonged lactation (Short 1984). In both women and primates, long-term administration is associated with a reduction of menstrual bleeding and amenorrhea. Although the amenorrhea is clearly related to degree of ovarian suppression, the majority of women who have incomplete ovarian suppression or sporadic ovulatory episodes report amenorrhea. It is possible that direct endometrial effects contribute significantly to the causation of mifepristone-induced amenorrhea. The association between dose, ovarian activity and vaginal bleeding pattern is reported in Table 1.7 (Brown, Cheng et al. 2002).

Table 1.7: Low-dose mifepristone for 120 days – degree of ovarian suppression and amenorrhea

<table>
<thead>
<tr>
<th>Group</th>
<th>Follicular activity</th>
<th>Ovulatory episodes</th>
<th>Amenorrhea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total suppression</td>
<td>Partial suppression</td>
<td>Continued activity</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Edinburgh</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mg</td>
<td>5 (19)</td>
<td>9 (35)</td>
<td>12 (46)</td>
</tr>
<tr>
<td>5 mg</td>
<td>9 (37)</td>
<td>10 (42)</td>
<td>5 (21)</td>
</tr>
<tr>
<td>Shanghai</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mg</td>
<td>12 (60)</td>
<td>7 (35)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>5 mg</td>
<td>14 (70)</td>
<td>3 (15)</td>
<td>3 (15)</td>
</tr>
</tbody>
</table>

Many women discontinue oestrogen-free progestogen-only contraception due to unpredictable vaginal bleeding. The mechanisms underlying these disturbances are still poorly understood and this has prompted gradually escalating effort to study all aspects of the clinical problem (Fraser and Hickey 2000). The activity and expression of a number of molecular systems has been shown to be altered in endometrium exposed to
prolonged progestagen treatment, however, it is not clear how these changes link together.

The endometrial histological response to progestogen treatment depends on the type and dose of progestogen and on the degree of suppression of ovarian activity, and will vary between individuals exposed to the same progestogen regimen and over time. The correlation between histology and menstrual bleeding pattern is poor except for severe atrophy which typically predicts amenorrhea. Endometrial angiogenesis has been the focus of ongoing research in endometrium exposed to long-term progestagen treatment. An increase in vascular density has been reported in following long-term progestagen treatment (Hickey, Simbar et al. 1999) and density was shown to be increased in atrophic endometrium compared to other histological patterns (Hickey, Simbar et al. 1999). We have reported a proliferative histology (Baird, Brown et al. 2003) and high degree of amenorrhea (Brown, Cheng et al. 2002) on long-term antiprogestogen treatment. This provides a unique opportunity to correlate histology, angiogenesis and bleeding patterns.

Endothelial cells are difficult to distinguish morphologically and immunohistochemistry staining is more sensitive than histology (Blackwell and Fraser 1988). Although there are numerous markers, no single marker can guarantee identification of all endothelial cells under all circumstances. CD34 is a glycosylated transmembrane glycoprotein expressed on the surface of hematopoietic stem cells and stromal cells, which binds to vascular endothelium, particularly on the membrane processes that interdigitate between endothelial cells (Stella, Cazzola et al. 1995). In most tissues, CD34 is expressed in endothelial cells of both large and small vessels,
including capillaries (Traweek, Kandalafit et al. 1991). However, CD34 has the disadvantage that it may also stain vascular basement membrane and connective tissue (Rees, Heryet et al. 1993). Also, CD34 may fail to identify larger endometrial vessels (Fina, Molgaard et al. 1990; Song, Markham et al. 1995). An alternative marker is platelet-endothelial cell adhesion molecule (PECAM, or CD31), expressed on endometrial endothelial cells, mainly at intercellular junctions. CD31 is more precise than CD34, vWF, and CD36 in identifying endometrial endothelial cells using immunostaining (Rees, Heryet et al. 1993).

**Vascular density** is reported as number of vessels per unit area of endometrium. Density has been shown to increase following long-term progestogen-induced endometrial atrophy (Hickey, Simbar et al. 1999), whereas, a previous study by the same group had reported a very small increase or no change following conditions of spontaneous or induced endometrial atrophy (Hickey, Lau et al. 1996). In paraffin embedded samples, it is not possible to distinguish between an increase in the total number of endometrial vessels and an increase in their tortuosity because of stromal and glandular regression. If vessels lengthen in atrophic endometrium, they may become more tortuous and thus appear to be denser in a small biopsy sample. Most studies have failed to correct the vascular density for changes in the stromal density and glands.

Locally produced vasoactive substances probably play a key role in regulating endometrial angiogenesis, although, these are influenced substantially by different dosage regimens and routes of administration of contraceptive steroids (Smith 2001). Vascular endothelial growth factor (VEGF), a key angiogenic factor, promotes
microvascular endothelial cell proliferation, migration and assembly into new vessels (Ferrara and Davis-Smyth 1997). Oestrogen promotes angiogenesis by regulating the expression of VEGF (Albrecht, Babischkin et al. 2003). The role of glucocorticoids in modulating vascular function has been investigated. Prostaglandins influence contractility of endometrial vessels and their permeability (Albrecht, Babischkin et al. 2003). An increase in the local concentration of prostaglandins in the endometrium is involved in the mechanism of mifepristone-induced vaginal bleeding in the luteal phase (Hapangama, Critchley et al. 2002). Glucocorticoids modulate prostaglandin production in endometrial stromal cells and fibroblasts (Pakrasi, Cheng et al. 1983; Schatz, Markiewicz et al. 1986; Neulen, Zahradnik et al. 1989; Delvin, Gagnon et al. 1990; Illouz, Boubli et al. 2000) and they may have an angiostatic role (Small, Hadoke et al. 2005).

With clear health benefits of mifepristone-induced amenorrhea, especially to women who suffer menstrual dysfunction, a better understanding of underlying endometrial mechanisms can help in the development of techniques to treat or prevent the unpredictable bleeding with existing and novel contraception.
Chapter 2

ANTI-PROLIFERATIVE EFFECT OF MIFEPRISTONE

Mifepristone and other progesterone receptor antagonists exert anti-proliferative on primate (van Uem, Hsiu et al. 1989; Wolf, Hsiu et al. 1989; Hodgen, van Uem et al. 1994; Katkam, Gopalkrishnan et al. 1995; Zelinski-Wooten, Slayden et al. 1998) and human (Croxatto, Salvatierra et al. 1993; Cameron, Critchley et al. 1996; Croxatto, Kovacs et al. 1998) endometrium. Since these compounds have virtually no affinity for the oestrogen receptor, the mechanism of this ‘no-competitive anti-oestrogenic activity’ (Wolf, Hsiu et al. 1989) remains largely unknown. Although various hypotheses have been proposed, it is becoming increasingly clear that the androgen receptor (AR) may mediate these effects.

AR is up-regulated in human endometrium following 21-30 days of low-dose mifepristone treatment (Slayden, Nayak et al. 2001), but there is no data on the expression following long-term (> 30 days) treatment. The exact underlying mechanism of AR up-regulation following mifepristone treatment is unknown, however, endometrial samples from an-ovulatory PCOS women suggest an increase secondary to unopposed oestrogen (Apparao, Lovely et al. 2002).

Endometrial samples were obtained from other studies (Cameron, Critchley et al. 1996; Brown, Cheng et al. 2002; Cameron, Glasier et al. 2003) and I carried out all the laboratory work and data analysis.
AIMS & OBJECTIVES

The aim of the present study was

1. To investigate endometrial proliferation following treatment with low-dose mifepristone using phospho-histone H3 mitosis marker.

2. To investigate steroid receptor content and distribution in women following treatment with low-dose mifepristone.

3. To compare endometrial proliferation and steroid receptor expression in mifepristone-treated samples with control groups whose endometrium was exposed to unopposed oestrogen.

MATERIAL AND METHODS

Human endometrial samples were obtained from three different patient groups. The local ethics committees (Institutional Review Board) approved each of the studies and all the women provided written informed consent.

Mifepristone group:

A subset of 16 subjects who had paraffin-embedded endometrial tissue sufficient for further analysis, were recruited at random from 58 volunteer subjects whose baseline characteristics, endocrine and endometrial findings have been reported previously (Brown, Cheng et al. 2002; Baird, Brown et al. 2003). Subjects were randomly allocated to receive 2 and 5 mg of mifepristone daily for the 120 treatment days. Subjects had a mean age of 30.5 years and a mean body mass index (BMI) of 24.5 kg/m². Endometrial biopsies were collected using a Pipelle endometrial sampling
device (Prodimed, Neuilly-en-Thelle, France) in the late follicular phase of the pre-treatment cycle (day 12), after 60 days of mifepristone treatment and after 120 days of treatment. Specimens were fixed in normal buffered formalin, processed and embedded in paraffin wax.

Control groups:

Two groups of subjects whose endometria were exposed to unopposed oestrogen were chosen as controls. Since unopposed oestrogen gives rise to ‘persistent proliferative’ endometrium, the endometrium from these subjects could be compared with that of subjects who remained an-ovulatory with mifepristone.

A group of subjects (n=6) with an-ovulatory polycystic ovarian syndrome (PCOS) participating in a study (Cameron, Critchley et al. 1996) evaluating the effects of low dose mifepristone on endometrial maturation and proliferation were recruited. Subjects had a mean age of 25 years (range 23-38 years) and a mean BMI of 24.5 kg/m$^2$ (range 18.7-27.6 kg/m$^2$) and all had biochemical and ultrasound evidence of polycystic ovaries. All women had an endometrial biopsy taken 21-23 days following a progestogen-induced withdrawal bleed. The study design, endocrine and endometrial findings have been reported (Cameron, Critchley et al. 1996)

A second group of postmenopausal subjects (1 year of amenorrhea or using hormone replacement therapy for 2 years, n=5) participating in a study (Cameron, Glasier et al. 2003) evaluating effects of onapristone on postmenopausal endometrium were recruited. Subjects had a mean age of 54.4 years (range 49-54 yrs) and a mean BMI of 26.6 Kg/m$^2$ (range 20.9-33.8 Kg/m$^2$). None of the women had used a hormone preparation within the preceding six weeks. Women were instructed to take daily 2 mg
17β oestradiol valerate (Schering UK, West Sussex) orally for 8 weeks (56 days). Endometrial biopsy was performed in the 8th week of treatment (n=4). One subject refused endometrial sampling. Endometrial histology was reported as proliferative in all samples. The study design, endocrine and endometrial findings have been reported (Cameron, Glasier et al. 2003).

**Immunohistochemistry (IHC):**

IHC was performed for the following proteins of interest: phospho-histone H3 (rabbit anti-pH3: Upstate Biotechnology, Lake Placid NY), Oestrogen Receptor (mouse anti-ER: Dako ER clone 1D5, Glostrup Denmark), Progesterone Receptor (mouse anti-PR: Abbott-PR ICA, North Chicago, USA) and Androgen Receptor (mouse anti-AR: F39, Biogenex). ER, PR, and AR IHC protocols followed those previously established in our laboratory (Wang, Critchley et al. 1998; Critchley, Brenner et al. 2001; Slayden, Nayak et al. 2001) whereas pH3 protocol followed that established by Brenner et al (Brenner, Slayden et al. 2003).

All antibodies were mouse monoclonal except pH3 which was rabbit polyclonal. All antibodies were tested individually at a range of dilutions and different antigen retrieval conditions to determine the protocol which gave the least background and highest specific staining (Table 2.1). Positive and negative controls were included and in most cases the negative controls were performed by adding a matched IgG antibody (mouse IgG, Sigma, Poole, Dorset, UK; rabbit IgG, Vector Laboratories, Peterborough, UK) of the same species and same antibody concentration as the primary antibody.

The generic IHC protocol was as follows:
1. Preparation and rehydration of slides: 5μm paraffin embedded tissue sections were dewaxed in Histoclear (National Diagnostics, UK), and rehydrated in descending grades of alcohol to distilled water (dH2O).

2. Epitope retrieval: The tissue sections were subjected to epitope retrieval in a pressure cooker (05 minutes) or microwave (10 minutes) using 0.01M Sodium Citrate at pH 6 (Table 2.1) and were then allowed to cool for 20 minutes.

3. Endogenous peroxidise block: Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide (BDH, Poole, UK) in methanol for 30 minutes at room temperature.

4. Avidin-Biotin block: For AR only, slides were incubated with avidin (Vector Laboratories, Peterborough, UK) and biotin (Vector Laboratories, Peterborough, UK) for 15 minutes each at room temperature.

5. Serum block: Non-specific binding of the primary antibody was blocked by incubating the sections for 20 minutes at room temperature in a 1:5 dilution of non-immune serum (Autogen Bioclear, Holly Ditch Farm, Wilts, UK) in buffer containing 5% bovine serum albumin (BSA).

6. Primary antibody: Sections were incubated with primary and control antibodies. The temperature and duration of incubation varied according to protein of interest (pH3 = overnight at room temperature; ER and PR = 1 hour at 37°C; AR = overnight at 4°C).

7. Secondary antibody: The sections were incubated in biotinylated secondary antibody (Vector Laboratories, Peterborough, UK) in non-immune serum.

8. Detection system: Sections were incubated with avidin biotin peroxidase complex (Vectastain HRP and Vectastain Elite PK 6101, Vector Laboratories,
Peterborough, UK) for 30-60 minutes each at room temperature. The peroxidase substrate 3,3 diamino-benzadine (DAB) (DAKO) was used as chromogen.

9. Counterstain, dehydration and slide preparation: Sections were then counterstained with haematoxylin, dehydrated in ascending grades of alcohol to xylene and mounted using Pertex (Cellpath plc. Hemel Hampstead, UK).
<table>
<thead>
<tr>
<th>Protein</th>
<th>Buffer</th>
<th>Epitope Retrieval</th>
<th>Blocking Serum</th>
<th>Detection System</th>
<th>Antibodies</th>
<th>Stock Solution</th>
<th>Dilution</th>
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<td>pH3</td>
<td>PBS/PBST</td>
<td>Pressure Cook</td>
<td>Normal Goat Serum</td>
<td>ABC-HRP</td>
<td>Rabbit anti-pH3</td>
<td>1 mg/ml</td>
<td>1:1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60 minutes</td>
<td>Rabbit IgG</td>
<td>1 mg/ml</td>
<td>1:1000</td>
</tr>
<tr>
<td>ER</td>
<td>PBS/PBST</td>
<td>Microwave</td>
<td>Normal Horse Serum</td>
<td>ABC-HRP</td>
<td>Mouse anti-ER</td>
<td>0.23 mg/ml</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60 minutes</td>
<td>Mouse IgG1</td>
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</tr>
<tr>
<td>PR</td>
<td>PBS/PBST</td>
<td>Microwave</td>
<td>Normal Horse Serum</td>
<td>ABC-HRP</td>
<td>Mouse anti-PR</td>
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<td>30 minutes</td>
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<td>Normal Horse Serum</td>
<td>ABC-Elite</td>
<td>Mouse anti-AR</td>
<td></td>
<td>1:480</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60 minutes</td>
<td>Mouse IgG</td>
<td>1 mg/ml</td>
<td>1:600</td>
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</tbody>
</table>
Immunohistochemistry score:

Semi-quantitative score: Location and intensity of immunostaining was measured using a semi-quantitative scoring system. Sections were scored blind by two observers (blind to study groups and to other’s results). Immunostaining intensity and distribution of epitopes in all tissue sections were assessed on an arbitrary four point scale; 0 = no staining, 1 = mild staining, 2 = moderate staining, and 3 = intense staining. This method of semi-quantitative scoring has been previously validated in our laboratory (Wang, Critchley et al. 1998). A high correlation has been demonstrated between objectively measured immunoreactivity (image analysis) and subjective semi-quantitative scoring of immunostaining patterns (Wang, Critchley et al. 1998).

Quantitative score: pH3 immunoexpression was assessed separately for glands and stroma using image analysis. The system used a Carl Zeiss Axistop 2 microscope (x 40 objective) connected to a MacIntosh G3 computer, using Openab 2.08 image analysis software (Improvision, Coventry, UK). At least 12 fields of view were selected at random from each tissue section. The glands and stroma from each digitised image were interactively dissected. Using Openlab colour discrimination software, the total number of pH3 expressing cells (brown product) and those not expressing pH3 (blue hematoxylin) were measured separately for each digitised image. The number of pH3 expressing cells is reported as a percentage of total cells (brown and blue) per sample. This method of image analysis has previously been described and validated in our laboratories (Critchley, Kelly et al. 1996; Wang, Critchley et al. 1998).
Statistical method:

Statistical analysis was performed using SPSS (SPSS, Inc., Chicago, IL, USA) and Excel 2002 (Microsoft Corporation). Continuous data are expressed as mean with standard errors and categorical data as median with range. Wilcoxon Signed Rank test was used to analyse repeated measures of IHC scores on same subjects (treatment versus pre-treatment) and the Mann-Whitney test was used to analyse independent measures of IHC scores between mifepristone and PCO/PM groups. The risk of spurious statistical results arising from multiple testing [4 statistical tests (2 repeated measures, 2 independent measures) for each IHC protein per endometrial compartment] was corrected for by using Bonferroni correction. The reported P value is the unadjusted value; the bonferroni adjusted value is reported as P_b.

RESULTS

Phospho-histone H3 mitosis marker (pH3)

Mitotic activity as indicated by antibody to pH3 showed a highly significant decrease (P≤0.001) in the endometrium by day 60 of treatment (mean ± sem - 0.89 ± 0.14, glands; 0.48 ± 0.09, stroma) (Figure 2.1) as compared to pre-treatment proliferative endometrium (mean ± sem - 3.48 ± 0.42, glands; 1.57 ± 0.16, stroma) (Figure 2.2 a, b). This decrease was demonstrable in both glands and stroma and was maintained at 120 days (mean ± sem - 0.96 ± 0.13, glands; 0.55 ± 0.11, stroma) (Figure 2.2 c). Endometrium from PCOS women showed higher mitotic activity in the glands (mean ± sem - 4.72 ± 0.74, P=0.168, NS) whereas post-menopausal (PM) women had a significantly lower mitotic activity in the stroma (mean ± sem - 0.65 ± 0.13, P=0.011,
S) compared to proliferative day 12 pre-treatment endometrium (Figure 2.1, 2.2 d). The mitotic activity in the stromal compartment (PCO group mean ± sem - 1.21 ± 0.22) and glands (PM group mean ± sem - 2.00 ± 0.55) were not significantly different to proliferative samples.

**Oestrogen receptor**

There was no significant change in ER expression in surface epithelium, stroma and glands (P<0.136) following treatment with mifepristone (Table 2.2; Figure 2.2 m, n, o). There was no difference between women treated with 2 or 5 mg mifepristone. The endometrium in both the control groups demonstrated strong ER staining in all three endometrial compartments (Figure 2.2 p). This pattern was similar to that seen in day 12 pre-treatment proliferative phase samples.

**Progesterone receptor**

There was a reduction in PR expression in surface epithelium, glands and stroma by day 60, which was maintained at 120 days (Table 2.3; Figure 2.2 i, j, k). There was no difference between women treated with 2 or 5 mg mifepristone. There was strong PR staining in all three endometrial compartments, surface epithelium, glands and stroma in PCOS and postmenopausal groups (Figure 2.2 l). This pattern was similar to that seen in day 12 pre-treatment proliferative phase samples.

**Androgen receptor**

Pre-treatment proliferative phase endometrium showed a strong expression of AR in the stroma and minimal or absent expression in glands and surface epithelium (Table 2.4, Figure 2.2 e). There was a significant increase in AR expression in surface epithelium, glands and stroma following treatment with mifepristone compared to that
seen in the proliferative phase pre-treatment sample. This increase occurred as early as 60 days (P<0.05) (Figure 2.2 f) and was maintained at day 120 (Figure 2.2 g). The increase was most marked in the glandular compartment where a virtual absence of expression in the proliferative pre-treatment sample (Figure 2.2 e) was replaced by intense immunostaining in post-treatment samples (P<0.01) (Figure 2.2 f, g). There was no difference between women treated with 2 or 5 mg mifepristone. Endometrium from PCOS and oestrogen treated post-menopausal women showed a strong AR expression in the stroma with minimal expression in glands and surface epithelium (Figure 2.2 h). This pattern was similar to that seen in day 12 pre-treatment proliferative phase samples. Expression in glands was significantly greater in the post-mifepristone treatment samples than samples from both PCOS and post-menopausal groups (P<0.05).
Table 2.2: Oestrogen Receptor immunoexpression expressed as mean (median) in endometrium before and after treatment with daily mifepristone; comparison with control groups, Polycystic Ovary (PCO) and Postmenopausal (PM).

<table>
<thead>
<tr>
<th></th>
<th>MIFEPRISTONE group (n=16)</th>
<th>PCO group (n=6)</th>
<th>PM group (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 12 pre-treatment</td>
<td>day 60 mifepristone</td>
<td>day 120 mifepristone</td>
</tr>
<tr>
<td>Surface</td>
<td>2.56 (2.5)</td>
<td>2.63 (3)</td>
<td>2.53 (3)</td>
</tr>
<tr>
<td>Glands</td>
<td>2.94 (3)</td>
<td>2.88 (3)</td>
<td>2.56 (3)</td>
</tr>
<tr>
<td>Stroma</td>
<td>2.56 (3)</td>
<td>2.62 (3)</td>
<td>2.50 (3)</td>
</tr>
</tbody>
</table>

No significant change in ER expression following mifepristone treatment.

No difference between the Mifepristone, PCO and PM groups.
Table 2.3: Progesterone Receptor immunoexpression expressed as mean (median) in endometrium before and after treatment with daily mifepristone; comparison with control groups, Polycystic Ovary (PCO) and Postmenopausal (PM).

<table>
<thead>
<tr>
<th></th>
<th>MIFEPRISTONE group (n=16)</th>
<th>PCO group (n=6)</th>
<th>PM group (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 12 pre-treatment</td>
<td>day 60 mifepristone</td>
<td>day 120 mifepristone</td>
</tr>
<tr>
<td>Surface</td>
<td>2.40 (2.5)</td>
<td>1.88 (2)</td>
<td>1.86 (2) ^a</td>
</tr>
<tr>
<td>Glands</td>
<td>2.81 (3)</td>
<td>2.13 (2) ^a</td>
<td>1.80 (2) ^a</td>
</tr>
<tr>
<td>Stroma</td>
<td>2.38 (2)</td>
<td>1.63 (2) ^a</td>
<td>1.60 (1) ^a</td>
</tr>
</tbody>
</table>

^a – P<0.05, significant decrease, mifepristone-treated endometrium versus pre-treatment (Wilcoxon Signed Rank Test)
Table 2.4: Androgen Receptor immunoexpression expressed as mean (median) in endometrium before and after treatment with daily mifepristone; comparison with control groups, Polycystic Ovary (PCO) and Postmenopausal (PM).

<table>
<thead>
<tr>
<th></th>
<th>MIFEPRISTONE group (n=16)</th>
<th>PCO group (n=6)</th>
<th>PM group (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 12 pre-treatment</td>
<td>day 60 mifepristine</td>
<td>day 120 mifepristine</td>
</tr>
<tr>
<td>Surface</td>
<td>0.93 (1)</td>
<td>2.27 (2)\textsuperscript{a}</td>
<td>2.14 (2)\textsuperscript{a}</td>
</tr>
<tr>
<td>Glands</td>
<td>0.44 (0)</td>
<td>2.25 (2)\textsuperscript{a}</td>
<td>2.06 (2)\textsuperscript{a}</td>
</tr>
<tr>
<td>Stroma</td>
<td>2.25 (3)</td>
<td>2.69 (3)\textsuperscript{a}</td>
<td>2.44 (3)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} - P<0.01, significant increase, mifepristone-treated endometrium versus pre-treatment (Wilcoxon Signed Rank Test)

\textsuperscript{b} - P<0.05, significant increase, mifepristone-treated endometrium versus PCO/PM controls (Mann Whitney Test)
Figure 2.1: Percentage of endometrial cells immunostaining for phospho H3 in glands (a) and stroma (b) before and after treatment with daily mifepristone; comparison with control groups, Polycystic Ovary (PCO) and Postmenopausal (PM). The values are expressed as median (horizontal bar), mean (square dot) and box plots showing 50% of values (box) with range (whiskers).

X - P<0.001, significant decrease, mifepristone day 60 & 120 treatment endometrium versus day 12 pre-treatment (Wilcoxon Signed Rank Test)

Y - P=0.01, significant decrease, day 56 E2 treated postmenopausal endometrium versus day 12 pre-treatment (Mann Whitney Test)
Figure 2.1: pH3 expression

- **a:** glands
- **b:** stroma

Day 12 Prolif.  Day 60 Mife.  Day 120 Mife.  Day 21 PCO  Day 56 PM

Immunoscore (% cells)
Figure 2.2: Immunoexpression of phospho H3 (a,b,c), androgen receptor (AR) (e,f,g), progesterone receptor (PR) (i,j,k) and estrogen receptor (ER) (m,n,o) in endometrial glands (Gl.) and stroma (Str.) of a woman before and after treatment with daily 5 mg mifepristone; comparison with endometrium of a postmenopausal woman on 56 days of unopposed estrogen (HRT) (2 mg 17β estradiol) (d,h,l,p); scale bar (d) = 100 microns; positive immunoexpression = brown, negative immunoexpression = blue.

Significant reduction in immunoexpression of phospho H3 mitosis marker (arrows) following treatment with 60 (b) and 120 days (c) mifepristone compared to follicular pre-treatment day 12 endometrium (a); endometrium from post-menopausal woman on unopposed estrogen showing ongoing mitosis (d).

Significant increase in AR immunoexpression following treatment with 60 (f) and 120 days (g) mifepristone compared to follicular pre-treatment day 12 endometrium (e); and endometrium from post-menopausal woman on unopposed estrogen (HRT) (h).

Significant decrease of PR immunoexpression following treatment with 60 (j) and 120 days (k) compared to follicular pre-treatment day 12 endometrium (i); endometrium from a post-menopausal woman on unopposed estrogen (HRT) showing a similar distribution as pre-treatment sample (l).

Immunoexpression of ER in follicular pre-treatment day 12 endometrium of woman (m) and following treatment with 60 days (n) and 120 days (o); endometrium from a post-menopausal woman on unopposed estrogen (HRT) (p).
Figure 2.2: IHC of pH3, AR, PR and ER
DISCUSSION

We confirm the anti-proliferative effects of low-dose mifepristone with a new mitosis marker, phospho-histone H3 along with a significant increase in glandular AR expression. Our study shows for the first time that following prolonged treatment with mifepristone (120 days), there is a significant down-regulation of PR.

Endometrial effects

Complex histological effects have been reported in mifepristone-treated endometrium; and these vary according to study methodology, the progesterone receptor antagonist (PRA) ligand and background endocrine milieu. The characteristic finding in endometrial samples following daily low-dose treatment is proliferative histology and marked reduction in cellular proliferation (Baird, Brown et al. 2003). Even where significant suppression of ovarian function and oestradiol production results in endometrial atrophy, a small number of samples continue to show cystic changes (Baird, Brown et al. 2003), thus suggesting that some of the observed endometrial changes are native to dose and type of PRA ligand. Although sporadic incidence of hyperplastic changes have been noted following low-dose treatment (Croxatto, Kovacs et al. 1998; Baird, Brown et al. 2003), persistent changes have only been reported following daily-doses ≥ 10 mg (Murphy, Kettel et al. 1995; Eisinger, Meldrum et al. 2003; Eisinger, Bonfiglio et al. 2005). Even when massive hyperplasia has been reported following very high daily dose of 400 mg, there was no cytological atypia (Newfield, Spitz et al. 2001). Our findings using pH3, a specific marker of mitosis, confirm that at the doses tested there is no cellular evidence of endometrial hyperplasia. Although a proportion of the endometrial samples show cystic dilatation,
the glands were lined by atrophic inactive epithelium in contrast to the pseudo-stratified appearance in typical cystic glandular hyperplasia (Baird, Brown et al. 2003). One subject showed complex hyperplasia following 60 days of treatment (Baird, Brown et al. 2003). Reassuringly, her endometrium regressed to an inactive state at 120 days.

Studies in animal models generally support data in human endometrial samples. Although, oestrogenic effects have been observed in the rats receiving long-term PR antagonist treatment (Rumpel, Michna et al. 1993; Bigsby and Young 1994), the non-human primate endometrium demonstrates endometrial atrophy and evidence of anti-oestrogenic activity (Chillik, Hsiu et al. 1986; Koering, Healy et al. 1986; Wolf, Hsiu et al. 1989; Ishwad, Katkam et al. 1993; Neulen, Williams et al. 1996; Slayden, Zelinski-Wooten et al. 1998).

Primate endometrium

The principal target for PRA in the primate endometrium is the spiral arteriole (Slayden, Zelinski-Wooten et al. 1998) which traverses almost the full thickness of the endometrium whereas in human tissue, terminates at the myometrial-endometrial interface (Chwalisz, Brenner et al. 2000). The peri-arteriolar degeneration of primate endometrial spiral arteries leading in turn to their atrophy during prolonged treatment may specifically reduce blood flow in the superficial endometrial layer, producing a nutritionally deprived endometrium that cannot respond to oestrogen. Another mechanistic pathway in the primate model is profound suppression of VEGF expression (Greb, Heikinheimo et al. 1997; Greb, Kiesel et al. 1999). We report a
small decrease in stromal VEGF expression in endometrial samples following low-dose mifepristone treatment (refer chapter 3).

Role of the androgen receptor

Exogenous and endogenous androgens can inhibit oestrogen-induced endometrial proliferation (Futterweit and Deligdisch 1986; Miller, Bedard et al. 1986; Rose, Dowsett et al. 1988; Tanaka, Umesaki et al. 1999; Tuckerman, Okon et al. 2000; Zang, Sahlin et al. 2007).

The role of endometrial AR is not clear, but, stromal AR would mediate any possible effects of normal levels of endogenous androgens. Although low-dose mifepristone treatment does not modulate circulating androgen levels, it up-regulates AR in glands and hence androgens could have direct effects on glands in addition to stroma. An increase in AR levels in mifepristone treated tissues could lead to an increased binding of androgens, which might antagonize the effects of estrogens on endometrial growth. Treatment with flutamide, a pure anti-androgen, blocked the anti-proliferative effect of the PR antagonist ZK 137 316 in the non-human primate thus adding strong support to the hypothesis that this effect of PR antagonist is mediated through changes in AR (Slayden and Brenner 2003). It is possible that mifepristone itself directly mediates these effects by interacting with AR for which the relative binding affinity is 13% (Elger, Bartley et al. 2000).

We report a lack/minimal glandular AR expression in proliferative phase control endometrial samples and this agrees with other reports (Mertens, Heineman et al. 1996; Adesanya-Famuyiwa, Zhou et al. 1999; Slayden, Nayak et al. 2001). Salyen et al confirmed this using ligand binding, immunohistochemistry and in-situ hybridisation
on the same set of endometrii (Slayden, Nayak et al. 2001). They reported an increase in endometrial AR, notably in the glandular compartment, after 21-24 days of low-dose (2 mg/day) mifepristone (Slayden, Nayak et al. 2001). We report a sustained increase in glandular AR expression at 30-40 days (chapter 4) and at 60 and 120 days of low-dose mifepristone treatment (present study).

**Mechanism of AR up-regulation**

The factors regulating the expression of AR in the endometrium are not clear. Our observations in post-menopausal women treated with oestrogen, and in women with PCOS, are in keeping with reports in women and non-human primates that oestrogen induces AR expression in the endometrium (Fujimoto, Nishigaki et al. 1995; Slayden, Zelinski-Wooten et al. 1998; Slayden, Nayak et al. 2001). Increased AR expression has been reported in both stroma and glands of endometrium from women with PCO (Apparao, Lovely et al. 2002). The expression was higher in those with persistent proliferative endometrium and may merely reflect the effect of prolonged exposure to unopposed oestrogen. Although this probably contributes to the changes in women treated with long term mifepristone, it is likely that the massive up-regulation of AR which occurs especially in the glands as early as 21 days after starting treatment is a specific effect of the PR antagonist (Slayden, Nayak et al. 2001). In our study the endometrial biopsy, from women with PCO, collected 21 days after a progestagen induced menses, showed a small increase in AR expression in glands and a similar observation was made in oestrogen-treated women.

**ER and PR expression**
Short term treatment with PR antagonist, either during the menstrual cycle, or with combined oestrogen therapy, leads to elevations of the two main uterine steroid receptors, ER and PR (Neulen, Williams et al. 1990; Slayden and Brenner 1994; Cameron, Critchley et al. 1996; Slayden, Zelinski-Wooten et al. 1998) whereas expression following chronic treatment has been shown to be increased, decreased or unchanged depending on the dose and duration of treatment (Murphy, Kettel et al. 1993; Murphy, Kettel et al. 1995; Danielsson, Swahn et al. 1997; Slayden, Zelinski-Wooten et al. 1998). We have demonstrated a down-regulation of PR after 60 days treatment. ER expression however remained unchanged in all endometrial compartments. The mechanisms involved are poorly understood, but could result from either chronic anti-mitotic activity (Heikinheimo, Hsiu et al. 1996) affecting cellular protein synthesis or androgen-AR interactions.

PR-A is transcriptional repressor of PR-B and ER. Similarly ERβ is a transcriptional repressor of ERα (proliferative activity). Hence the ratio of ER and PR isoforms may determine the balance of oestrogenic and anti-oestrogenic endometrial effects in mifepristone treated samples. The ER antibody used in this study (Clone 1D5, Dako) immunostains ERα and expression decreased in glands (P=NS), however was unchanged in the stroma. We have investigated ER-isoform expression after 30-40 days of 5mg mifepristone daily (chapter 4). Both ERα and ERβ were strongly expressed in endometrium following 30-40 days of treatment; however there was no pre-treatment control biopsy for comparison. More data is obviously needed, however, it is unlikely that the observed endometrial changes which are reported within 21 days of treatment, are secondary to a modulation of ER isoforms. The role of ER and PR isoforms in mifepristone-treated endometrium needs to be elucidated further.
Chapter 3

MIFEPRISTONE AND AMENORRHEA

Women often discontinue oestrogen-free contraceptive methods such as progestogen-only pill (POP) due to a high incidence of break through bleeding (Belsey and Farley 1988). In contrast the majority of women who take mifepristone have no periods and many women now regard the absence of periods as a desirable side-benefit (Glasier, Smith et al. 2003). The mechanism underlying these disturbances in endometrial function is poorly understood and not clearly related to levels of endogenous or exogenous steroid hormones (Fraser, Hickey et al. 1996).

Mifepristone-induced amenorrhea is clearly related to degree of ovarian suppression (Brown, Cheng et al. 2002), but, a more direct modulation of vascular function cannot be ruled out. Endometrial vascular function is regulated by locally produced vasoactive substances such as VEGF and these are influenced substantially by different dosage regimens and routes of administration of contraceptive steroids (Smith 2001). Glucocorticoids are other potential modulators of angiogenesis and vascular function either through direct angiostatic (Small, Hadoke et al. 2005) or indirect prostaglandin-modulated (Pakrasi, Cheng et al. 1983; Schatz, Markiewicz et al. 1986; Neulen, Zahradnik et al. 1989; Delvin, Gagnon et al. 1990; Illouz, Boubli et al. 2000; Albrecht, Babischkin et al. 2003) mechanisms.

Endometrial samples were obtained from a previous study (Brown, Cheng et al. 2002) and I carried out all the laboratory work and data analysis.
AIMS AND OBJECTIVES

The aim of this study was

1. To investigate the effects of low-dose mifepristone on endometrial microvessel density, VEGF and GR expression
2. To correlate endometrial microvessel density, VEGF, GR expression with endometrial histology and bleeding patterns.

MATERIAL AND METHODS

A subset of 16 subjects who had paraffin-embedded endometrial tissue sufficient for further analysis, were recruited at random from 58 volunteer subjects whose baseline characteristics, endocrine and endometrial findings have been published previously (Brown, Cheng et al. 2002; Baird, Brown et al. 2003). These 16 subjects were the same subset as for study 1. The local ethics committees (Institutional Review Board) approved the study and all subjects provided written informed consent. Subjects were randomly allocated to receive 2 (n = 8) and 5 mg (n = 8) of mifepristone daily for the 120 treatment days. Subjects had a mean age of 30.5 years (range 24 to 40) and a mean BMI of 24.5 kg/m² (range 21 to 34). Endometrial biopsies were collected using a Pipelle endometrial sampling device (Prodimed, Neuilly-en-Thelle, France) in the late follicular phase of the pre-treatment cycle (day 12), after 60 days of mifepristone treatment and after 120 days of treatment. Specimens were fixed in normal buffered formalin, processed and embedded in paraffin wax.

Immunohistochemistry (IHC):
Immunohistochemistry was performed for the following proteins: Vascular Endothelial Growth Factor (rabbit anti-VEGF: SantaCruz Biotechnology, California, USA), CD31 (mouse anti-CD31: Novacastra Laboratories, Newcastle upon Tyne, UK) and Glucocorticoid Receptor (mouse anti-GR: Novocastra Laboratories, Newcastle upon Tyne, UK). IHC protocols followed those previously published (Nayak, Critchley et al. 2000; Bamberger, Milde-Langosch et al. 2001).

All antibodies were mouse monoclonal except VEGF which was rabbit polyclonal and antibodies were tested individually at a range of dilutions and different antigen retrieval conditions to determine the protocol which gave the least background and highest specific staining (Table 3.1). Positive and negative controls were included and in most cases the negative controls were performed by adding a matched IgG antibody (mouse IgG, Sigma, Poole, Dorset, UK; rabbit IgG, Vector Laboratories, Peterborough, UK) of the same species and same antibody concentration as the primary antibody.

The generic IHC protocol was as follows:

1. Preparation and rehydration of slides: 5µm paraffin embedded tissue sections were dewaxed in Histoclear (National Diagnostics, UK), and rehydrated in descending grades of alcohol to distilled water (dH₂O).

2. Epitope retrieval: The tissue sections were subjected to epitope retrieval in a pressure cooker (05 minutes) or microwave (10 minutes) using 0.01M Sodium Citrate at ph 6 (Table 3.1) and were then allowed to cool for 20 minutes.
3. Endogenous peroxidise block: Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide (BDH, Poole, UK) in methanol for 30 minutes at room temperature.

4. Avidin-Biotin block: slides were incubated with avidin (Vector Laboratories, Peterborough, UK) and biotin (Vector Laboratories, Peterborough, UK) for 15 minutes each at room temperature.

5. Serum block: Non-specific binding of the primary antibody was blocked by incubating the sections for 20 minutes at room temperature in a 1:5 dilution of non-immune serum (Autogen Bioclear, Holly Ditch Farm, Wilts, UK) in buffer containing 5% bovine serum albumin (BSA).

6. Primary antibody: Sections were incubated at 4°C with primary and control antibodies overnight.

7. Secondary antibody: The sections were incubated in biotinylated secondary antibody (Vector Laboratories, Peterborough, UK) in non-immune serum.

8. Detection system: Sections were incubated with avidin biotin peroxidase complex (Vectastain HRP and Vectastain Elite PK 6101, Vector Laboratories, Peterborough, UK) for 30-60 minutes each at room temperature. The peroxidase substrate DAB (DAKO) was used as chromogen.

9. Counterstain, dehydration and slide preparation: Sections were then counterstained with haematoxylin, dehydrated in ascending grades of alcohol to xylene and mounted using Pertex (Cellpath plc. Hemel Hampstead, UK).
<table>
<thead>
<tr>
<th>Protein</th>
<th>Buffer</th>
<th>Epitope Retrieval</th>
<th>Blocking Serum</th>
<th>Detection System</th>
<th>Antibodies</th>
<th>Stock Solution</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>TBS/TBST</td>
<td>Microwave</td>
<td>Normal Goat Serum</td>
<td>ABC-Elite</td>
<td>rabbit anti-VEGF</td>
<td>0.2 mg/ml</td>
<td>1:600</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 minutes</td>
<td>VEGF pre-absorbed</td>
<td>0.02 mg/ml</td>
<td>1:60</td>
</tr>
<tr>
<td>GR</td>
<td>PBS/PBST</td>
<td>Pressure Cook</td>
<td>Normal Rabbit Serum</td>
<td>ABC-HRP</td>
<td>mouse anti-GR</td>
<td>0.124 mg/ml</td>
<td>1:40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60 minutes</td>
<td>mouse IgG2a</td>
<td>1.0 mg/ml</td>
<td>1:320</td>
</tr>
<tr>
<td>CD31</td>
<td>PBS/PBST</td>
<td>Pressure Cook</td>
<td>Normal Horse Serum</td>
<td>ABC-Elite</td>
<td>mouse anti-CD31</td>
<td>0.1 mg/ml</td>
<td>1:800</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 minutes</td>
<td>mouse IgG1</td>
<td>1.0 mg/ml</td>
<td>1:8000</td>
</tr>
</tbody>
</table>
Semi-quantitative IHC score:

Location and intensity of immunostaining was measured using a semi-quantitative scoring system. Sections were scored blind by two observers (blind to study groups and to other’s results). Immunostaining intensity and distribution of epitopes in all tissue sections were assessed on an arbitrary four point scale; 0 = no staining, 1 = mild staining, 2 = moderate staining, and 3 = intense staining. This method of semi-quantitative scoring has been previously validated in our laboratory (Wang, Critchley et al. 1998) and high correlation has been demonstrated between objectively measured immunoreactivity (image analysis) and subjective semi-quantitative scoring of immunostaining patterns (Wang, Critchley et al. 1998).

Vessel counts and Stromal density:

Vessel counts and stromal density were measured using image analysis. The system used a Carl Zeiss Axistop 2 microscope (x 40 objective) connected to a MacIntosh G3 computer, using Openab 2.08 image analysis software (Improvision, Coventry, UK). At least 12 fields of view were selected at random from each tissue section at a magnification of x 40. The glands and stroma from each digitised image were interactively dissected.

All immunostained (brown) structures were considered positive and counted for each digitised image, even if lumen was not identified. A similar methodology has been described to study changes in vessel density following norplant use (Hickey, Simbar et al. 1999). The results were averaged and expressed as vessels per square mm. Vessel density was corrected for an increase in stromal density which subjectively appears to be increased following mifepristone treatment (Baird, Brown et al. 2003; Baird, Brown
et al. 2003). The total number of cells not expressing CD31 (blue hematoxylin) were measured separately for each digitised image using Openlab colour discrimination software and results are expressed as vessels per 1000 stromal nuclei. This method of image analysis has previously been described and validated in our laboratories (Critchley, Kelly et al. 1996; Wang, Critchley et al. 1998).

Stromal density was expressed as the total number of stromal nuclei (brown CD31 and blue hematoxylin) per square millimetre of endometrial tissue.

Statistical methods:

Statistical analysis was performed using SPSS (SPSS, Inc., Chicago, IL, USA) and Excel 2002 (Microsoft Corporation). Continuous data are expressed as mean with standard errors and categorical data as median with range. Wilcoxon Signed Rank test was used to analyse repeated measures of IHC scores and stromal and vessel density (treatment versus pre-treatment). The risk of spurious statistical results arising from multiple testing [2 statistical tests for each IHC protein per endometrial compartment] was corrected for by using Bonferroni correction. The reported P value is the unadjusted value; the bonferroni adjusted value is reported as P_b.

RESULTS

Endometrial histology, serum oestradiol and bleeding patterns

All 16 pre-treatment endometria were sampled in the proliferative phase. Following treatment 9/16 endometria were still proliferative at 60 and 120 days respectively. 4 endometria showed cystic dilatation of the glands (Figure 3.2 e) and 5 inactive
epithelium at 60 days and 120 days of treatment respectively. Serum oestradiol levels remained in the mid-proliferative range following treatment (mean±sem- 511±70.21 pmol/l, pre-treatment; 459±90.94 pmol/l, mifepristone 60 days; 290±63.39 pmol/l, mifepristone 120 days). There was no significant change in the level of oestradiol following treatment (P>0.05, Bonferroni correction). 7/8 women on 2 mg mifepristone and all 8 women on 5 mg of mifepristone were completely amenorrheic during treatment. There was no significant difference (P>0.05, Mann-Whitney test) between women treated with 2 or 5 mg mifepristone for any parameters studied except GR expression in the vascular endothelium at 60 days of treatment [median (range) at 60 days = 3 (3), 2mg group; 2 (2-3), 5 mg group; P = 0.012, Bonferroni correction, Mann-Whitney Test]. The 2 and 5 mg datasets are therefore combined and results reported are for all 16 subjects.

Stromal density

An average of 0.5 (sem = 0.05) square millimetre of endometrial stroma was examined in the pre-treatment samples and 0.37 (sem=0.04) square millimetres in the treatment samples. Stromal density as expressed as nuclei per square millimetre of endometrial tissue increased significantly (24 %) by day 60 of treatment (P<0.001) and the increase (18%) was maintained at 120 days (P<0.05, Figure 3.1; mean ± sem – 8057 ± 355, pre-treatment; 10003 ± 355, day 60; 9510 ± 351, day 120).

Microvessel density

Vessel density per square millimetre of endometrial tissue increased (47 %) by day 60 of treatment (P<0.01, Figure 3.1, Figure 3.2 b). This increase was highly significant and was maintained (49%) at 120 days (P<0.01, Figure 3.2 c; mean ± sem – 267 ± 18,
pre-treatment; 392 ± 32, day 60; 398 ± 37, day 120). To compensate for an increase in stromal density, microvessel density was calculated per total nuclei in each endometrial sample. There was a modest (P=0.08, NS) increase (15 %) by day 60 and a significant (P<0.01) increase (34 %) by day 120 of treatment (Figure 3.1, Figure 3.2 b, c; mean ± sem – 33 ± 1.9, pre-treatment; 38 ± 2.1, day 60; 42 ± 2.75, day 120.

**VEGF**

VEGF protein was strongly expressed in the cytoplasm of endometrial glands and surface epithelium. Expression in the stroma was patchy and faint (Table 3.2, Figure 3.2 e). VEGF expression remained unchanged in the glandular [median (range) = 2(1-3) pre-treatment, 1(1-3) day 60 and 1(0-2) day 120], surface epithelium [median (range) = 1(0-3) pre-treatment, 1(0-3) day 60 and 1(0-2) day 120] and endothelial cells [median (range) = 1(0-2) pre-treatment, 0.5(0-2) day 60 and 1(0-1) day 120] following treatment with mifepristone. There was a small decrease (P<0.01) in stromal expression [median (range) = 1(0-2) pre-treatment, 0(0-2) day 60 and 0(0-1) day 120] of VEGF following 120 days of treatment (Table 3.2, Figure 3.2 f, g).

**GR**

The proliferative phase pre-treatment endometrial samples showed strong immunoexpression in the nuclei of stromal [median (range) = 2(0-3)] and endothelial [median (range) = 3(1-3); Table 3.3, Figure 3.2 p] cells and a complete absence of expression in the surface epithelium [median (range) = 0(0-0); Figure 3.2 i] and glands [median (range) = 0(0-0); Table 3.3, Figure 3.2 i, j]. Following treatment with mifepristone, nuclear immunoexpression was induced in glands [median (range) = 0(0-2) day 60 and 1(0-2) day 120, P<0.05] and surface epithelium [median (range) = 1(0-3)
day 60 and 1(1-3) day 120, P<0.01]. This was evident by 60 days and maintained at 120 days (Table 3.3, Figure 3.2 k, l, m, n). Immunoexpression in the stroma [median (range) = 2(1-3) day 60 and 2(1-3) day 120] and endothelial cells [median (range) = 3(2-3) day 60 and 3(2-3) day 120; Figure 3.2 q, r] was unchanged.
Table 3.2: VEGF immunoexpression expressed as mean (median) in endometrium before and after treatment with daily mifepristone.

<table>
<thead>
<tr>
<th></th>
<th>MIFEPRISTONE group</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 12 pre-treatment</td>
<td>day 60 mifepristone</td>
<td>day 120 mifepristone</td>
<td></td>
</tr>
<tr>
<td>n=</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td><strong>Surface</strong></td>
<td>1.43 (1)</td>
<td>1.40 (1)</td>
<td>1.40 (1)</td>
<td></td>
</tr>
<tr>
<td><strong>Glands</strong></td>
<td>1.63 (2)</td>
<td>1.40 (1)</td>
<td>1.25 (1)</td>
<td></td>
</tr>
<tr>
<td><strong>Stroma</strong></td>
<td>0.94 (1)</td>
<td>0.33 (0)</td>
<td>0.31 (0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Endothelium</strong></td>
<td>0.69 (1)</td>
<td>0.64 (0.5)</td>
<td>0.44 (0)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> P=0.01 (Wilcoxon Test), significant decrease, mifepristone-treated versus pre-treatment.
Table 3.3: Glucocorticoid receptor immunoeexpression expressed as mean (median) in endometrium before and after treatment with daily mifepristone.

<table>
<thead>
<tr>
<th>MIFEPRISTONE group</th>
<th>day 12 pre-treatment</th>
<th>day 60 mifepristone</th>
<th>day 120 mifepristone</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=16</td>
<td>0 (0)</td>
<td>0.13 (1) a</td>
<td>1.47 (1) a</td>
</tr>
<tr>
<td>Surface</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glands</td>
<td>0 (0)</td>
<td>0.44 (0) b</td>
<td>0.69 (1) a</td>
</tr>
<tr>
<td>Stroma</td>
<td></td>
<td>1.75 (2)</td>
<td>1.94 (2)</td>
</tr>
<tr>
<td>Endothelium</td>
<td></td>
<td>2.47 (3)</td>
<td>2.56 (3)</td>
</tr>
</tbody>
</table>

- P<0.01, significant increase (Wilcoxon Test), mifepristone-treated endometrium versus pre-treatment
- P<0.05, significant increase (Wilcoxon Test), mifepristone-treated endometrium versus pre-treatment

a, b - P<0.01, significant increase (Wilcoxon Test), mifepristone-treated endometrium versus pre-treatment

n=16
Figure 3.1: Density of endometrial stroma (a) and microvessel (b, c) in women before and after treatment with mifepristone.

The values are expressed as median (horizontal bar), mean (square dot) and box plots showing 50% of values (box) with range (whiskers).

- **a** - p < 0.01, significant increase, mifepristone day 60 & 120 treatment endometrium versus day 12 pre-treatment (Wilcoxon Signed Rank Test)

- **b** - p < 0.05, significant increase, mifepristone day 60 & 120 treatment endometrium versus day 12 pre-treatment
Figure 3.2: Immunoexpression of CD31, VEGF and GR in endometrial glands (Gl.), stroma (Str.), surface epithelium (surf.) and vascular endothelium (Ves.) before and after treatment with mifepristone. Scale bar (a, j, p) = 50 microns; positive immunoexpression = brown

Significant increase in microvessel (arrows) density following treatment (b,c), significant decrease in stromal VEGF following treatment for 120 days (g)) and significant increase of GR immunoexpression following treatment for 60 (k,l) and 120 days (m,n) compared to follicular pre-treatment day 12 endometrium; note absence of GR immunoexpression in glands during pre-treatment day 12 endometrium (j) and significant expression following treatment (l,n); also note strong expression of GR in vascular endothelium (p,q,r); negative controls are included.
Figure 3.2: IHC of CD31, VEGF and GR
DISCUSSION

Mifepristone-induced amenorrhea has obvious health benefits. Investigation of potential underlying mechanism shows an increase in glandular GR and microvessel density and a decrease in stromal VEGF.

Endometrial histology and vascular density

Histological appearance of the endometrium does not correlate well with menstrual patterns except the development of extreme histological atrophy, which typically predicts amenorrhea. In the primate model, continuous mifepristone induces marked endometrial atrophy and the spiral arteries are the primary targets that are damaged or inhibited by progesterone antagonists like mifepristone (Chwalisz, Brenner et al. 2000). Microvessel density shows a variable increase in conditions of spontaneous (post-menopausal) and induced (norplant, danazol and goserelin) endometrial atrophy and the mechanisms involved may vary according to the nature of the atrophic stimulus (Hickey, Lau et al. 1996; Hickey, Simbar et al. 1999). In women exposed to high and medium dose progestogens and long-term users of levonorgestrel-releasing intrauterine system (Mirena), a decrease in endometrial vascular density has been observed (Song, Markham et al. 1995; Oliveira-Ribeiro, Petta et al. 2004). In the present study over 90% women were amenorrhoeic. The endometrium was proliferative and there was a significant increase in microvessel density. We used CD31 to identify the vessels as preliminary studies with the alternate immunohistochemical marker CD34 were unsatisfactory. The use of CD31 may explain the discrepancy in the numerical count of vessels per area of endometrium between our study and that by other investigators (Hickey, Lau et al. 1996; Lau,
Moller et al. used CD31 to identify vessels in endometrial samples of 16 normally menstruating pre-menopausal women and reported wide individual variation in vessel density (Moller, Rasmussen et al. 2001). There was no consistent variation over the menstrual cycle.

Vasoactive compounds

VEGF is a major regulator of endothelial cell proliferation, angiogenesis, vasculogenesis and capillary hyperpermeability (Ferrara and Davis-Smyth 1997; Ferrara 1999; Smith 2001). The presence of VEGF mRNA, protein and its receptors has been demonstrated in the human and primate endometrium throughout the menstrual cycle (Torry, Holt et al. 1996; Ferrara and Davis-Smyth 1997; Meduri, Bausero et al. 2000; Nayak, Critchley et al. 2000; Moller, Lindblom et al. 2002; Nayak and Brenner 2002; Sugino, Kashida et al. 2002). Proliferative endometrium demonstrates prominent glandular immunoreactivity and faint, inconsistent staining in stromal cells similar to observations in the present study (Sugino, Kashida et al. 2002). Mifepristone abolished VEGF expression in the endometrial glandular epithelium of cynomolgous monkeys (Greb, Heikinheimo et al. 1997) and this might represent a mechanism for the suppression of angiogenesis and severe endometrial atrophy observed following mifepristone treatment in the primate model. In the present study, VEGF immunoexpression was significantly reduced in the stroma following mifepristone treatment. There was a non-significant reduction in glandular immunoexpression and microvessel density was significantly increased following treatment. It has been suggested that VEGF may not be the primary regulator of endothelial cell proliferation in the human endometrium (Gargett, Lederman et al. 1995).
Besides VEGF, other factors such as angiopoietin and fibroblast growth factor also regulate endometrial angiogenesis (Smith 2001). VEGF regulates vascular permeability and the decrease in stromal VEGF may explain the increase in stromal density following treatment with mifepristone (Ferrara 1999; Ferrara 1999).

Role of GR and glucocorticoids

The most striking observation in our study was the change in the expression of GR and its location. In the normal menstrual cycle GR is located only in the nuclei of endometrial stromal and endovascular cells and is absent in the glands (Bamberger, Milde-Langosch et al. 2001). In our study, the pre-treatment proliferative phase samples showed a strong nuclear receptor expression in the stroma and a complete absence in the glands and surface epithelium. Following treatment with low-dose mifepristone, there was a significant induction of nuclear GR expression in both glands and surface epithelium. Although the presence of GR has been reported in the luminal (surface) epithelium of rat uterus (Korgun, Dohr et al. 2003) this is the first study to report the presence of nuclear glucocorticoid receptors in endometrial gland cells. Mifepristone binds strongly to GR and PR but more weakly to AR (Spitz and Bardin 1993) and we have shown (chapter 2) that chronic treatment with low-doses mifepristone up regulates AR and down regulates PR in the endometrium (Narvekar, Cameron et al. 2004). The underlying cellular mechanisms are poorly understood, but, the striking temporo-spatial up-regulation of AR and GR in the glands and surface epithelium suggest that they share common mechanistic pathways.

The exact physiological role of GR and glucocorticoids in the human endometrium is not clear. The expression pattern points to a functional role in the complex process of
decidualisation (Bamberger, Milde-Langosch et al. 2001). Several effects of glucocorticoids on endometrial cells have been reported. Uterine events such as menstruation, implantation, cervical softening and parturition share similarity with non-reproductive inflammatory situations (Kelly 1996). At high concentrations, glucocorticoids inhibit most immunological responses and are well-known anti-inflammatory agents. Prostaglandins have a pivotal role in menstruation and endometrial bleeding (Baird, Cameron et al. 1996) and glucocorticoids have been shown to suppress PGF2α production (Schatz, Markiewicz et al. 1986; Neulen, Zahradnik et al. 1989; Delvin, Gagnon et al. 1990; Illouz, Boubli et al. 2000) and phospholipase A2 (Pakrasi, Cheng et al. 1983), one of the enzymes considered to be rate-limiting in generating free arachidonic acid for prostaglandin synthesis. An increase in the local concentration of prostaglandins in the endometrium has been postulated in the mechanism of bleeding observed following administration of single-dose mifepristone in the mid-luteal phase (Hapangama, Critchley et al. 2002). The expression of GR in endothelial cells suggests a role for glucocorticoids in modulation of angiogenesis in the endometrium as has been reported in the rat aorta (Small, Hadoke et al. 2005). Endogenous and exogenous glucocorticoids, exert tonic inhibition whereas treatment with a glucocorticoid receptor antagonist enhances angiogenesis in the mice model (Small, Hadoke et al. 2005). An increase in GR expression could potentate the effects of circulating glucocorticoids on the endometrium thereby suppressing local prostaglandin concentrations and inhibiting angiogenesis. Contrary to theoretical expectations, we have demonstrated a small increase in microvessel density. This increase may represent a counting artefact in endometrial sections following stromal compaction and gland dilatation. A better understanding of the role
of glucocorticoids and androgens and their receptors in the endometrium is needed to reconcile the different endometrial effects into a working hypothesis.

Glucocorticoids and fertility

Glucocorticoids may also modulate human fertility. Elevated levels of glucocorticoids disrupt normal uterine development and implantation (Campbell 1978; Monheit and Resnik 1981; Bigsby 1993; Hicks, Duran-Reyes et al. 1994). Mechanisms by which glucocorticoids may influence implantation include their known effects on actin polymerization, lysosomal activity, PG synthase, PGE nitric oxide synthase, and matrix metalloproteinases (Salamonsen 1996), all of which have known roles in implantation. Excess glucocorticoid exposure can disturb the normal pattern of growth and differentiation of the primate fetus (Arcuri, Battistini et al. 1997). On the other hand, cortisol, may modulate local immunosuppressive activity within the decidua by inhibiting the production of anti-inflammatory cytokines, such as IL-1 (Snyder and Unanue 1982) thus protecting the developing blastocyst from maternal immune rejection (Ricketts, Verhaeg et al. 1998). Although low-dose mifepristone is contraceptive by inhibiting ovulation in up to 90% subjects (Brown, Cheng et al. 2002), an increase in surface (luminal) and glandular glucocorticoid receptor expression may play a role in the endometrial anti-fertility effects of mifepristone.
Chapter 4

EFFECTS OF MIFEPRISTONE ON THE VAGINA

The state of the vaginal micro-environment affects a woman's risk of human immunodeficiency virus (HIV) transmission. Oestrogen-induced surface keratinisation and hyperplasia protects Rhesus monkeys against SIV inoculation, whereas oestrogen-deficient women such as those who are post-menopausal or on long-acting progestagen treatment, are at increased risk of HIV, presumably as a result of vaginal thinning. Numerous other factors such as vaginal microflora, immune cell populations and natural anti-microbials modulate innate defences of the reproductive tract.

The effects of hormonal contraception on vaginal defences to sexually-transmitted infections have not been well investigated. The data on vaginal effects of mifepristone is limited to a few primate studies.

I was fully involved in study design, recruitment and analysis of data. I recruited 5 out of 8 subjects and carried out all the preliminary laboratory work on vaginal samples which included methodology for vaginal thickness measurement, RNA extraction and optimisation of immunohistochemistry protocols.
AIMS & OBJECTIVES

Since progestagen treatment increases and oestrogen treatment decreases HIV/SIV transmission, the aim of the present study was to investigate whether the anti-progestagen mifepristone modulates underlying mechanisms (vaginal morphology, steroid receptor and natural anti-microbial [Secretory Leukocyte Protease Inhibitor (SLPI)] which may modulate transmission.

MATERIAL AND METHODS

Study design: A single-centre, open, single-group study in female volunteers.

Subjects: Eight healthy subjects with mean age of 35 years (range 27-39 years), mean body mass index (BMI) of 23 Kg/m2 (range 17.3 - 28.2 Kg/m2) and regular menstrual cycles (25-42 days).

Inclusion criteria:

1. Women aged 18-40 inclusive.
2. Women with regular menstrual cycles.
3. Willing and able to take part in the study.
4. Prepared to use barrier contraception for the duration of the study, or those who are sterilised or whose partner is sterilised.
5. Agree to refrain from vaginal medications during study period.
6. Agree to refrain from sexual intercourse 48 hours prior to vaginal biopsy.
7. Negative βHCG test before commencing the study.
8. Written informed consent.
Exclusion criteria:

1. Those who have used hormonal contraception in the past three months, or depot hormones within six months of entering the trial.
2. Those who have breastfed in the past three months.
3. Abnormal vaginal discharge, clinically significant vaginitis, cervicitis.
4. Sexually transmitted diseases.
5. Those who have had an IUD in-situ in the past three months.
8. History of cervical surgery which may make endometrial biopsy impossible.
10. Vaginal bleeding of unknown aetiology or inter-menstrual bleeding.
11. Other significant disease, e.g. Cardiovascular, renal or liver disease or malignancy, sufficient to interfere with the evaluation of the study.
12. Clinically significant abnormal findings in the pre-entry laboratory screening not related to the primary diagnosis or any other established or stable conditions.
13. Chronic alcoholism, drug abuse or any other condition associated with poor patient compliance.
14. Treatment with an investigational drug within one month of inclusion.
15. Current treatment with corticosteroids.
16. Known allergy to mifepristone.
Ethical approval: The proposal was approved by the Local Ethical Committee (Institutional Review Board). All subjects gave written informed consent.

Study visits: (Figure 4.1)

All subjects were screened before entering the study. Screening included a full medical, gynaecological history and examination including measurement of height, weight, blood pressure, and pulse. Blood samples were collected for measurement of routine clinical chemistry and haematology (liver function tests, urea and electrolytes, glucose, full blood count). \( \beta \)HCG was measured to exclude pregnancy before entering the trial. Subjects were studied for one pre-treatment cycle, one cycle of treatment (approximately 33 days), and for one post-treatment cycle. Each subject was reviewed on day 12 of the pre-treatment menstrual cycle (Visit 1), at the end of treatment (Visit 2), and on day 12 of the post-treatment menstrual cycle (Visit 3). Subjects were given a menstrual record card and asked to record all vaginal bleeding. All women received planned treatment for at least 5 days beyond expected date of menstruation (33 days) so that any post-treatment vaginal bleeding was due to withdrawal of mifepristone rather than a spontaneous menstruation.
Assessment of ovarian function:

Ovarian function was monitored by measurement of ovarian steroids in urine and plasma and by transvaginal sonography. All subjects collected twice-weekly samples of early morning urine during the study period, starting in the early follicular phase (day 1–5) of the pre-treatment cycle. Aliquots were frozen and stored at -20 C until assayed for estrone glucuronide (E1G), pregnanediol glucuronide (PdG), and creatinine (Cr). PdG was measured using a direct enzyme immunoassay, and E1G by direct immunoassay. Ovarian follicular activity during treatment was compared with that during follicular phase of the pre-treatment cycle and the activity was scored as complete suppression, partial suppression and continued follicular activity. Ovulation
was deemed to have occurred if the excretion of PdG exceeded 0.5 mmol/mol Cr and was at least 3-fold higher than that in the preceding week. A detailed description of this methodology is given in our previous report (Brown, Cheng et al. 2002). Blood samples were collected at all study visits, and assayed for oestradiol (E2) and progesterone (P4) using radioimmunoassay (RIA). Assay characteristics and methodology have been described in our previous reports (Brown, Cheng et al. 2002). A transvaginal ultrasound scan was carried out at all study visits and ovarian dimensions, follicle number and diameter, and presence of ovarian cysts were recorded.

**Vaginal biopsy:**

A full thickness vaginal biopsy was taken from the lateral vaginal wall 4 cm proximal to the hymeneal ring on day 12 of the pre-treatment cycle (Visit 1) and again after completion of treatment (Visit 2). 1 ampoule of Citanest with Octapressin (3%, prilocaine hydrochloride 30 mg/mL, felypressin 0.03 unit/mL, Dentsply) was injected into the lateral vaginal wall as a local anaesthetic and haemostatic agent. This also elevated the target vaginal tissue sufficiently to permit easy access for a biopsy. Vaginal biopsy was performed using a long Shumaker forceps. Vaginal tissues were stored in RNAlater (Applied Ltd, Cambridgeshire, UK, RNAlater is an aqueous storage reagent to stabilise and protect RNA) and neutral buffered formalin (NBF; for future preparation of paraffin embedded tissue for immunohistochemistry). A second biopsy was taken if adequate sample was not obtained with the first biopsy. Vaginal bleeding from the biopsy site was controlled using either silver nitrate or if necessary a vicryl 3-0 suture (Ethicon, UK), depending on the amount of bleeding.
An endometrial biopsy was collected using Pipelle endometrial sampler (Prodimed, Neuilly-en-Thelle, France) at end of treatment (Visit 2), fixed in NBF, and embedded in paraffin. Endo-cervical and posterior fornix swabs were collected at all study visits to screen for infection which might influence the parameters studied in target vaginal tissues.

Safety parameters:

At each study visit, blood pressure and pulse were measured, and blood was taken for measurement of routine clinical chemistry and haematology. In addition, each subject was asked to report any health problems or adverse events that had occurred since the last visit.

Vaginal thickness measurement:

Vaginal tissue samples were embedded in paraffin and serial 5μm sections were cut at 90 degree angle to the vaginal surface epithelial layers. The sections were stained with haematoxylin and eosin and digital images were captured at 10 eyepiece magnification using a Spot microscope connected to a Windows PC computer. Image Proplus 4.5 (Media Cybernetics, Silver Spring, USA) software was calibrated to match the eyepiece used to capture the image. The surface and basement membrane of vaginal epithelium was outlined using a trace tool within the software (Figure 4.2). The software automatically calculated the average distance between the two traced outlines.
Immunohistochemistry (IHC):

IHC was carried out on both the vaginal and endometrial biopsies for the following proteins of interest: oestrogen receptor alpha (mouse anti-ERα: Novocastra, Newcastle-upon-Tyne, UK), oestrogen receptor beta (mouse anti-ERβ: Serotec, Oxford, UK), progesterone receptor (mouse anti-PR: Novocastra, Newcastle-upon-Tyne, UK), androgen receptor (rabbit anti-AR: Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), phospho-histone H3 (rabbit anti-pH3: Upstate Biotechnology, Poole,
and secretory leukocyte protease inhibitor (mouse anti-SLPI: Hycult Biotechnology, Cambridge, UK). All antibodies used were mouse/rabbit monoclonal except pH3 which was a rabbit polyclonal. They were tested individually at a range of dilutions and different antigen retrieval conditions to determine the protocol which gave the least background and highest specific staining (Table 4.1). Positive and negative controls were included in every run. In most cases negative controls were performed by adding a matched IgG control antibody (mouse IgG, Sigma, Poole, Dorset, UK; rabbit IgG, Vector Laboratories, Peterborough, UK) of the same species and at the same antibody concentration as the primary antibody. Protocols were either carried out on the bench or using a Bond-X automated immunohistochemistry staining machine (Vision Biosystems, Newcastle, UK).

The generic IHC protocol was as follows:

1. Preparation and rehydration of slides: 5μm paraffin embedded tissue sections were dewaxed in Histoclear (National Diagnostics, UK), and rehydrated in descending grades of alcohol to distilled water (dH2O).

2. Epitope retrieval: The tissue sections were subjected to epitope retrieval either by heating the sections in a microwave oven (setting high), or in a Tefal Clipso pressure cooker (setting 2/high, Tefal, Nottingham, UK). The buffer concentration and duration of antigen retrieval varied dependent on protocol (Table 4.1). Sections were then allowed to cool for 20 minutes.

3. Endogenous peroxidise block: Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide (BDH, Poole, UK) in methanol for 30 minutes at room temperature.
4. Avidin-Biotin block: For AR only, slides were incubated with avidin (Vector Laboratories, Peterborough, UK) and biotin (Vector Laboratories, Peterborough, UK) for 15 minutes each at room temperature.

5. Serum block: Non-specific binding of the primary antibody was blocked by incubating the sections for 20 minutes at room temperature in a 1:5 dilution of non-immune serum (Autogen Bioclear, Holly Ditch Farm, Wilts, UK) in buffer containing 5% bovine serum albumin (BSA).

6. Primary antibody: Sections were incubated at 4°C with primary and control antibodies overnight.

7. Secondary antibody: The sections were incubated in biotinylated secondary antibody (Vector Laboratories, Peterborough, UK) in non-immune serum.

8. Detection system: Sections were incubated with avidin biotin peroxidase complex (Vectastain HRP and Vectastain Elite PK 6101, Vector Laboratories, Peterborough, UK) for 30-60 minutes each at room temperature. The peroxidase substrate DAB (DAKO) was used as chromogen.

9. Counterstain, dehydration and slide preparation: Sections were then counterstained with haematoxylin, dehydrated in ascending grades of alcohol to xylene and mounted using Pertex (Cellpath plc. Hemel Hampstead, UK).
Table 4.1: Immunohistochemistry protocol for ER, PR, AR, pH3 & SLPI

<table>
<thead>
<tr>
<th>Protein</th>
<th>IHC method</th>
<th>Detection system</th>
<th>Antigen retrieval</th>
<th>Tissue</th>
<th>Primary antibody</th>
<th>Negative Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>Bond-X machine-ABC detection</td>
<td>Biotinylated secondary and ABC detection (Vision biosystems, UK)</td>
<td>Pressure Cook, 0.01M sodium citrate pH 6, 5 minutes</td>
<td>Vagina</td>
<td>1:100 3 hrs room temp.</td>
<td>MigG1 1:1300</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Endometrium</td>
<td>1:1000 3 hrs room temp.</td>
<td>MigG1 1:1300</td>
</tr>
<tr>
<td>ERβ</td>
<td>Biotinylated secondary-ABC-streptavidin</td>
<td>Biotinylated rabbit anti-mouse antibody and ABC-streptavidin (DAKO, Cambridgeshire, UK)</td>
<td>Pressure Cook: 0.05M glycine/EDTA pH8, 7 minutes</td>
<td>Vagina</td>
<td>1:40 overnight at 4°C</td>
<td>Serum</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Endometrium</td>
<td>1:80 37°C for 60 minutes</td>
<td>MigG1 1:2000</td>
</tr>
<tr>
<td>PR</td>
<td>Goat anti-mouse envision system</td>
<td>Goat anti-mouse envision system (DAKO, Cambridgeshire, UK)</td>
<td>Microwave: 0.01M sodium citrate pH 6, 10 minutes</td>
<td>Vagina</td>
<td>1:400 overnight at 4°C</td>
<td>RlgG 1:2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Endometrium</td>
<td>1:1000 overnight at room temp.</td>
<td>RlgG 1 1:1000</td>
</tr>
<tr>
<td>AR</td>
<td>Biotinylated secondary-ABC-Elite</td>
<td>Biotinylated goat anti-rabbit antibody and ABC-Elite (Vector, Peterborough, UK)</td>
<td>Pressure Cook: 0.01M sodium citrate pH 6, 5 minutes</td>
<td>Vagina</td>
<td>1:50 overnight at 4°C</td>
<td>MigG 1:500</td>
</tr>
<tr>
<td>pH3</td>
<td>Goat anti-rabbit envision system</td>
<td>Goat anti-rabbit envision system (DAKO, Cambridgeshire, UK)</td>
<td>Pressure Cook: 0.01M sodium citrate pH 6, 5 minutes</td>
<td>Vagina</td>
<td>1:50 overnight at 4°C</td>
<td>MigG 1:500</td>
</tr>
<tr>
<td>SLPI</td>
<td>Biotinylated secondary-ABC-Elite</td>
<td>Biotinylated horse anti-mouse antibody and ABC-Elite (Vector, Peterborough, UK)</td>
<td>Microwave: 0.01M sodium citrate pH 6, 10 minutes</td>
<td>Vagina</td>
<td>1:50 overnight at 4°C</td>
<td>MigG 1:500</td>
</tr>
</tbody>
</table>

+ The anti h-ERβ antibody has been previously pre-absorbed with the peptide to which it had been raised (Saunders, Millar et al. 2000)
**Immunohistochemistry analysis:**

We used a descriptive methodology as there is no quantitative or semi-quantitative methodology established for analysis of vaginal tissue samples in our laboratory. The intensity and distribution of immunostaining for ERα, ERβ, PR, AR, pH3 and SLPI is described for vagina (epithelium and stroma) and endometrium (glands, stroma and surface epithelium) and differences between pre and post-treatment samples are analysed.

**RNA extraction:**

Tissue was minced using a standard sterile surgical scalpel blade and immersed in 2 millilitres of TRI-reagent (Sigma-Aldrich, St Louis, USA). The mixture was homogenised for 60 seconds and incubated overnight at 4 degree Celsius. The following day, tissue sample was warmed to room temperature and 200 micro litre of bromochloropropane was added. The mixture was centrifuged at 14000 rpm at 4 degrees Celsius for 15 minutes and aqueous phase RNA (supernatant) was transferred to a fresh tube. 500 micro litre of isopropanol was added and incubated at 4 degree Celsius for 60 minutes. The mixture was centrifuged for 10 minutes, supernatant discarded and pellet washed with 1 millilitre of 70% ethanol. The mixture was centrifuged for 5 minutes and supernatant discarded allowing the pellet to dry for 5 minutes. The pellet was re-suspended in 20 micro litre of RNA solution. To standardise measurements between the various biopsy specimens, the same amount of RNA was assessed in each sample. The amount of specific amplicon is related to ribosomal 18S, which is constant relative to the amount of cDNA present, and subsequently, to an experimental internal control. The RNA was reverse transcribed
(TaqMan Reverse Transcription Reagents Kit, Applied Biosystems) and PCR (Polymerase Chain Reaction) amplified (TaqMan Universal Master Mix, No Amp Erase UNG, Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. PCR amplification of cDNA was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Specific forward and reverse primers (300 nmol/l) and probe (200 nmol/l, all synthesised by BioSource UK, Nivelles, Belgium) for the natural antibiotic were also added. Ribosomal 18S cDNA was measured using TaqMan Ribosomal RNA Control Reagents (VIC dye, Applied Biosystems) in each sample as an internal control following the manufacturer's protocol. Samples were measured in triplicate and no template controls were included in all runs. Primers and probes for quantitative PCR were designed using the PRIMER EXPRESS program (Applied Biosystems, Table 4.2) (King, Critchley et al. 2000; King, Fleming et al. 2002; Fleming, King et al. 2003; King, Critchley et al. 2003; King, Fleming et al. 2003; King, Morgan et al. 2003) and probes were fluorescently labelled with the proprietary dyes FAM (5') and TAMRA (3').
Table 4.2: PCR primer and probe sequence for SLPI

<table>
<thead>
<tr>
<th>SLPI</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLPI</td>
<td>GCATCAAATGCCTGGATCCT</td>
<td>GCATCAAAACATTGGCCATAAGTC</td>
<td>TGACACCCAAACCCCAACAAGGAGG</td>
</tr>
</tbody>
</table>

All probes were labelled with 5' FAM and 3' TAMRA fluorophores
**Statistical analysis:**

Statistical analysis was performed using SPSS (SPSS, Inc., Chicago, IL, USA) and Excel 2002 (Microsoft Corporation, Reading, UK). Sex steroid and vaginal thickness are expressed as mean with either standard error of mean or standard deviation. Menstrual cycle data are expressed as mean and range. Wilcoxon Signed Rank test was used to compare repeated measures of sex steroid level, menstrual data, vaginal thickness and natural anti-microbial RNA content before and after treatment.

**RESULTS**

All eight subjects completed the study. The subjects took mifepristone 5mg/day orally for an average of 33 days (range 28-40). The average length of the control menstrual cycle was 27 (range 24-29) and that of the control menstrual period 5.7 days (range 4-9). All eight women reported amenorrhoea during ingestion of mifepristone. The time from discontinuing the mifepristone treatment to the next menstrual period was 17 days (range 10-23), thus the length of the mifepristone cycle was 50 days (range 38-63). Average length of the menstrual period post discontinuation of mifepristone was 5.1 days (range 4-7).

During the treatment with mifepristone seven of the eight subjects experienced either complete suppression of ovarian activity (3/8 women, Figure 4.3 a, subject 08) or persistent follicular activity but no ovulation (4/8 subjects, Figure 4.3 b, subject 04). In the remaining subject (subject 07, Figure 4.3 c) there was a 3 fold rise in the excretion of pregnanediol in the first 10 days of treatment suggesting the formation of a corpus luteum (07). However there was no menstrual bleeding when the level of pregnanediol
dropped 14 days after starting the mifepristone. In this subject a persistent ovarian cyst of 42 mm diameter was detected at completion of treatment (day40) when the level of progesterone (14nmol/) was slightly raised consistent with a persistent unruptured partially luteinised follicle.

Multiple follicles were detected by transvaginal ultrasound (diameter of the largest ranged from 10 to 29mm) in all 8 subjects. The concentrations of E2 in blood samples collected at visit 2 on the last day of mifepristone treatment were compatible with persistent follicular activity (496 ± 57 pmol/l). In 7/8 women the concentration of progesterone was <10nmol/l (3 ± 1 nmol/l) indicating lack of ovulation.

Endometrial histology

Endometrial biopsies obtained at the end of mifepristone treatment displayed inactive or weakly proliferative endometrium in 7 of the 8 subjects. In one subject (subject 09) tortuous glands with evidence of intra-luminal secretion were seen. As expected there was strong immunostaining of ERα andβ, PR and AR in both glands and stroma. Histological evidence of mitosis was absent or infrequent in all samples.

Vaginal histology and vaginal thickness

Vaginal biopsy was obtained in all subjects without complications both before and after mifepristone administration. The pre-treatment vaginal biopsy was performed in the follicular phase prior to ovulation (mean 12 menstrual day, range 7-16 menstrual day) in 7 of the 8 subjects (estradiol mean+sem- 659±141 pmol/l, progesterone mean+sem- 4.3±1 nmol/l). In the remaining subject (subject 05) ovulation had already occurred on the day of biopsy (day 14 of menstrual cycle) as indicated by high
circulating concentration of progesterone (44nmol/l). Post-treatment vaginal biopsy was performed on day 33 (range 28-40) of treatment.

The histology of the vagina showed the expected basal layer of epithelium mounted by up to 20 layers of more superficial desquamating cells (Figure 4.2). Vaginal thickness was not altered during administration of mifepristone (342 ± 40 micron vs. 303 ± 69 micron, P=0.2, NS.)

Steroid receptor expression in the vagina (ERα, ERβ, AR, PR)

There was nuclear staining of ERα, ERβ, and AR in both vaginal stroma and in the epithelium. Immuno-reactivity extended through the basal and intermediate layers but not the superficial layer of vaginal epithelium (Figure 4.4). Staining was far more evident in the epithelium as compared to the stroma. There was no significant difference in sex steroid receptor immunostaining after mifepristone administration.

Apart from a few scattered nuclei in the stroma the basal layer of vaginal epithelium immunostaining for PR was confined to stroma (Figure 4.4). There was no significant difference in PR immuno-reactivity after administration of mifepristone.

pH3 immuno-reactivity

pH3 immunostaining was confined to scattered nuclei in the basal layers of the vaginal epithelium (Figure 4.4). There was no significant change in immuno-expression following treatment with mifepristone.

SLPI mRNA and protein expression

SLPI mRNA was present in vagina and this epitope was localised to the superficial layer of the vaginal epithelium both before and after mifepristone administration.
SLPI immunostaining was also demonstrated in the superficial layers of luminal and glandular epithelium of the endometrium (Figure 4.4). SLPI mRNA expression was unchanged following treatment with mifepristone (P>0.05, Wilcoxon test).

**Safety parameters**

There was no derangement of heart rate, blood pressure or of haematology and biochemistry parameters including liver function tests during the study. Vaginal and cervical bacteriology swab tests cultured negative for all subjects. Vaginal biopsy was well tolerated by all women. One woman had possible low-grade endometritis following endometrial biopsy and was successfully treated with a 7 day course of antibiotics.
Figure 4.3: Excretion of metabolites of ovarian steroids in women taking 5mg of mifepristone for 31-36 days

Estrone (E1G) and Pregnanediol (PdG); x axis = timescale, day 0 = start of treatment; pre-treatment (negative values), treatment (boxed) and follow-up cycle are shown; black bars = menstrual episodes; arrow = vaginal biopsy; a: complete suppression of ovarian activity; b: persistent follicular activity but no ovulation; c: single ovulatory episode and no menstruation following fall in pregnanediol levels
Figure 4.3: Urinary steroid profile

(a) Mifepristone 5 mg/d

(b) Mifepristone 5 mg/d

(c) Mifepristone 5 mg/d
Figure 4.4: Immunohistochemical (IHC) localisation of estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), progesterone receptor (PR), androgen receptor (AR), phospho-Histone H3 (pH3) and serine leukocyte protease inhibitor (SLPI) in the vagina [epithelium (epth), stroma (str)] and endometrium [gland (glnd) and stroma (str)] before and after treatment with 5 mg mifepristone.

Scale bar = 100 microns; inserts = negative controls; strong expression of ERα (a,b), ERβ (d,e), and AR (j,k) in the basal and para-basal layers of epithelium and a relative lack of PR (g,h) in the epithelium, no observed change following treatment; pH3 expression in the basal layers (m,n; arrows) and endometrium (post mifepristone – o; arrows); strong expression of all steroid receptors in the post-treatment endometrium (c,f,i,l); SLPI confined to superficial layers of vaginal epithelium (p,q) and endometrial glands (r).
Figure 4.4: IHC of ER, PR, AR, pH3 and SLPI

<table>
<thead>
<tr>
<th>Pre-mifepristone</th>
<th>Post-mifepristone</th>
<th>Endometrium</th>
</tr>
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<tbody>
<tr>
<td><strong>ER alpha</strong></td>
<td></td>
<td></td>
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<tr>
<td>a</td>
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<td>c</td>
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<td><strong>ER beta</strong></td>
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<td>g</td>
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<td><strong>PR</strong></td>
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<td><strong>AR</strong></td>
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<td>p</td>
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<td>r</td>
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<tr>
<td><strong>SLPI</strong></td>
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</table>

Legend:
- **epith.**
- **str.**
- **str.**
- **gland.**

120
Discussion

The vagina is a key portal of entry for HIV and other STIs. We report the effect of mifepristone on different parameters involved in the natural defences of the vagina to infection. Vaginal epithelial thickness, steroid receptor and SLPI and distribution were unchanged following treatment with mifepristone for 30-40 days.

Vaginal epithelial thickness

Vaginal epithelial thickness is regulated by the levels of circulating oestrogen. (Sjoberg, Cajander et al. 1988; Ma, Lu et al. 2001; Farage and Maibach 2006). There is a significant reduction in circulating oestrogen levels following long-term progestagen treatment (Miller, Patton et al. 2000) and severe vaginal atrophy has been demonstrated in primate studies and this clearly increases the risk of SIV transmission (Marx, Spira et al. 1996). However the response of human vaginal epithelium to progestagen-induced hypo-oestrogenism is variable with most studies reporting no change (Mauck, Callahan et al. 1999; Bahamondes, Trevisan et al. 2000), one study reporting a small but significant decrease (10 %) (Ildgruben, Sjoberg et al. 2005) and another, paradoxically, an increase (Ildgruben, Sjoberg et al. 2003).

It is difficult to reconcile all available data into a working hypothesis due a difference in study designs and methodology. The vagina has diverse embryological origins (Ulfelder and Robboy 1976) and Ildgruben et al used a cross-sectional study design and sampled lateral vaginal fornices, whereas we used a longitudinal study design and sampled lateral mid-vaginal wall in keeping with other reports (Mauck, Callahan et al. 1999; Bahamondes, Trevisan et al. 2000).
Although vaginal epithelium clearly responds to circulating oestrogen, the underlying cellular and molecular mechanisms are poorly understood. We localised pH3, a marker for mitosis and cellular proliferation to a few scattered nuclei in the basal layers of the vaginal epithelium. The strong nuclear expression of ER and AR in the basal and para-basal layers suggests a role in the regulation of epithelial proliferation. Oestrogen treatment induces surface keratinisation and hyperplasia of primate vaginal epithelium and we expected similar change in mifepristone-treated vaginal samples due to unopposed oestrogen effect. The observed epithelial thickness in control samples in the present study (mean pre-treatment thickness 342 microns) is comparable to other reports (Mauck, Callahan et al. 1999; Bahamondes, Trevisan et al. 2000; Ildgruben, Sjoberg et al. 2003). Thickness was unchanged following mifepristone treatment and this agrees with similar work in cynomolgus monkeys (Grow, Williams et al. 1996).

There are several possible explanations for our findings. Firstly, mifepristone treatment did not have any effect on level of circulating steroid hormones. Secondly, PR was localised to a few scattered nuclei in the basal layers of vaginal epithelium and hence mifepristone, a high-affinity PR ligand, did not have any direct epithelial effects.

**Steroid receptor content and distribution**

Consistent with previous reports we have demonstrated a strong immuno-expression of ER, AR and a relative lack of PR in the vaginal epithelium compared to sub-epithelial stroma (MacLean, Nicol et al. 1990; Hodgins, Spike et al. 1998; Blakeman, Hilton et al. 2000; Pelletier and El-Alfy 2000; Gebhart, Rickard et al. 2001; Traish, Kim et al. 2002; Berman, Almeida et al. 2003; Fu, Rezapour et al. 2003). The role of AR in the genital tract is unclear, but AR may play a role in regulating endometrial proliferation.
(Brenner, Slayden et al. 2003; Slayden and Brenner 2003; Narvekar, Cameron et al. 2004), modulating vaginal blood flow (Berman, Almeida et al. 2003) and female genital sexual arousal (Traish, Kim et al. 2002).

**Endometrial findings**

The endometrium sampled at end of mifepristone treatment showed persistent proliferative histology and strong ER, PR and AR expression which is consistent with our previous reports (Baird, Brown et al. 2003; Narvekar, Cameron et al. 2004).

**SLPI**

The expression, regulation and role of natural anti-microbial compounds in the female reproductive tract is extensively reviewed elsewhere (King, Critchley et al. 2003). SLPI has been shown to play an important role in limiting transmission of HIV (McNeely, Dealy et al. 1995; Pillay, Coutsoudis et al. 2001; Farquhar, VanCott et al. 2002) and other lower genital tract infection (Draper, Landers et al. 2000). SLPI mRNA has been demonstrated in vaginal fluid previously (Draper, Landers et al. 2000; Pillay, Coutsoudis et al. 2001). We have demonstrated mRNA in vaginal tissue and immuno-localised the protein to the superficial layers of the vaginal epithelium. The expression in superficial layers of surface and glandular endometrium is in keeping with previous reports (King, Critchley et al. 2000). Natural anti-microbial expression is modulated by hormonal treatment and up-regulation of endometrial SLPI by progesterone is attenuated in presence of mifepristone (King, Morgan et al. 2003). Reassuringly, expression and distribution of SLPI was unchanged following mifepristone treatment.
Chapter 5

DEVELOPING MIFEPRISTONE AS A ONCE-A-MONTH CONTRACEPTIVE PILL

Women across different continents and cultures have expressed a desire for a once-a-month contraceptive pill (Glasier, Smith et al. 1999). Mifepristone has shown great promise to be developed as a once-a-month contraceptive pill, however, a practical and reliable method of timing administration is not available.

The peri-ovulatory 'window of opportunity' to administer mifepristone is 3 days and existing technology to identify this window is complex, expensive, cumbersome and not infallible. Women with regular menstruation may be able to estimate this window by a simple calendar approach by recording the dates of menstrual bleeding and take the pill at the appropriate time thus allowing the method to be easily used at home.

This multi-centre study was designed and co-ordinated by the Contraceptive Development Network. 399 women in 5 international centres participated in the study. I recruited all 80 women in the Edinburgh centre and was involved in the design, co-ordination, collation and analysis of data from all centres.
AIMS AND OBJECTIVES

The aim of the study was

1. To test the feasibility of using a calendar approach (12\textsuperscript{th} day before next menses as calculated from the length of previous menstrual cycles) to identify the correct time for administration of mifepristone as a once-a-month contraceptive pill.

2. To investigate the effect of three different doses of mifepristone (10mg, 25mg or 200mg) on the length of the menstrual cycle and pattern of menstrual bleeding.

MATERIAL AND METHODS

Setting: 5 family planning centres across the world [Edinburgh (Scotland), Sagamu (Nigeria), Cape Town (South Africa), Hong Kong and Shanghai (Peoples Republic of China)].

Study Design: double-blind, parallel group, randomised controlled trial. The effect of three different doses of mifepristone (10mg, 25mg or 200mg) on the length of the menstrual cycle and pattern of menstrual bleeding was compared with placebo.

The rationale for testing the three doses of mifepristone was as follows. Contraceptive potential of 10 and 200 mg mifepristone has been reported. A dose of 200mg has been shown to be contraceptive, but given at the wrong time (too early or too late) will frequently result in menstrual irregularity (Swahn, Johannisson et al. 1988). A dose of 10mg mifepristone has been shown to be effective when used for emergency contraception (WHO 1999) but has an effect on endometrial development which is less
marked than the effect of 200 mg (Marions, Hultenby et al. 2002). This dose may be less likely to cause cycle disruption but also less likely to be effective. An intermediary dose of 25mg was chosen as an empirical compromise between anti-nidatory endometrial effects, efficacy and potential for cycle disruption by mifepristone. All classical dose finding studies are designed to span the lowest possible effective dose (even if it may be too low) and the highest dose deemed as safe.

Subjects: The intention was to recruit a total of 400 subjects (80 per centre) from women aged 18-40 years attending family planning clinics.

Inclusion Criteria:

1. female volunteers aged 18-40 years inclusive
2. prepared to use barrier methods for the duration of the study, already have an IUD in-situ, previously sterilized (subject or partner), or not requiring contraception.
3. regular menstrual cycles of between 25-35 days with no greater than 3 day’s variation in the past 3 months.
4. willing to provide written informed consent.

Exclusion Criteria:

1. those who have used any type of hormonal contraception within 3 months of starting the trial.
2. clinically relevant abnormal findings during the physical/gynaecological examination.
3. those who have breastfed in the past three months.
4. current treatment with corticosteroids.
5. treatment with an investigational drug within one month of inclusion.
6. long term use of any prescription drugs for a significant medical condition.
7. chronic alcoholism, drug abuse or any other condition associated with poor patient compliance.
8. undiagnosed vaginal bleeding.
9. other significant disease eg. cardiovascular, renal or liver disease or malignancy, sufficient to interfere with the evaluation of the study.

**Ethical approval:** The proposal was approved by the local Ethical Committee (Institutional Review Board) in each centre. Written informed consent was obtained from all subjects.

**Study visits:**

A full medical and gynaecological history and examination were obtained at recruitment (Visit 1). The duration of menstrual bleeding was determined in the same manner. For the duration of the study all episodes of vaginal bleeding were recorded on a menstrual diary card and spotting and bleeding were differentiated. Women contacted the investigators in the first week of the intervention cycle and attended the clinic 12 days before the anticipated date of next menses as estimated from their normal cycle length (Visit 2). Pregnancy was excluded by a urine pregnancy test and subjects were randomised to receive 10 mg, 25mg, or 200mg mifepristone, or placebo. A blood sample was collected for the measurement of LH and progesterone. Women were reminded to record any menstrual bleeding or adverse events on the record card. The subject contacted the investigators and returned the record card after onset of menses in the cycle that followed treatment cycle (Visit 3).
**Study medication and randomisation:**

The study medication was dispensed as 10, 25, 200mg of mifepristone or placebo tablets provided by the Shanghai Hualian Pharmaceutical Co Ltd, (370 Jiang Wan Road West, Shanghai). The mifepristone tablets came in three different sizes so three sizes of placebo were produced to match the size and colour of mifepristone tablets. Each subject received a bottle containing one mifepristone (10 or 25 or 200 mg) plus 2 different sized placebo tablets. Women in the placebo group received all three placebo tablets. The study was double-blind. Randomisation at visit 2 was performed by blocked computer-generated randomisation for each centre to ensure a good balance of numbers in the different treatment groups. Sealed envelopes were used to conceal the treatment allocation until the women were registered as definite participants.

**Blood samples, assays and analysis:**

Blood samples collected by venepuncture on day of drug administration (Visit 2) were centrifuged and the serum was stored at -20°C until analysis. Standard radioimmunoassay techniques were used in each centre for the measurement of LH and progesterone. Based on the laboratory normal values the samples were classified as originating from three stages of the ovarian cycle:

1. Follicular - LH < 10 IU/l and progesterone < 5 nmol/l
2. Luteal - LH < 10 IU/l and progesterone > 20 nmol/l
3. Peri-ovulatory - LH > 10 IU/l and/or progesterone 5-20 nmol/l
Definitions:

Patterns of vaginal bleeding are an important factor in the acceptability of contraceptive methods. The analysis of data obtained from daily menstrual diary records is a major methodological problem to which no satisfactory solution exists. The WHO recommended reference period method, introduced to avoid the arbitrary rules and definitions required for an analysis based on the concept of a menstrual cycle, is widely used and its application and limitations are reviewed elsewhere (Belsey and Farley 1988). The minimum reference period for analysis is 90 days and this methodology is not appropriate for our study design (short study period and single ‘one-off’ intervention). However, definitions of various menstrual endpoints have been adapted from those recommended by WHO (Rodriguez, Faundes-Latham et al. 1976; Belsey, Machin et al. 1986; Belsey and Farley 1988).

A menstrual period was defined as 2 or more days of blood loss (bleeding or spotting) with at least 1 day of bleeding and bounded at each end by ≥ 2 bleeding/spotting-free days (Belsey and Farley 1988). Isolated days of bleeding, or runs of spotting without bleeding, were excluded. If there was ≤ 1 day separating two sets of blood loss, this was counted as a single menstrual period.

Normal cycle lengths were defined as follows. Cycle length and range were calculated from the dates of the last three menstrual periods for women who kept a written record (retrospective normal cycle length). Women who did not provide a record of the dates of menstruation but claimed to have very regular cycles were asked to give an estimate of normal cycle length and a range of variation (reported normal cycle length). Women who did not keep a record of their cycles and who were unsure of their normal cycle
length kept a prospective record of the next three cycles before the intervention cycle (prospective normal cycle length).

**Statistical methods:**

The length of the intervention cycle was compared to that of the normal cycle length (as defined earlier) and to the cycle length of women treated with placebo. The primary outcome measure was a lengthening or shortening of the normal menstrual cycle length by more than five days following administration of the drug. A cut off of five days was considered clinically significant as this degree of disruption would be likely to result in a woman seeking advice (particularly if her period was delayed) and would be sufficient to jeopardize the contraceptive effectiveness of the regimen. The proportion of cycles disrupted by more than 5 days was compared between adjacent randomized groups (i.e. placebo versus 10mg, 10mg versus 25mg and 25mg versus 200mg) by chi-squared tests, with Yates' correction at 5% level of significance. Based on an estimated incidence of delayed cycles of 5% in the placebo group, the study had > 80% power to detect significant differences of the order of 20% between adjacent pairs of actively treated groups.

A secondary analysis of the incidence of lengthened cycles was carried out across all four study groups using Chi-squared tests for trends. Chi-squared tests were used to compare the outcomes in different stages of the cycle based on hormone measurements.
RESULTS

Starting from December 2001, a total of 399 women were recruited; 79 from Hong Kong and 80 from each of the other four centres. Recruitment finished in April 2003. They were randomised into four equally sized groups who were given a single dose of placebo, 10, 25, or 200mg mifepristone. 391 of the 399 women were followed up after the administration of drug. Eight women were withdrawn; six became pregnant prior to first post-drug menstrual episode and two did not supply any diary data. Two women became pregnant in the cycle following the intervention cycle and were included in primary analysis.

Baseline characteristics are summarised in Table 5.1, 5.2. The four groups were similar in age, parity and body mass index. Mean values (ranges) were 31 (18-40) years for age, 1.2 (0-6) for parity and 24 (15-48) for BMI.

Current methods of contraception and duration of use are summarised in Table 5.3. The majority of subjects used barrier methods of contraception (46%) followed by intrauterine device (IUD) (20%) and sterilisation (16%). 15% of women were not sexually active and there was no difference in contraceptive usage across the treatment groups. Mean (SEM) duration of use was 38 (8.5), 45 (6.2) and 51 (8.9) months for IUD, barrier and sterilisation.

Although the overall mean cycle length was similar between the centres (29.3 ± 5.4 days), there was considerable variation both between and within women [mean range 1.94 (s.d. 2.18) days based on 281 prospective menstrual episodes] in whom repeated measurements were available prior to intervention cycle. Similarly the length of the intervention cycle varied among women in the placebo-treated group. Only 85/97
(89%) women had intervention cycle lengthened or shortened by more than 5 days (Table 5.4).

There was a statistically significant, dose-related tendency for disruption of cycle length (of more than ± 5 days) in the women who received mifepristone (Table 5.4) from 16% of women treated with 10 mg, to 52% of women treated with 200 mg of mifepristone ($\chi^2=23.88$; df=3; P<0.001). However the difference between adjacent drug groups in respect of menstrual delay was not significantly different (Table 5.4) (for placebo versus 10 mg ($\chi^2=2.97$; df=1; P=0.08); for 10 mg versus 25 mg ($\chi^2=1.88$; df=1; P=0.17); and for 25 mg versus 200 mg ($\chi^2=1.54$; df=1; P=0.21). A dose-related increase in the proportion of women experiencing menstrual cycles of a shorter duration was also demonstrated (Table 5.4) but the difference between placebo and mifepristone only reached statistically significance in the group treated with 200mg (25%) ($\chi^2=19.34$; df=1, P<0.001).

More than half of study subjects provided an estimate of normal cycle length and a range of variation (reported normal cycle length, n=199, 51%), the remainder provided either a retrospective record of three menstrual periods (retrospective normal cycle length, n = 87, 22%) or kept a prospective record of the next three cycles before the intervention cycle (prospective normal cycle length, n = 105, 27%). There was no difference in the principal outcome measure (variation in cycle length of more than 5 days) for all the three groups.

Hormone data were used to classify the time of the ovarian cycle when the drug was given in the 389 women for whom the data were available (Table 5.5). When all the groups and centres were combined, treatment (mifepristone or placebo) was given in
the peri-ovulatory period (the correct time) in only 179/389 (46%) of cycles. In 64/389 (16%) it was given in the follicular phase (before ovulation, too early) and in the remainder (38%) in the luteal phase (after ovulation, too late).

The degree of cycle disruption was much less in the women who were given mifepristone in the peri-ovulatory period (Table 5.5). Hundred and one of the 137 women in this group (74%) had their next menses within five days of the expected time. In contrast, in the 200mg group 75% women who took the mifepristone in the follicular phase had delay of more than five days in the onset of the next menses, while in 59% of those taking it in the luteal phase, the menstrual cycle was shortened (Table 5.5). Menstrual characteristics of women who took mifepristone in the peri-ovulatory phase are further classified by the type of normal cycle length in Table 5.6.

A few women (0, 2, 1 and 14 percent women in the placebo, 10, 25 and 200 mg groups respectively) menstruated within a few days of administration of tablets and experienced a second bleeding episode at the anticipated time of next menstruation. This bleeding pattern was strongly associated (P<0.001) with administration of drug in the luteal phase of the cycle.

In total, 8 women became pregnant during the study, 6 in the intervention cycle and 2 in the subsequent cycle. Relevant study characteristics of pregnant women are summarised in Table 5.7. Endocrine data was available in only 3 subjects.
<table>
<thead>
<tr>
<th></th>
<th>PLACEBO</th>
<th>10 MG</th>
<th>25 MG</th>
<th>200 MG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n= 97</td>
<td>98</td>
<td>99</td>
<td>98</td>
</tr>
<tr>
<td><strong>Mean (SEM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age Trial Entry</td>
<td>31.34 (0.63)</td>
<td>31.65 (0.61)</td>
<td>31.10 (0.59)</td>
<td>31.03 (0.64)</td>
</tr>
<tr>
<td>BMI (Kg/m2)</td>
<td>23.99 (0.64)</td>
<td>23.72 (0.50)</td>
<td>23.84 (0.55)</td>
<td>23.26 (0.50)</td>
</tr>
<tr>
<td>Gravidity</td>
<td>1.88 (0.17)</td>
<td>1.89 (0.15)</td>
<td>2.03 (0.17)</td>
<td>1.55 (0.16)</td>
</tr>
<tr>
<td>Parity</td>
<td>1.40 (0.15)</td>
<td>1.37 (0.13)</td>
<td>1.45 (0.14)</td>
<td>1.04 (0.12)</td>
</tr>
<tr>
<td>Terminations</td>
<td>0.36 (0.07)</td>
<td>0.42 (0.08)</td>
<td>0.45 (0.07)</td>
<td>0.36 (0.08)</td>
</tr>
<tr>
<td>Miscarriages</td>
<td>0.11 (0.04)</td>
<td>0.12 (0.05)</td>
<td>0.10 (0.04)</td>
<td>0.13 (0.05)</td>
</tr>
<tr>
<td>Ectopic</td>
<td>0.01 (0.01)</td>
<td>0.01 (0.01)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
</tr>
</tbody>
</table>
Table 5.2: Distribution of subjects according to centre and type of normal cycle length

<table>
<thead>
<tr>
<th>Study Centre</th>
<th>PLACEBO</th>
<th>10 MG</th>
<th>25 MG</th>
<th>200 MG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>N</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>Edinburgh</td>
<td>19</td>
<td>19</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Cape town</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>19</td>
<td>18</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>Shanghai</td>
<td>19</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Nigeria</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Normal cycle length</th>
<th>PLACEBO</th>
<th>10 MG</th>
<th>25 MG</th>
<th>200 MG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reported</td>
<td>50</td>
<td>49</td>
<td>48</td>
<td>52</td>
</tr>
<tr>
<td>Retrospective</td>
<td>22</td>
<td>21</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>Prospective</td>
<td>25</td>
<td>27</td>
<td>31</td>
<td>22</td>
</tr>
</tbody>
</table>

135
Table 5.3: Current method of contraception and duration of use [Mean SEM (standard error of mean) and number (n)]

<table>
<thead>
<tr>
<th></th>
<th>PLACEBO</th>
<th>10 MG</th>
<th>25 MG</th>
<th>200 MG</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=</td>
<td>97</td>
<td>98</td>
<td>99</td>
<td>98</td>
</tr>
<tr>
<td>n</td>
<td>Duration</td>
<td>n</td>
<td>Duration</td>
<td>n</td>
</tr>
<tr>
<td>IUD</td>
<td>17</td>
<td>52.06</td>
<td>11.72</td>
<td>16</td>
</tr>
<tr>
<td>Barrier</td>
<td>51</td>
<td>42.31</td>
<td>5.83</td>
<td>43</td>
</tr>
<tr>
<td>Sterilisation</td>
<td>16</td>
<td>51.75</td>
<td>6.73</td>
<td>17</td>
</tr>
<tr>
<td>Natural</td>
<td>0</td>
<td>na</td>
<td>na</td>
<td>2</td>
</tr>
<tr>
<td>Other</td>
<td>2</td>
<td>24.00</td>
<td>12.00</td>
<td>0</td>
</tr>
<tr>
<td>Abstinence</td>
<td>11</td>
<td>na</td>
<td>na</td>
<td>20</td>
</tr>
</tbody>
</table>
Table 5.4: Percentage of subjects with length of intervention cycle shortened or lengthened by more than 5 days

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>n</th>
<th>Within 5 days</th>
<th>Shortened</th>
<th>Lengthened</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>97</td>
<td>89</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>10 mg</td>
<td>97</td>
<td>84</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>25 mg</td>
<td>99</td>
<td>72</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>200 mg</td>
<td>98</td>
<td>48</td>
<td>26</td>
<td>26</td>
</tr>
</tbody>
</table>
Table 5.5: Phase of cycle in relation to ovulation during which subjects received treatment and number (percentage) of intervention cycles shortened or lengthened by more than 5 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peri-ovulatory</th>
<th>Follicular</th>
<th>Luteal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within 5 Shorten</td>
<td>Lengthen</td>
<td>n Within 5 Shorten</td>
</tr>
<tr>
<td>Placebo*</td>
<td>42 38(91)</td>
<td>2(5)</td>
<td>16 14(88)</td>
</tr>
<tr>
<td>10 mg</td>
<td>40 35(88)</td>
<td>0(0)</td>
<td>13 7(54)</td>
</tr>
<tr>
<td>25 mg</td>
<td>43 31(72)</td>
<td>4(9)</td>
<td>23 12(52)</td>
</tr>
<tr>
<td>200 mg</td>
<td>54 35(65)</td>
<td>5(9)</td>
<td>12 2(17)</td>
</tr>
</tbody>
</table>

* 2 subjects missing due to lack of hormonal data for analysis
Table 5.6: Type of normal cycle length and the number (percentage) of intervention cycles shortened or lengthened by more than 5 days in subjects administered drug in the peri-ovulatory phase

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Reported</th>
<th>Retrospective</th>
<th>Prospective</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Within ± 5 days</td>
<td>Shorten</td>
</tr>
<tr>
<td>Placebo</td>
<td>21</td>
<td>19(91)</td>
<td>1(5)</td>
</tr>
<tr>
<td>10 mg</td>
<td>15</td>
<td>11(73)</td>
<td>0(0)</td>
</tr>
<tr>
<td>25 mg</td>
<td>19</td>
<td>15(79)</td>
<td>1(5)</td>
</tr>
<tr>
<td>200 mg</td>
<td>23</td>
<td>14(61)</td>
<td>2(9)</td>
</tr>
</tbody>
</table>
Table 5.7: Characteristics of pregnant subjects

<table>
<thead>
<tr>
<th>TIMING OF PREGNANCY</th>
<th>TREATMENT GROUP</th>
<th>TYPE OF MENSTRUAL RECORD</th>
<th>ENDOCRINE STATUS</th>
<th>CURRENT CONTRACEPTION</th>
<th>DURATION OF CURRENT CONTRACEPTION (MONTHS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pregnant during intervention cycle</td>
<td>200 mg</td>
<td>reported</td>
<td>no data</td>
<td>barrier</td>
<td>96</td>
</tr>
<tr>
<td>pregnant during intervention cycle</td>
<td>200 mg</td>
<td>reported</td>
<td>no data</td>
<td>abstinence</td>
<td></td>
</tr>
<tr>
<td>pregnant during intervention cycle</td>
<td>10 mg</td>
<td>reported</td>
<td>no data</td>
<td>IUD</td>
<td>3</td>
</tr>
<tr>
<td>pregnant during intervention cycle</td>
<td>25 mg</td>
<td>reported</td>
<td>no data</td>
<td>barrier</td>
<td>36</td>
</tr>
<tr>
<td>pregnant during intervention cycle</td>
<td>Placebo</td>
<td>reported</td>
<td>no data</td>
<td>abstinence</td>
<td></td>
</tr>
<tr>
<td>pregnant during intervention cycle</td>
<td>Placebo</td>
<td>retrospective</td>
<td>peri-ovulatory</td>
<td>IUD</td>
<td>60</td>
</tr>
<tr>
<td>pregnant after intervention cycle</td>
<td>200 mg</td>
<td>prospective</td>
<td>peri-ovulatory</td>
<td>barrier</td>
<td>60</td>
</tr>
<tr>
<td>pregnant after intervention cycle</td>
<td>10 mg</td>
<td>prospective</td>
<td>peri-ovulatory</td>
<td>abstinence</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

The ideal time to administer mifepristone is just after the start of LH surge and before endometrium is primed for a progesterone-withdrawal bleed and a three day ‘window of opportunity’ around the time of ovulation has been identified by biochemical and ultrasound monitoring of menstrual cycle (Brown, Williams et al. 2003). In the present study, we attempted to identify this peri-ovulatory window by prediction of length of menstrual cycle with knowledge of each woman’s previous menstrual pattern. This study clearly demonstrates that such a calendar approach to timing the administration of once-a-month mifepristone is not practical.

Timing of administration

Even with the flexibility of a three day window, treatment was mistimed in over half the cycles. Although normal cycle length and the length of pre-intervention cycle correlated well, there was unexpected variation in the length of intervention cycle with 11 % of women in the placebo group demonstrating a variation of more than 10 days. This was significantly more than our pre-study assumption (5 %) and may explain why mifepristone was mistimed. A possible explanation may be menstrual cycle disruption due to participation in research study (Brown, Williams et al. 2003). Seven women with regular menstrual cycles were studied during a control cycle and then in a second cycle when 200 mg mifepristone was given in the peri-ovulatory phase. The intervention cycle was slightly longer (30.2 ± 0.7 days) than the control cycle (28.2±0.9 days, P = 0.09) due to
prolongation of the follicular phase (16.50 ± 1.0 days vs. 14.3 ± 0.6 days, P = 0.06) (Brown, Williams et al. 2003).

A large number of studies describing bleeding patterns have observed discrepancies between women’s claims of a regular menstrual cycle of usually 28 days and the actual regularity of such patterns revealed by prospective recording. Regardless of the way in which we calculated normal cycle lengths (retrospective recording, prospective recording and reported normal length), there was no difference in the principal outcome measure (variation in cycle length of more than 5 days) for all the three groups. Moreover, the proportions of women with reported, retrospective or prospective (51, 22 and 27 % respectively) records of menstrual cycle lengths are likely to be representative of women attending for contraceptive advice in different family planning settings.

Pattern of menstrual bleeding

The effect of treatment on cycle length was related to both the dose of mifepristone and to the timing of administration in relation to ovulation. Administration in the follicular phase prolonged the cycle presumably by delay in onset of LH surge (Liu, Garzo et al. 1987; Shoupe, Mishell et al. 1987; Swahn, Johannisson et al. 1988; Batista, Cartledge et al. 1992; Batista, Cartledge et al. 1994). Only the highest dose (200 mg) of mifepristone caused a significant shortening of cycle by inducing a withdrawal bleed when administered in the luteal phase (Shoupe, Mishell et al. 1987). More than one in ten women treated with 200 mg of mifepristone experienced two bleeding episodes, one progesterone-withdrawal and the other at the expected time of menstruation. Even women
who received treatment during the peri-ovulatory phase of the menstrual cycle showed a significant dose-dependent trend in cycle disruption (lengthening). Although the exact functional form of these dose-bleeding relationships cannot be defined, such dose-related effects on menstrual cycle length based on timing of administration in relation to ovulation have not been reported previously. Results from a large randomised multi-centre trial investigating the efficacy of 10, 50 and 600 mg of mifepristone for emergency contraception (WHO 1999) confirm considerable disruption in the cycle if mifepristone is given on random days of the cycle.

Limitations of the study

Our study was not sufficiently powered to detect small (<20%) differences between adjacent groups. The reported (16%) disruption in the 10 mg group is within confidence interval of the estimate for the placebo group (mean 11%, 95% CI 4.7-17.3%). However, the group sizes are small, so the precision of estimates is low. Although a larger study, finding the same disruption rates (11% vs 16%) may demonstrate non-inferiority of the 10 mg dose, the variation in cycle length is such that fewer than 50% women would take the compound at the correct time. Also, published literature suggests that the 10 mg dose is unlikely to disrupt endometrium sufficiently to support effective contraception.

Due to its inherent unreliability, any dose of mifepristone administered using the calendar method is unlikely to have a better contraceptive efficacy than a similar dose taken at random (emergency contraception).
SUMMARY

The progesterone receptor antagonist, mifepristone, has tremendous therapeutic potential as a safe and effective oestrogen-free contraceptive pill. Although there are many social and political hurdles in its development, the scientific community is awaiting long-term data on safety and efficacy.

The current studies investigated various effects of mifepristone on the hypothalamic-pituitary-ovarian axis and reproductive tissues.

Endometrial proliferation and amenorrhea

Daily low-dose mifepristone is an effective contraceptive pill. Although concerns have been raised of possible hyperplastic endometrial effects, the compound markedly inhibits cellular proliferation despite proliferative histology. The majority of women also report amenorrhea in contrast to other oestrogen-free progestogen only contraception.

We investigated potential endometrial mechanisms underlying the reported endometrial anti-proliferative effects and amenorrhea. The results are as follows:

1. Anti-proliferative effect: Mifepristone down-regulates pH3, a mitosis marker, thus confirming the cellular anti-proliferative effect previously demonstrated by traditional markers such as mitosis count, PCNA and Ki67 which have technical and logistic limitations.

2. Steroid hormones: Mifepristone up-regulates glandular AR and GR; down-regulates stromal PR and has no effect on expression of ER.
a. Up-regulation of AR by mifepristone has been reported before in human and primate models, however, we are the first to report the presence of glandular GR in the human model. The role of endometrial AR and GR is unclear; however, their temporal and spatial expression suggests common mechanistic pathways of regulation.

b. Mifepristone is an in-vitro antagonist of PR, AR and GR, however, in contrast to AR and GR there is down-regulation of stromal PR. The underlying mechanistic pathways are unclear and the impact on progesterone dependent endometrial molecular functions needs to be characterised.

c. There is a strong expression of ER (ER α, β at 30-40 days; ER α at 60 and 120 days) and it is unlikely that a modulation of ER-isoforms mediates observed endometrial effects and function following mifepristone treatment.

3. VEGF and micro-vessel density: The decrease in expression of stromal VEGF which agrees with primate data may explain the increase in stromal density, a characteristic finding in mifepristone-treated endometrium. Unlike the severe vascular atrophy observed in the primate model, we demonstrated an in microvessel density and this may be related to a counting artefact following stromal compaction and gland dilatation.

4. GR and vascular endothelium: We demonstrated strong expression of GR in endometrial endothelium. An induction of glandular GR should theoretically
potentiate the tonic effect of glucocorticoids on angiogenesis. Although, there was no numerical effect on micro-vessel density, the effect of glucocorticoid-GR on other aspects of vascular function is unknown.

Effects on the vagina

Long-term progestagen treatment increases transmission risk of HIV and other STI’s, presumably as a consequence of vaginal thinning and modulation of defence mechanisms. There was no change in vaginal thickness and proliferation, steroid receptor and SLPI content in the human vagina following 30-40 days of low-dose mifepristone (anti-progestagen) treatment. However, these findings are reassuring for future development of this compound for contraception and other gynaecological uses.

Once-a-month pill

Preliminary studies indicate that once-a-month administration of mifepristone is an effective and much anticipated approach to contraception. The timing of administration is crucial to success and a 3-day ‘window of opportunity’ has been identified in the peri-ovulatory phase of the menstrual cycle. Our study demonstrates that a calendar-based estimation of this therapeutic window cannot be used to identify the correct time to administer mifepristone as a once-a-month contraceptive pill. It is difficult to envisage how an easy and reliable way of defining the correct timing could be devised.

There was a greater variation in menstrual cycles in our subjects than previously reported in similar or other populations and there is a dose-related lengthening or shortening of menstrual cycle if mifepristone is given in the follicular or luteal phase respectively. The
10 mg dose did not disrupt menstruation when compared to placebo, however, underlying endometrial mechanisms to support contraceptive efficacy need to be investigated.
CONCLUSIONS

1. AR may have a key role in mediating the anti-proliferative effect of low-dose mifepristone.

2. Mifepristone-induced amenorrhea may be related to GR-mediated regulation of vascular function.

3. Low-dose mifepristone has no appreciable effect on the vaginal thickness and defences.

4. Mifepristone causes a dose-related disruption of menstruation and a calendar-based approach to time administration of once-a-month contraception is ineffective.
DIRECTION FOR FUTURE RESEARCH

The role of AR and GR in endometrium needs to be elucidated and the content and distribution of steroid receptor (ER, PR) isoforms needs to be investigated in mifepristone-treated endometrium.

The endometrial effects of very low (< 10 mg) single doses of mifepristone need to be investigated and further work is needed to correlate degree of endometrial effect and contraceptive efficacy. Since mifepristone disrupts the endometrium in a dose-dependent manner, the lowest single dose which has sufficient anti-nidatory endometrial effect needs to be established. Finding a simple and reliable timing methodology is still enigmatic!


ACKNOWLEDGEMENTS

I express my sincere thanks and gratitude to Professors David Baird, Anna Glasier and Hilary Critchley for providing extensive supervision, inputs and corrections throughout the course of this dissertation.

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Last but not the least I am forever indebted to the subjects who volunteered for this study, without whom the work would be incomplete.
Low-Dose Mifepristone Inhibits Endometrial Proliferation and Up-Regulates Androgen Receptor

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Mifepristone in daily low doses has contraceptive potential by inhibiting ovulation. follicular development is maintained, and although the endometrium is exposed to unopposed estrogen, there are no signs of hyperplasia or atypia. The mechanism of this antiestrogenic action is unknown. We have investigated the effect of daily low-dose mifepristone on proliferation markers and steroid receptors in surface epithelium, glands, and stroma of the endometrium. Endometrial biopsies were collected from 16 women before (late proliferative) and 60 and 120 d after taking 2 or 5 mg mifepristone daily for 120 d. Endometrial proliferation (H3 mitosis marker) and steroid (estrogen, progesterone, and androgen) receptor content were studied using standard immunocytochemistry techniques. There was a significant decrease in the expression of H3 mitosis marker (P < 0.001) and progesterone receptor (P < 0.05) in endometrial glands and stroma by d 60 of treatment. In contrast, the expression of androgen receptor increased (P < 0.01) in glands, surface epithelium, and stroma compared with the pretreatment sample. These changes were maintained at 120 d of treatment. The expression of estrogen receptor was unchanged in stroma and surface epithelium; however, there was a significant decrease in expression after 120 d of treatment (P = 0.034). As androgens can antagonize estrogen action, enhanced glandular androgen receptor expression induced by mifepristone could play a role in its antiproliferative effects. (J Clin Endocrinol Metab 89: 2491-2497, 2004)

PROGESTERONE RECEPTOR (PR) antagonists have many potential uses, including the treatment of endometriosis, fibroids, breast cancer, and meningiomas (1). Mifepristone is now licensed in many countries for medical termination of pregnancy (2, 3). In low daily doses it can serve as a novel, estrogen-free, contraceptive pill (4-6). Because follicular development is maintained, the endometrium is exposed to estrogen for prolonged periods unopposed by progesterone, raising concerns about potential risks of endometrial hyperplasia (7-9). It has been demonstrated in studies in nonhuman primates (10-14) and women (5, 6, 15, 16) that mifepristone and other PR antagonists exert antiproliferative effects on the endometrium. Such antiproliferative properties carry enormous importance for the sustained development of these promising compounds for long-term use. As mifepristone has no direct effect on estrogen receptor (ER), the mechanism of this noncompetitive, antiestrogenic activity still remains largely unknown (10).

There is substantial evidence that exogenous androgen can have inhibitory effects on the female reproductive tract (17-20). It has been suggested that the androgen receptor (AR) could play an important role in the noncompetitive antiproliferative actions of PR antagonists (21). Slayden et al. (21) demonstrated a significant up-regulation of AR in endometrium after 21-30 d of treatment with PR antagonists. However, there are no data on the expression of AR after long-term treatment with mifepristone.

We previously reported that daily doses of 2 and 5 mg mifepristone for 120 d has contraceptive potential by suppressing ovulation and endometrial cyclicity (4). A striking feature of the endometrium after treatment with low dose mifepristone was a significant reduction in mitotic count and Ki67 immunostaining compared with endometrium on d 12 of control cycles (6). Because Ki67 protein is expressed during several phases of the cell cycle, e.g. G1, S, G2, and M, counts of Ki67-positive nuclei in paraffin sections are numerically greater than mitotic counts, although the Ki67 is usually well correlated with the mitotic index (22, 23). Mifepristone may block completion of the cell cycle at G2-M interphase (24), so that Ki67 protein persists for some time after cell division has been arrested. Therefore, Ki67 counts may fail to reveal that mifepristone treatment suppressed estrogen-dependent proliferation (5). Direct counting of mitotic cells in hematoxylin/eosin-stained sections is a time-consuming process that requires highly skilled observers (25). We evaluated a new marker of phosphorylated proteins associated with mitosis, phospho-H3. This marker has been validated in paraffin-embedded endometrial tissues and shows an excellent correlation with the mitotic count (26). The aim of the present study was to examine endometrial proliferation and steroid receptor content and distribution in women after treatment with low dose mifepristone, with particular emphasis on the role of the AR in mediating endometrial antiproliferative effects.

Abbreviations: AR, Androgen receptor; BMI, body mass index; DAB, 3,3'-diaminobenzidine; ER, estrogen receptor; NGS, nonimmune goat serum; NHS, nonimmune horse serum; PCOS, polycystic ovarian syndrome; PR, progesterone receptor.

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Subjects and Methods

Human endometrial samples were obtained from three different patient groups. The local ethics committees (institutional review board) approved each of the studies, and all women provided written informed consent.

Mifepristone group

A subset of 16 women, aged 18–40 yr, with regular menstrual cycles (25–35 d) were studied for one pretreatment, four treatment, and one posttreatment cycles from 58 volunteer women from Edinburgh previously reported (4, 6). Subjects were randomly allocated to receive 2 and 5 mg mifepristone daily for the 120 treatment d. Subjects had a mean age of 30.5 yr and a mean body mass index (BMI) of 24.5 kg/m². Endometrial biopsies were collected using a Pipelle endometrial sampling device (Prodimed, Neuilly-en-Thelle, France) in the late follicular phase of the pretreatment cycle (d 12), after 60 d of mifepristone treatment, and after 120 d of treatment. Specimens were fixed in normal buffered formalin, processed, and embedded in paraffin wax. Endocrine and endometrial findings have been reported previously (4, 6).

Control groups

Two groups of women whose endometrium was exposed to unopposed estrogen were chosen as controls. As unopposed estrogen gives rise to persistent proliferative endometrium, the endometrium from these women could be compared with that of subjects who remained anovulatory with mifepristone. A group of women (n = 6) with anovulatory infertility due to polycystic ovarian syndrome (PCOS) participating in a study evaluating the effects of low-dose mifepristone on endometrial maturation and proliferation were recruited (5). Subjects had a mean age of 25 yr (range, 22–38 yr) and a mean BMI of 24.5 kg/m² (range, 18.7–27.6 kg/m²), and all had biochemical and ultrasound evidence of polycystic ovaries (27). All women had an endometrial biopsy taken 21–23 d after a progestogen-induced withdrawal bleed.

A second group of postmenopausal women (1 yr of amenorrhea or using hormone replacement therapy for 2 yr; n = 5) participating in a study evaluating the effects of oanpristone on postmenopausal endometrium were recruited. The details of the study along with endocrine and endometrial findings have been reported separately (28). Subjects had a mean age of 54.4 yr (range, 49–54 yr) and a mean BMI of 26.6 kg/m² (range, 20.9–33.8 kg/m²). None of the women had used a hormone preparation within the preceding 6 wk. Women were instructed to take daily 2 mg 17β-estradiol valerate (Schering UK, Burgess Hill, West Sussex, UK) orally for 8 wk (56 d). Endometrial biopsy was performed in the eighth week of treatment (n = 4). One subject refused endometrial sampling. Endometrial histology was reported as proliferative in all samples.

Immunocytochemistry

Immunocytochemistry was performed for the immunolocalization of phospho-H3 (Upstate Biotechnology, Inc., Lake Placid, NY), Estrogen receptor (ER clone ID5, DAKO, Glostrup Denmark), PR (Abbott-PR ICA, Abbott Laboratories, Inc, North Chicago, IL) and AR (FS9, BiogeneX Laboratories, San Ramon, CA). ER, PR, and AR immunostaining procedures followed the methods previously described (21, 25, 30). The phospho-H3 immunostaining procedures followed those described by Brenner et al. (26).

Phospho-H3 mitosis marker

Paraffin sections (5 μm) were dewaxed in Histoclear (National Diagnostics, Atlanta, GA), rehydrated through a series of alcohols, and washed with PBS. The slides were then subjected to microwave antigen retrieval in 0.01 m sodium citrate buffer at pH 6 for 10 min. Endogenous peroxidase activity was blocked by immersing in 3% hydrogen peroxide (Merck & Co.) in distilled water for 10 min at room temperature. Nonspecific binding of the primary antibody was blocked by incubating the sections for 20–30 min at room temperature in nonimmune goat serum (NGS; Vector Laboratories, Inc., Peterborough, UK). A variety of dilutions (0.66, 1, and 2 μg/ml) of the primary antibody in NGS were assessed in preliminary studies. The best compromise between signal to noise (specific staining vs. background) for phospho-H3 was 1 μg/ml (1:1000 dilution). Slides were then incubated overnight at room temperature with rabbit polyclonal phospho-H3 antibody (1:1000 dilution in NGS) or similarly with a control rabbit IgG antibody (1:1000 dilution in NGS). An avidin-biotin peroxidase system was used as the detection system.

The slides were incubated in biotinylated horse antirabbit secondary antibody (Vector Laboratories, Inc.) in NGS, followed by the avidin-biotin peroxidase complex (Vestacain HRP, Vector Laboratories, Inc.), for 60 min each at room temperature. The peroxidase substrate 3,3′-diaminobenzidine (DAB; Vector Laboratories, Inc.) was used to visualize the reaction.

AR

Paraffin sections (5 μm) were dewaxed in Histoclear (National Diagnostics), rehydrated through a series of alcohols, and washed with PBS. The slides were then subjected to pressure cooker antigen retrieval in 0.01 m sodium citrate buffer at pH 6 for 5 min. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide (Merck & Co.) in distilled water for 10 min at room temperature. Nonspecific binding of the primary antibody was blocked by incubating the sections for 20–30 min at room temperature in nonimmune horse serum (NHS; Vector Laboratories, Inc.). Slides were then incubated at 4°C with monoclonal antihuman AR antibody F9.4 overnight (1:800 dilution in PBS/BSA gel) or similarly with a control mouse IgG antibody (1:600 dilution in PBS/BSA gel). An avidin-biotin peroxidase system was used as the detection system. The slides were incubated in biotinylated horse antimouse secondary antibody (Vector Laboratories, Inc.) in NHS, followed by avidin-biotin peroxidase complex (Vestacain Elite FK 6101, Vector Laboratories, Inc.), for 60 min each at room temperature. The peroxidase substrate DAB (Vector Laboratories, Inc.) was used to visualize the reaction.

PR

Paraffin sections (5 μm) were dewaxed in Histoclear (National Diagnostics), rehydrated through a series of alcohols, and washed with PBS. The slides were then subjected to microwave antigen retrieval in 0.01 m sodium citrate buffer at pH 6 for 10 min. Endogenous peroxidase activity was blocked by incubating the sections for 20–30 min at room temperature in NHS (Vector Laboratories, Inc.). Slides were then incubated at 37°C for 1 h with mouse monoclonal PR antibody (1:40 dilution in PBS/BSA gel) or similarly with a control mouse IgG antibody (1:1000 dilution in NHS). An avidin-biotin peroxidase system was used as the detection system. The slides were incubated in biotinylated horse antimouse secondary antibody (Vector Laboratories, Inc.) in NHS, followed by avidin-biotin peroxidase complex (Vestacain Elite FK 6101, Vector Laboratories, Inc.) for 30 min each at room temperature. The peroxidase substrate DAB (Vector Laboratories, Inc.) was used to visualize the reaction.

ER

Paraffin sections (5 μm) were dewaxed in Histoclear (National Diagnostics), rehydrated through a series of alcohols, and washed with PBS. The slides were then subjected to microwave antigen retrieval in 0.01 m sodium citrate buffer at pH 6 for 10 min. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide (Merck & Co.) in distilled water for 10 min at room temperature. Nonspecific binding of the primary antibody was blocked by incubating the sections for 20–30 min at room temperature in NHS (Vector Laboratories, Inc.). Slides were then incubated at 37°C for 1 h with mouse monoclonal ER antibody (1:25 dilution in NHS) or similarly with a control mouse IgG subtype 1 antibody (1:150 dilution in PBS/BSA gel). An avidin-biotin peroxidase system was used as the detection system. The slides were incubated in biotinylated horse antimouse secondary antibody (Vector Laboratories, Inc.) in NHS, followed by avidin-biotin peroxidase complex (Vestacain Elite FK 6101, Vector Laboratories, Inc.), for 60 min each at room temperature. The peroxidase substrate DAB (Vector Laboratories, Inc.) was used to visualize the reaction.
oxidase substrate DAB (Vector Laboratories, Inc.) was used to visualize the reaction.

**Immunocytochemistry score**

**Semiquantitative score.** The location and intensity of immunostaining were measured using a semiquantitative scoring system. Sections were scored blind by two observers (blind to study groups and to the other’s results). This scoring system is a standard method used in previous studies (29, 30). A high correlation has been demonstrated between objectively measured immunoreactivity (image analysis) and subjective semiquantitative scoring of immunostaining patterns (29). Immunostaining intensity and distribution of epitopes in all tissue sections were assessed on an arbitrary four-point scale: 0 = no staining, 1 = mild staining, 2 = moderate staining, and 3 = intense staining. This method of semiquantitative scoring has been previously validated in our laboratory (29).

**Quantitative score.** Phospho-H3 mitosis marker immunostaining was assessed separately for glands and stroma using image analysis. The system used a Axioskop 2 microscope (×40 objective; Carl Zeiss, Inc., New York, NY) connected to a Macintosh G3 computer (Apple Computer, Cupertino, CA), using Openlab 2.08 image analysis software (Improvision, Coventry, UK). At least 12 fields of view were selected at random from each tissue section. The glands and stroma from each digitized image were interactively dissected. Using Openlab color discrimination software, the total number of phospho-H3-expressing cells (brown product) and the number of those not expressing phospho-H3 (blue hematoxylin) were measured separately for each digitized image. The number of phospho-H3-expressing cells is reported as a percentage of total cells (brown and blue) per sample. This method of image analysis has previously been described and validated in our laboratories (29, 31).

**Statistical methods**

Statistical analysis was performed using SPSS (SPSS, Inc., Chicago, IL) and Excel 2002 (Microsoft Corp, Redmond, WA). Continuous data are expressed as the mean ± SE, and categorical data are expressed as the median and range. Nonparametric tests (Friedman’s test, Wilcoxon’s signed rank test, and Mann-Whitney test), with and without Bonferroni correction, were used to compare immunostaining scores at various time points. Where there were significant differences in the conclusions, these are noted.

**Results**

**Phospho-H3 mitosis marker**

Mitotic activity as indicated by antibody to phosphorylated histone H3 showed a highly significant decrease ($P \leq 0.001$) in the endometrium by d 60 of treatment (mean ± SEM; glands, 0.89 ± 0.14; stroma, 0.48 ± 0.09; Fig. 1) compared with pretreatment proliferative endometrium (glands, 3.48 ± 0.42; stroma, 1.57 ± 0.16; Fig. 2, A and B). This decrease was demonstrable in both glands and stroma and was maintained at 120 d (glands, 0.96 ± 0.13; stroma, 0.55 ± 0.11; Fig. 2C). Endometrium from PCOS women showed higher mitotic activity in the glands (4.72 ± 0.74; $P = 0.168$, not significant), whereas postmenopausal women had a significantly lower mitotic activity in the stroma (0.65 ± 0.13; $P = 0.011$) compared with proliferative d 12 pretreatment endometrium (Figs. 1 and 2D). The mitotic activity in the stromal compartment (PCO group, 1.21 ± 0.22) and glands (postmenopausal group, 2.00 ± 0.55) was not significantly different from that in proliferative samples.

AR

Pretreatment proliferative phase endometrium showed a strong expression of AR in the stroma and minimal or absent expression in glands and surface epithelium (Table 1 and Fig. 2E). There was a significant increase in AR expression in surface epithelium, glands, and stroma after treatment with mifepristone compared with that seen in the proliferative phase pretreatment sample. This increase occurred as early as d 60 ($P < 0.05$; Fig. 2F) and was maintained on d 120 (Fig. 2G). The increase was most marked in the glandular compartment, where a virtual absence of expression in the proliferative pretreatment sample (Fig. 2E) was replaced by intense immunostaining in posttreatment samples ($P < 0.01$; Fig. 2, F and G). There was no difference between women treated with 2 or 5 mg mifepristone. Endometrium from PCOS and estrogen-treated postmenopausal women showed strong AR expression in the stroma, with minimal expression in glands and surface epithelium (Fig. 2H). This pattern was similar to that in d 12 pretreatment proliferative phase samples. Expression in glands was significantly greater in the

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**Note:** The image includes a figure (Fig. 1) that is not described in the text, but it is essential for understanding the results. The figure shows the percentage of endometrial cells immunostaining for phospho-H3 in glands (A) and stroma (B) before and after treatment with daily mifepristone; comparison with control, polycystic ovary (PCO) and postmenopausal (PM) groups. The values are expressed as median (horizontal bar), mean (square dot), and box plots showing 50% of values (bar) with range (whiskers). X, $P = 0.001$, significant decrease, mifepristone d 60 and 120 treatment endometrium vs. d 12 pretreatment by (Wilcoxon’s signed rank test). Y, $P = 0.01$, significant decrease, d 56 estradiol-treated postmenopausal endometrium vs. d 12 proliferative (by Mann-Whitney U test).

**Table 1:**

<table>
<thead>
<tr>
<th>Day</th>
<th>Prolif.</th>
<th>Mife.</th>
<th>Mife.</th>
<th>PCO</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
postmifepristone treatment samples than in samples from both PCOS and postmenopausal women ($P < 0.05$).

**PR**

There was a reduction in PR expression in surface epithelium, glands, and stroma by d 60, which was maintained on d 120 (Table 2 and Fig. 2, I–K). There was no difference between women treated with 2 or 5 mg mifepristone. There was strong PR staining in all three endometrial compartments (surface epithelium, glands, and stroma) in PCOS and postmenopausal groups (Fig. 2L). This pattern was similar to that in d 12 pretreatment proliferative phase samples.
TABLE 2. PR immunoeexpression expressed as the mean (median) in endometrium before and after treatment with daily mifepristone; comparison with control, polycystic ovary (PCO), and postmenopausal (PM) groups

<table>
<thead>
<tr>
<th>Mifepristone group (n = 16)</th>
<th>d 12 pretreatment</th>
<th>d 60 mifepristone</th>
<th>d 120 mifepristone</th>
<th>PCO group (n = 6), d 21</th>
<th>PM group (n = 4), d 56</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface</td>
<td>2.40 (2.5)</td>
<td>1.88 (2)</td>
<td>1.86 (2)*</td>
<td>2.00 (1)</td>
<td>2.25 (2)</td>
</tr>
<tr>
<td>Glands</td>
<td>2.51 (3)</td>
<td>2.13 (2)*</td>
<td>1.80 (2)*</td>
<td>2.50 (3)</td>
<td>2.70 (3)</td>
</tr>
<tr>
<td>Stroma</td>
<td>2.58 (2)</td>
<td>1.63 (2)*</td>
<td>1.60 (1)*</td>
<td>2.39 (2)</td>
<td>2.50 (3)</td>
</tr>
</tbody>
</table>

* P < 0.05, significant decrease, mifepristone d 60 and 120 treatment endometrium vs. d 12 pretreatment (Wilcoxon signed rank test).

TABLE 3. ER immunoeexpression expressed as the mean (median) in endometrium before and after treatment with daily mifepristone; comparison with control, polycystic ovary (PCO), and postmenopausal (PM) groups

<table>
<thead>
<tr>
<th>Mifepristone group (n = 16)</th>
<th>d 12 pretreatment</th>
<th>d 60 mifepristone</th>
<th>d 120 mifepristone</th>
<th>PCO group (n = 6), d 21</th>
<th>PM group (n = 4), d 56</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface</td>
<td>2.56 (2.5)</td>
<td>2.83 (3)</td>
<td>2.53 (3)</td>
<td>2.5 (2)</td>
<td>2.75 (3)</td>
</tr>
<tr>
<td>Glands</td>
<td>2.94 (3)</td>
<td>2.88 (3)</td>
<td>2.56 (3)*</td>
<td>3.00 (5)</td>
<td>3.00 (3)</td>
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<tr>
<td>Stroma</td>
<td>2.56 (3)</td>
<td>2.62 (3)</td>
<td>2.50 (3)</td>
<td>2.83 (3)</td>
<td>2.25 (2)</td>
</tr>
</tbody>
</table>

* P = 0.034, significant decrease, mifepristone d 120 treatment endometrium vs. d 12 pretreatment (Wilcoxon signed rank test).

ER

There was no significant change in ER expression in surface epithelium and stroma after treatment with mifepristone (Table 3 and Fig. 2, M-O). Expression in glands was decreased after 120 d (P = 0.034). This failed to reach significance after using Bonferroni's correction (P < 0.102). There was no difference between women treated with 2 or 5 mg mifepristone. The endometrium in both control groups demonstrated strong ER staining in all three endometrial compartments (Fig. 2P). This pattern was similar to that in d 12 pretreatment proliferative phase samples.

Discussion

This study extends our previous report of the effects of daily low-dose mifepristone on endometrial development (4-6). We have confirmed antiproliferative effects of low dose mifepristone with a new mitosis marker, phospho-H3, along with a significant increase in glandular AR expression. Our study shows for the first time that after prolonged treatment with mifepristone (120 d), there is a significant down-regulation of PR.

Concern has been expressed previously that long-term use of PR antagonists may lead to endometrial hyperplasia and possible malignancy due to exposure of the endometrium to the effects of unopposed estrogen (7-9). Evidence of estrogenic stimulation of the endometrium has been observed in the rats receiving long-term PR antagonist treatment (32, 33). The nonhuman primate endometrium, however, demonstrates endometrial atrophy and evidence of antioestrogenic activity (10, 34-38). In women, after high doses of mifepristone (25 and 50 mg/d) variable effects, such as atypical cystic changes, have been described in eutopic endometrium (8, 39). In a study in which women with pelvic endometriosis were treated with 50 mg mifepristone/d for 6 months, there was evidence of endometrial hyperplasia and numerous mitotic figures (8). The occurrence of endometrial gland dilatation in 34% of women receiving chronic treatment with mifepristone (1 mg/d given for 150 d) has also been reported (15). We reported previously that 18-23% of women treated with 2 or 5 mg mifepristone/d developed cystic changes in the endometrium, although the cysts were lined with inactive glandular tissue (6). There is a case report of an adolescent girl, aged 13 yr, with Cushingoid features and morbid osteoporosis who was treated with high doses (400 mg/d) of mifepristone for its antiglucocorticoid effect (7). However, with each of the two 6-month courses of mifepristone, given 9 months apart, she developed massive simple endometrial hyperplasia. There was no evidence of atypia, and ER and PR concentrations were in the normal range. This abnormality resolved on cessation of treatment. Eisenger et al. (9) investigating effects of 5 and 10 mg mifepristone daily for 6 months on uterine leiomyomatous noted simple endometrial hyperplasia in 28% of subjects. No atypical hyperplasia was noted. Our findings using phospho-H3, a specific marker of mitosis, confirm that at the doses tested, there is no evidence of endometrial hyperplasia. Although a proportion of the endometrial samples show cystic dilatation, the glands are lined by atrophic inactive epithelium in contrast to the pseudo-stratified appearance in typical cystic glandular hyperplasia (6).

In agreement with previous reports (21, 40, 41), proliferative phase control endometria demonstrated absent or minimal AR expression. Treatment with a PR antagonist enhances stromal and induces glandular AR expression (21). Slayden et al. (21) used ligand binding, immunocytochemistry, and in situ hybridization on the same set of endometria to ascertain the regulation of and localization of AR during normal and hormonally regulated cycles and to evaluate changes in AR in women and nonhuman primates treated with PR antagonist (mifepristone or ZK 137 316). Treatment of macaques with estradiol implants for 28 d significantly increased AR mRNA in stromal cells, but not in the glands. The highest levels of AR mRNA in both stroma and glands were detected after combined treatment with estradiol and PR antagonist (mifepristone or ZK 137 316) treatment. At all stages of the human menstrual cycle, AR staining was localized predominantly in the endometrial stroma, with no or barely detectable staining in the glands. After mifepristone treatment (2 mg/d for 21-24 d), there were distinct and notable increases in AR staining of the glands and surface.
epithelium plus some enhancement of stromal AR staining (21).

Short-term treatment with PR antagonist, either during the menstrual cycle or with combined estrogen therapy, leads to elevations of the two main uterine steroid receptors, ER and PR (5, 42, 43). Expression after chronic treatment has been shown to be increased, decreased, or unchanged depending on the dose and duration of treatment (8, 38, 39, 44). We have demonstrated a down-regulation of PR after 60 d of treatment. ER expression decreased in glands, but remained unchanged in surface epithelium and stroma. The mechanisms involved are poorly understood, but could result from either chronic antiestimocytotic activity (24) affecting cellular protein synthesis or androgen-AR interactions.

The endometrium is a target tissue for androgen action. There is ample evidence of antigestonic effects of exogenous androgens in vivo (17-20), and androstenedione can inhibit human endometrial cell growth and secretory activity in vitro (45). Currently, the role of endogenous androgens in the endometrium is not clear, but stromal AR would mediate any possible effects of normal levels of endogenous androgens. Treatment with mifepristone up-regulates AR in glands; hence, androgens could have direct effects on glands in addition to stroma. An increase in AR levels in mifepristine-treated tissues could lead to an increased binding of androgens, which might antagonize the effects of estrogens on endometrial growth. Treatment with flutamide, a pure antiandrogen, blocked the antiproliferative effect of the PR antagonist ZK 137 316 in the nonhuman primate, adding strong support to the hypothesis that this effect of PR antagonist is mediated through changes in AR (46). It is possible that mifepristone itself directly mediates these effects by interacting with AR, for which the relative binding affinity is 13% (47).

The factors regulating the expression of AR in the endometrium are not clear. In the normal cycle, AR is confined to the stroma, with little or no expression in the glands (21, 40, 41). Our observations in postmenopausal women treated with estrogen and in women with PCOS are in keeping with reports in women and nonhuman primates that estrogen stimulates AR expression in the endometrium (38, 48). Increased AR expression in both stroma and glands of endometrium from women with PCO have been reported by others (49). The expression was higher in those with persistent proliferative endometrium and may merely reflect the effect of prolonged exposure to unopposed estrogen. Although this probably contributes to the changes in women treated with long-term mifepristone, it is likely that the massve up-regulation of AR that occurs, especially in the glands, as early as 21 d after starting treatment is a specific effect of the PR antagonist (21). In our study the endometrial biopsy from women with PCO, collected 21 d after progestagen-induced menses, showed little, if any, AR expression in glands and was similar to that observed in estrogen-treated women.

**Summary**

In summary, we have shown that low dose mifepristone treatment has a significant antiproliferative effect on the endometrium. There is down-regulation of PR and ER and up-regulation of AR. The mechanisms involved are poorly understood. An increase in glandular AR content could mediate the antigestonic, antiproliferative effects of PR antagonists. Whether androgens mediate all of these effects or whether PR antagonists interact in concert with other factors remains to be established.

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**References**

48. Chilliki CF, Hsii JG, Acosta AA, van Uem JF, Hodgen GD 1986 RU486-
Effects of onapristone on postmenopausal endometrium

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Abstract

The progesterone antagonist mifepristone (RU486, Exelgyn) has been shown to exert a paradoxical agonist effect on postmenopausal endometrium. We conducted a study to investigate the effects of the 'pure' antiprogestin onapristone (ZK98 299, Schering AG) on postmenopausal endometrium. Seventeen postmenopausal subjects (45–62 years), took 2 mg of oestradiol and either placebo, 1 mg onapristone or 10 mg of onapristone, daily for 56 days. An endometrial biopsy was performed during the final week of treatment and assessed for histology and immunohistochemistry for oestrogen receptors (ER), progesterone (PR), androgen receptors (AR) and the cell proliferation marker Ki 67. FSH fell in all 14 subjects who completed the study, consistent with the effect of oestradiol treatment. There was a dose-dependent additive effect of onapristone on suppression of gonadotrophins. All endometrial biopsies showed proliferative endometrium. A similar pattern and intensity of immunostaining of ER, PR and Ki 67 was observed in all groups, with positive immunoreactivity in both glands and stroma. AR immunostaining was observed in both glands and stroma from all subjects, but there was an increase in intensity of immunostaining within the glandular epithelium of women receiving 10 mg onapristone. The antiprogestin onapristone, in contrast to mifepristone, is not agonistic on postmenopausal endometrium and does not exert obvious antiproliferative effects. It does however cause a dose dependent suppression of FSH and LH release.

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Keywords: Antiprogestin; Progesterone antagonist; Endometrium; Antiproliferative; Androgen receptor

1. Introduction

It has previously been demonstrated in both animal models and in human studies, that the antiprogestin mifepristone (RU486, mifegyne, Exelgyn, Paris) exerts antiproliferative effects on endometrium [1–5]. In oestradiol-treated castrate monkeys, treatment with low doses of mifepristone induced secretory changes in the endometrium, but with higher doses endometrial proliferation was inhibited [1,2]. An increase in apoptosis was also observed in both glands and stroma [6]. In women of reproductive age, short term studies (1 month) of continuous administration of low doses of mifepristone (2–10 mg) have shown that despite anovulation and continued oestrogen secretion, the endometrium appears ‘hyporeactive’ and devoid of mitoses [5,7]. It has been suggested that the mechanism of such antiproliferative effects may be mediated through an inhibitory effect on the progression of cells through the mitotic phase of the cell cycle [5]. Clearly, such antiproliferative properties have enormous importance with regards long-term treatment with antiprogestins and the implications for their potential use for prevention and treatment of endometrial hyperplasia. In pre-menopausal women, it has been suggested that continuous administration of low doses (2–5 mg) of mifepristone could be used as an oestrogen-free contraceptive pill which would preserve endogenous oestrogen secretion, but would also protect the endometrium against hyperplasia [5,8,9].

Mifepristone has also been shown to exert antiproliferative effects on the endometrium of postmenopausal women [10]. In the latter study, mifepristone was observed to exert a paradoxical progesterone ‘agonist’ effect, inducing secretory changes in oestrogen primed endometrium, in addition to suppressing markers of endometrial proliferative activity. This has led to suggestions that antiprogestins (in conjunction with oestradiol treatment) could be developed as a novel bleed-free form of hormone replacement therapy, that may be devoid of the side-effects and break-through bleeding typically associated with progestogen use.

The identification of mitoses on morphological examination or the immunohistochemical demonstration of cell cycle-related antigens such as Ki 67 are accepted methods.
of assessing endometrial proliferative activity. The immunolocalisation of steroid receptors can also provide useful information regarding endometrial proliferative status. Oestrogen and progesterone receptors are upregulated by oestrogen and down regulated by progesterone [11]. Furthermore, androgen receptor expression is maximal in proliferative endometrium [12]. Thus, the presence or absence of steroid receptors within the endometrium may give a useful measure of net oestrogenic activity within the tissue.

Onapristone (ZK 98 299, Schering AG, Berlin), is an antiprogestin which differs in several respects from mifepristone. Not only does it have a much shorter half-life, but it is generally considered to be a pure progesterone antagonist [13]. When administered in the immediate postovulatory period to women with regular menstrual cycles, it has been shown to produce similar effects to mifepristone, in that it does not disrupt the length of the luteal phase but retards the development of a secretory endometrium [14]. In order to investigate whether onapristone might exhibit similar antiproliferative activity on postmenopausal endometrium to that of mifepristone, we conducted a study to examine the effects of daily administration of onapristone in conjunction with oestradiol treatment in postmenopausal volunteers.

The effects of treatment on histology and the immunolocalisation of oestrogen receptors (ER), progesterone receptors (PR), androgen receptors (AR) and the cell proliferation marker Ki 67 were chosen as markers of endometrial status and proliferative activity.

2. Experimental

2.1. Materials and methods

Seventeen healthy postmenopausal women (with either ≥1 year of amenorrhoea or using hormone replacement therapy for ≥2 years) with an intact uterus, volunteered for the study. Women were recruited from a community menopause clinic at Dean Terrace Centre, Edinburgh. Subjects had a mean age of 54.4 years (range 45–62 years) and a mean body mass index of 25.5 Kg/m² (range 21–34 Kg/m²). None of the women had used a hormone preparation within the preceding 6 weeks. All women underwent a thorough clinical assessment prior to commencing the study, which consisted of a gynaecological and general physical examination, together with measurement of height, weight and blood pressure. A transvaginal ultrasound was performed and a cervical smear was taken if none had been performed within the last year. Blood was also collected for estimation of haemoglobin, blood cell count, prothrombin time, serum urea and electrolytes, creatinine, urate and tests of liver function (bilirubin, alkaline phosphatase, alanine transaminase and aspartate transaminase). In addition, blood was also collected for measurement of follicle stimulating hormone (FSH), luteinising hormone (LH), progesterone, androstenedione and cortisol. These blood tests were repeated on completion of the study. Subjects were issued with a menstrual calendar on which they recorded details of any bleeding episodes throughout the study period. The protocol was approved by the local ethical subcommittee and all volunteers gave written informed consent.

2.2. Study design

All subjects received tablets of 2 mg 17β oestradiol daily and were randomised to receive in addition either (i) placebo tablets, or identical tablets of (ii) 1 mg onapristone or (iii) 10 mg onapristone, for 56 days. Subjects attended the research centre at the beginning of treatment (baseline) and after 2, 4 and 8 weeks of treatment. A further ‘follow-up’ visit was made 4 weeks after cessation of study medication. At each visit, vital signs were recorded and a sample of blood was collected. The plasma was separated immediately at 4 °C and then frozen at −20 °C until assayed for FSH, LH, oestradiol, progesterone, cortisol and androstenedione by radioimmunoassay [15]. Intra- and inter-assay coefficients of variation were 8.4, 6.9, 8.0, 8.0, 4.0 and 10% respectively; inter-assay coefficients of variation were 9.7, 8.8, 11.0, 10.0, 6.0 and 10% respectively. Transvagal ultrasonoraphy (Siemens Sonoline S I-250 machine with a 7.5/5 MHz vaginal probe) was performed at the initial visit and after 4 and 8 weeks of treatment to measure endometrial thickness. This was measured as the maximum antero-posterior diameter of the endometrium in a plane through the central longitudinal axis of the uterine body [16].

During the last week of study medication (week 8), an endometrial biopsy was also performed using a pipelle sampler (Prodimed, Eurosurgical, UK). A portion of endometrium was fixed immediately in (a) Bouin’s solution for histological assessment and (b) neutral buffered formalin for immunohistochemistry, for an overnight fixation period at 4 °C. Specimens were then processed, paraffin-embedded and 5 μm thick sections were cut. Sections for histological assessment were stained with haematoxylin-eosin and assessed by an experienced gynaecological pathologist who was unaware of the treatment type.

Immunohistochemistry was performed for the immunolocalisation of oestrogen receptor (IDS; Dako, Glostrup, Denmark), progesterone receptor (ICA; Abbott, North Chicago, IL, USA), androgen receptor (F-39, BioGenex) and the cell cycle proliferation marker Ki 67 (NCL-Ki 67-MM1; Novoceastra, Newcastle, UK).

2.2.1. Oestrogen receptor (ER) immunostaining

Oestrogen receptor immunostaining was conducted as previously described using a microwave method of antigen retrieval [5]. A mouse monoclonal antibody (IDS; Dako was used as the primary antibody (dilution 1:25)) and an avidin-biotin horseradish peroxidase (ABC) system was used as the detection system. The chromogen was diaminobenzidine tetrahydrochloride (DAB), and slides were lightly counterstained with Harris’s haematoxylin.
2.2.2. Progesterone receptor (PR) immunostaining

PR immunostaining was detected using the method as previously described in detail [5]. The staining protocol used a rat monoclonal antibody (Abbot-PR ICA) as the primary antibody (dilution 1:10) together with an alkaline phosphatase anti-alkaline phosphatase (APAAP) detection system. The chromogen was vector red (Vector SK 5100) and no counterstain was applied.

2.2.3. Androgen receptor (AR) immunostaining

AR immunostaining was detected using a previously described procedure [12]. A pressure cooker method of antigen retrieval was used, placing slides in 0.01 M sodium citrate buffer at pH 6 for 5 min. The staining protocol used a monoclonal antihuman AR antibody (F-39, BioGenex, Lab Inc.) as the primary antibody (dilution 1:480) together with an avidin-biotin horseradish peroxidase (ABC) detection system. The chromogen was diaminobenzidine tetrahydrochloride (DAB), and slides were lightly counterstained with Harris’s haematoxylin.

2.2.4. Ki 67 immunostaining

The immunohistochemistry protocol used has previously been described in detail [5]. Briefly, a microwave method of antigen retrieval was used together with a mouse monoclonal primary antibody (NCL-Ki 67-MM1, Novo Castra) (dilution 1:100) in conjunction with an ABC detection system. The chromogen was DAB, and slides were lightly counterstained with Harris’s haematoxylin.

2.2.5. Quantification

The intensity and distribution of ER, PR and AR immunostaining was evaluated subjectively by two observers (blinded to the treatment types) using a light microscope. The immunostaining was assessed as none, mild, moderate or intense staining. Assessment of Ki 67 immunostaining was performed using an image analysis system (Colour vision, Improvision, UK), as previously described [5]. Ten fields of view (×20 objective) were selected at random from each tissue section. The ratio of the total area of positively staining nuclei to the total area of all nuclei within glandular and stromal compartments was generated. The mean percentage immunostaining within glands and stroma in each tissue section was calculated to provide an index of cell proliferation.

2.2.6. Statistical analysis

Comparisons were performed using Students t-test, analysis of variance (ANOVA), Tukey HSD pairwise comparisons, or Mann Whitney as appropriate.

3. Results

Only fourteen of the seventeen subjects completed the study. The study was terminated prematurely due to growing concern about the incidence of elevated liver function tests, in a separate study of women with breast carcinoma receiving high doses of onapristone. Of the 14 subjects who completed the study, there were the following numbers in each treatment group: 2 mg oestradiol and placebo (n = 5), 2 mg oestradiol and 1 mg onapristone (n = 5), and 2 mg oestradiol and 10 mg onapristone (n = 4).

3.1. Effect on haematology and biochemistry

The haematological and biochemical tests were repeated at the end of the study. In addition, tests of liver function were also performed during treatment, in the fourth week of study medication. In all subjects, haematological parameters were within the normal range. Serum biochemistry was normal in all but one subject (Subject no. 16) who had elevated transaminases during the fourth week of treatment (AST 128; normal range 5–45). Liver function tests in this subject returned to normal on repeat testing 11 days after stopping study medication.

3.2. Effect on gonadotrophins and ovarian hormones

There was a significant fall in FSH concentration in all subjects over time (P = 0.0002). A further suppression in FSH levels was seen however in the group of women receiving 10 mg of onapristone (P = 0.029) (Fig. 1). In the groups receiving placebo and 1 mg onapristone, there was no significant change in LH levels over the course of the study. LH was however suppressed over time in the group receiving 10 mg onapristone (P < 0.001 with respect to time, P = 0.003 with respect to treatment) (Fig. 2).

In all subjects, concentrations of plasma progesterone remained in the postmenopausal range throughout the study (Progesterone <3 nmol/l). During the course of study treatment, oestradiol levels rose significantly in all groups (P <

![Fig. 1. The mean ± S.E.M. concentrations of FSH in all treatment groups at baseline, 2, 4 and 8 weeks of treatment. FSH is significantly decreased over time in all groups *P = 0.0002. Suppression of FSH is significantly greater in 10 mg onapristone group than other groups *P = 0.029.](image)
Table 1
Mean ± S.E.M. concentrations of cortisol and androstenedione concentrations at baseline (0 week) and end of study (8 weeks) in each group

<table>
<thead>
<tr>
<th></th>
<th>Cortisol (nmol/l)</th>
<th>Androstenedione (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 week</td>
<td>8 weeks</td>
</tr>
<tr>
<td>Placebo</td>
<td>441.6 ± 94.2</td>
<td>458.9 ± 47.5</td>
</tr>
<tr>
<td>1 mg Onapristone</td>
<td>372.2 ± 55.9</td>
<td>512.6 ± 79.9</td>
</tr>
<tr>
<td>10 mg Onapristone</td>
<td>319.7 ± 40.3</td>
<td>476.8 ± 25.7</td>
</tr>
</tbody>
</table>

Fig. 2. The mean ± S.E.M. concentrations of LH in all treatment groups at baseline, 2, 4 and 8 weeks of treatment. LH is significantly suppressed over time in group receiving 10 mg onapristone (*P < 0.01). LH is significantly suppressed in 1 mg onapristone group compared to other groups (*P = 0.003).

0.01). Mean (±S.E.M.) concentrations of oestradiol during the eighth week of treatment were 443.4 ± 122.7 498.8 ± 207.1 and 546.7 ± 249.9 pmol/l (for placebo, 1 and 10 mg of onapristone groups respectively). There was no significant difference between treatment type and oestradiol concentrations.

3.3. Effect on pituitary adrenal axis- cortisol and androstenedione

There was no significant change in the concentrations of cortisol or androstenedione during the course of treatment (Table 1).

3.4. Effect on endometrium

Two subjects (both receiving 10 mg onapristone) experienced bleeding during the course of the study. One of these women (subject no. 4) bled during the last 2 weeks of treatment, for a total of 9 days. The bleeding was initially light but subsequently heavy. Transvaginal ultrasound performed at this time, demonstrated an endometrial thickness of 18 mm. Endometrial histology in this subject displayed features of late proliferative endometrium. In the other woman (subject no. 6), light bleeding occurred during the first week of treatment and lasted 4 days. No further bleeding occurred during treatment and endometrial histology at the end of the study was that of weakly proliferative endometrium.

There was considerable inter-subject variability in endometrial thickness (as measured using transvaginal ultrasound) within each treatment group, at the end of the study (8 weeks). The range of the endometrial thickness in each group was 2–13, 2–8 and 8–18 mm for placebo, 1 mg onapristone and 10 mg onapristone respectively. There was no statistically significant difference in endometrial thickness between treatment groups, with 9 out of 14 subjects having an endometrial thickness of >4 mm.

3.5. Histology

Out of the fourteen women who completed the study, endometrial biopsies were obtained from eleven. In two women, no tissue was obtained at biopsy and in one case, the subject refused endometrial sampling. Out of these 11 samples, the following numbers where obtained from each treatment group: placebo (n = 4), 1 mg onapristone (n = 3) and 10 mg onapristone (n = 4).

![Histology](image-url)

Fig. 3. Histology of endometrium from oestradiol and placebo group (a), oestradiol and 1 mg onapristone (b) and oestradiol and 10 mg onapristone (c). All endometrium shows similar proliferative appearance. Scale bar = 50 μm.
3.6. Steroid receptor immunostaining

Positive immunoreactivity for ER was visualised as dark brown nuclear staining and that of PR as bright red nuclear staining. Both the distribution and the intensity of immunostaining, for ER and PR was similar in endometrium from...
all treatment types, with all endometrial biopsies displaying moderate positive glandular and stromal immunoreactivity (Fig. 4).

Positive immunoreactivity for AR was identified as dark brown nuclear staining. All biopsies displayed either moderate or intense AR immunostaining within the stroma. Mild AR immunostaining was observed in the glands of both oestradiol-only and 1 mg onapristone exposed endometrium, but intense AR immunostaining but observed within the glands of endometrium from the group receiving 10 mg onapristone. (Fig. 5).

3.7. Ki 67 immunostaining

Positive immunoreactivity for Ki 67 was visualised as dark brown nuclear staining. The distribution and intensity of immunostaining was similar within endometrium from all treatment types, with moderate intensity of immunostaining in both glands and stroma. There was no significant difference in the proliferation index (% of positively staining nuclei) as assessed using image analysis, between treatment groups in either glandular or stromal compartments (Fig. 5).

4. Discussion

It has previously been reported that mifepristone exerts progesterone ‘agonist’ effects on postmenopausal endometrium [10]. In addition to inducing histological features of secretory changes, markers of oestrogenic proliferative activity in the endometrium were suppressed, including decreased activity of the enzyme DNA polymerase α and increased activity of oestradiol dehydrogenase [10]. In contrast, in our study, onapristone was not observed to exhibit either progesterogenic or antiproliferative effects on the endometrium of postmenopausal subjects. This may reflect real differences in the properties of the two antiprogestins, which include different half-lives and mechanisms of binding to the progesterone receptor [17,18]. Furthermore, whereas animal studies have consistently demonstrated antiproliferative effects of mifepristone on endometrium, it has been reported that onapristone exerts oestrogenic effects in the rodent uterus [19]. There are however several differences in study design between our study and the original study using mifepristone in postmenopausal women, which could possibly account for some of the differences in findings [10]. In the mifepristone study, endometrial assessments were made following 6 days of combined mifepristone (100 or 200 mg) and oestriadiol therapy, after a period of 9 days of unopposed oestrogen exposure. In our study, endometrial assessments were made in the eighth week of combined onapristone (1 or 10 mg) and oestriadiol therapy, without any prior period of oestrogen priming. When one compares the oestriadiol only-treated endometrium with those who received onapristone, there was no significant difference in endometrial thickness, histology, or in the pattern of distribution of immunostaining for ER, PR or Ki 67. This would suggest that onapristone, was unable to antagonise the mitogenic effects of oestradiol. Reassuringly there was no evidence of endometrial hyperplasia, although this is perhaps not surprising in view of the relatively short duration of treatment (8 weeks) with oestrogen and the small numbers of biopsies available for analysis. Indeed, use of unopposed oestradiol for 10–12 weeks (followed by 2 weeks of progestogen therapy) forms the basis of many commercially available ‘long-cycle’ or ‘quarterly’ hormone replacement therapy regimes for menopausal women. Studies of such regimes have reported rates of simple hyperplasia of around 15% after 10 weeks of unopposed oestradiol therapy [20].

The finding of an increase in AR immunostaining intensity within the glandular epithelium in endometrium of women treated with 10 mg onapristone, does however suggest that there may be a subtle effect of onapristone on the endometrium, which may be dose-related. Increased androgen receptor expression within glandular epithelium has previously been demonstrated in association with mifepristone use in women of reproductive age [12,21]. It has even been suggested that this may be a possible mechanism for the antiproliferative effect of mifepristone, since there is both in both in vivo and in vitro evidence that androgens can inhibit endometrial cell growth [12,22].

Unfortunately the study had to be terminated prematurely due to concern about elevation of liver function tests in a separate study using onapristone, and so fewer endometrial samples than anticipated were available for assessment. While we observed one subject in our study to have raised serum transaminases, elevated liver function tests have also been reported in association with the antiprogestin mifepristone [23–25].

Prolonged administration of onapristone did not significantly affect serum concentrations of either cortisol or androstenedione as measured throughout the study. This suggests that onapristone in the doses used in this study, exerts no significant anti-glucocorticoid activity. This is consistent with previous reports in pre-menopausal women, which demonstrated an absence of significant glucocorticoid activity using onapristone in either a single 400 mg dose or in a daily doses of 5–50 mg [14,24].

A suppression in FSH levels was observed in all subjects throughout the study consistent with the effect of oestradiol treatment. However, the additive suppressive effect of 10 mg onapristone on FSH concentrations, together with the suppressive effect of this dose on serum LH concentrations was an unexpected finding. It has previously been reported that mifepristone potentiates the suppression of gonadotrophins in postmenopausal women receiving oestradiol [10]. Whether such an effect represents a progesterone agonist effect at the level of the hypothalamo-pituitary axis, or is an intrinsic property of antiprogestins is unknown.

In conclusion therefore, the antiprogestin onapristone, in contrast to mifepristone, did not exert progesterone-agonist effects on postmenopausal endometrium. Furthermore, it
did not exhibit obvious antiproliferative effects on the endometrium, as assessed by measurements of endometrial thickness, histology or immunohistochemistry for either the proliferation marker Ki 67, or ER and PR immunostaining. Subtle dose-related effects of onapristone on the endometrium cannot however be excluded in view of a subjective increase in intensity of immunostaining for AR within glandular epithelium associated with 10 mg onapristone treatment.

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References

Mifepristone-induced amenorrhoea is associated with an increase in microvessel density and glucocorticoid receptor and a decrease in stromal vascular endothelial growth factor

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ABSTRACT: We have previously shown that the progesterone antagonist mifepristone is a contraceptive when given in a dose of 2 or 5 mg per day. The majority of women experience amenorrhoea rather than the irregular breakthrough bleeding usually occurring with other estrogen-free contraceptive pills, such as progestogen-only pill (POP). We investigated the effects of low-dose mifepristone on endometrial parameters which may be associated with changes in endometrial function, such as microvasculature, vascular endothelial growth factor (VEGF) and glucocorticoid receptor (GR) content. METHODS AND RESULTS: Endometrial biopsies were collected from 16 women before (late proliferative phase) and 60 and 120 days after taking 2 or 5 mg mifepristone daily for 120 days. Seven of the eight women who received 2 mg mifepristone and all eight women who received 5 mg were amenorrhoeic during the study. Mean estradiol (E₂) concentrations remained in the mid-proliferative range, and the majority (9/16) of women showed proliferative endometrial histology at 60 and 120 days following treatment. There was a significant increase in the density of the endometrial stroma (P < 0.05) and microvessels (P < 0.01) following 120 days of treatment. Immunocytochemistry showed that GR, hitherto localized specifically in endometrial stroma, was up-regulated in the nuclei of glands (P < 0.05) and surface (luminal) epithelium (P < 0.01) by 60 days and maintained at 120 days. There was a significant reduction in stromal VEGF protein expression by day 120 of treatment (P ≤ 0.01). CONCLUSION: The high incidence of amenorrhoea in women taking mifepristone may be related to changes in the regulation of vascular function.

Keywords: contraception/endometrium/glucocorticoid receptor/mifepristone/vascular endothelial growth factor

Introduction

Mifepristone is a potent antagonist of both progesterone and glucocorticoids. In daily low doses, it acts as a contraceptive by inhibiting ovulation and by altering endometrial function (Brown et al., 2002; Baird et al., 2003a,b). Currently used estrogen-free contraceptive methods such as progestogen-only pill (POP) are often discontinued because of a high incidence of troublesome side effects, such as breakthrough bleeding (Belsky and Farley, 1988). In contrast, the majority of women who take mifepristone have no problems. Although traditionally amenorrhoea has been classified as a disadvantage, many women now regard the absence of periods as a desirable side benefit (Glasier et al., 2003). The mechanisms underlying these disturbances in endometrial function are poorly understood and not clearly related to levels of endogenous or exogenous steroid hormones (Fraser et al., 1996).

Locally produced vasoactive substances probably play a key role in regulating endometrial angiogenesis, although these are influenced substantially by different dosage regimens and routes of administration of contraceptive steroids (Smith, 2001). Vascular endothelial growth factor (VEGF) promotes microvascular endothelial cell proliferation, migration and assembly into new vessels (Ferrara and Davis-Smyth, 1997), and estrogen has been shown to promote angiogenesis by regulating the expression of VEGF (Albrecht et al., 2003). Prostaglandins (PGs) influence contractility of endometrial vessels and their permeability (Albrecht et al., 2003). An increase in the local concentration of PGs in the endometrium is involved in the mechanism of mifepristone-induced vaginal bleeding in the luteal phase (Hapangama et al., 2002). Glucocorticoids modulate PG production in endometrial stromal cells and fibroblasts (Pakrasi et al., 1983; Schatz et al., 1986; Neulen et al., 1989; Delvin et al., 1990; Illouz et al., 2000). Glucocorticoid
receptor (GR) is strongly expressed in the nuclei of endometrial cells in the stromal compartment of human endometrium during the menstrual cycle (Bamberger et al., 2001; Henderson et al., 2003). Although the exact physiological role of glucocorticoids and GR in the human endometrium is unknown, it has been suggested that they have an angiostatic role (Small et al., 2005).

We have previously reported the effects of daily low-dose mifepristone on the histology of the endometrium, sex hormone receptors and various proliferation markers (Baird et al., 2003a,b; Narvekar et al., 2004). Mifepristone treatment is associated with a striking up-regulation of the expression of androgen receptors (ARs) in the glands and with an increase in stromal density. The aim of this study was to look at the effects of low-dose mifepristone on endometrial microvessel density, VEGF and GR protein expression and correlate with endometrial histology and bleeding patterns.

Materials and methods

Human endometrial samples were obtained from 16 women with regular menstrual cycles (25–35 days), who were a subset of 58 volunteers women from Edinburgh, as previously reported (Brown et al., 2002; Baird et al., 2003a; Narvekar et al., 2004). The local ethics committees (Institutional Review Board) approved the studies, and all the women provided written informed consent. Subjects were randomly allocated to receive 2 (n = 8) or 5 mg (n = 8) of mifepristone daily for the 120 treatment days. Subjects had a mean age of 30.5 years (range 24–40) and a mean BMI of 24.5 kg/m² (range 21–34). Endometrial biopsies were collected using a Pipelle endometrial sampling device (Prodimed, Neully-on-Thelle, France) in the late follicular phase of the pretreatment cycle (day 12), after 60 days of mifepristone treatment and after 120 days of treatment. Specimens were fixed in normal buffered formalin, processed and embedded in paraffin wax. Endocrine and endometrial findings have been reported previously (Brown et al., 2002; Baird et al., 2003a; Narvekar et al., 2004).

Immunocytochemistry

Immunocytochemistry was performed for the immunolocalization of VEGF, CD31 (endothelial marker) and GR. Immunostaining procedures followed those previously published (Nayak et al., 2000; Bamberger et al., 2001). All antibodies were tested individually at a range of dilutions and different antigen retrieval conditions to determine the protocol which gave the least background and highest specific staining (Table I). Positive and negative controls were included. All tissue sections were initially prepared in a similar manner. Five-micrometre paraffin-embedded tissue sections were dewaxed in Histoclear (National Diagnostics, UK) and rehydrated in ascending grades of alcohol to distilled water. The tissue sections were subjected to antigen retrieval in a pressure cooker (5 min) or microwave (10 min) using 0.01 M sodium citrate at pH 6 (Table I) and were then allowed to cool to room temperature. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide (BDH, Poole, UK) in methanol for 30 min at room temperature. Non-specific binding of the primary antibody was blocked by incubating the sections for 20 min at room temperature in 1:5 dilution of normal immune serum (Autoimmune Bioclear, Holly Ditch Farm, Wils, UK) in buffer containing 5% bovine serum albumin. Sections were incubated at 4°C with primary and control antibodies overnight. An avidin–biotin–peroxidase system was used as the detection system. The sections were incubated with biotinylated secondary antibody (Vector Laboratories, Peterborough, UK) in normal immune serum followed by avidin–biotin–peroxidase complex (Vectastain horse-radish peroxidase and Vectastain Elite PK 6101, Vector Laboratories) for 30–60 min each at room temperature. All tissue sections underwent an identical epitope visualization step. The peroxidase substrate 3,3'-diaminobenzidine (Dako) was used as chromogen. Sections were then counterstained with haematoxylin, dehydrated in ascending grades of alcohol to xylene and mounted using Pertex (Cellpath plc., Hemel Hempstead, UK).

Table I. Immunocytochemistry protocols for vascular endothelial growth factor (VEGF), glucocorticoid receptor (GR) and the endothelial marker CD31

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Buffer</th>
<th>Antigen retrieval</th>
<th>Non-immune serum</th>
<th>Antibodies</th>
<th>Stock solution (mg/ml)</th>
<th>Dilution</th>
<th>Detection system</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>TBS/TBST</td>
<td>Microwave</td>
<td>Normal goat serum</td>
<td>VEGF</td>
<td>0.2</td>
<td>1:60</td>
<td>ABC-Elite 30 min</td>
</tr>
<tr>
<td>GR</td>
<td>PBS/PBST</td>
<td>Pressure cook</td>
<td>Normal rabbit serum</td>
<td>VEGF pre-absorbed</td>
<td>0.02</td>
<td>1:60</td>
<td>ABC-Elite 30 min</td>
</tr>
<tr>
<td>CD31</td>
<td>PBS/PBST</td>
<td>Pressure cook</td>
<td>Normal horse serum</td>
<td>Mouse IgG2a</td>
<td>1.0</td>
<td>1:320</td>
<td>ABC-Elite 30 min</td>
</tr>
</tbody>
</table>

CD31 Sections were incubated at 4°C either with mouse anti-human CD31 (Novocast Laboratories, Newcastle upon Tyne, UK) overnight at a 1:800 dilution in normal horse serum (NHS) or similarly with a control mouse immunoglobulin (IgG1) antibody at 1:8000 dilution in NHS.

VEGF Sections were incubated overnight at 4°C either with polyclonal rabbit anti-human VEGF (Santa Cruz Biotechnology, CA, USA) antibody at 1:600 dilution in normal goat serum (NGS) or similarly with a control VEGF preabsorbed antibody at 1:60 dilution in NHS.

GR Non-specific binding of avidin and biotin was blocked by incubating with avidin and biotin for 15 min each followed by non-immune rabbit serum (NRS) for 20–30 min at room temperature. Sections were incubated at 4°C either with monoclonal mouse anti-human GR IgG2a (Novocast Laboratories, Newcastle upon Tyne, UK) at a dilution of 1:40 in NRS or similarly with a control mouse IgG2a antibody at 1:320 dilution in NRS.

Semi-quantitative immunocytochemistry score

Location and intensity of immunostaining were measured for VEGF and GR using a semi-quantitative scoring system. Sections were scored blind by two observers (blind to study groups and to other's results). This scoring system is a standard method used in previous studies (Wang et al., 1999; Crichtley et al., 2001; Narvekar et al., 2004), and a
High correlation has been demonstrated between objectively measured immunoreactivity (image analysis) and subjective semi-quantitative scoring of immunostaining patterns (Wang et al., 1998). Immunostaining intensity and distribution of epitopes in all tissue sections were assessed on an arbitrary four-point scale: 0 = no staining, 1 = mild staining, 2 = moderate staining and 3 = intense staining.

Vessel counts and stromal density

Vessel counts and stromal density were measured using image analysis. The system used a Carl Zeiss Axistop 2 microscope (×40 objective) connected to a MacIntosh G3 computer, using Openlab 2.08 image analysis software (Improvision, Coventry, UK). At least 12 fields of view were selected at random from each tissue section at a magnification of ×40. The glands and stroma from each digitized image were interactively dissected.

CD31 immunoexpression was used to identify endothelial cells (Narvekar et al., 1993). All immunostained (brown) structures were considered positive and counted for each digitized image, even if lumen has not been identified. A similar methodology has been described to study changes in vessel density following Norplant use (Hickey et al., 1999). The results were averaged and expressed as vessels per square millimetre. Endometrial stromal density appears to be increased following daily low-dose mifepristone (Baird et al., 2003a). To compensate for this, the total number of cells not expressing CD31 (blue haematoxylin) was measured separately for each digitized image using Openlab colour discrimination software, and the results are also expressed as vessels per 1000 stromal nuclei. This method of image analysis has previously been described and validated in our laboratories (Critchley et al., 1996; Wang et al., 1998).

Stromal density was expressed as the total number of stromal nuclei (brown CD31 and blue haematoxylin) per square millimetre of endometrial tissue.

Statistical methods

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) and Excel 2002 (Microsoft Corporation). Continuous data are expressed as mean with SE and categorical data as median with range. Non-parametric tests (Friedman test, Wilcoxon-signed rank test and Mann–Whitney test), with and without Bonferroni correction, were used to compare study variables at various time points.

Results

Endometrial histology, serum estradiol and bleeding patterns

All 16 pretreatment endometria were sampled in the proliferative phase. Following treatment, 9/16 endometria were still proliferative at 60 and 120 days. Four endometria showed cystic dilatation of the glands and five inactive epithelium at 60 and 120 days of treatment, respectively. Serum estradiol (E2) levels remained in the mid-proliferative range following treatment (511 ± 70.21 pmol/l, pretreatment; 459 ± 9.94 pmol/l, mifepristone 60 days; 290 ± 63.39 pmol/l, mifepristone 120 days). There was no significant change in the level of E2 following treatment (P > 0.05, Bonferroni correction). Seven of the eight women on 2 mg mifepristone and all eight women on 5 mg of mifepristone were completely amenorrhoeic during treatment. Detailed description of menstrualphysiology, endocrinology and endometrial histology has been reported previously (Brown et al., 2002; Baird et al., 2003a).

There was no significant difference (P > 0.05, Mann–Whitney test) between women treated with 2 or 5 mg mifepristone for any parameters studied except for the degree of GR protein expression in the vascular endothelium at 60 days of treatment [median (range) at 60 days = 3 (3), 2-mg group; 2 (2–3), 5-mg group].

Figure 1. Density of endometrial stroma (a) and microvessel (b and c) in women before and after treatment with mifepristone. The values are expressed as median (horizontal bar), mean (square dot) and box plots showing 50% of values (box) with range (whiskers). (a) P ≤ 0.01 (Wilcoxon test), significant increase, mifepristone treatment versus pretreatment; (b) P < 0.05 (Wilcoxon test), significant increase, mifepristone treatment versus pretreatment.
group; \( P = 0.012 \), Bonferroni correction, Mann–Whitney test].

As the GR expression was identical by 120 days in the two groups and there were no differences in any of the other 36 parameters tested, it was considered that this difference could have occurred by chance. Therefore, the 2- and 5-mg data sets were combined, and results reported are for all 16 subjects.

### Stromal density

An average of 0.5 mm\(^2\) (SE = 0.05) of endometrial stroma was examined in the pretreatment samples and 0.37 mm\(^2\) (SE = 0.04) in the treatment samples. Stromal density, expressed as nuclei per square millimetre of endometrial tissue, increased significantly (24%) by day 60 of treatment \((P < 0.001)\), and the increase (18%) was maintained at 120 days \((P < 0.05\), Figure 1a; 8057 ± 355, pretreatment; 10 003 ± 355, day 60; 9510 ± 351, day 120).

### Microvessel density

Vessel density per square millimetre of endometrial tissue increased (47%) by day 60 of treatment \((P \leq 0.01\), Figures 1b and 2). This increase was highly significant and was maintained (49%) at 120 days \((P < 0.01\), Figure 1b; 267 ± 18, pretreatment; 392 ± 32, day 60; 398 ± 37, day 120). To compensate for an increase in stromal density, microvessel density was calculated per total nuclei in each endometrial sample. There was a modest \((P = 0.08\), ns) increase (15%) by day 60 and a significant \((P < 0.01)\) increase (34%) by day 120 of treatment (Figure 1c; and Figure 2b and c; 33 ± 1.9, pretreatment; 38 ± 2.1, day 60; 42 ± 2.75, day 120).

### VEGF

VEGF protein was strongly expressed in the cytoplasm of endometrial glands and surface epithelium. Expression in the stroma was patchy and faint (Table II, Figure 2e). VEGF expression remained unchanged in the glandular [median (range) = 2 (1–3) pretreatment, 1 (1–3) day 60 and 1 (0–2) day 120], surface epithelium [median (range) = 1 (0–3) pretreatment, 1 (0–3) day 60 and 1 (0–2) day 120] and endothelial cells [median (range) = 1 (0–2) pretreatment, 0.5 (0–2) day 60 and 1 (0–1) day 120] following treatment with mifepristone. There was a decrease \((P \leq 0.01)\) in the stromal expression [median (range) = 1 (0–2) pretreatment, 0 (0–2) day 60 and 0 (0–1) day 120] of VEGF following 120 days of treatment (Table II, Figure 2f and g).

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**Figure 2.** Immunoeexpression of the endothelial marker CD31, vascular endothelial growth factor (VEGF) and glucocorticoid receptor (GR) in endometrial glands (Gl.), stroma (Str.), surface epithelium (surf.) and vascular endothelium (Ves.) before and after treatment with mifepristone. Scale bar (a, j and p) = 50 \(\mu\)m; positive immunoeexpression = brown. Significant increase in microvessel (arrows) density following treatment (b and c), significant decrease in stromal VEGF following treatment for 120 days (g) and significant increase of GR immunoeexpression following treatment for 60 (k and l) and 120 days (m and n) compared to follicular pretreatment day 12 endometrium; note the absence of GR immunoeexpression in glands during pretreatment day 12 endometrium (j) and significant expression following treatment (l and n); also note the strong expression of GR in vascular endothelium (p, q and r); negative controls are included.
Vascular endothelial growth factor immunoexpression was assessed as mean (median) in endometrium before and after treatment with daily mifepristone.

<table>
<thead>
<tr>
<th>Mifepristone group (n = 16)</th>
<th>Day 12 pretreatment</th>
<th>Day 60 mifepristone</th>
<th>Day 120 mifepristone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface</td>
<td>1.43 (1)</td>
<td>1.40 (1)</td>
<td>1.40 (1)</td>
</tr>
<tr>
<td>Lands</td>
<td>1.63 (2)</td>
<td>1.40 (1)</td>
<td>1.25 (1)</td>
</tr>
<tr>
<td>Stroma</td>
<td>0.94 (1)</td>
<td>0.33 (0)</td>
<td>0.31 (0)*</td>
</tr>
<tr>
<td>Endothelium</td>
<td>0.69 (1)</td>
<td>0.64 (0.5)</td>
<td>0.44 (0)</td>
</tr>
</tbody>
</table>

P = 0.004 (Wilcoxon test), significant decrease, day 120 mifepristone treatment versus day 12 pretreatment.

The pretreatment proliferative phase endometrial samples showed strong GR immunoexpression in the nuclei of stromal cells (median (range) = 2 (0–3)) and endothelial (median (range) = 3 (1–3); Table III, Figure 2p) cells and a complete absence of expression in the surface epithelium (median (range) = 0 (0–0); Figure 2i) and glands (median (range) = 0 (0–0); Table III, Figure 2i and j). Following treatment with mifepristone, nuclear immunoexpression was induced in glands (median (range) = 0 (0–2) day 60 and 1 (0–1) day 120, P < 0.05) and surface epithelium (median (range) = 1 (0–3) day 60 and 1 (1–3) day 120, P < 0.01). This was evident by 60 days and maintained at 120 days (Table III, Figure 2k–n). Immunoexpression in the stroma (median (range) = 2 (1–3) day 60 and 2 (1–3) day 120) and endothelial cells (median (range) = 3 (2–3) day 60 and 3 (2–3) day 120; Figure 2q and r) was unchanged.

**Discussion**

This study extends our previous report of the effects of daily low-dose mifepristone on endometrial development (Brown et al., 2002; Baird et al., 2003a,b; Narvekar et al., 2004). Here, we show that mifepristone-induced amenorrhea is associated with an increase in glandular GR and microvessel density and a decrease in stromal VEGF.

Histological appearance of the endometrium does not correlate well with menstrual patterns, except with the development of extreme histological atrophy, which typically predicts amenorrhea. In the primate model, continuous mifepristone induces marked endometrial atrophy, and the spiral arteries are the primary targets that are damaged or inhibited by progestosterone antagonists such as mifepristone (Chwalisz et al., 2000). Microvessel density increases in conditions of spontaneous (post-menopausal) and induced (Norplant, danazol and goserelin) endometrial atrophy, and the mechanisms involved may vary according to the nature of the atrophic stimulus (Hickey et al., 1996, 1999). In women exposed to high- and medium-dose progestogens and long-term users of levonorgestrel releasing intrauterine system (Mirena), a decrease in endometrial vascular density has been observed (Song et al., 1995; Oliveira-Ribeiro et al., 2004). In this study, over 90% women were amenorrheic. The endometrium was proliferative, and there was a significant increase in microvessel density. We used CD31 to identify the vessels because preliminary studies with the alternative immunohistochemical marker CD34 were unsatisfactory. The use of CD31 may explain the discrepancy in the numerical count of vessels per area of endometrium between our study and that by other investigators (Hickey et al., 1996; Lau et al., 1999; Schindl et al., 2001). A study that used CD31 to identify vessels in endometrial samples of 16 normally menstruating premenopausal women showed wide individual variation in vessel density and no clear trend or difference over the menstrual cycle (Moller et al., 2001).

VEGF is a major regulator of endothelial cell proliferation, angiogenesis, vasculogenesis and capillary hyperpermeability (Ferrara and Davis-Smyth, 1997; Ferrara, 1999a,b; Smith, 2001). The presence of VEGF mRNA, protein and its receptors has been demonstrated in the human and primate endometrium throughout the menstrual cycle (Torry et al., 1996; Ferrara and Davis-Smyth, 1997; Meduri et al., 2000; Nayak et al., 2000; Moller et al., 2002; Nayak and Brenner, 2002; Sugino et al., 2002), and proliferative endometrium demonstrates prominent glandular immunoreactivity and faint, inconsistent staining in stromal cells, similar to observations in this study (Sugino et al., 2002). Mifepristone abolished VEGF expression in the endometrial glandular epithelium of cynomolgous monkeys (Grub et al., 1997), and this might represent a mechanism for the suppression of angiogenesis and severe endometrial atrophy observed following mifepristone treatment in the primate model. In this study, VEGF immunoexpression was significantly reduced in the stroma following mifepristone treatment. There was a non-significant reduction in glandular VEGF immunoexpression, whereas microvessel density was significantly increased following treatment. It has been suggested that VEGF may not be the primary regulator of endothelial cell proliferation in the human endometrium (Garrett et al., 1999). Besides VEGF, other factors such as angiopoietin and fibroblast growth factor also regulate endometrial angiogenesis (Smith, 2001). VEGF regulates vascular permeability, and the decrease in stromal VEGF may explain the increase in stromal density following treatment with mifepristone (Ferrara, 1999a,b).

The most striking observation in this study was the change in the expression of GR protein and its location. In the normal menstrual cycle, GR is located only in the nuclei of endometrial stromal and endovascular cells and is absent in the glands (Bumberger et al., 2001). In this study, the pretreatment proliferative phase samples showed a strong nuclear receptor expression in the
stroma and a complete absence in the glands and surface epithelium. Following treatment with low-dose mifepristone, there was a significant induction of nuclear GR protein expression in both glands and surface epithelium. Although the presence of GR has been reported in the luminal (surface) epithelium of rat uterus (Korgun et al., 2003), this is the first study to report the presence of nuclear GRs in endometrial gland cells. Mifepristone binds strongly to GR and progesterone receptor but more weakly to AR (Spitz and Bardin, 1993), and we have previously reported that chronic treatment with low-dose mifepristone up-regulates AR and down-regulates progesterone receptor in the endometrium (Narvekar et al., 2004). The underlying cellular mechanisms are poorly understood, but the striking tempor-spatial up-regulation of AR and GR in the glands and surface epithelium suggests that they share common mechanistic pathways. However, it is unlikely that the changes described in this study are the result of a change in the levels of cortisol because this dose of mifepristone has no demonstrable effect on the pituitary–adrenal axis (Brown et al., 2002).

The exact physiological role of GR and glucocorticoids in the human endometrium is not clear. The expression pattern points to a functional role in the complex process of decidualization (Bamberger et al., 2001). Several effects of glucocorticoids on endometrial cells have been reported. Uterine events such as menstruation, implantation, cervical softening and parturition share similarity with non-reproductive inflammatory situations (Kelly, 1996). At high concentrations, glucocorticoids inhibit most immunological responses and are well-known anti-inflammatory agents. PGs have a pivotal role in menstruation and endometrial bleeding (Baird et al., 1996), and glucocorticoids have been shown to suppress PGF2 alpha production (Schatz et al., 1986; Neulen et al., 1989; Delvin et al., 1990; Illouz et al., 2000) and phospholipase A2 (Pakrasi et al., 1983), one of the enzymes considered to be rate-limiting in generating free arachidonic acid for PG synthesis. An increase in the local concentration of PGs in the endometrium has been postulated in the mechanism of bleeding observed following administration of mifepristone in the mid-luteal phase (Hapangama et al., 2002). The expression of GR in endothelial cells suggests a role for glucocorticoids in modulation of angiogenesis in the endometrium as has been reported in the rat aorta (Small et al., 2005). Endogenous and exogenous glucocorticoids exert tonic inhibition, whereas treatment with a GR antagonist enhances angiogenesis in the mouse model (Small et al., 2005). An increase in GR expression could potentiate the effects of circulating glucocorticoids on the endometrium, thereby suppressing local PG concentrations and inhibiting angiogenesis. Contrary to theoretical expectations, we have demonstrated a small increase in microvessel density. This increase may represent a counting artefact in endometrial sections following stromal compaction and gland dilatation. A better understanding of the role of glucocorticoids and androgens and their receptors in the endometrium is needed to reconcile the different endometrial effects into a working hypothesis. Glucocorticoids may also modulate human fertility. Elevated levels of glucocorticoids disrupt normal uterine development and implantation (Campbell, 1978; Monheit and Resnik, 1981; Bigsby, 1993; Hicks et al., 1994). Mechanisms by which glucocorticoids may influence implantation include their known effects on actin polymerization, lysosomal activity, PG synthesis, PGF nitric oxide synthase and matrix metalloproteinases (Salamonsen, 1996), all of which have known roles in implantation. Excess glucocorticoid exposure may disturb the normal pattern of growth and differentiation of the primate fetus (Arcuri et al., 1997). On the other hand, cortisol may modulate local immunsuppressive activity within the decidua by inhibiting the production of anti-inflammatory cytokines, such as interleukin-1 (Snyder and Unanue, 1982), thus protecting the developing blastocyst from maternal immune rejection (Ricketts et al., 1998). Although low-dose mifepristone acts as a contraceptive by inhibiting ovulation in up to 90% subjects (Brown et al., 2002), an increase in surface (luminal) and glandular GR expression may play a role in the endometrial anti-fertility effects of mifepristone.

Summary

The most striking observation in this study was the change in the expression of GR protein and its location. Glucocorticoids and GR modulate angiogenesis. We have demonstrated an increase in endometrial vessel density and VEGF. The high incidence of amenorrhoea in women taking mifepristone may be related to the regulation of vascular function.

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References


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Original research article

Changes in vaginal morphology, steroid receptor and natural antimicrobial content following treatment with low-dose mifepristone

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Methods: In a pilot study, eight women were given mifepristone 5 mg/day for an average of 33 days. Ovarian function was assessed by measurement of estradiol and progesterone in blood and their metabolites in urine and by serial ultrasound of their ovaries. Vaginal biopsies were collected before (late proliferative) and after taking mifepristone.

Results: All subjects showed a similar pattern of descending serum concentrations of mifepristone. The elimination phase half-life was 18±5.1 h (mean±SD). Mean C<sub>max</sub> measured at 1 h was 641.7 nmol/L (range, 502–740 nmol/L). All eight women reported amenorrhea for the duration of treatment and seven of eight women showed biochemical and ultrasound evidence of anovulation. There was no significant change in vaginal thickness following treatment [342±40 μm pretreatment, 303±69 μm posttreatment (mean±SEM); p>0.05]. Estrogen (ER<sub>a</sub>, ER<sub>β</sub>) and androgen receptor were expressed in both vaginal epithelium and subepithelial stroma, whereas progesterone receptor was expressed predominantly in the subepithelial stroma. There was no change in receptor content and distribution following mifepristone treatment. Natural antimicrobial mRNA [secretory leukocyte protease inhibitor, human beta defensins mRNA (HBD1, HBD2, HBD3, HBD5), granulysin and elafin] was extracted from the vaginal tissues, and the content was unaffected by mifepristone treatment.

Conclusion: The absence of changes in vaginal thickness, steroid receptor and natural antimicrobial content and its distribution in this preliminary study suggests that in contrast to other estrogen-free contraceptives, mifepristone is unlikely to be associated with the increased risk of transmission of HIV and other sexually transmitted infections.

Keywords: Mifepristone; Vaginal thickness; Steroid receptors; Natural antimicrobial; Pharmacodynamics

1. Introduction

The state of the vaginal microenvironment affects a woman’s risk of human immunodeficiency virus (HIV) transmission. Several human and nonhuman primate studies have shown that long-acting gestagen treatment increases the transmission of HIV [1–3], simian immunodeficiency virus (SIV) [4] and other sexually transmitted infections (STIs) [5,6]. The underlying mechanisms are poorly understood. Experiments on hysterectomized rhesus monkeys suggest that the vagina, rather than the cervix or the uterus, is the main portal of viral entry [7,8].

Epithelial thickness and integrity modulate the ease of access of virus to immune cells and subepithelial vasculature [9]. Estrogen-induced surface keratinization...
and hyperplasia protect rhesus monkeys against SIV inoculation [10,11], whereas estrogen-deficient women such as those who are postmenopausal [12,13] or on long-acting gestagens are at increased risk of HIV, presumably as a result of vaginal thinning. Other biological variables such as vaginal microflora [14-16], immune cell populations [17,18] and natural antimicrobials [19-21] also play an important role in innate defenses of the reproductive tract.

Mifepristone, a potent antagonist of progesterone (P), has the potential to be developed for contraception and other gynecological uses [22-24]. Daily low-dose treatment inhibits ovulation but maintains follicular development, thus exposing reproductive tissues to unopposed estrogen. Since gestagen treatment increases and estrogen treatment decreases HIV/SIV transmission, the aim of the present study was to investigate whether the antigestagen mifepristone modulates underlying mechanisms involved in transmission. We investigated vaginal morphology, steroid receptor and natural antimicrobial [secretory leukocyte protease inhibitor (SLPI), human beta defensins mRNA (HBD1, HBD2, HBD3, HBD5), granulysin and elafin] content. We also report the pharmacokinetics and pharmacodynamics of 5 mg mifepristone supplied by Hualian Pharmaceuticals Co. Ltd. (Shanghai, China), which has been used in the present study as well as in previous studies [23].

2. Subjects and methods

We report two pilot studies using 5 mg mifepristone — the study on vaginal epithelium that was carried out in Edinburgh and the pharmacokinetic study in Helsinki.

2.1. Effects of mifepristone on vagina

A single-center, open, single-group study concerning female volunteers was undertaken. Eight healthy subjects with a mean age of 35 years (range, 27-39 years) and a mean body mass index (BMI) of 23 kg/m² (range, 17.3-28.2 kg/m²) who had regular menstrual cycles (25-42 days) were recruited to the study. The women agreed to refrain from the use of vaginal medications during the study period or from sexual intercourse 48 h prior to vaginal biopsy. Women who had breast-fed or had taken hormonal contraception less than 3 months prior to the study and those with vaginal or pelvic infections (current or past) were excluded. The proposal was approved by the local ethics committee (institutional review board). All women gave written informed consent before enrolment and were screened before entering the study. Screening included a full medical, gynecological history and examination, including measurement of height, weight, blood pressure and pulse. Blood samples were collected for measurement of routine clinical chemistry and hematology (liver function tests, urea and electrolytes, glucose, full blood count). β-HCG was measured to exclude pregnant women from the study. Subjects were studied for one pretreatment cycle, one cycle of treatment (approximately 33 days) and one posttreatment cycle. Each subject was reviewed on Day 12 of the pretreatment menstrual cycle (Visit 1), at the end of treatment (Visit 2) and on Day 12 of the posttreatment menstrual cycle (Visit 3). Subjects were given a menstrual record card and were asked to record all vaginal bleeding.

2.2. Assessment of ovarian function

Ovarian function was monitored by measurement of ovarian steroids in urine and plasma and by transvaginal sonography. All subjects collected twice weekly samples of early morning urine during the study period, starting in the early follicular phase (Days 1-5) of the pretreatment cycle. Aliquots were frozen and stored at -20°C until assayed for estrone glucuronide (E1G), pregnanediol glucuronide (PdG) and creatinine (Cr). PdG was measured using a direct enzyme immunoassay, while E1G was measured by direct immunoassay. Ovarian follicular activity during treatment was compared with that during the follicular phase of the pretreatment cycle, and the activity was scored as complete suppression, partial suppression or continued follicular activity. Ovation was deemed to have occurred if the excretion of PdG exceeded 0.5 mmol/mol Cr and was at least threefold higher than that in the preceding week. A detailed description of this methodology is given in our previous report [23]. Blood samples were collected at all study visits and assayed for estradiol (E2) and P using radioimmunoassay (RIA). Assay characteristics and methodology have been described in our previous reports [23]. A transvaginal ultrasound scan was carried out at all study visits, and ovarian dimensions, follicle number and diameter and presence of ovarian cysts were recorded.

2.3. Vaginal biopsy

A full-thickness vaginal biopsy was taken from the lateral vaginal wall 4 cm proximal to the hymeneal ring on Day 12 of the pretreatment cycle (Visit 1) and, again, after completion of treatment (Visit 2). One ampoule of Citanest with octapressin (3%; prilocaine hydrochloride, 30 mg/mL; felypressin, 0.03 U/mL; Dentasy) was injected into the lateral vaginal wall as a local anesthetic and hemostatic agent. This also elevated the target vaginal tissue sufficiently to permit easy access for a biopsy. Vaginal biopsy was performed using a long Schumacher forceps. Vaginal tissues were stored in RNAlater (Applied Ltd., Cambridgeshire, UK; RNA later is an aqueous storage reagent to stabilize and protect RNA) and neutral-buffered formalin (NBF; for future preparation of paraffin-embedded tissue for immunohistochemistry). A second biopsy was taken if adequate sample was not obtained from the first biopsy. Vaginal bleeding from the biopsy site was controlled using either silver nitrate or, if necessary, a Vicryl 3-0 suture (Ethicon, UK), depending on the amount of bleeding.

An endometrial biopsy was collected using Pipelle endometrial sampler (Prodimed, Neuilly-en-Thelle, France) at end of treatment (Visit 2), fixed in NBF, and embedded in paraffin. Endocervical and posterior fornix swabs were
collected at all study visits and cultured for pathogenic organisms, for example, gonococcus, trichomons and streptococcus, which might influence the parameters studied in target vaginal tissues.

2.4. Safety parameters

At each study visit, blood pressure and pulse were measured, and blood was taken for measurement of routine clinical chemistry and hematology. In addition, each subject was asked to report any health problems or adverse events that had occurred since the last visit.

2.5. Vaginal thickness measurement

Vaginal tissue samples were embedded in paraffin, and serial 5-μm sections were cut at a 90° angle to the vaginal surface epithelial layers. The sections were stained with hematoxylin and eosin, and digital images were captured at x10 eyepiece magnification using a Spot microscope connected to a Windows PC computer. Image Proplus 4.5 (Media Cybernetics, Silver Spring, CO, USA) software was calibrated to match the eyepiece used to capture the image. The surface and basement membranes of vaginal epithelium were outlined using a trace tool within the software (Fig. 1). The software automatically calculated the average distance between the two traced outlines.

2.6. Immunohistochemical (IHC) localization of estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), progesterone receptor (PR), and androgen receptor (AR) and proliferation marker phospho-histone H3 (PH3)

Immunohistochemistry was carried out on both the vaginal and endometrial biopsies for the following proteins of interest: ERα (Novocastra, Newcastle-upon-Tyne, UK), ERβ (Serotec, Oxford, UK), PR (A+B) (Novocastra), AR (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), PH3 (Upstate Biotechnology, Poole, UK) and SLPI (Hycult Biotechnology, Cambridge, UK). All antibodies used were mouse monoclonal, except for PH3, which was a rabbit polyclonal. They were tested individually at a range of dilutions and at different antigen retrieval conditions to determine the protocol that gave the least background and

**Table 1** Immunohistochemistry protocol

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tissue</th>
<th>IHC method</th>
<th>Antigen retrieval</th>
<th>Primary antibody</th>
<th>Negative control</th>
<th>Detection system</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>Vagina</td>
<td>Bond-X machine and ABC detection</td>
<td>Pressure cook: 0.01 M sodium citrate, pH 6, 5 min</td>
<td>1:100 mouse anti-ERα</td>
<td>MlgG1 1:1300</td>
<td>Biotinylated secondary and ABC detection (Vision Biosystems)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 h at room temperature</td>
<td>1:1000 mouse anti-ERα</td>
<td>MlgG1 1:13,000</td>
<td>Serum*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 h at room temperature</td>
<td>1:40 mouse anti-ERβ</td>
<td>Goat antimouse and ABC-streptavidin (both from DAKO, Cambridgeshire, UK)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Overnight at 4°C</td>
<td>Rabbit anti-H3</td>
<td>Goat anti-histone H3 (BioGenex)</td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>Endometrium</td>
<td>Goat antimouse antibody and ABC-streptavidin</td>
<td>Microwave: 0.01 M sodium citrate, pH 6, 10 min</td>
<td>1:80 mouse anti-PR</td>
<td>MlgG1 1:2000</td>
<td>Goat antimouse antibody and ABC-streptavidin (both from DAKO, Cambridgeshire, UK)</td>
</tr>
<tr>
<td>AR</td>
<td>Endometrium</td>
<td>Goat antimouse antibody and ABC-Elite</td>
<td>Pressure cook: 0.01 M sodium citrate, pH 6, 5 min</td>
<td>1:400 rabbit anti-AR</td>
<td>RlgG1 1:2000</td>
<td>Goat anti-histone H3 (BioGenex)</td>
</tr>
<tr>
<td>H3</td>
<td>Endometrium</td>
<td>Goat antimouse antibody and ABC-Elite</td>
<td>Pressure cook: 0.01 M sodium citrate, pH 6, 5 min</td>
<td>1:1000 rabbit anti-H3</td>
<td>RlgG1 1:1000</td>
<td>Goat anti-histone H3 (BioGenex)</td>
</tr>
<tr>
<td>SLPI</td>
<td>Endometrium</td>
<td>Biotinylated secondary antibody and ABC-Elite</td>
<td>Microwave: 0.01 M sodium citrate, pH 6, 10 min</td>
<td>1:50 mouse anti-SLPI</td>
<td>MlgG1 1:500</td>
<td>Biotinylated horse antimouse antibody and ABC-Elite (both from Vector Laboratories)</td>
</tr>
</tbody>
</table>

* The anti-ERβ antibody has been previously preabsorbed with the peptide to which it had been raised [25].
highest specific staining (Table 1). Positive and negative controls were included in every run. In most cases, negative controls were performed by adding a matched IgG control antibody (mouse IgG, Sigma, Poole, Dorset, UK; rabbit IgG, Vector Laboratories, Peterborough, UK) of the same species and at the same antibody concentration as the primary antibody. Protocols were carried out either on the bench or with the use of a Bond-X automated immunohistochemistry staining machine (Vision Biosystems, Newcastle, UK).

All tissue sections were initially prepared in a similar manner. Five-micron paraffin-embedded tissue sections were dewaxed in Histoclear (National Diagnostics, Hesle, UK) and rehydrated in descending grades of alcohol to distilled water (dH2O). Antigen retrieval was then carried out by heating the sections either in a microwave oven (setting: high) or in a Tefal Clipso pressure cooker (Setting 2/high, Tefal, Nottingham, UK). The buffer concentration and duration of antigen retrieval varied depending on protocol (Table 1). Sections were left to cool in both cases for 20 min. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide (BDH, Poole, UK) in methanol for 30 min at room temperature. For AR only, sections were incubated in avidin for 15 min at room temperature (Vector Laboratories), followed by incubation in biotin (Vector Laboratories), also for 15 min at room temperature. Nonspecific binding of the primary antibody was blocked by incubating the sections for 20 min at room temperature in a 1:5 dilution of nonimmune serum (Autogen Bioclear, Holly Ditch Farm, Wilt, UK) in phosphate-buffered saline containing 5% bovine serum albumin. All immunostaining methods employed a detection system dependent on visualization of the reaction using a horseradish peroxidase enzyme and the chromagen 3,3-diaminobenzidine (DAB). After the DAB step, the sections were counterstained in hematoxylin before dehydrating them in ascending grades of alcohol and mounting them from xylene with Pertex (Cellpath plc., Hemel Hempstead, UK). Full details of each individual protocol are given in Table 1.

2.7. IHC analysis

We used a descriptive methodology as there is no quantitative or semiquantitative methodology established for analysis of vaginal tissue samples in our laboratory. The intensity and distribution of immunostaining for ERα, ERβ, PR, AR, PH3 and SLPI are described for vagina (epithelium and stroma) and endometrium (glands, stroma and surface epithelium), and differences between pre- and posttreatment samples were analyzed.

2.8. RNA extraction

Tissue was minced using a standard sterile surgical scalpel blade and immersed in 2 mL of Tris-buffered saline (Sigma-Aldrich, St. Louis, MO, USA). The mixture was homogenized for 60 s and incubated overnight at 4°C. The following day, tissue sample was warmed to room temperature and 200 μg of bromochloropropane was added. The mixture was centrifuged at 14,000 rpm for 15 min, and aqueous-phase RNA (supernatant) was transferred to a fresh tube. Five hundred microliters of isopropanol was added and incubated at 4°C for 60 min. The mixture was centrifuged for 10 min; the supernatant was discarded, and the pellet was washed with 1 mL of 70% ethanol. The mixture was centrifuged for 5 min, and the supernatant was discarded, allowing the pellet to dry for 5 min. The pellet was resuspended in 20 μL of RNA solution. To standardize measurements between the various biopsy specimens, we assessed the same amount of RNA in each sample. The amount of specific amplicon is related to ribosomal 18S, which is constant relative to the amount of cDNA present and, subsequently, to an experimental internal control. The RNA was reverse transcribed (TagMan Reverse Transcription Reagents Kit, Applied Biosystems, Foster City, USA) and polymerase chain reaction (PCR) amplified (Tag-Man Universal Master Mix, No Amp Erase UNG, Applied Biosystems) according to the manufacturer’s instructions. PCR amplification of cDNA was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Specific forward and reverse primers (300 nmol/L) and probe (200 nmol/L, all synthesized by BioSource UK, Nivelles, Belgium) for the natural antibiotic were also added. Ribosomal 18S cDNA was measured using TagMan Ribosomal RNA Control Reagents (VIC dye, Applied Biosystems) in each sample as an internal control following the manufacturer’s protocol. Samples were measured in triplicate, and no template controls were included in all runs. Primers and probes for quantitative PCR were designed using the PRIMER EXPRESS program (Applied Biosystems;

Table 2

<table>
<thead>
<tr>
<th>Sequences of quantitative PCR primers and probes for natural antimicrobials</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLPI</td>
<td>GCCATCAAATGCTGAGCATCTT</td>
<td>GCCATCCAAATTTGGCCATAGTTC</td>
<td>TGACACCCCAAAACAAACAAGAGG</td>
</tr>
<tr>
<td>HBD1</td>
<td>TCGCCGCTGGGAGGCAATG</td>
<td>CCTCTGATACAGCTGCTTGAAT</td>
<td>CTTACTTCTGCTGGCATTTCTTACCAA</td>
</tr>
<tr>
<td>HBD2</td>
<td>CTGATGCTTCTCTCGAGTGTGTTT</td>
<td>CCTGAGTCAATATTGCCCTACCTCTC</td>
<td>AAGGCGGATACAGAAGTGGCCCTAACCCAA</td>
</tr>
<tr>
<td>HBD3</td>
<td>CGAGGCGGCGGCAGTGT</td>
<td>CGAGCAGCTTGGCCATGTTT</td>
<td>CGTGCGTCAGCTGGCTCCAAAGA</td>
</tr>
<tr>
<td>HBD4</td>
<td>GGCATGCTAGATACACATACTTT</td>
<td>TGGCTGACTTATTGCCTTTTCTTCTT</td>
<td>GTCCAAATTGCTCATTCAGTCCACTGGA</td>
</tr>
<tr>
<td>HBD5</td>
<td>ACCTCAAGGTTCAGAGCAAGAAG</td>
<td>AGAGGGCAGCTGAGGCGTGCA</td>
<td>CTGGATTGCAGTGCTCCCATCACCCA</td>
</tr>
<tr>
<td>Granuloma</td>
<td>CAGGCTGTTGAAAGGAGCATCTCA</td>
<td>GAGGCGTACGCCTGCAAGGA</td>
<td>CGGTCGTCGCCCACATGCC</td>
</tr>
<tr>
<td>Elafin</td>
<td>TGGCTTGGCTGGCTCATCT</td>
<td>CAGATCTTTGAAAGGAGGCGTGGTAT</td>
<td>ATCCCGCGGCGGCIAGTCCAGCTCC</td>
</tr>
</tbody>
</table>

All probes were labeled with 5' FAM and 3' TAMRA fluorophores.
2.9. Pharmacokinetic study

A pharmacokinetic study of 5 mg of mifepristone was carried out among the same cohort of women who had previously participated in another pharmacokinetic study [32]. The study was approved by the Institutional Review Board of the Helsinki Central Hospital and the Finnish National Agency for Medicines; all subjects signed an informed consent document. Subject characteristics, methodology, collection of sample, analysis of serum levels of mifepristone and calculation of pharmacokinetic parameters were as described previously [32]. In brief, six healthy women, with regular menstrual cycles (23–36 days), a mean age of 32 years (range, 21–45) and a BMI that ranges from 19 to 26 kg/m², volunteered for the study. The 10-mg mifepristone tablets supplied by Hualian Pharmaceuticals Co. Ltd. were halved, and a dose of 5 mg, po, was ingested on Day 10 or 11 of the menstrual cycle. Blood samples were collected at 0, 1, 2, 4 and 8 h and, thereafter, daily for the next 6 days and on Day 10 following mifepristone ingestion. Mifepristone was measured in serum by RIA after extraction with n-hexane:ethyl acetate and separation by column chromatography using Chromosorb® [33]. The detection limit is 0.36 nmol/L, and the intra-assay and interassay coefficients of variation were 8.4% and between 10.3% and 13.6%, respectively.

2.10. Statistical analysis

Statistical analysis was performed using SPSS (SPSS, Inc., Chicago, IL, USA) and Excel 2002 (Microsoft Corporation, Reading, UK). Sex steroid, vaginal thickness and pharmacokinetic data are expressed as mean with either standard error of the mean or standard deviation. Menstrual cycle data are expressed as mean and range. Nonparametric tests (Friedman's test, Wilcoxon Signed Rank Test and Mann-Whitney test) were used to compare sex steroid level, menstrual data, vaginal thickness and natural antimicrobial RNA content before and after treatment.

3. Results

3.1. Effects of mifepristone on vagina

All eight subjects completed the study. The subjects took mifepristone 5 mg/day, po, for an average of 33 days (range, 28–40).

The average length of the control menstrual cycle was 27 days (range, 24–29), while that of the control menstrual period was 5.7 days (range, 4–9). All eight women reported amenorrhea during ingestion of mifepristone. The time from discontinuing the mifepristone treatment to the next bleeding episode was 17 days (range, 10–23); thus, the length of the mifepristone cycle was 50 days (range, 38–63). Average length of the bleeding episode after discontinuation of mifepristone was 5.1 days (range, 4–7).

During the treatment with mifepristone, seven of the eight subjects experienced either complete suppression of ovarian activity (3/8 women, Fig. 2A, Subject 8) or persistent follicular activity but no ovulation (4/8 subjects, Fig. 2B, Subject 4). In the remaining subject (Subject 7, Fig. 2C), there was a threefold rise in the excretion of pregnanediol in the first 10 days of treatment, suggesting the formation of a corpus luteum. However, there was no menstrual bleeding when the level of pregnanediol dropped 14 days after starting the mifepristone. In this subject, a persistent ovarian cyst with a 42-mm diameter was detected at completion of treatment (Day 40) when the level of P (14 nmol/L) was slightly raised, which was consistent with a persistent unruptured partially luteinized follicle.
Multiple follicles were detected by transvaginal ultrasound (diameter of the largest ranged from 10 to 29 mm) in all eight subjects. The concentrations of E2 in blood samples collected at Visit 2 on the last day of mifepristone treatment were compatible with persistent follicular activity (496±57 pmol/L). In seven of eight women, the concentration of P was <10 nmol/L (3±1 nmol/L), indicating lack of ovulation.
3.2. Endometrial histology

Endometrial biopsies obtained at the end of mifepristone treatment displayed inactive or weakly proliferative endometrium in seven of the eight subjects. In one subject (Subject 9), tortuous glands with evidence of intraluminal secretion were seen. As expected, there was strong immunostaining of ERα and ERβ as well as of PR (A+B) and AR in both glands and stroma. Histological evidence of mitosis was absent or infrequent in all samples.

3.3. Vaginal histology and vaginal thickness

Vaginal biopsy was obtained in all subjects without complications both before and after mifepristone administration. In seven of eight subjects the pretreatment vaginal biopsy was performed between Day 6 and Day 16 of the follicular phase prior to ovulation as confirmed by the hormone levels (mean±SEM: E2, 659±141 pmol/L, P, 4.3±10 nmol/L). In the remaining subject (Subject 5), ovulation had already occurred on the day of biopsy (Day 14 of menstrual cycle) as indicated by high circulating concentration of P (44 nmol/L). Posttreatment vaginal biopsy was performed on Day 33 (range, 28-40) of treatment.

The histology of the vagina showed the expected basal layer of epithelium mounted up to 20 layers of more superficial desquamating cells (Fig. 1). Vaginal thickness was not altered during administration of mifepristone (342±40 μm vs. 303±69 μm; p=.2, NS).

3.4. Steroid receptor expression in the vagina (ERα, ERβ, AR and PR)

There was nuclear staining of ERα, ERβ and AR in both vaginal stroma and in the epithelium. Immunoreactivity extended through the basal and intermediate layers but not through the superficial layer of vaginal epithelium (Fig. 3). Staining was far more evident in the epithelium as compared with the stroma. There was no significant difference in sex steroid receptor immunostaining after mifepristone administration.

Apart from a few scattered nuclei in the stroma, the basal layer of vaginal epithelium immunostaining for PR was confined to stroma (Fig. 3). There was no significant difference in PR immunoreactivity after administration of mifepristone.

3.5. PH3 immunoreactivity

PH3 immunostaining was confined to scattered nuclei in the basal layers of the vaginal epithelium (Fig. 3). There was no significant change in immunoexpression following treatment with mifepristone.

3.6. Natural antimicrobial mRNA and protein expression

SLPI mRNA was present in vagina, and this epitope was localized to the superficial layer of the vaginal epithelium both before and after mifepristone administration (Fig. 3). SLPI immunostaining was also demonstrated in the superficial layers of luminal and glandular epithelium of the endometrium (Fig. 3). SLPI mRNA expression was unchanged following treatment with mifepristone (p>.05, Wilcoxon test). HBD1, HBD2, HBD3, HBD5, granulysin and elafin mRNA were present in the vagina. The expression was unchanged following treatment with mifepristone (p>.05, Wilcoxon test).

3.7. Safety parameters

There was no derangement in heart rate, blood pressure or in hematology and biochemistry parameters including liver function tests during the study. Vaginal and cervical bacteriology swab tests cultured negative for pathogenic organisms in all subjects. Vaginal biopsy was well tolerated by all women. One woman had possible low-grade endometritis following endometrial biopsy and was successfully treated with a 7-day course of antibiotics.

3.8. Pharmacokinetics of 5 mg mifepristone

Serum levels (mean±SD) of mifepristone following ingestion of 5 mg mifepristone are summarized in Fig. 4. Mean C_{max} measured at 1 h was 641.7 nmol/L (range, 502-740 nmol/L). All subjects showed a similar pattern of descending serum concentrations of mifepristone. The elimination phase half-life was 18±5.1 h (mean±SD). The mean (SD) areas-under-concentration-curves \text{AUC}_{0-24} \text{h} and \text{AUC}_{0-24} \text{h} were 2.4 (0.5) and 4.8 (1.3) μmol/L, respectively.

4. Discussion

The vagina is a key portal of entry for HIV and other STIs. In this article, we report the effect of a potential new contraceptive pill on different parameters involved in the

Fig. 4. Serum levels (mean±SD) of mifepristone following ingestion of 5 mg. The data are depicted on both linear (lower) and semilogarithmic (insert) scales.
natural defenses of the vagina to infection. Vaginal epithelial thickness, steroid receptor and natural antimicrobial content and distribution were unchanged following treatment with mifepristone for 30–40 days. Vaginal epithelial thickness is regulated by the levels of circulating estrogen. Epithelial thickness is maximal at time of ovulation [34–37] and decreases in the luteal phase and postmenopause [36]. There is a significant reduction in circulating estrogen levels following long-term gestagen treatment [37]. Severe vaginal atrophy has been demonstrated in primate studies, and this clearly increases the risk of HIV transmission [4]. However, the response of human vaginal epithelium to gestagen-induced hypoestrogenism is variable. A small but significant decrease (10%) in thickness has been demonstrated in one study [37], whereas most other studies have demonstrated no change [38,39]. Paradoxically, vaginal epithelial hyperplasia has been reported in users of depot medroxyprogesterone acetate (DMPA), oral contraceptive pill and P implants [39–41]. E2 levels in all three treatment groups were significantly lower compared with normal menstruating women. The steroid receptor content and distribution were similar except for PR, which was suppressed in the DMPA group.

It is difficult to reconcile all available data into a working hypothesis due to differences in study designs and methodology. The vagina has diverse embryological origins [42]; lidgren et al. used a cross-sectional study design and sampled lateral vaginal fornices, whereas we used a longitudinal study design and sampled lateral midvaginal wall in keeping with other reports [41].

Although vaginal epithelium clearly responds to circulating estrogen, the underlying cellular and molecular mechanisms are poorly understood. We localized PH3, a marker for mitosis and cellular proliferation, to a few scattered nuclei in the basal layers of the vaginal epithelium. The strong nuclear expression of ER and AR in the basal and parabasal layers suggests a role in the regulation of epithelial proliferation. Estrogen treatment induces surface keratinization and hyperplasia of primate vaginal epithelium, and we expected a similar change in mifepristone-treated vaginal samples due to unopposed estrogen effect. The observed epithelial thickness in control samples in the present study (mean pretreatment thickness, 342 µm) is comparable to other reports [38–40]. Thickness was unchanged following mifepristone treatment, and this agrees with similar work in cynomolgus monkeys [43]. There are several possible explanations for our findings. Firstly, mifepristone treatment did not have any effect on level of circulating steroid hormones. Secondly, PR was localized to a few scattered nuclei in the basal layers of vaginal epithelium and, hence, mifepristone, a high-affinity PR ligand, did not have any direct epithelial effects.

Consistent with previous reports, we have demonstrated a strong immunoreexpression of ER and AR as well as a relative lack of PR in the vaginal epithelium compared to subepithelial stroma [44–51]. The distribution of steroid receptors has obvious implications for the development of topical and parenteral steroid treatment. Available data, including the present study, indicate that it is likely that estrogen preparations will act via epithelial ER, whereas P preparations are likely to have an endocrine and paracrine effect after binding with the subepithelial PR. The role of AR in the genital tract is unclear, but AR may play a role in regulating endometrial proliferation [52–54] and in modulating vaginal blood flow [50] and female genital sexual arousal [49].

The endometrium sampled at the end of mifepristone treatment showed persistent proliferative histology and strong ER, PR and AR expression, which is consistent with our previous reports [54,55] and which demonstrates that the mifepristone preparation used in this study is comparable to that we have used previously from another source (Exelgyn, Paris).

The expression, regulation and role of natural antimicrobial compounds in the female reproductive tract are extensively reviewed elsewhere [29]. We have investigated natural antimicrobials that regulate innate protection at mucosal interfaces. SLPI has been shown to play an important role in limiting transmission of HIV [20,56,57] and other lower genital tract infection [19]. SLPI mRNA has been demonstrated in vaginal fluid previously [19,20]. In the present article, we demonstrate mRNA in vaginal tissue and immunolocalize the protein to the superficial layers of the vaginal epithelium. The expression in superficial layers of surface and glandular endometrium is in keeping with previous reports [26]. HBD1 [58] and elafin [59] mRNA have been reported in human vagina. We show that HBD2, HBD3, HBD5 and granulysin mRNA are present in vaginal tissue. HBD4 mRNA was not previously demonstrated. Natural antimicrobial expression is modulated by hormonal treatment, and up-regulation of endometrial SLPI by P is attenuated in the presence of mifepristone [31]. Reassuringly, expression and distribution of natural antimicrobials were unchanged following mifepristone treatment.

Low-dose mifepristone suppressed menstruation and ovulation in a majority of women in the present study. This adds to similar observations in our previous reports [23,60] and also demonstrates the biological activity of mifepristone supplied by Hualian Pharmaceuticals Co. Ltd. The pharmacokinetic properties of the 5-mg dose from the above supplier follow a first-order linear kinetic pattern [32,61]. The half-life of 18 h is shorter than the 20 h previously reported for 10 mg mifepristone, whereas the AUC0–24 h was half that of the 10-mg dose [32]. All women received planned treatment for at least 5 days beyond the expected date of menstruation (33 days) so that any posttreatment vaginal bleeding was due to withdrawal of mifepristone rather than to a spontaneous menstruation. Fortuitously, Subject 7 was administered mifepristone for 40 days. She did not bleed, although there was evidence of ovulation and formation of corpus luteum followed by a significant decrease in pregnanediol levels. This supports our previous
reported findings that mifepristone-induced amenorrhea is a result of direct endometrial effects that are possibly mediated by a rise in glandular and luminal glucocorticoid and AR [62].

In summary, we have shown that low-dose mifepristone does not influence vaginal thickness and proliferation. We have demonstrated that ER (ERα and ERβ), PR, AR, SLPI and other natural antimicrobials are present in the human vagina. There is no change in epitope expression following mifepristone treatment. The presence and distribution of vaginal steroid receptors has implications for the development of topical and systemic preparations to modulate this sex steroid responsive organ in health and disease.

Acknowledgments

We are grateful to Ann Kerr, Heather Graham and Susan Morrow in Edinburgh for help in running and coordinating this study; Neil Hollow for endocrine assays; Teresa Henderson for technical assistance; Rob Elton for statistical support; and Meg Anderson for secretarial help. Mifepris- tone was supplied by Hualian Pharmaceuticals Co. Ltd., 370 Jiang Wan Road West, Shanghai, China.

References


Toward developing a once-a-month pill: a double-blind, randomized, controlled trial of the effect of three single doses of mifepristone given at midcycle on the pattern of menstrual bleeding

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aContraceptive Development Network, Centre for Reproductive Biology, Edinburgh, Scotland; bCenter for Research in Reproductive Health, Ogun Remo University Teaching Hospital, Sagamu, Nigeria; cDepartment of Obstetrics and Gynaecology, University of Cape Town, Medical School, Cape Town, South Africa; dDepartment of Obstetrics and Gynaecology, University of Hong Kong, Queen Mary Hospital, Hong Kong, People’s Republic of China; and eShanghai Institute of Family Planning Technical Instruction, International Peace Maternity and Child Health Hospital, China Welfare Institute, Shanghai, People’s Republic of China.

Objective: To test the feasibility of timing the administration of mifepristone as a once-a-month contraceptive pill on the 12th day before the next menses, as calculated from the length of the previous menstrual cycles.

Design: Double-blind, randomized, controlled trial.

Setting: Five family planning centers across the world.

Patient(s): Three hundred ninety-nine women attending family planning clinics.

Intervention(s): Randomized to receive 10, 25, or 200 mg of mifepristone or a placebo.

Main Outcome Measure(s): Lengthening or shortening of the normal menstrual cycle length following administration of the drug by at least 5 days.

Result(s): The menstrual period came within 5 days of the predicted date in 88% of women receiving the placebo, 84% of women receiving 10 mg, 72% of women receiving 25 mg of mifepristone, and only 48% of women treated with 200 mg of mifepristone. Increasing the dose of mifepristone was associated with an increased chance of having a delayed period (P<.001). Only 45% of women were in the peri-ovulatory phase of the cycle according to LH and P measurements on the day of drug administration. Women treated before ovulation were more likely to have delayed menses with all three doses of mifepristone.

Conclusion(s): Because of the disruption in cycle length, it appears unlikely that mifepristone administered once a month, at a calendar-based time, would provide a reliable method of contraception. (Fertil Steril® 2006;86: 819–24. ©2006 by American Society for Reproductive Medicine.)

Key Words: Mifepristone, once-a-month pill, pattern of menstrual bleeding

The anti-P mifepristone, given once per month, is an effective method of contraception (1–3). In 1999, we performed a questionnaire study in Edinburgh, Cape Town, Hong Kong, and Shanghai to examine women’s attitudes toward the concept of a once-a-month pill. We concluded that a once-a-month pill would be a popular form of contraception to many women in different cultures (4).

For successful development as a once-a-month contraceptive pill, the timing of administration of mifepristone is critical. Administered 48 hours after the LH surge, shortly after ovulation, 200 mg of mifepristone does not alter the length of the menstrual cycle and bleeding is not usually induced (5, 6). This dose markedly retards endometrial development to a degree that is likely to prevent implantation (5–8). It also changes uterine contractility to a pattern that is usually observed in the late luteal phase (9), perhaps adding to the contraceptive effect.

However, if it is administered during the follicular phase of the menstrual cycle (i.e., too early), mifepristone inhibits follicular development, delays the midcycle LH surge, and prolongs the length of the cycle (10, 11). Administration of mifepristone in the midluteal to late luteal phase (i.e., too
late) induces bleeding within a few days, and this is often followed by a second bleed at the time of the expected menses (10, 12). Thus, a single dose of mifepristone administered outside the critical time results in irregular bleeding, which many women find unacceptable.

In the two studies that demonstrated the efficacy of the method, the administration of mifepristone was timed accurately by identifying the midcycle peak of LH in the urine. In Sweden, this was determined with urine test sticks (13); in Edinburgh, Scotland, home-use fertility monitors were used (14). Both approaches are expensive and probably too demanding on user compliance to be used routinely. In the Edinburgh study for example, 11.2% of LH surges were missed because of imperfect use of the monitor (15).

We have recently demonstrated that mifepristone given on the day of the LH surge (LH+0), also retards endometrium sufficiently to prevent implantation, without interfering with ovulation (16). This observation widens the potential “window of opportunity” for administering mifepristone as a once-a-month contraceptive to at least 3 days (LH + 0 to LH + 2). It prompted us to design a study to test the feasibility of timing once-a-month mifepristone administration without the assistance of technology. Instead, we used a calendar approach, giving a single dose of mifepristone on day of menses minus 12, which was estimated from the mean of the subject’s previous menstrual cycles. The effect of three different doses of mifepristone (10, 25, or 200 mg) on the length of the menstrual cycle and pattern of menstrual bleeding was compared with the placebo.

The rationale for testing the three doses of mifepristone was as follows. Contraceptive potential of 10 and 200 mg of mifepristone has been reported. Doses <10 mg do not inhibit ovulation (17–19), and although the endometrium is disrupted, the pregnancy rate is disappointingly high (18, 19). A dose of 200 mg is reportedly contraceptive, but if it is given at the wrong time (i.e., too early or too late), it will frequently result in menstrual irregularity (10). A dose of 10 mg of mifepristone is reportedly effective when used for emergency contraception (20), but it has an effect on endometrial development that is less marked than the effect of 200 mg (21). This dose may be less likely to cause cycle disruption but is also less likely to be effective. A dose of 25 mg appeared to be a sensible compromise between the antifertility effects on the endometrium, efficacy, and potential for cycle disruption by mifepristone.

MATERIALS AND METHODS
This was a multicenter, double-blind parallel group study in female volunteers. The intention was to recruit a total of 400 subjects (80 per center) from women 18–40 years old attending family planning clinics in five centers: Edinburgh (Scotland), Sagamu (Nigeria), Cape Town (South Africa), and Hong Kong and Shanghai (People’s Republic of China). The women were required to use barrier methods for the duration of the study or have a copper intrauterine device (IUD) in situ, or were relying on sterilization (subject or partner). Recruitment criteria included a history of regular menstrual cycles of 25–35 days varying within ±3 days in the last 3 months. Women who had a significant medical condition, who were breastfeeding, or had used hormonal contraception within 3 months before the study were excluded. The local ethical committee (institutional review board) approved the proposal in each center. Written informed consent was obtained from all subjects.

A full medical and gynecological history and examination were obtained at recruitment (visit 1). Normal cycle lengths were defined as follows. Cycle length and range was calculated from the dates of the last three menstrual periods for women who kept a written record (retrospective normal cycle length, n = 87). Women who did not provide a record of the dates of menstruation but claimed to have very regular cycles were asked to give an estimate of normal cycle length and a range of variation (reported normal cycle length, n = 199). Women who did not keep a record of their cycles and who were unsure of their normal cycle length kept a prospective record of the next three cycles before the intervention cycle (prospective normal cycle length, n = 105).

The duration of menstrual bleeding was determined in the same manner. For the duration of the study, all episodes of vaginal bleeding were recorded on a menstrual diary card, and spotting and bleeding were differentiated. Women contacted the investigators in the first week of the intervention cycle and attended the clinic 12 days before the anticipated date of next menses, as estimated from their normal cycle length (visit 2). Pregnancy was excluded by a urine pregnancy test, and subjects were randomized to receive 10 mg, 25 mg, or 200 mg of mifepristone or a placebo. A blood sample was collected for the measurement of LH and P. Women were reminded to record any menstrual bleeding or adverse events on the record card. At approximately the expected time of the onset of menses following treatment, the subject was contacted (usually by phone) and asked to return the record card to the clinic (visit 3) after the onset of menses.

The study medication was dispensed as 10, 25, or 200 mg of mifepristone or placebo tablets provided by the Shanghai Hualian Pharmaceutical Company Ltd. (Shanghai, People’s Republic of China). The mifepristone tablets came in three different sizes; therefore, three sizes of placebo were produced to match the size and color of mifepristone tablets. Each subject received a bottle containing one mifepristone (10, 25, or 200 mg) plus two different-sized placebo tablets. Women in the placebo group received all three placebo tablets. This was a double-blind study. Randomization at visit 2 was performed by blocked computer-generated randomization for each center to ensure a good balance of numbers in the different treatment groups. Sealed envelopes were used to conceal the treatment allocation until the women were registered as definite participants.
Blood samples collected by venepuncture on day of drug administration (visit 2) were centrifuged and the serum was stored at −20°C until analysis. Standard radioimmunoassay techniques were used in each center for the measurement of LH and P. On the basis of the laboratory normal values, the samples were classified as originating from three stages of the ovarian cycle: follicular LH <10 IU/L and P <5 nmol/L; luteal LH <10 IU/L and P >20 nmol/L; peri-ovulatory LH >10 IU/L and/or P 5–20 nmol/L.

Statistical Analysis
The main aim of the study was to investigate the effect of mifepristone on the time interval between the day of administration of the drug and the start of the next menses (i.e., on the length of the menstrual cycle).

A menstrual period was defined as ≥2 days of blood loss (i.e., bleeding or spotting), with at ≥1 day of bleeding, and bounded at each end by ≥2 bleeding- and spotting-free days (22) Isolated days of bleeding, or runs of spotting without bleeding, were excluded. If there was ≤1 day separating the two sets of blood loss, this was counted as a single menstrual period.

The length of the intervention cycle was compared with that of the normal cycle length (as defined earlier) and to the cycle length of women treated with the placebo. The primary outcome for this study was a shortening of ≥5 days in the normal menstrual cycle length following administration of the drug. A cutoff of 5 days was considered clinically significant because this degree of disruption would likely result in a woman seeking advice (particularly if her period was delayed) and would be sufficient to jeopardize the contraceptive effectiveness of the regimen.

The proportion of cycles significantly disrupted was compared between adjacent randomized groups (i.e., placebo vs. 10 mg, 10 vs. 25 mg, and 25 vs. 200 mg) by χ² tests, with Yates’ correction at 5% level of significance. In addition, a secondary analysis of the incidence of lengthened cycles was performed across all four study groups using χ² tests for trends. The χ² tests were used to compare the outcomes in different stages of the cycle on the basis of hormone measurements. Pearson correlation and two-sample t-tests were used to test for associations between demographic factors and length of cycles, and paired t-tests were used to compare the lengths of different cycles. The number of times each patient reported adverse events of specific types was tested against the study drug dose using χ² tests for linear association and compared between centers by Kruskal-Wallis tests. This was calculated on the basis of an estimated incidence of delayed cycles of 5% in the placebo group. With 73 women in each group, the study had >80% power to detect significant differences on the order of 20% between adjacent pairs of actively treated groups. A total of 100 women were recruited to allow for dropouts.

RESULTS
Starting from December 2001, a total of 399 women were recruited: 79 from Hong Kong and 80 from each of the other four centers. Recruitment finished in April 2003. The women were randomized into four equally sized groups; they were given a single dose of a placebo or 10, 25, or 200 mg of mifepristone; 391 of the 399 women were observed after the administration of drug. Eight women were withdrawn; six became pregnant before the first postdrug menstrual episode, and two did not supply any diary data. Two women became pregnant in the cycle following the intervention cycle and were included in primary analysis. The four groups were similar in age, parity, and body mass index (BMI). Mean values (ranges) were 31 (18–40) years for age, 1.2 (0–6) for parity, and 24 (15–48) for BMI. Although the overall mean cycle length was similar between the centers (29.3 ± 5.4 days), there was considerable variation both between and within women in whom repeated measurements (mean range 1.94 [SD 2.18] days on the basis of 281 prospective menstrual episodes) were available before the intervention cycle. Similarly, the length of the intervention cycle varied among women in the placebo-treated group. Only 85 (87.6%) of 97 women had intervention cycle lengths ± 5 days of normal cycle length (Table 1).

There was a statistically significant, dose-related tendency for disruption of cycle length (of more than ± 5 days) in the women who received mifepristone (Table 1), from 16% of women treated with 10 mg to 52% of women treated with 200 mg of mifepristone (χ² = 23.88; P<.001). However, the

| Table 1 |

Percent of subjects with length of intervention cycle within and outside 5 days of preintervention cycle.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Within ± 5 days</th>
<th>Shortened (&lt;5 days)</th>
<th>Lengthened (&gt;5 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo, % (range)</td>
<td>89 (61–94)</td>
<td>8 (4–15)</td>
<td>3 (1–9)</td>
</tr>
<tr>
<td>10 mg, % (range)</td>
<td>84 (75–90)</td>
<td>6 (3–13)</td>
<td>10 (6–18)</td>
</tr>
<tr>
<td>25 mg, % (range)</td>
<td>72 (63–81)</td>
<td>10 (6–18)</td>
<td>18 (12–27)</td>
</tr>
<tr>
<td>200 mg, % (range)</td>
<td>48 (38–58)</td>
<td>26 (18–35)</td>
<td>26 (19–36)</td>
</tr>
</tbody>
</table>

Narvekar. Mifepristone and once-a-month contraceptive pill.

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difference between adjacent drug groups with respect to menstrual delay was not significantly different (Table 1) for placebo versus 10 mg ($\chi^2 = 2.97; P = .08$); for 10 mg versus 25 mg ($\chi^2 = 1.88; P = .17$); and for 25 mg versus 200 mg ($\chi^2 = 1.54; P = .21$). A dose-related increase in the proportion of women experiencing menstrual cycles of a shorter duration was also demonstrated (Table 1), but the difference between placebo and mifepristone only reached statistical significance in the group treated with 200 mg (25%) ($\chi^2 = 19.34; P < .001$).

This degree of cycle disruption was unexpected and led us to suspect that the administration of mifepristone in relation to ovulation was mistimed. The hormone data were therefore used to classify the time of the ovarian cycle when the drug was given in the 389 women for whom the data were available (Table 2). When all the groups and centers were combined, treatment (i.e., mifepristone or placebo) was given in the periovulatory period, which was the optimum time, in only 179 (46%) of 389 of cycles. In 64 (16%) of 389, it was given in the follicular phase (i.e., before ovulation). In the remainder (38%), it was given too late in the luteal phase. The degree of cycle disruption was much less in the women who were given mifepristone in the periovulatory period (Table 2). Ninety-five of the 137 women in this group (69%) had their next menses within 5 days of the expected time. In contrast, in the 200-mg group, 83% of the women who took the mifepristone in the follicular phase had a delay of more than 5 days in the onset of the next menses, whereas in 59% of those taking it in the luteal phase, the menstrual cycle was shortened (Table 2).

The mean value ($\pm$ SD) for the length of the preintervention cycle (L1) based on available diary data (n = 159 subjects) was 29.3 $\pm$ 5.4 days, and this did not differ significantly from that of the normal cycle length of 29.2 $\pm$ 2.0 days or from that of the length of intervention cycle (L2) in the placebo group (mean 28.8 $\pm$ 3.9 days). Thus, no evidence was found of an effect off the placebo on cycle length. The L1 correlated with the length of the normal cycle as reported or recorded retrospectively by the women ($P < .001$), and in most cases, the two were within 3 days of each other. Ten cases were between 4 and 10 days discrepant, and only four cases were more than 10 days different. There was a borderline significant negative correlation between L1 and age ($r = -0.14; P = .007$), and normal cycle length also indicated a negative correlation with age ($r = -0.17; P < .001$). The L1 also correlated with heart rate ($r = +0.20; P < .01$) but not with height, weight, blood pressure, center, race, or parity. The mean duration of preintervention menstrual episodes was 5.0 $\pm$ 1.8 days (based on 637 episodes) and of bleeding-free intervals was 25 $\pm$ 3.2 days (based on 279 episodes).

A few women (0, 2%, 1%, and 14% of women in the placebo, 10-, 25-, and 200-mg groups, respectively) menstruated within a few days of administration of the tablets and experienced a second bleeding episode at the anticipated time of next menstruation. This bleeding pattern was strongly associated ($P < .001$) with administration of the drug in the luteal phase of the cycle.

**DISCUSSION**

To be effective as a once-a-month contraceptive pill, mifepristone must be administered in a dose sufficient to prevent implantation and at the correct time in the cycle to minimize menstrual disruption. The ideal time to administer mifepristone is just after the start of the LH surge and before the endometrium is primed for a P-withdrawal bleed. We have previously identified a 3-day window of opportunity around the time of ovulation (16).

In the present study, we have attempted to identify this critical time of administration on the basis of a prediction of the length of menstrual cycle. This study clearly demonstrates that such a calendar approach to timing the administration of once-a-month mifepristone is not practical.

Even with the 3-day window of opportunity, treatment was mistimed in more than half the cycles. Although normal cycle length and length of the preintervention cycle correlated well, an unexpected variation in the length of interven-
tion cycle occurred, with 11% of women in the placebo group demonstrating a variation of >10 days. This was significantly more than our pre-study assumption (5%) and may explain why mifepristone was mistimed.

Large databases of menstrual diaries (23, 24) have demonstrated that each woman has her own central trend and variation in menstrual cycle lengths. It may be possible to devise a more accurate formula to predict the length of the menstrual cycle if more menstrual data were available. It was previously reported that when data pertaining to the previous 12 menstrual cycles is available, prediction of the onset of the next menstrual cycle within the range of previous cycles is possible in ≥90% of women (25). Nevertheless, this requires a complex mathematical formula, and such methodology defeats the very purpose of our study, which was to find a reliable and simple method of timing administration. A large number of studies describing bleeding patterns have observed discrepancies between women's claims of a regular menstrual cycle of usually 28 days and the actual regularity of such patterns revealed by prospective recording. Regardless of the way in which we calculated normal cycle lengths (i.e., retrospective recording, prospective recording, and reported normal length), no difference was found in the principal outcome measure (variation in cycle length of >5 days) among the three calculated groups.

The effect of treatment on cycle length was related to both the dose of mifepristone and to the timing of administration in relation to ovulation. Even the lowest dose (10 mg) caused cycle disruption in 16% of women, and disappointingly, a 25-mg dose disrupted the cycle in over one in four women. In contrast, although treatment with a placebo was associated with some change in cycle length, almost 90% of women menstruated within the expected time. Administration in the follicular phase prolonged the cycle, presumably by delay in the onset of LH surge (10, 11, 26-28). Only the highest dose (200 mg) of mifepristone caused a significant shortening of cycle by inducing a withdrawal bleed when administered in the luteal phase (12). More than 1 in 10 women treated with 200 mg of mifepristone experienced two bleeding episodes: one at P withdrawal and the other at the expected time of menstruation. Even women who received treatment during the peri-ovulatory phase of the menstrual cycle indicated a significant dose-dependent trend in cycle disruption (lengthening). Although the exact functional form of these dose-bleeding relationships cannot be defined, such dose-related effects on menstrual cycle length, on the basis of the timing of administration in relation to ovulation, have not been reported previously.

We used cross-sectional data on LH and P to classify women into ovulatory categories. This is almost certainly less accurate than composite longitudinal data on the basis of follicle growth tracking (by ultrasound), and serum and urinary LH and P and their metabolites. Results from a large, randomized, multicenter trial investigating the efficacy of 10, 50, and 600 mg of mifepristone for emergency contra-

ception (20) confirm considerable disruption in the cycle if mifepristone is given at random days of the cycle.

Women with irregular cycles or cycles with a normal variation in length of more than 3 days were excluded from this study. If a calendar-based approach to timing the administration of mifepristone is inaccurate in women with very regular cycles, it would not be a very practical contraceptive for routine use. Many women find irregular bleeding intolerable; it is the most common reason for discontinuation of all hormonal methods of contraception (22). Menstruation is often regarded as an inconvenience, and unpredictable bleeding is even worse. Moreover, women worry that something is wrong or that they may be pregnant. Mifepristone disrupts menstruation even if given at the correct periovulatory time in the cycle and, as such, may be unacceptable for practical use, even if a better and simpler method of timing administration is devised.

In summary, we have demonstrated that it is unlikely that mifepristone administered once a month at a calendar-based time would provide a reliable method of contraception. There was a greater variation in menstrual cycles in our subjects than previously reported in similar or other populations, and a dose-related shortening or shortening of menstrual cycle occurs if mifepristone is given in the follicular or luteal phase, respectively. Even correct periovulatory administration of single 10- and 25-mg mifepristone doses disrupted menstruation. It is difficult to envisage how an easy and reliable way of defining the correct timing could be devised. Even if such a method was devised, considerable dose-related disruption may render mifepristone unacceptable as a once-a-month pill for practical use.

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REFERENCES

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